DETECTION OF INDUCIBLE CLINDAMYCIN RESISTANCE ALONG WITH E - TEST IN *Staphylococcus aureus* ISOLATED FROM CLINICAL SAMPLES

A Dissertation Submitted to **Department of Microbiology**, **GoldenGate International College**, Tribhuvan University, Kathmandu, Nepal, in partial Fulfilment of the Requirements for the Award of the Degree of **Master of Science in Microbiology (MEDICAL)**

By

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2019

RECOMMENDATION

This is to certify that Ms. Sonia Subedi has completed this dessertation work entitled **"Detection of inducible clindamycin resistance along with E-Test in *Staphylococcus aureus* isolated from clinical samples"** as a partial fulfilment of M.Sc Degree in Microbiology (Medical) under our supervision. To the best of our knowledge, this is her original research work and has not been submitted for any other degree.

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ACKNOWLEDGEMENT

Foremost, I would like to express my sincere gratitude to my supervisors **Mr. Milan Kumar Upreti**, Head, Department of Microbiology, GoldenGate International College and **Mr. Krishna Govinda Prajapati**, Microbiologist B & B Hospital, Lalitpur, for their motivation, assiatance and continuous guidance. Without their guidance throughout the entire period of my thesis work, this woukd have never been accomplished.

I am also grateful to **Associate Prof. Dr. Binod Lekhak**, Tribhuvan University, for his continuous support and guidance. I would like to express my special thanks to **Mr. Nabin Kisor Bimali** (former lecturer),GoldenGate International College for their expert guidance and intellectual support in completion of my dissertation. I am feeling immense pleasure to express my thanks to all the laboratory staffs of B & B hospital for their co-operation and help during my study period and work.

I am equally grateful to my family, dear friends and all those people who directly or indirectly helped me in completing this work.

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ABSTRACT

Staphylococcus aureus (S.aureus) is commonly isolated pathogen from clinical specimen with increasing trend of antimicrobial resistance. The main aim of this study was to isolate *Staphylococcus aureus* from different clinical sample and to determine their susceptibility patterns. This study was conducted from August 2017 to January 2018. During this time period, a total of 3893 different clinical samples were processed by standard microbiological techniques. Following identification, isolates were subjected to antibiotic susceptibility testing using modified Kirby- Bauer disc diffusion method and further admitted for screening of methicillin- resistant *Staphylococcus aureus* (MRSA), inducible clindamycin resistant *Staphylococcus aureus* and MIC performed by E- test.

Among 3893 different clinical isolates processed in this study, *S.aureus* was isolated from 97 samples (7.60%) which constituted 62 (63.9%) from male and 35 (36.1%) from female; 60 (61.9%) from inpatient and 37 (38.1%) from outpatient. 59 (60.8%) isolates were obtained from MRSA. In this study, all the MRSA isolates were obtained multidrug resistant (MDR), whereas 71.05% that means out of 27 of 38 of MSSA isolates were MDR. Although none of the isolates shows showed constitutive resistance to clindamycin, there were 35 inducible clindamycin resistant isolates; 12 from MSSA and 23 from MRSA and the isolates were found susceptible to vancomycin by E Test method. On conclusion, observation of D- effect among some isolates provokes the necessities for development of new strategies.

Keywords: *Staphylococcus aureus*, MRSA, Inducible clindamycin resistance,

E test

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PHOTOGRAPHS

LIST OF APPENDICES

ABBREVIATIONS

AMR	Antimicrobial Resistance
AST	Antimicrobial Susceptibility test
BA	Blood Agar
CDC	Centre for Disease Control
CA MRSA	Community acquired methicillin resistance Staphylococcus aureus
CLSI	Clinical Laboratory Standard Institute
DNA	Deoxyribonucleic Acid
FDA	Food And Drug Administration
HA MRSA	Hospital Acquired Methicillin Resaitant Staphylococcus aureus
ICR	Inducible Clindamycin Resiatant
LAT	Lattex Agglutination Test
MRSA	Methicillin Resistant Staphylococcus aureus
MSSA	Methicillin Sensitive Staphylococuus aureus
MDR	Multidrug Resistant
MA	MacConkey Agar
MIC	Minimum Inhibitory Concentration
PCR	Polymerase Chain Reaction
PVL	Panton- Valentine Leukocidin
RNA	Ribonucleic Acid
SCC*mec*	Staphylococcal cassette chromosome *mec*
TSB	Tryptic Soy Broth
VISA	Vancomycin Intermediate Staphylococcus aureus
VRSA	Vancomycin Resistant staphylococcus Aureus

CHAPTER I
INTRODUCTION AND OBJECTIVE

1.1 Introduction

Drug resistance microorganism has been recognized as a growing danger to the global society. They endanger people in prosperous societies to poor nations (Levy et al, 2005). Antimicrobial resistance (AMR) is a long-standing problem with magnitude and speed its spread becoming the global most serious current public health problem. Until the discovery of antimicrobials in the middle of twentieth century,the human kind was overwhelmed by infectious diseases. The ability to manage infectious diseases has been greatly improved because of discovery of various antimicrobial agents (Deyno et al, 2017). However, the beginning of the era of AMR were recorded soon after the discovery of penicillin in which a number of treatment failures and occurrence of some bacteria no longer sensitive to penicillin started being noticed (Kirby, 1994).

Staphylococcus aureus (S. aureus), a gram positive bacterium, is naturally susceptible to virtually every antibiotic that has ever been developed (Chamber and DeLeo, 2009). The development of resistance to many antibiotics by *S. aureus* has involved acquisition of determinants by horizontal gene transfer of mobile genetic elements (Jensen and Lyon, 2009). These determinants may have evolved in antibiotic producers to protect them from potentially inhibitiory molecules or in their competitors (Foster TJ, 2017).Antibiotic resistance strains of *S. aureus*, methicillin resistant *S. aureus* (MRSA) and vancomycin resistant *S. aureus* (VRSA) have become successful to register themselves in “threat level of serious” and “ threat level of concern”in US (CDC, 2013). CDC 2013 has warned that MRSA may reach to the “threat level of urgent” if its infection rates increases or its strain becomes more resistant to other antibiotic agents.

Bacteria have developed mechanisms for resisting the effects of antibiotics since the introduction of antimicrobials. The emergence of multidrug resistance in

Gram positive bacteria is a particularly an important development. *S. aureus* is of great concern because of its intrinsic virulence, its ability to cause an array of life threatening conditions and to adapt different environmental conditions (Karchmer, 2006). The importance of *S. aureus* as a human pathogen,is its extraordinary potential to develop an antimicrobial resistance apart from its ability to cause a life threatening infection in hospital as well as in community settings (Uzunovic et al, 2013). *S. aureus* is known to be notorious in acquisition of resistance to new drugs and continue to defy attempts at medical control. Many strains of *S. aureus* carry a wide variety of multidrug resistant genes on plasmids, which aid the spread of resistance even among different species (Nsofor et al, 2016).

As a first evidence of antimicrobial resistance, antibiotic (penicillin) resistance in S. *aureus* was observed in 1942 (Deurenberg and Stobberingh, 2008) and spread to community to become pandemic in early 1950s (Roundtree and Freeman, 1956). Although, vancomycin was developed for the treatment of penicillin resistant *S. aureus* in 1958, it was soon superseded by new beta lactams (beta–lactamase resistant analogues of methicillin such as cloxacillin etc) (Foster, 2004). Unfortunately, MRSA were detected within one year of development of methicillin and became a significant problem of clinics during in 1970s. Vancomycin was again brought into extense usefor the treatment of MRSA which resulted into development of VISA and VRSA (Walsh and Howe, 2002). VISA and VRSA has been reported from different parts of world. 13 cases of VRSA reported from U.S. since 2002 (CDC, 2013).

The overall burden of staphylococcal disease, particularly that caused MRSA, is increasing in many countries in both healthcare and community settings. MRSA was detected in early 1960s in clinical settings(Hospital- acquired (HA) MRSA) but detected in community in early 1990s in Western Australia has subsequently been reported worldwide (Chua kyra et al, 2011). The rapidity and extent to which CA-MRSA strain spread have been remarkable. CA-MRSA strains have been reported from United States as well as from Canada, Asia, South America,

Australia and throughout Europe and countries with historically low prevalence of MRSA. Globally, CA-MRSA strains have shown vast diversity in number of different clones (Chambers and DeLeo, 2009). Both strains of MRSA are genetically different (David and Daum, 2010). Nearly all MRSA strains, which carries the methicillin resistance determinant *mecA* gene, contains the staphylococcal cassette chromosome *mec (SCCmec)* element (Katayama et al, 2000). HA-MRSA is typically associated with *SCCmec* type I-III whereas CA-MRSA is associated with type IV and V (David and Daum, 2010). CA-MRSA strain also carry Panton- Valentine leukocidin (PVL) virulence gene which differentiate them from HA-MRSA (Fogarty et al, 2015).

Clindamycin, a lincosamide antibiotic,serves as one such alternative for treating both methicillin susceptible *S. aureus* (MSSA) and methicillin resistant *S. aureus* (MRSA) infections, due to its excellent pharmacokinetic properties.However, due to widespread use of these antibiotic has led to a large number of staphylococcal strains resistant to it (Adhikari et al, 2017). It remains effective for many infections caused by CA-MRSA;CA-MRSA strains are more susceptible to clindamycin (Flora GM, 2013). However, due to increased use of these antimicrobials may drive the emergence of new subclones of CA-MRSA that may be multidrug resistant (Diep et al, 2008). Diep et al 2008 described a multidrug resistant USA300 isolate that had accumulated multiple resistance gene. The failure of treatment of *S. aureus* infections due to inducible clindamycin resistance(ICR) has raised an discussion that use of clindamycin must be closely monitored for signs of treatment failure or relapse of infection (Shrestha et al, 2014);the clindamycin susceptible strain may turn to ICR strain during treatment (Woods, 2009).

There are several researches conducted in Nepal on *S. aureus*. Varying prevalence of MRSA has been reported from different parts of Nepal such as26.1% in Dharan, 68% in Chitwan and 57.1% in Birgunj(Raut et al, 2017). Lower prevalence was reported by Subedi and Brahmadathan (15.4%) and Baral et al. (26%) and higher prevalence was reported byKhanal and Jha (68%) and Tiwari et

al. (69.1%).A high proportion of MDR isolates were reported. However,in Nepal, very few reports on prevalence of inducible clindamycin resistance and vancomycin resistance among *S. aureus* have been published(Adhikari et al, 2017).The high resistance of *S. aureus* against antimicrobials inflame the necessities of continuous monitoring of their susceptibility pattern.so that new strategies can be developed. This study provides an insight about the antimicrobial resistant phenotype pattern of *S. aureus* that were assayed during the study period.

1.2 Objective

1.2.1 General objective

The objective of this research is to perform the antimicrobial resistance of *S. aureus* isolates from clinical samples.

1.2.2 Specific objective

- To study the antimicrobial resistance pattern of *S. aureus* by Modified Kirby Bauer disc diffusion method.
- To determine the rate of MRSA.
- To perform screening of inducible clindamycin resistant *S. aureus* using D-test.
- To perform E-test of isolates against vancomycin.

CHAPTER II
LITERATURE REVIEW

Sir Alexander Ogston (1882), who was a Scottish surgeon, named the clustered micrococci “staphylococci”, from the Greek staphyle, meaning “bunch of grapes”. He observed the organism in stained smear of pus in his patient. Later, Anton J. Rosenbach,a German surgeon, isolated two strains of staphylococci, which he named for the pigmented appearance of their colonies: *Staphylococcus aureus*, from the Latin aurum for gold and *Staphylococcus albus*(now called *epidermidis*), from the Latin albus for white (Orenstein, 1998).

S. aureus is agram positive bacterium that appears in clusters. It is catalase and oxidase positive and unlike other staphylococcal species, it is a coagulase positive organism (Cheesebrough, 2006). They donot form gas from carbohydrate. They prefer aerobic environment but can also grow in absence of oxygen i.e facultative anaerobes. The range of temperature for their growth is 6-44 ˚C but optimum at 37˚C and range of pH is 4.2-9.3 (optimum at 7) (Deyno et al, 2017). They are non-motile and usually non-capsulated (Humphreys, 2007). They are the hardest nonspore forming bacteria and can survive many non physiologic environmental conditions (Reddy et al, 2015). Some strains of *S. aureus* produces beta-hemolytic colonies on 5% sheep blood agar. Mannitol Salt Agar (MSA) is used as selective agar media to recover *S. aureus* from various samples (Cheesebrough, 2006).

2.1 Epidemiology

S. aureus belongs to genus *Staphylooccus*, which has more than 20 species. *S. aureus* is a normal flora which is associated with skin, skin glands and mucous membrane of almost all wram blooded animals. About 30% of the human population is colonized by *S. aureus* (Tadesse et al, 2018). It is also a leading cause of life- threatening blood stream infection, osteoarticular, skin, soft tissue and respiratory tract, device-associated and surgical site infections particularly in

immune compromised, young and elderly patients (Tong et al, 2015). Humans are a natural reservoir for *S. aureus* and asymptomatic colonization is far more common than infections (Chambers, 2001).Colonization may occur in various body parts, mainly nasopharynx, perineum, or skin, particularly if the cutaneous barrier has been disrupted and may occur shortly after birth and may recur anytime thereafter (Payne et al, 1996). Commensal bacteria have to balance between efficient surface adherence, to avoid removal by mechanical forces, not being recognized and destroyed by host immune system (Sollid et al, 2014). For *S. aureus* this is being challenged by their own ability to express virulence factors (Vandenesch et al, 2012); so virulence has to be tightly down regulated during colonization. Gene product being beneficial for colonization such as adhesion and immune evasion proteins (Burain et al, 2010) andprobably also gene products that protect against the reactive oxygen species and desiccation have to be up-regulated (Cosgrove et al, 2007).

S. aureus is one of the most successful and adaptable human pathogens which can easily adapt in the environment (Taddesse et al, 2014). It has its remarkable ability to acquire antibiotic resistance mechanism (Zetola et al, 2005). It has its capacity to adapt in the new environment and advances in patients care partially; are responsible for the increase in incidence of infections (Boucher and Corey, 2008). And today, MRSA is aproblem in hospitals worldwide and is increasingly recovered from nursing homes and the community (Enright et al, 2002). It is being associated with substantial rates of morbidity and mortality (Ansari et al, 2014).

The emergence of the community- acquired MRSA (CA MRSA) inaugurated a new debate in the epidemiology of *S. aureus*. Methicillin Resistant *S. aureus* infections were only isolated from hospital settings until 1990s, when CA MRSA isolates were detected in community members with no risk factors for HA-MRSA (Pantosti, 2012). Though CA-MRSA isolates were considered more virulent than HA- MRSA, HA-MRSA isolates harbours a greater number of AMR determinants (Hau et al, 2018). CA-MRSA strains are also associated with greater

toxin production, compared with HA-MRSA strains; many CA-MRSA strains carry the Panton-Valentine leukocidin (PVL) genes. PVL, regarded as molecular marker of CA-MRSA, which encode cytotoxins that can cause tissue necrosis and leukocyte destruction (Naber, 2009). MRSA which was identified in community infrequently, have now been documented as increase in community acquired MRSA, which may suggest a changing in epidemiology (Naghavi-Behzad M et al, 2015). It has started encroaching in the hospitalsettings, may be of its rapid multiplication rate than of HA- MRSA (Okuma et al, 2002). The studies showing a significant decrease in MRSA resistance as well as a significant increase in number of infections reflects the appearance of CA-MRSA strains in hospitals (Klevens et al, 2006).

2.2 Pathogenesis

S. aureus is both commensal as well as pathogenic organism. It is a notorious pathogenic organism which has well established itself in the hospital settings, as well as community (Okuma et al, 2002). As a commensal, its most common habitat is anterior nares: 20% individuals are persistently nasally colonized with *S. aureus* and remaining intermittently colonized (Mulcahy et al, 2012). Nasal colonization may be the source of the infecting organism in the hospital settings as well as community. On comparison of patients' nasal colonization with MRSA and MSSA, patients colonized with MRSA are 4 times more likely to develop invasive infections (Safdar and Bradley, 2008). *S. aureus* may also spread from person to person, usually through directcontact or in the form of aerosols during speaking, sneezing, or coughing (Humphreys, 2007).

2.3 Virulence factors:

S. aureus strains can express a wide array of potential virulence factors including surface proteins that promote adherence to damaged tissue (Foster and Hook, 1998), binds proteins in blood to help evade antibody mediated immune response and promote in iron uptake (Foster, 2004). The organism also expresses a number of membrane damaging toxins and superantigen toxins that can cause damage to

tissue and symptom of septic shock respectively (Fedtke et al, 2004). There is a growing realization that *S. aureus* has multiple mechanisms for evading both innate immunity, which is mediated by polymorphonuclear leukocytes and induced immunity, which is mediated by both B and T cells (Goodyear and Silverman, 2003). Some virulence factors are expressed by genes that are located on mobile genetic elements called pathogenicity islands e.g. Toxic Shock SyndromeToxin–1 and some enterotoxins or lysogenic bacteriophages e.g. Panton-Valentine leucocidin (PVL) (Novic, 2003) and factors associated with suppressing innate immunity such as the chemotaxis inhibitory protein and staphylokinase, which are integrated in the bacterial chromosome (Foster, 2004).

Once the organism adheres to the host tissue, it protects itself from the host immune response by several ways. It can produce extracellular slime layer, i.e. biofilm, which facilitates adherence and may limit the access of antibiotics, and may reduce the host's inflammatory response (Mahto et al, 2014). *S. aureus* has also been reported to produce antiphagocytic capsule. Various enzymes such as protease, lipases, elastases, staphylokinases, produced by *S. aureus* enable it to invade and destroy the host tissue and spread to other sites (Brooks et al, 2007). Host factors are also important for the susceptibility of individual to the staphylococcal infections. Carriers are found to be infected with their own colonizing strains, but sometimes they are benefited also. A study byWertheim et al (2004) shows that bacteremia in non-colonized patients has higher mortality than in colonized patients.

2.4 Antimicrobial resistance of *S. aureus*:

Over the past three decades,the synthesis of large number of antibiotics has caused complacency about the threat of bacterial resistance (Rubin et al, 1999). Due to indiscriminate use of antibiotics,strains of *S. aureus* have developed resistance to antibiotics (Deyno et al, 2017). Bacteria have become resistant to antimicrobial agents as a result of chromosomal changes or the exchange of genetic material via plasmids and transposons (Naghavi Behzad et al, 2015)

Resistance of microorganisms to antimicrobials is of different types. It may be intrinsic, constitutive or induced. In intrinsic resistance, the organisms are resistant to the antimicrobial due to their innate metabolic characteristics while constitutive resistance is a type of acquired resistance. And inducible resistance is resistance phenotype which is only displayed when organisms comes in contact with antimicrobial which induces its expression (Smith and Jarvis, 1999). In *S. aureus,* conjugation, transduction, or transformation may be involved as horizontal gene transfer mechanism; however the later two are rare mechanisms (Al-Masaudi et al, 1991). Chromosomal mutation usually results into altered antimicrobial target of action, but also can affect bacterial membrane permeability to an antimicrobial, or enzymatically inactivate it (Hickey and Nelson, 1997). Conjugation may bring resistance by transfer of chromosomal or plasmid-borne genes. Plasmid is extrachromosomal DNA that has capacity to self-replicate. Acquisition of resistance by plasmid has two advantages to the organisms. First, plasmid may bear multiple resistance genes beneficial to organisms. Second, plasmid does not remove the organism'schromosomal material, but there is only addition of extra genetic material (Smith and Jarvis, 1999).

2.4.1 Methicillin Resistance in *S .aureus*:

MethicillinResistant *Staphylococcus aureus* (MRSA) is a strain of *Staphylococcus aureus* that is resistant to a large group of antibiotics known as β lactams, which includes penicillins and cephalosporins (Kayastha et al, 2007). In 1940s, for the infections caused by *S. aureus,* Penicillin G was the choice of treatment. However, since 1960s, *S. aureus* strains resistant to penicillinase resistant penicillins, as represented by the original member of the class, methicillin, have gradually emerged worldwide (Aliffe, 1997 ; Chambers, 2001).They are referred as methicillin resistant *Staphylococcus aureus* (MRSA) and are resistant to β lactam agent (Feng et al, 2008). Globally, the burdenof infections caused by them is increasing among different populations and were initially associated with hospital settings. In 1990s, it has been isolated from healthy individuals in communities who had no previous history of hospitalization (Udo et al, 1993).

These type of MRSA were described as community acquired MRSA (CA-MRSA) and since then, has been major cause of infections worldwide (Udo and Boswihi, 2017).

Methicillin resistance is mediated by the *mecA* gene, which confers resistance to all beta-lactam antibiotics, located on a mobile genetic island called staphylococcal cassette chromosome *mec* (SCC *mec*).PBP 2a is encoded by *mecA* gene which is located in SCC*mec* (Hiramatsu, 2004). Chromosomal resistance from a random mutation in genes encoding for other PBPs, also rarely occur (Henze and Berger-Bachi, 1995; Hackbarth et al, 1995). SCC*mec* has been characterized as novel, mobile resistance element that differs from both transposons and bacteriophages. HA- MRSA and CA- MRSA differ from each other in consisting different allotypes of SCC*mec*. SCC*mec* types I, II, and III are typically associated to HA MRSA strains while SCC*mec* types IV and V are associated with CA MRSA (Aydiner et al, 2012). Other distinctive properties of CA MRSA compared to HA MRSA include more antimicrobial susceptible phenotype due to presence of much smaller SCC*mec* type, and the presence of different gene profiles that include Panton-Valentine Leukocidin (PVL) toxin gene (Niami et al, 2003). CA- MRSA strains are resistant to β- lactams in contrast to HA MRSA which are also resistant to tetracyclines, clindamycin, and trimethoprim-sulphamethoxazole in addition to β- lactams (Niami et al, 2003; Okuma et al, 2002). *mecA* gene can be transmitted between *S. aureus* and other staphylococci, however MRSA typically spreads through clones (Berger-Bachi and Rohrer, 2002).

Briefly, *mecA* gene along with regulatory sequences *mecI* and *mecR* form *mecA* complex. *mecR* gene encodes a cytoplasmic membrane receptor. Binding of β-lactam antibiotics to this receptor generates a signal cascade that leads to proteolysis of the product of repressor gene *mecI* (Niemeyer et al, 1996). If repression is loss, there is uninhibited production PBP 2a by *mecA* gene. Strains that have completely functional *mecI* remain susceptible to the β- lactam antibiotics. Mutation in the repressor gene *mecI* leads to the loss of repressor

function. However, the methicillin-resistance (by production of PBP 2a) in the presence of intact *mecI* gene has also been reported (Niemeyer et al, 1996), may be due to mutations in other repressor genetic elements such as *blaR2* (Bal and Gould, 2005).

Methicillin resistance in *S. aureus* is expressed in a stepwise fashion (Hiramatsu, 2004). In the pre-MRSA that has the *mecA* gene together with regulators genes *mecI* and *mecR1*, *S. aureus* strains do not express methicillin resistance. In heteroresistance the *mecI*-mediated repression is released by mutation and the strain start to resist low concentration of methicillin but still remains susceptible to higher concentrations. Finally, a homogeneous MRSA develops that has homogeneously high resistance (Appelbaum, 2007).

2.4.1.1 Screening of methicillin resistance *S. aureus*:

CLSI guideline (2012) has focused on detection of oxacillin and cefoxitin disk diffusion method,for the detection of methicillin resistance in *S. aureus*. Resistant organism observed by these techniques should be reported resistant to all beta-lactams or should not be reported.Latex agglutination test and PCR can be performed for the detection of methicillin resistance. Study conducted by Mohanasoundaram and Lalitha (2006), they have concluded that " conventional methods for detection of methicillin resistance like disc screening, disc diffusion and MIC are cost effective but time consuming. Latex agglutination though expensive is rapid and can be a good preliminary screen with high sensitivity andspecificity…multiplex PCR is a good confirmatory test though expensive".

2.4.2 Clindamycin resistance

Clindamycin, a derivative of lincomycin, the lincosamide antibiotic that inhibits protein synthesis by target modification; act by binding to the 23S rRNA component of 50S ribosomal subunit (Woods, 2009). It is useful for treatment of skin and soft tissue infection and infection caused by *Staphylococcus spp*. Especially MRSA. It has excellent tissue and bone penetration and accumulates in and no requisition of renal dosing adjustment, which make it an important

therapeutic agent (Shrestha and Rana, 2014).Clindamycin has several advantages but major barrier in its usage is development of resistance especially inducible resistance (Sande, 2015).

Lincosamide (e.g clindamycin), macrolide (e.g erythromycin), streptogramin (e.g quinupristin- dalfopristin) have antimicrobial agents which are collectively termed as MLS_B, has been used to treat staphylococcal infections (Zelazny et al, 2005). Because of good pharmacokinetics properties such as good oral and excellent tissue penetration, clindamycin is most used antibiotic, but over use of MLS_B in the treatment procedure of infections has been led to increase of resistance to these antibiotics (Moosavian et al, 2014). They are chemically distinct but have similar inhibitory effects on bacterial protein synthesis. The genes that cause resistance to one of MLS_B antibiotic can lead to the development of cross resistance to the other members of groups as well (Kaskatepe and Yildiz, 2014).Resistance to MLS_B antibiotics occur by many different mechanism: an active efflux pump which is encoded by *msrA* gene, enzymatic inactivation of antibiotic and ribosomal target modification that is the major mechanism of resistance and affects these antibiotics (Yilmaz et al, 2007) The induciblеresistance of clindamycin, which is a cross resistance due to sharing of same target by MLS_B antibiotics, is due to the enzymatic modification.*Erm* gene encode enzyme that confer inducible resistance or not to MLS_B antimicrobial agent via methylation of the 23S rRNA , reducing binding by MLS_B agents to the ribosome(Kaskatepe and Yildiz, 2014).

The most common mechanism include target site modification which is mediated by *erm* gene; can be expressed either constitutively ($cMLS_B$ phenotype) or inducibly ($iMLS_B$ phenotype). The erm genes codes for methylase enzyme which methylates and alters the target site of MLSB antibiotics i.e. the 23S ribosomal RNA (Adhikari et al, 2017). In constitutive resistance (cMLSB phenotype), it is resistance to both clindamycin and erythromycin which is observed during susceptibility testing, whereas, in inducible resistance (iMLSB phenotype)

resistance to erythromycin is observed and clindamycin is observed to remain susceptible during susceptibility testing (Upadhya and Birada, 2011).

Due to lack of identity of inducible clindamycin resistance,it has lead to false laboratory reports and could lead to clinical failure when clindamycin is used therapeutically and cause treatment problems (Chelae et al, 2009).The inducible resistance cannot be detected by routine susceptibility test but can be distinguished by erythromycin-clindamycin disk approximation test i.e. D-test according to therecommendation of the Clinical and Laboratory Standards Institute. Isolates with constitutive resistance are resistant to erythromycin and clindamycin, while isolates with inducible resistance are resistant to erythromycin but appear susceptible to clindamycin (Schreckenberger et al, 2004). D-test is a double-disk test where erythromycin is placed at a distance of about 15-20 mm from clindamycin disk to induce the resistance mechanism (Woods, 2009).

Clindamycin resistance is common among health care-associated MRSA strains than community associated MRSA strain (Woods, 2009). It remained effective treatment of various types of infections caused by CA- MRSA. But, clindamycin resistance is on increasing trend (Hulten et al, 2006) and they may be no longer an appropriate for empiric treatment of serious infections. Emergence of multidrug resistant CA-MRSA, involving resistance to erythromycin and clindamycin, makes the drug unsuitable for the empiric therapy (Chen et al, 2005).

2.4.2.1 Screening of inducible clindamycin resistance:

D-test: As already mentioned, inducible clindamycin resistance (ICR) can't beobserved in routinely used susceptibility testing. CLSI guideline (2012) hasrecommended performing D-test for it. In this test two antibiotic discs,erythromycin and clindamycin are placed at a distance of about 15-20 mm.The flattening of the zone of inhibition adjacent to erythromycin disk isreferred to as inducible clindamycin resistance. Broth microdilution test canalso be performed for screening ICR (CLSI, 2012).

2.4.3 Vancomycin

Vancomycin is a glycopeptides which is produced by Amycolatopsis orientalis (actinomycete) and is widely used in medical practice especially after the appearance of resistant strains of *S. aureus* to methicillin. However, the emergence of molecular mechanism of resistance to vancomycin and appearance of phenotypic resistance profile in heterogenous sub populations of staph aureus as described susceptible have hindered the use of glycopeptides as first choice agent in antibiotic therapy (Micek, 2007). Vancomycin inhibits the synthesis of bacterial cell wall, that binds to subunits of the peptide side chain (D-alanyl-D-alanine). Despite of having large molecular size, it occupies a sizable area and thus, prevents further synthesis of the peptidoglycan polymer around the bacterium. For clinical use, it is often considered as bacteriostatic (Leung, 2014).

The emergence of MRSA in late 1970s has dramatically increased the use of vancomycin. Over 20 years, intravenous vancomycin has been the standard of care for serious MRSA infections. By 1996, the first isolate of MRSA with reduced susceptibility to vancomycin was reported (Jacob and DiazGranados, 2013). Vancomycin is an antimicrobial agent, to treat life- threatening infections with MRSA. The true mechanism of vancomycin resistance in *S. aureus* is not known. It was initially feared that *S. aureus* would acquire the van gene that code for vancomycin resistance in *Enterococcus spp* (Tiwari and Sen, 2006). Showsh et al, (2001) have demonstrated the presence of sex pheromone in *S. aureus* that promotes plasmid transfer in *Enterococcus spp*. Release of these pheromones by *S. aureus* with proximity to vancomycin-resistant enterococci causes the transfer of plasmids encoding van gene to the *S. aureus*.

Diminished susceptibility due to acquisition of *VanA* gene was expected but the actual involved mechanism was unusual thickening of cell wall containing dipeptides, which is capable of binding vancomycin, thereby reducing availability of the drug for intracellular target molecules (Cui et al, 2003). The predicted mechanism of *VanA* gene plasmid-mediated transfer from enterococci was later

observed for the first time in 2002- the first description of vancomycin-resistant *S. aureus* (VRSA) (CDC, 2002; Howden et al, 2010).

The high prevalence of MRSA led to the increased use of vancomycin which resulted into emergence of multiple phenotype with reduced susceptibility (Rehm and Tice, 2010): different phenotypes are heterogeneous vancomycin-intermediate *S. aureus* (hVRSA), vancomycin-intermediate *S. aureus* (VISA), and vancomycin-resistant *S. aureus* (VRSA). CLSI guideline (2012) categorize *S.aureus* as vancomycin-susceptible *S. aureus* (VSSA), VISA, or VRSA when the vancomycin minimum inhibitory concentration (MIC) is ≤2 μg/mL, 4 to 8 μg/mL, or ≥16 μg/mL, respectively. The MICs of hVISA may fall within the susceptible range when tested by routine methods; these strains stably produce subsets of cells in the intermediate range. Those organisms in the intermediate range are assumed precursors of VISA (Hiramatsu et al, 1997). However, the breakpoints that differentiate hVISA strains from VSSA have not been established (Hu et al, 2013).

The resistance mechanisms for both VISA and hVISA appear to have common features. However, it is always observed in patterns that distinguish them from Vancomycin-Susceptible *Staphylococcus aureus* (VSSA). Although the gene expression of hVISA or VISA during expo-sure to vancomycin denotes some patterns, subtle differences in the contribution of transcripts of these two phenotypes are found. A basic evidence regarding decrease in susceptibility is related to the cell wall thickening in bacterial cells (Zheng and Zhang, 2012). VRSA resist vancomycin by synthesizing an alternative cellwall terminal peptide (D-ala-D-lac), rather than the normal terminal peptide (D-ala-D-ala). Vancomycin could not bind to the former peptide. Whereas, reduced susceptibility to vancomycin in VISA/hVISA is due to the unusual thickening of cell wall containing dipeptides (D-ala-D-ala): the dipeptides is capable of binding vancomycin, thereby reducing the availability of the drug for intracellular target molecules (Lowy, 1998; Howden et al, 2006; Walsh and Howe, 2002). hVISA refers to *S. aureus* strains in which subpopulations display variable susceptibility

to vancomycin (Hiramatsu et al, 1997). hVISA and VISA have been reported predominately in MRSA strains (Kirby et al, 2010; Richter et al, 2011). However, they are also present among MSSA, suggesting the screening of both MRSA and MSSA isolates to get actual prevalence (Liu and Chambers, 2003; Pilai et al, 2009).

There are a number of types of susceptibility test available. Traditionally, agar disk diffusion has been used to measure glycopeptide susceptibility, but now this method is not regarded as standard method. This method is not suitable for large antibiotic molecules, such as glycopeptides which diffuse too slowly into agar. An alternative method for measuring glycopeptide MIC is broth microdilution which is the gold standard test for measuring antibiotic MICs. E test has been developed as an accurate and easier agar plate method. An E test strip, which contains a gradient of antibiotic, is placed on an inoculated agar plate and the pattern of bacterial growth is examined after 24 hours. Since the Etest uses a gradient of antibiotic concentration, it has greater precision than disc diffusion methods, allowing better ascertainment of the actual MIC (Joana et al, 2013)

2.4.3.1 Detection of VISA/VRSA

Interpretive criteria for vancomycin disk for susceptibility technique have not been recommended. However CLSI (2012) provide the MIC breakpoint of vancomycin for detection of *S. aureus* as susceptible, intermediate or resistant, ≤4 µg/mL, 4-8 µg/mL, ≥16 µg/mL respectively. BHI agar with 6 µg/mL vancomycin concentration can also be used to detect the reduced susceptibility of *S. aureus* to vancomycin. Further confirmation need to done by MIC method taking a colony that grows on BHI vancomycin agar. The vancomycin MIC testing can also be performed by brothmicro dilution method and E-test.

2.5 Nepalese scenario of *S. aureus* infections:

Similar to global scenario, *S. aureus* istaken as one of the major pathogen in Nepal. It has been frequently isolated from different wards of hospital and from different clinical samples including pus and wound swab, blood and other body

fluids, urine, sputum etc. But, morbidity and mortality due to *S. aureus* has not found a proper documentation. There are research activities performed in Nepal to find out the prevalence of the *S. aureus*. In a study conducted by Adhikari et al (2017), 25.1% were methicillin resistant *S. aureus*.The incidence of MRSA was reported to be 20% in 2001, 15.4% in 2005, 26.14% in 2008, 39.6% in 2010 and 42.4% in 2013 in Nepal (Ansari et al, 2014).

2.6 Laboratory identification of *S. aureus*:

Proper identification of organism reflects the quality of laboratory and skilled manpower employed. Generally, identifications start with culture in appropriate media. Colony characteristics and gram staining were done for identification purpose. The use of differential media allows rapid and conventional identification of organism. Staphylococci are cocci shaped which retain the color of crystal violet under microscope after gram staining.Coagulase test is performed to differentiate *S. aureus* from other staphylococci.

The following biochemical test provide the differentiating characteristics of *S. aureus* from other species (Cheesebrough, 2006):

Coagulase test: It is coagulase positive. Two types of coagulase are produced by most strains: free coagulse which convert fibrinogen to fibrin and bound coagulase (clumping factor).

Catalase test: It help to differentiate *S. aureus* (catalase positive)from streptococci.

DNA-ase test: *S. aureus* is DNA-*ase* positive.

S. aureus is sensitive to novobiocin .

2.6.1 Other method for identification of *S.aureus*:

Chromogenic media:

Different chromogenic media are available commercially for the identification of *S. aureus*. In *S. aureus* ID (bioMérieux, La Balme Les Grotte France) *S. aureus* produces green colonies due to production of alpha-glucosidase (Perry et al,

2003). Another medium CHROMagar *Staph* provides significantly higher sensitivity than that of the conventional method (Galliot et al, 2000). In this chromogenicmedium *S. aureus* produce mauve colonies after 24 hours of incubation at 37°C.

Latex agglutination tests (LAT):

Kits like Accu-Staph and Staphaurex arecommercially used for the identification of *S. aureus*. They work on theprinciple of simultaneous detection of clumping factor and protein A (Kuuselaet al, 1994). Since they had problem with the *S. aureus* strains which produce little or no clumping factor and protein A,the later formulation of latex tests include protein A and/or clumping factorbut also detect various surface antigens, which improved the sensitivity of thetests (Brown et al, 2005).

2.7 Antimicrobial susceptibility test(AST):

Various methods have been employed for AST which include

- Dilution method
- Disc diffusion method
- Antimicrobial gradient method (E test)
- Automated instrument systems

2.7.1 Dilution method:

Dilution methods are used to determine the minimal inhibitory concentration (MIC) of antimicrobial to inhibit or kill the microorganisms (Jorgensen and Ferraro, 2009). Dilutionmethod provides both quantitative results (MIC value) and qualitative results: the MIC value can be interpreted as susceptible, intermediate, or resistant. There are two methods which include:

- Broth dilution
- Agar dilution

2.7.2 Disc diffusion method:

It is simple, practical and well standardized method but this method only provide qualitative data i.e. the organism being tested can be determined as susceptible, intermediate, or resistant, but MIC value can't be determined (Jorgensen and Ferraro, 2009 ; CLSI, 2012).

2.7.3 Antimicrobial gradient method:

This method uses the principle of establishment of antimicrobial concentration gradient in an agar medium as means of determining MIC (Jorgensen and Ferraro, 2009).The Etest method (AB Biodisk, Solna, Sweden) gives an MIC result and has an advantage over other MIC methods.It is a quantitative test technique, for determining the minimum inhibitory concentration MIC of anti microbial agents, against microorganisms and for detection of resistance mechanisms.It consists of plastic strip calibrated with a MIC scale in µg/ml and codes to identify the antimicrobial agent (Flora GM, 2013).

2.7.4 Automated instrument systems:

Automated instrument systems like Microscan, WallkAway (Siemens Healthcare Diagnostics), BD Phoenix Automated Microbiology System (BD Diagnostics), Vitek 2 System (bioMerieux), and Sensititre ARIS 2X (Trek Diagnostics Systems) are approved by FDA. They consist of photometer or fluorimeter system to determine growth development and susceptibility testing. They also have enhanced computer software used to interpret susceptibility result including "expert systems" for analyzing test results for atypical patterns and unusual resistance phenotypes (Richter and Ferraro, 2007).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials and equipments:

Materials, equipments and reagents used in different stages of this study have been included in appendix I.

3.2 Methodology:

The study was conducted prospectively at B & B Hospital, Gwarko, Lalitpur in collaboration with GoldenGate International College, Battisputali, Kathmandu. The study was conducted from August 2017 to Janaury 2017.

3.2.1 Study Population:

The study included patients of all age group who were visiting hospital for their routine culture.

3.2.2 Ethical Approval:

Ethical approval was taken from institutional Review Committee at B & B Hospital Pvt. Ltd. After giving brief information about this research, written informed consent was obtained from patients prior to sampling. In case of illiterate participants, information as provided by reading the consent form in presence of witness.

3.2.3 Sample Size:

A total of 3893 different clinical sample were collected during the study period. The samples included urine, wound pus, sputum and catheter tip. Those samples were then sent for routine culture and antibiotic susceptibility test and then processed during study period.

3.2.4 Sample collection and transportation:

The samples were collected by medical personnel only by using aseptic procedure. During collection of samples, special care was applied to avoid the contamination by commensal organisms. After collection, the samples were properly covered by sterile capped and then properly labeled. After labeling, the samples were transported to microbiological lab promptly.

3.2.5 Inclusion and Exclusion criteria:

Only those samples which were properly collected and labeled were included with no visible sign of contamination. Those sample which were not collected by medical personnel or self collected by patients were not included in the study.

3.3 Sample Procesing:

3.3.1 Isolation of *S. aureus*:

The samplesurine, sputum, wound pus and catheter tips, were inoculated to MacConkey Agar (MA), Blood Agar(BA) and Mannitol Salt Agar(MSA). The plates were then incubated at 37˚C for 18 - 24 hours. The composition and preparation of different media is mentioned in Appendix II.

3.3.1.1 Culture of the specimen:

Urine Sample:

A loopful of sample was inoculated into MA and BA plates and the plates were incubated at 37˚C for 24 hours. The growth was observed on the plate next day. Growth of the isolates in culture plate was identified and then susceptibility test was performed. In case of contamination, the sample was asked for re-collection.

WoundPus Sample:

The sample were inoculated into MA and BA plates and incubated at 37˚C for 24 hours.

Sputum Sample:

The samples were inoculated MA, BA and CA plates. In BA 5µg optochin disc and in CA 10µg bacitracin disc were added to screen out *Streptococcus pneumonae* and *Haemophilus influenza*. CA plates were incubated at 37˚C anaerobically in 5-10% CO_2 environment and MA and BA were incubated at 37˚C aerobically.

Catheter tips:

Such tips were held with sterile forceps and rolled over the surface of MA and BA plates and streaked. The plates were incubated at 37˚C for 24 hours and growth of the isolates were observed and identified.

3.3.2 Identification of *S. aureus*:

The culture plates were identified on the basis of standard microbiological procedure which includes colony characteristics, gram staining and conventional method (Cheesbrough, 2000).

Colony characteristics: The colony morphology of *S. aureus* on different plates were described in Appendix III.

Gram staining: The suspected colonies were performed for gram staining procedure. Those organism which give gram positive cocci in grape like cluster were identified as staphylococci and further identification was performed.

Biochemical test: The pure culture was obtained from primary culture and then it was used for biochemical tests. Coagulase test, catalase test and oxidase test, oxidative – fermentative test were performed for identification. The procedures were mentioned in Appendix III.

Purity of plates:

It was used to ensure that the inoculation used for biochemical test was pure culture and also used to see whether biochemical test was performed in aseptic condition or not. Thus, while performing biochemical tests, the same inoculums were sub cultured in respective medium and incubated. The media were checked for appearance of pure growth of organism.

3.3.3 Preservation of *S. aureus*:

Pure culture of *S. aureus* isolates were inoculated into tryptic soy broth(TSB) containing 20% glycerol and preserved at -8˚C.

3.4 Antimicrobial susceptibility test:

Antibiotic susceptibility tests of the *S. aureus* isolates were performed by modified Kirby-Bauer disk diffusion method in compliance with Clinical and Laboratory Standards Institute (CLSI) guidelines using Mueller- Hinton agar standard media (CLSI, 2012). AST were performed by following antibiotics: amoxicillin (10µg), amikacin (30µg), ciprofloxacin (30µg), ofloxacin (5µg), gentamycin (30µg), chloramphenicol(30µg), erythromycin(15µg), clindamycin(2µg), linezolid(30µg), azithromycin(30µg), nitrofurantion(10µg), oxacillin(5µg), cefoxitin (30µg). The detail procedure is mentioned in AppendixIII.

3.5 Screening of methicillin resistant *S.aureus*:

This method was performed by cefoxitin disc diffusion method and result was interpreted according to CLSI (2012) guideline.

Result interpretation: isolates which gave zone of inhibition (ZOI)≥22 mm were identified as methicillin susceptible(MSSA) and isolates with ZOI ≤ 21 mm were identified as methicillin resistant (MRSA).

3.6 Detection of inducible clindamycin resistance(ICR):

D test: Isolates that were erythromycin resistant were performed for inducible resistance by 'D test' as per CLSI guideline (CLSI 2012). In this test, two discs, namely erythromycin (15µg) and clindamycin(2µg) were placed in a 15 mm away edge to edge on MHA plate which were previously inoculated with bacterial suspension. The plates were then incubated at 37˚C for 18-24 hours and result was interpreted.

Result interpretation: flattening of zone of inhibition adjacent to erythromycin disk (referred to as D- zone) or hazy growth within the zone of inhibition around

clindamycin (even if no D-zonevis apparent) is regarded as positive i.e. Inducible CLindamycin Resistance (CLSI 2012).

3.7 MIC determination:

MIC was performed for vancomycin by applying a technique known as E test. E test is simple, cost effective and rapid method for determining MIC of single antimicrobial agents. The test is based on diffusion of an antibiotic gradient from a plastic strip on inoculated agar media. The resulting elliptical zone of bacterial inhibition is read at the point of intersection of the ellipse with an MIC scale on the strip (Ngui-yen et al 1992). All tests were incubated at 37˚C for 18-24 hours and results were interpreted.

Result interpretation: elliptical zone of bacterial inhibition ≤ 2 is read as sensitive;4-8 is read as intermediate:≥16 is read as resistance.

3.8Analysis of data:

All the results were recorded in worksheet and later data were analyzed by using SPSS version 21 software. Major data were expressed in percentage and significance of test was performed by using Chi-square test.P- value less than 0.05 were considered as statistically significant.

3.9 Quality Control

3.9.1Regular monitoring of the laboratory equipments, reagent and media:

Laboratory equipment like incubator, autoclave, refrigerator, hot air oven were monitored regulary. Temperature was also noted .Reagents and biochemical media were also monitored by checking their manufacture and expiry date along with their proper storage. Sterility testing and performance testing were also performed by using standard control strains.

3.9.2 Quality control during isolation and identification:

Agar plates that passed the quality control i.e. sterility and performance test, were used. Pure culture of isolated organism was used for identification purpose. The fresh plasma was used for coagulase test i.e for both slide and tube test.

3.9.3 Quality control during AST:

Muller-Hinton agar and antibiotic discs were used for performing AST. The media and antibiotic disc were checked for lot number, manufacture and expire date and stored properly. Control strain of *E.coli* (ATCC 25922) and *Staph aureus*(ATCC 25923) were used for the standardization of AST.

CHAPTER IV

RESULTS

In this study, both in-patients and out-patients, visiting the microbiology laboratory of B & B Hospital with request form for clinical sample culture, were considered as the target participant group. A total of 3893 clinical samples were processedduring the study period. *S. aureus*, however, was obtained only from 97 samples. Those isolates were further investigated for their antimicrobial susceptibility pattern, methicillin-resistance, inducible clindamycin resistance and vancomycin resistance.

4.1 Pattern of growth

Out of 3893 different clinical samples studied, 1276 (30.2%) showed growth, while 2617 (69.8%) showed no growth. The result is shown in figure 1.

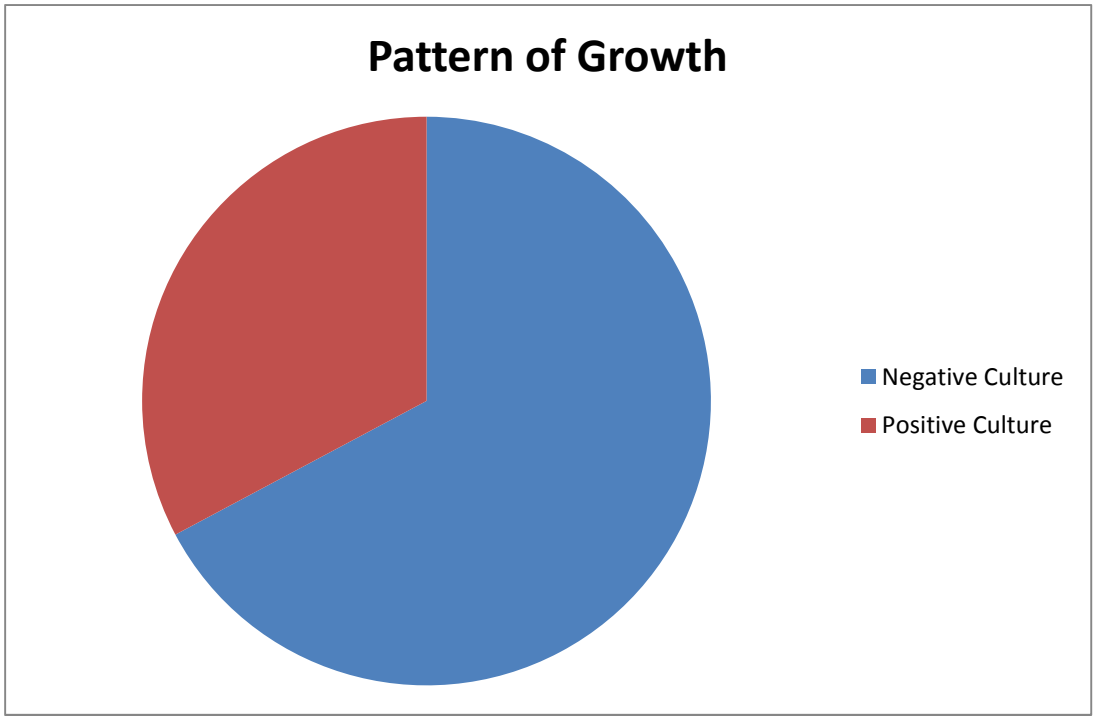

Figure 1: Chart showing pattern of growth among clinial isolates

4.2 Distributions of organism in different clinical samples:

Among 3893 samples processed, 1276 showed culture positive results in which *Escherichia coli* (510) were found to be most predominant organism followed by *Klebsiella pneumonia* (209),*Pseudomonas aeruginosa* (137), Coagulase negative staphylococci(90), *Staphylococcus aureus* (97), *Acinetobacter spp* (60), *Enterococcus spp* (54), *Enterobacter spp* (45), *Streptococcuspyogens* (18), other non haemolytic streptococcus (17), *klebsiella oxytoca* (15), *Proteus mirabilis* (14),*Citrobacter freundii* (3), *Morganella morganii* (3), *Haemophilus influenza* (3) and *Proteusvulgaris* (1). The result is shown in table 1:

Table 1: Distribution of organism in different clinical samples

Organism isolated	Number of isolates	Percentage (%)
Escherichia coli	510	39.96
Klebsiella pneumonia	209	16.37
Pseudomonas aeruginosa	137	10.73
CONS	90	7.05
Staphylococcus aureus	97	7.60
Acinetobacter spp	60	4.70
Enterococcus spp	54	4.23
Enterobacter spp	45	3.52
Streptococcus pyogens	18	1.41
NHS	17	1.33
Klebsiella oxytoca	15	1.17
Proteus mirabilis	14	1.09
Citrobacter freundii	3	0.23
Morganella morganii	3	0.23
Haemophilus influenza	3	0.23
Proteus vulgaris	1	0.07
Total	1276	100

4.3 Distribution of *S. aureus* according to sex

Out of 3893 sample processed, *S. aureus* was obtained from specimen of 97 participant; 62 (63.9%) isolates from male patients and 35 (36.1%) from female patients (Table 2).

Table 2: Distribution of *S. aureus* according to Sex

Sex	Growth(%)	P value
Male	62 (63.9)	0.73
Female	35 (36.1)	

4.4 Distribution of *S. aureus* according to patient types

Sixty (61.9%) of those were sample obtained from inpatients whereas the remaining i.e 37 (38.1%) were from outpatients (Table 3).

Table 3: Distribution of *S. aureus* according to patient types

Patient type	Growth (%)	P value
Inpatient	60 (61.9)	0.67
Outpatient	37 (38.1)	

4.5 Age wise distribution of *S. aureus*

Among 97 isolates of *S. aureus*, the infection caused by organism was found higher in age group 31- 45 (32%) and 16- 30 (28.9%) while least in age group 60 above (9.2).

Table 4: Age wise distribution of isolates

Age group	Growth (%)
0-15	14 (14.4)
16-30	28 (28.9)
31-45	31 (32)
46-60	15 (15.5)
60 above	9 (9.2)

4.6 Distribution of *S. aureus* according to sample

The occurrence of organism was higher in wound pus (88.65%) followed by urine (6.2%), sputum (3.1%) and catheter tip (2.05%).

Table 5: Distribution of isolates according to sample

Sample	Frequency	Percentage
Wound/ pus	86	88.65
Urine	6	6.2
Sputum	3	3.1
Catheter tip	2	2.05

4.7 Distribution of *S. aureus* according to age and sex:

Among 97 isolates of *S. aureus*, predominant age of the patient infected by *S. aureus* belonged to age group 31-45 (n=31; 32%) and least belonged to age group 60 above (n=9;9.2%). The results are shown in table 6.

Table 6: Distribution of *S. aureus* according to age and sex

Age	Male (%)	Female (%)	Total (%)
0-15	7 (11.3)	7 (20)	14 (14.4)
16-30	20 (32.3)	8 (22.9)	28 (28.9)
31-45	20 (32.3)	11 (31.4)	31 (32)
46-60	10 (16.1)	5 (14.3)	15 (15.5)
60 above	5 (8.1)	4 (11.4)	9 (9.2)

4.8 Antibiotic Susceptibility Pattern

Antimicrobial Susceptibility Testing (AST) was performed by modified Kirby-Bauer disc diffusion method. A total of 13 antibiotics were included in AST. Isolates were screened for methicillin resistance by interpretation of the zone of inhibition (ZOI) produced by each isolates with cefoxitin (30µg) disc (CLSI 2012). There were 59 (60.8%) cefoxitin-resistant (MRSA) isolates in this study. Number (percentage) of isolates susceptible or resistant to each antibiotic, among MRSA and MSSA, is listed in Table 7 and Table 8

Table 7: Antimicrobial Susceptibility Pattern of MSSA:

Antibiotics	MSSA(n=38)	
	Sensitive (%)	**Resistant (%)**
AK	27 (71.1)	11 (28.9)
AMX	6 (15.8)	32 (84.2)
AZM	15 (39.5)	23 (60.5)
CIP	10 (26.3)	28 (73.7)
C	36 (94.7)	2 (5.3)
CX	38 (100)	0
CD	30 (78.9)	8 (21.1)
E	3 (7.9)	35 (92.1)
GEN	28 (73.7)	10 (26.3)
LZ	35 (92.1)	3 (7.9)
OF	17 (44.7)	21 (55.3)
OX	25 (65.8)	13 (34.2)
NIT	3 (100)	0

Table 8: Antimicrobial Susceptibility Pattern of MRSA:

Antibiotics	MRSA (n=59)	
	Sensitive (%)	**Resistant (%)**
AK	37 (62.7)	22 (37.3)
AMX	14 (23.7)	45 (76.3)
AZM	17 (28.8)	42 (71.2)
CIP	8 (13.6)	51 (86.4)
C	53 (89.8)	6 (10.2)
CX	0	59 (100)
CD	43 (72.9)	16 (27)
E	0	59 (100)
GEN	33 (55.9)	26 (44.1)
LZ	58 (98.3)	1 (1.7)
OF	7 (11.9)	52 (88.1)
OX	3 (5.1)	56 (94.9)
NIT	0	1 (100)

4.9Antimicrobial resistance of *S. aureus* isolates in relation to type of patients:

Out of 97 isolates of *S. aureus*, most of the isolates obtained from inpatients were resistant to tested antibiotics as compared to isolates from outpatient. However, there is insignificant association between antimicrobial resistance and type of patient. The antimicrobial resistance pattern and type of patient is shown in Table 9.

Table 9: Antimicrobial resistance of *S. aureus* with type of patient:

Antibiotics	Types of patient		p value
	Inpatient n = 60 (%)	**Outpatient n = 37 (%)**	
AK	20 (33.3)	13 (35.1)	
AMX	49 (81.7)	28 (75.7)	
AZM	42 (64.9)	23 (35.4)	
CIP	48 (60.8)	31 (39.2)	
C	4 (50)	4 (50)	
CX	37 (62.7)	22 (37.3)	0.83
CD	13 (54.2)	11 (45.8)	
E	58 (61.7)	36 (97.3)	
GEN	22 (36.7)	14 (37.8)	
LZ	1 (1.7)	3 (8.1)	
OF	45 (75)	28 (75.7)	
OX	45 (75)	24 (64.9)	
NIT	1 (25)	1 (25)	

4.10Antimicrobial resistance of *S. aureus* isolates with respect to sex:

The antimicrobial resistance pattern showed that the isolates obtained from male patient were more resistant to all antibiotics tested as compared to female. However, there is insignificant association between antimicrobial resistance and sex of patient. The antimicrobial resistance pattern and type of patient is shown in Table 10.

Table 10 :Antimicrobial resistance of *S. aureus* with respect to sex:

Antibiotics	Sex		P value
	Male n=62 (%)	Female n=35 (%)	
AK	25 (40.3)	8 (22.9)	
AMX	50 (80.6)	27 (77)	
AZM	42 (62.7)	23 (65.7)	
CIP	51 (82.3)	28 (80)	
C	8 (12.9)	0	0.59
CX	37 (59.7)	22 (62.9)	
CD	17 (27.4)	7 (20)	
E	60 (96.8)	34 (97.1)	
GEN	25 (40.3)	11 (31.4)	
LZ	1 (1.6)	3 (8.6)	
OF	45 (72.6)	28 (80)	
OX	42 (67.7)	27 (77)	
NIT	1 (33.3)	0	

4.11Multidrug resistance (MDR)

Multidrug resistance was identified on the basis of their antibiotic resistance pattern. The organism resistant to three or more than three antibiotics of different classes was considered as MDR. Out of 97 isolates, 27 of 38 isolates shows MDR in case of MSSA whereas all MRSA are considered as MDR which is illustrated via figure 2.

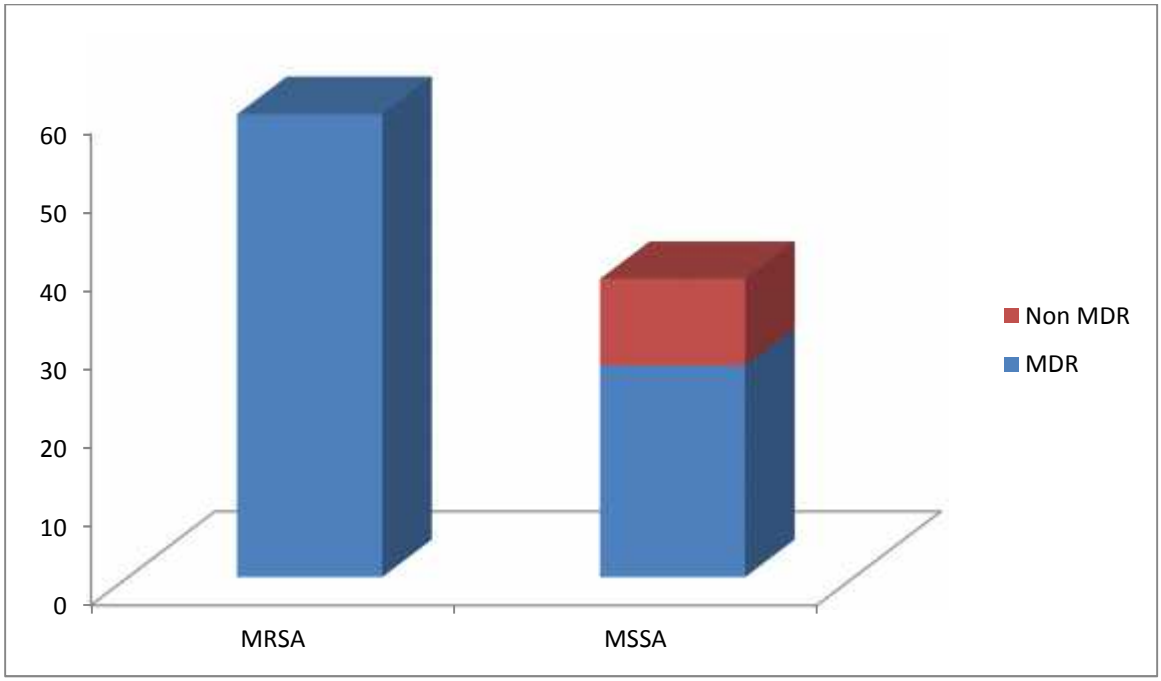

Figure 2: Comparative chart showing MDR and Non MDR in MRSA and MSSA

4.12Prevalance of MRSA isolates

Out of 97 isolates, 59 (60.8%) isolates were obtained resistant to cefoxitin (MRSA) by disc diffusion method.

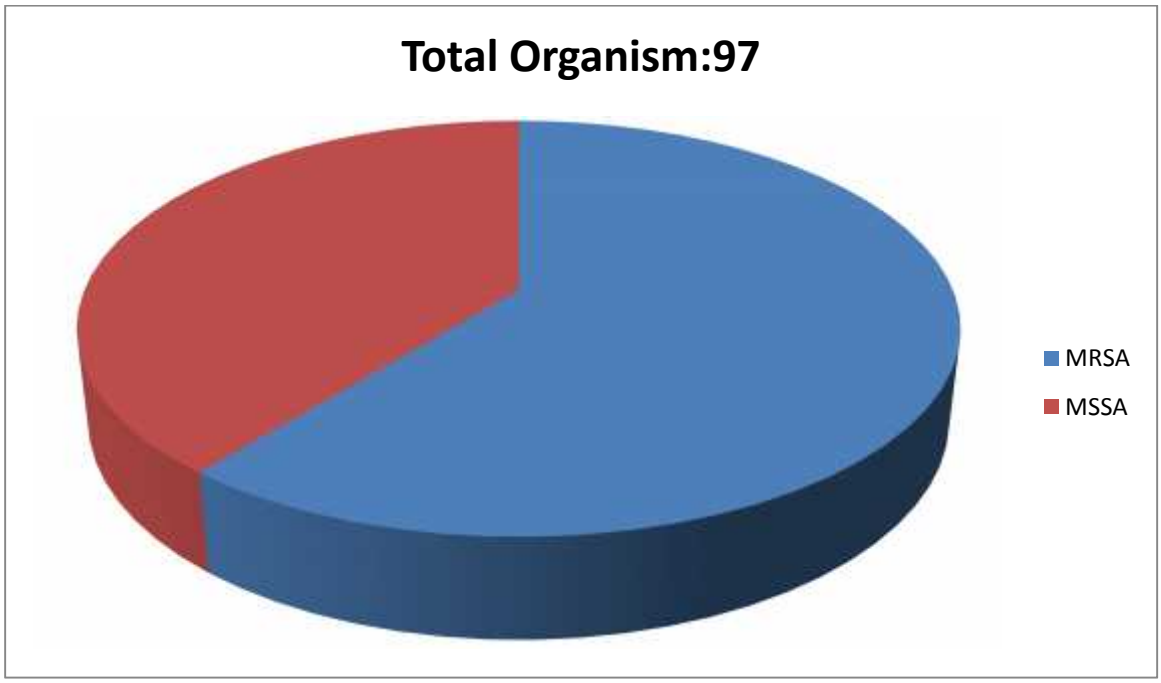

Figure 3: Chart showing prevalence of MRSA isolates among S.aureus

4.13 Frequency of clindamycin resistant isolates

There were no any observations of constitutive clindamycin resistance among *S.aureus* isolates. However, inducible clindamycin resistance (ICR) was observed among 35 isolates: twenty three of those ICR positive or D- effect positive (D+) isolates were resistance to cefoxitin (MRSA) and twelve were susceptible to cefoxitin (MSSA).However, distribution of inducible clindamycin resistance among MSSA and MRSA was statistically insignificant (p value >0.05).

Table 11: Distribution of inducible clindamycin resistance among MSSA and MRSA

Methicillin Resistance Screening	ICR Screening		Total	P value
	D+	D-		
MSSA	12	26	38	
MRSA	23	36	59	1
Total	35	62	97	

4.14 MIC against vancomycin by E test

Methicillin susceptible isolates as well as methicillin resistant isolates which gives positive D test were assayed for E test by using vancomycin strip for MIC method. All those were obtained to have their MIC below 2, i.e. susceptible to vancomycin.

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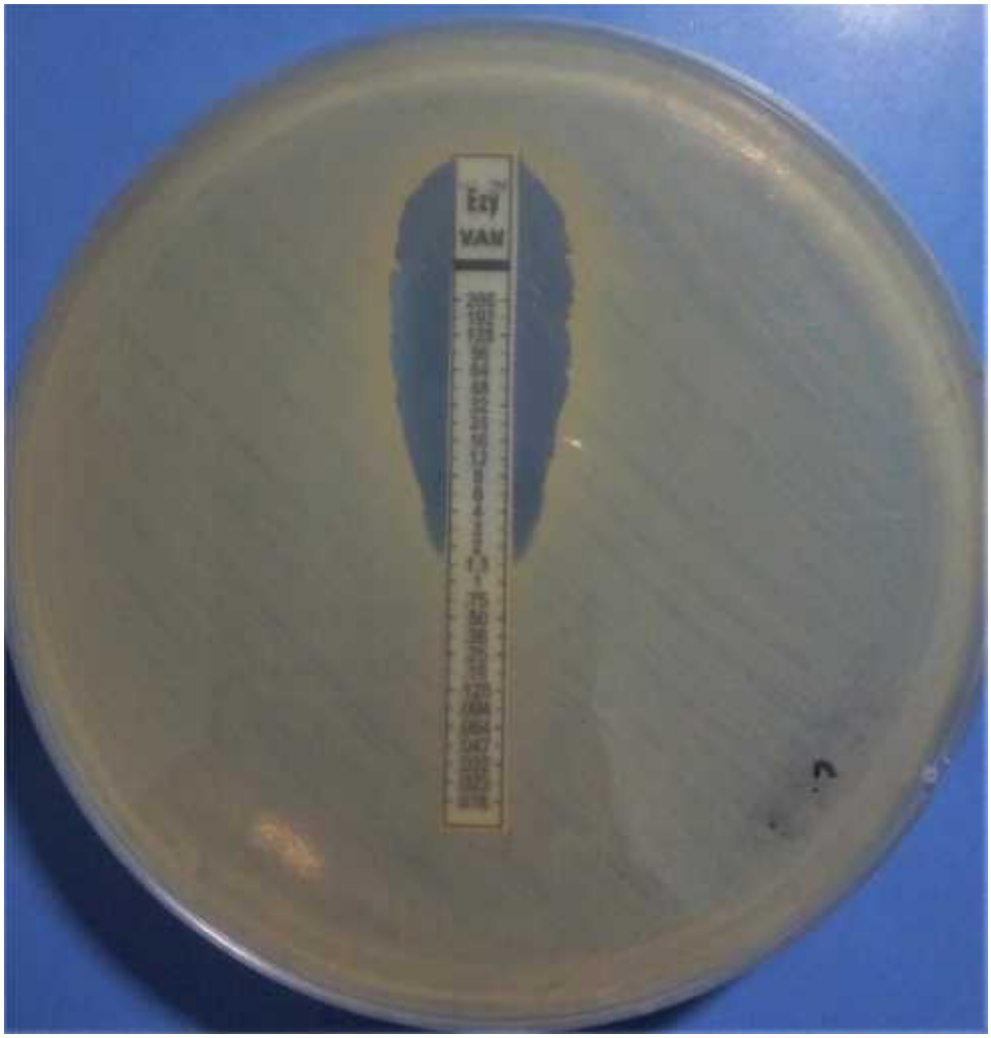
PHOTOGRAPHS

Photograph 1: *Staphylococcus aureus* in nutrient agar

(Typically raised, smooth, medium to large colonies (2-4mm), slightly translucent with cream yellow pigmentation)

Photograph 2: Screening test for inducible clindamycin resistant (D-test and methicillin resistant). Flattening of zone of inhibition adjacent to erythromycin disk reffered as D-zone whereas isolates eith ZOI ≤ 21mm were identified as ethicillin resistant (MRSA).



Photograph 3: E-test performed for *S.aureus* which shows sensitive to vancomycin. Ellipitical zone of bacterial inhibition ≤ 2 is read as sensitive.

Photograph 4: E-test performed for *S.aureus* which shows sensitive to vancomycin. Ellipitical zone of bacterial inhibition ≤ 2 is read as sensitive.

CHAPTER V
DISCUSSION

Antimicrobial resistance has been noticed as one of the paramount microbial threats of the twenty-first century. The multidrug resistance to most of the antibiotics used in infections caused by staphylococci is an increasing problem. The emergence of methicillin resistance among *Staphylococcus aureus* strains led to difficulties in the treatment of infections. Anitbiotic resistance has resulted in larger hospital stay of patients, resulting in economic burden as well as psyhological stress (Kumar et al, 2009). Therefore, surveillance on the antimicrobial susceptibility patterns of *Staphylococcus aureus* is of utmost importance in understanding new and emerging resistance trends as well as in the management of both hospital and community-acquired infections (Ansari et al, 2014).

A total of 3893 different clinical specimen including urine, wound pus, sputum, cathether tip and other sample from patients of OPD, various wards (medical, surgical, pediatric, post-operative and emergency) and intensive care unit of hospital that were sent for microbiological investigation were analysed. Of 3893, total samples, 1276 (30.2%) showed significant growth while 2617 (69.8%) showed no growth, which is in accordance with Benerjee et al (2018).

A total of 97 *S.aureus* were isolated from various clinical sample during the study period. Those *S.aureus* were assayed for methicillin resistance, inducible clindamycin resistance and vancomycin resistance. Among 97 isolates, 59 (60.8%) showed resistance to cefoxitin i.e MRSA and 35 of the isolates showed D- effect i.e they were inducible clindamycin resistant (ICR) strains. Those isolate which are methicillin resistant along with D- effect were found to be susceptible to vancomycin during MIC by E test method.

From Table 1, among culture positive sample, *E.coli* (510,39.96%) was found to be the predominant isolate which is in accordance with Karimzadeh et al (2017),

followed by *K.pneumoniae* (209,16.37%) and *P.aeruginosa*(137,10.73%), *Staphylococcus aureus* (7.60%), and others including Coagulase negative Staphylococci, *Enterococcus spp,Streptococcus pyogens*, Non-haemolytic Streptococcii, *Enterobacter spp, Klebsiella oxytoca, Proteus mirabilis, Citrobacter freundii, Morganella morganii, Haemophilus inflenzae* and *Proteus vulgaris*were found in the study. The prevalence rate was reported similar by Benerjee et al (2018) in case of *K.pneumoniae*(15%) and *P.aeruginosa* (10.2%), *Staph aureus* (6.6) but in contrast, lower in case of *E.coli* (11.2%).

From Table 2, out of 97 isolates of *S.aureus*, 62 (63.9%) were obtained from male patients whereas 35 (36.1%) were recovered from female patient which was similar to study conducted by Reddy et al (2015). Increased rate of infection among male patients may be attributed to their outdoor occupation which is more prone to injuries as well as by their personal habit also (Ansari et al, 2014). This might be also due to large number of male patients visiting and admitted to hospitals and more male suffering road accidents. However, no correlation studies have been done till now.

From Table 3 , 60 (61.9%) were recovered from inpatient while 37 (38.1%) from outpatient giving a probability of increased health care associated infections. From Table 4 , predominant age of the patient infected by *S. aureus* belonged to 31-45 (31, 32%), followed by age group 16-30 (28, 28.9%) but lower in age group above 60 (9,9.2%).

From Table 5, out of 97 isolates, distribution of *S. aureus* was higher in wound pus (88.65%) followed by urine (6.2%), sputum (3.1%) and cathether tip (2.05%) which is similar to the study conducted by Obiazi et al (2012) The higher distribution of isolation among this age may be due to involvement in various activities where they may get some injuries or wounds in their daily life or could be attributed to poor personal hygiene (Nsofor et al, 2016).

Antimicrobial susceptibility testing (AST) was performed by modified Kirby-Bauer disk diffusion method. Simultaneously, MRSA screening was also done by

using cefoxitin (30 µg) disc diffusion method (CLSI, 2012). 59 of the isolates were MRSA. From Table 7 and Table 8, among aminoglycosides, 37.3% resistant of MSSA and 28.9% resistant of MRSA was observed in amikacin whereas in gentamicin 26.3% resistant and 44.1% resistant was observed which is similar with Pandey et al (2012) but contrast with Ansari et al (2014) showing 4% resistant to amikacin.. Low level of susceptibility was observed in clindamycin, chloramphenicol of MRSA strain in comparison to MSSA strain. None of the MRSA isolates were susceptible to erythromycin whereas MSSA was susceptible to erythromycin. A worse situation was observed with ciprofloxacin, with resistance to 86.4% of isolates. This may be associated with the more use of this drug in different types of infections. Also the less susceptibility to ciprofloxacin, a fluoroquinolone, may be due to use of it without prescription during infection. The susceptible strain may acquire resistance 3-4 days after the start of therapy (CLSI 2012). In a study conducted by Pandey et al (2012), only 17.93% of *S.aureus* were resistant to ciprofloxacin where they have suggested it can be used as drug of choice for MRSA infection by limiting indiscriminate use.

The widespread use of antibiotics has accelerated in the evolution of MRSA and led to the emergence of strains that acquired multiple resistance genes (Stefani and Varaldo, 2003). With the current emergence of multidrug resistance MRSA in hospital and dramatically increased incidence of community acquired MRSA, MRSA has been able to evolve rapidly and created new clinical problem (Valentini et al, 2008). Increasing resistance of MRSA strains to vancomycin have created a challenging scenario for making clinical treatment decisions, although there is availability of other antibiotics, including daptomycin and linezolid that may be effective against MRSA (Boucher et al, 2010).

The MRSA prevalence rate may differ in different perspective i.e in different geographical regions, in between countries and in between hospitals (Tiwari et al, 2009). MRSA infections found to be varied in infections of different parts of body as well as status of patients (Pahadi et al, 2015). The percentage of MRSA isolated in this study is 60.8% which is as similar with Tiwari et al (2009)

(69.1%). Khanal and Jha found 68% MRSA from skin infected patients attending the hospitals in Chitwan Nepal. Among 59 MRSA isolated in this study, 22 (37.3%) were from outpatients and 37 (62.7%)was from inpatients. Similar to this study, the study done by Rajbhandari et al in Bir Hospital reported 55% MRSA infection in outpatients and 76%, in hospitalized patients. While Adebayo and Johnson and Dar et al. reported 26.9% and 54.85% prevalence of MRSA respectively in their studies. The study by Niraula reported 12.9% and 13.7% of MRSA in outpatients and inpatients respectively visiting Manmohan Memorial Community Hospital, Kathmanduin contrast, a high percentage of isolates were obtained from outpatients by Baral et al (2011); 23% of the isolates were from inpatients and remaining 77% were from outpatients; however, MRSA are more associated with hospital environment.70% of the isolates were from inpatients in a study conducted by Kumari et al (2008).

Salab et al reported 63% of MRSA isolates were multi drug resistant which shows contrast in our study (100% resistant) indicating the changing of MRSA to multi drug resistant MRSA. The rate of MDR-MRSA (100%) is higher than that of the result reported in the studies conducted by Tiwari et al. i.e. 40.1% and Pandey et al. i.e. 75.86%. Though this study is in accordance with the previous studies from Nepal and other countries showing high percentage of MDR among MRSA; >65% by Kumari et al., 93% by Rahimi et al. and 63% by Salah et al. (Kumari et al, 2008; Salah et al, 2012; Rahimi et al, 2013) (Lama et al, 2017). Among MSSA, 27 of 38 (31.4%) isolates were MDR in this study.

In this study, isolates were found susceptible to clindamycin during routine antimicrobial susceptibility testing (AST) i.e none of the isolates showed constitutive resistance. But when induction test was performed, 35 (36.08%) of the isolates showed D effect i.e they were inducible clindamycin resistant strains ($iMLS_B$ phenotype) as mentioned in Table 10. Twelve of those were from MSSA and twenty three were MRSA. Absence of constitutive resistance but still presence of 36.08% of inducible clindamycin provokes the importance of D test, if clinical failure is not anticipitated (Woods, 2009). And, observation of D effect

to be more among MRSA in comparision to MSSA indicates that clindamycin may not be suitable to be used as empirical therapy against MRSA. However, further study with larger sample size need to be conducted before reaching a conclusion.

Clindamycin is drug which is useful for treating skin and soft tissue infections which are caused by *Staphylococcus aureus.* It has excellent tissue penetration, accumulates in abscesses, is not impeded by high bacterial burden at the infection site and no renal dose adjustment are needed. Due to its good oral bioavailability, it is a good option for outpatients' therapy and change over after intravenous antibiotics (Bhoja et al, 2017): it has also been used in many cases as intravenous antibiotics (Siberry et al, 2003). However, the differentiation of inducible-clindamycin resistance (iMLS$_B$ phenotypes) from other type of resistance is a critical issue because of the therapeutic implications of using clindamycin to treat a patient with an inducible clindamycin-resistant *S.aureus* isolate. Clindamycin resistance may be either of *erm*-mediated or *msrA*-mediated. *msrA*-mediated resistance is due to efflux-pump mechanism. *ermA*-mediated resistance of clindamycin may be either constitutive or inducible (Fiebelkorn et al, 2003).

The importance of D test has provoked due to the treatment failure of some cases which were clindamycin sensitive. Actually those clindamycin-sensitive looking isolates may be inducible clindamycin-resistance which could not be observed by normal susceptibility testing. In the other hand, reporting all erythromycin-resistant staphylococci as clindamycin-resistant prevents the use of clindamycin in infections caused by truly clindamycin-susceptible staphylococcal isolates (Fiebelkorn et al, 2003).

Although there are limited literatures mentioning the clindamycin resistance in *S.aureus* in Nepal, there are several research articles reporting the clindamycin resistance in *S.aureus* in neighbor country India. In a similar study in India conducted by Deotale et al (2010) D test positive was observed in 14.5 % *S.aureus* isolates. They also observed constitutive clindamycin resistance in 3.6 %

of isolates; however no any constitutive resistance was observed in this present study. In other studies conducted in India there were similar results, Gupta *et al.* (2009), Ciraj et al. (2009),Deepa et al (2013) (Navidiania, 2015) and Kumar et al. (2012) reported D test positive in 18 % , 13.1 %, 33.6% and 16.9 % of isolates respectively. Study conducted in Iran by Seifi et al. (2012) reported D test positive in 11.67 % of *Staph aureus* isolates along with constitutive resistance in 26.07 % isolates. In the study conducted by Ujwol et al. (Bhomi et al, 2016), D-test positive isolates were found to be 18.03% and study also reported constitutive resistance in 36.06% of isolates.(Lama et al, 2017) In this study 39% of D test positive isolates were MRSA while 31.6% isolates were MSSA. Similar higher percentage of D test positive in MRSA in comparison to MSSA was also observed in above mentioned literature. A positive D-test indicates the presence of iMLSB genotype. This means that it is possible, but far from certain, that a sub- population of microbes resistant to clindamycin may emerge and lead to clinical failure or recrudescence (Woods, 2009). There are few reports about clinical failure of clindamycin. Due to these failures associated with D test positive strain, CLSI recommendto report D test positive isolates as resistant to clindamycin (CLSI, 2012)

In this study, none of the isolates were found to be resistant to vancomycin by performing E-test.The E-test gradient technology is based on a combination of the concepts of dilution and diffusion principles for susceptibility testing. E-test directly quantifies antimicrobial susceptibility in terms of discrete MIC values. The E-test is basically an agar diffusion method. It consists of plastic strip calibrated with a MIC scale in µg/ml and codes to identify the antimicrobial agent. A predefined concentration gradient antibiotic across 15 two–fold dilutions of a conventional MIC method, is immobilized on the other surface of the carrier. When E-test strip is applied on to an inoculated agar surface the preformed exponential gradient of antimicrobial agent or reagents are immediately transferred to the agar matrix. MIC is read directly from the scale in terms of µg/ml at the point where the edge of the inhibition ellipse intersects the strip other

growth patterns may also be seen for resistance detection method (Flora GM, 2013).

Limitations of the study

The main limitation of this study was the short duration of the study period and small sample size. The small number of cases may not be representative of hospital patient population under study. Because of the short duration of the study our observations might have been affected by seasonal trends of patient admission in different wards of hospital. Although phenotypic studies are simpler and cost effective to detect resistance, genotypic techniques may be needed for deeper understanding of mechanism lying behind the resistance. However, genotypic techniques could not be used in this study due to limitation of budget and time.

CHAPTER VI
CONCLUSION AND RECOMMENDATION

6.1 Conclusion

This study was performed on 97 clinical *S. aureus* isolates obtained from various clinical sample.The organisms were identified on the basis of standard microbiological and biochemical techniques. Upon creating resistance profile a high load of MDR, 31.4% and 100 % among MSSA and MRSA respectively, was obtained. Upon screening, methicillin-resistant *S. aureus* (MRSA), and inducible clindamycin-resistant *S. aureus* was found to be 60.8 %(n=59), and 36.08 % (n=35) respectively. All the MRSA isolates were susceptible to vancomycin upon performing MIC assay by E-test. This high load of MDR organism provokes the necessities of strictly performing susceptibility testing before starting antibiotic therapy, or there may be chance of clinical failure. Our study suggests the microbiology laboratory to perform D-test on all erythromycin-resistant isolates.

6.2 Recommendations

- D test should be performed on all erythromycin resistant clindamycin susceptible. Reporting of clindamycin should be on the basis of D test but not on susceptibility testing.
- Regular monitoring of multi drug resistance among the clinical isolates must be performed.
- The practice of self medication or blindly prescribing the antibiotics should be discouraged.

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APPENDICES

APPENDIX I

Clinical and Microbiological Profile of Patients

A) Clinical profile

Patient id: ……. Date: ……..

Age/sex: ……. Type of Patient: ……

History of antibiotic used: ……

B) Microbiological profile

Day 1 (…./…../…….)

Specimen: …….

Time of sample collection: ……

Receiving time at the laboratory: ……

Specimen incubation time: …….

Incubation temperature: ……..

Culture on: 1) …….. 2)…….. 3)…….

Day 2 (……/...../……)

Reading of culture plates:

Media used	Feature	Shape	Size	Colour	Texture	Opacity	Consistency

Gram staining results: ……

Catalase: …….. Oxidase: …….

Others: …….

Provisional identification of organisms: ……

Inoculation on: 1) …….. 2) …… 3) ……..

Day 3 (…../……/……)

Biochemical tests:

Results:

Coagulase test: ……

O/F test: ……

Others: ……

Antimicrobial Susceptibility Testing by Kirby- Bauer disc diffusion method:

Antibiotic used	**Concentration of antibiotics**	**Zone of inhibition**	**Interpretation**

MRSA: yes or no

D test: yes or no

Day 4 (……/…../…..)

MIC by E-test

APPENDIX II: Equipments and Materials:

1. Equipments:

Incubator

Autoclave

Hot air oven

Refrigerator

Microscope

Weighing machine

Gas burner

Glass equipments

Inoculating wire and loops

2. Microbiological media:

Nutrient Agar

MacConkey Agar

Blood Agar

Mannitol Salt Agar

Muller Hinton Agar

Hugh – Leifson's medium

3. Chemicals and Reagents:

Gram's stain reagent (crystal violet, gram's iodine, 70 % alcohol, safranin)

Catalase reagent (3 % hydrogen peroxide)

Oxidase reagent

4. Glasswares

Beakers

Measuring cylinder

Conical flask

Petri-dishes

Glass rods

Reagent bottles

Glass slides

Test- tubes

5. Antibiotic discs:

Amikacin

Chloramphenicol

Erythromycin

Ceftriaxone

Clindamycin

Ofloxacin

Linezolid

Gentamycin

Azithromycin

Cefotaxime

Nitrofurantion

Oxacillin

Cefoxitin

Appendix III: Bacteriological media and Reagent

A. Composition and preparation of different media

1. Nutrients agar (NA)

Composition	gram/liter
Peptic digest of animal tissue	5.00
Beef extract	1.50
Yeast extract	1.50
Sodium chloride	5.00
Agar	15.00
Final pH (at 25˚C)	7.4 ± 0.2

Preparations: 28 grams of medium was suspended in 1000 ml of distilled water and boiled to dissolve completely. Then, medium was autoclaved at 121˚C(15 lbs pressure) for 15 minutes. The sterilized medium was then poured into the sterilized petridishes and then allowed to cool.

2. Nutrient Broth

Composition	gram/litre
Peptic digest of animal tissue	5.00
NaCl	5.00
Beef extract	1.50
Yeast extract	1.50
Final pH at 25˚C	7.4±0.2

Prepatations: 13 gram of media was dissolved in 1000 ml of distilled water and was autoclaved at 121˚C(15 lbs pressure) for 15 minutes. The sterilized media was then cooled to room temperature.

3. MacConkey Agar

Composition	gram/liter
Peptic digest of animal tissue	20.00
Lactose	10.0

Sodium taurocholate	5.0
Neutral red	0.04
Agar	20.00
Final pH at 25˚C	7.4±0.2

Preparations: 51.53 gram of media was dissolved in 1000 ml of distilled water. The media was then sterilized by autoclaving at 15lbs pressure(121˚C) for 15 minutes.The sterilized media was then poured in sterilized petriplate then allowed to cool.

4. Blood Agar:

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

Composition	**gram/liter**
Beef heart infusion	500.00
Tryptose	10.0
NaCl	5.0
Agar	15
Final pH at 25˚C	7.4±0.2

Preparations: 42.5 grams of blood base agar base medium was suspended in 1000 ml of distilled water, dissolved by boiling and sterilized by autoclaving at 121˚C (15 lbs pressure) for 15 minutes. After cooling to 40-50˚C, 50 ml sterile defibrinated sheep blood was added aseptically and mixed well before pouring.

5.Muller Hinton Agar:

Compositions	**gram/liter**
Beef infusion form	300.0
Casein acid hydrolysate	17.50
Starch	1.50
Agar	17.00
Final pH at 25°	7.3 ±0.2

Preparation: 38gm of the media was dissolved in 1000ml of the distilled water. The media was then sterilized by autoclaving at 15lbs pressure (121°C) for

15min. The sterilized media was then poured in sterilized petriplate then allowed to cool.

6. Hugh and Leifson media

Composition	**gram.liter**
Peptic digest of animal tissue	2.00
NaCl	5.00
K2PO4	0.30
Glucose	10.00
BTB	0.05
Agar	2.00
Final pH at 25° C	6.8 ±0.2

Preparation: As directed by manufacturing company, 19.40gm of the mediawas dissolved in 1000ml of the distilled water. The media was then dispensedin test tubes and sterilized by autoclaving at 15lbs pressure (121°C) for 15min.

B. Composition and preparations of different reagent:

1. Gram staining reagents

i) Crystal violet solutions:

Compositions	**gram(ml)/liter**
Crystal violet	20.0
Ammonium oxalate	9.0
Ethanol or methanol	95.0ml
Distilled water 1 liter	

Preparations: 20 gram of crystal violet was weighed in a clean piece of paper and transferred to a clean brown bottle. Then 95 ml of ethanol was added and mixed until the dye gets completely dissolved. To the mixture, 9 gram of ammonium oxalate was dissolved in 200 ml of distilled water, finally the volume was made 1 liter by adding distilled water.

ii) Lugol's iodine:

Compositions	**gram/liter**
Potassium Iodide	20.00 g
Iodine	10.00 g
Distilled Water to make 1 liter	

Preparations: To 250 ml distilled water, 20 grams of Potassium Iodide was dissolved. Then 10 grams of iodine was mixed to it until it was dissolved completely. Finally, the volume was made 1 liter by adding distilled water.

iii) Acetone- Alcohol Decolorizer

Compositions	**ml**
Acetone	500 ml
Ethanol (absolute)	475 ml
Distilled Water	25.0 ml

Preparations: 475 ml of ethanol (absolute) was added to 25 ml of distilled water, mixed and transferred into a clean bottle.Then immediately, 500 ml of acetone was added tothe bottle and mixed well.

iv) Safranin (Counter Stain)

Composition	**per 100ml**
Safranin (2.5% solution in 95% ethyl alcohol	10.00 ml
Distilled Water	100.0 ml

Preparations:2.5 % of Safranin solution was prepared in 95%ethanol. 10 ml of this solution was then suspended in 100 ml of distilled water.

2. Catalase test (3%H_2O_2)

Hydrogen Peroxide	1ml
Distilled Water	9ml

Preparation*:* To 9ml ofdistilled water, 1ml of Hydrogen Peroxide was added and mixed well so as to make a3% solution of Hydrogen Peroxide.

3. Oxidase test:

Oxidase reagent (impregnated in Whatman's No.1 filter paper)

Tetramethyl *p*-phenylene diamine dihydrochloride(TPD)	1.00g
Distilled Water	100ml

Preparation*:* 1 gram of TPD was dissolved in 100ml of D/W. To this solution, strips of Whatman'sNo.1 filter paper were soaked and drained for about30 seconds. Then these strips were freeze dried andstored in a dark bottle tightly sealed with a screwcap.

4. Preparations of 0.5 Mc Farland solutions:

0.5 ml of 0.048 M $BaCl_2$ (1.17% w/v $BaCl_2.H_2O$) was added to 99.5 ml of 0.18 M H_2SO_4(1% w/v) with constant stirring. The McFarland standard was thoroughly mixed to ensure that it is evenly suspended. Using matched cuvettes with a 1 cm light path and water as blank standard, the absorbance was measured in a spectrophotometer at a wavelength of 625 nm. The acceptable range for the turbidity standard is 0.08-0.13. The standard was distributed into screw-cap tubes of the same size and volume as those used to prepare the test inoculum. The tubes were sealed tightly to prevent loss by evaporation and stored protected from light at room temperature. The turbidity standard was vigorously agitated on a vortex mixer before use. Standards may be stored for up to 6 months, after which time they should be discarded.

C. Biochemical test for identification of bacteria:

Catalase test:

It is used to detect the presence of enzyme catalase in a bacterium. The enzyme catalase catalyzes the breakdown of hydrogen peroxide with the release of free oxygen. It is present in most cytochrome containing aerobic and facultative anaerobic bacteria. It is user to differentiate *staphylococcus* and *streptococcus*.

A small amount of culture to be tested is picked up from a nutrient agar with sterile loop and this is inserted into hydrogen peroxide solutuion (3%) held on a

slide. Immediate production of air bubbles in solution denotes a positive test and no bubbles indicates a negative test.

Oxidase test:

This test is performed for the detection of cytochrome oxidase in bacteria which catalyzes the transport of electrons between electron donors. In the presence of redox dye Tetramethyl-*p*-phenylene diamine dihydrochloride, the cytochrome oxidase oxidizes it into a deep purple colored end product Indophenol which is detected in the test.

The dry filter paper is performed by impregnating strips of filter paper with 1% kovacs oxidase reagent. The paper is smeared with the bacterial colonies to be tested by a glass rod. In positive test, the paper turns into deep purple within 10 seconds. No color change indicates negative test.

Oxidation-Fermentation test

This test is done to determine the oxidative or fermentative metabolism of carbohydrate resulting in production of various organic acids as end product. Some bacteria are capable of metabolizingcarbohydrates (as exhibited by acid production) only under aerobic conditions, while others produce acid both aerobically and anaerobically. Most medical bacteria are facultative anaerobes.

The test organism was stabbed into the bottom of two sets of tubes with Hugh and Leifson's media, bromothymol blue being the pH indicator. The inoculated medium in one of the tubes was covered with a 10 mm deep layer of sterile paraffin oil. The tubes were then incubated at 37°C for 24 hours. After incubation the tubes were examined for carbohydrate utilization as shown by acid production.

Fermentative organism utilizes the carbohydrate in both the open and sealed tubes as shown by a change in color of the medium from green to yellow. Oxidative organisms, however, are able to use the carbohydrate only in the open tube.

Coagulase test:

This test is use to differentiate species within the genus *Staphylococcus*: *S. aureus* (usually positive) from *S. epidermidis* (negative). A positive coagulase test is usually the final diagnostic criterion for the identification of *Staphylococcus aureus.* Free coagulase and bound coagulase are thetwo types of coagulase possessed by this organism;most strains possess both free and bound coagulase.

Slide Coagulase Test:

Bound coagulase (Clumping Factor) is detected by slide test. The bound coagulase is bound to the bacterial cell wall and reacts directly with fibrinogen. This results in alteration of fibrinogen so that it precipitates on the staphylococcal cell, causing the cells to clump when a bacterial suspension is mixed with plasma.

Tube Coagulase Test

This test is carried out to detect production of free coagulase. Plasma contains coagulase reacting factor (CRF) which activates free coagulase. The activated coagulase acts upon prothrombin thus converting it to thrombin. Thrombin converts fibrinogen into fibrin which is detected as a firm gel (clot) in the tube test. Tube test is performed when negative or doubtful results are obtained in slide coagulase test.

APPENDIX IV(working procedure)

A. Colony morphology of *S. aureus* in different culture media

i. Typically raised, smooth, medium to large colonies (2-4 mm), slightly translucent with cream yellow pigmentation

ii. In blood agar, some strain are hemolytic

iii. In MacConkey agar, the colonies are small pin head sized about 0.1- 0.5 mm, pink or orange due to lactose fermentation

iv. In MSA, yellow colonies due to mannitol fermentation

B. Tests performed for identification of *S. aureus*

1. Gram staining

i. A thin film of material to be examined was prepared on a clean grease free slide and dried.

ii. The smear was heat fixed and allowed to cool before staining

iii. The slide was flooded with crystal violet stain and allowed to remain without drying for 1 minute.

iv. The slide was rinsed with tap water shaking excess water off the slide.

v. The slide was flooded with iodine solution and allowed to remain on the surface without drying for twice as long as the crystal violet was in contact with the slide surface.

vi. The slide was rinsed with tap water shaking excess water off the slide.

vii. The slide was flooded with alcohol aceton decolorizer for 10 seconds and rinsed immediately with tap water until no further color flows from the slide with decolorized. Thicker smear requires more aggressive decolorizing.

viii. The slide was flooded with counter stain (safranin) for 1 minute and washed off with water.

ix. The slide was blotted between two clean sheets of bibulous paper and examined microscopically under oil immersion at 100X.

2. Catalase test

i. 2-3 ml of 3 % hydrogen peroxide was taken in a test tube.

ii. Several colonies of organism were removed from the agar plated and immersed in the hydrogen peroxide.

iii. The immediate bubbling was recorded immediately.

3. Oxidase test

i. A piece of filter paper was placed in a clean petri dish and 2-3 drops of freshly prepared oxidase reagent was added.

ii. Using a glass rod, a colony of test organism was smeared on the filterpaper.

iii. Observation was done for the development of blue-purple color withinfew seconds.

4. Coagulase test

I. Slide test method(detects bound coagulase)

i. A drop of distilled water was placed on each end of a slide.

ii. A colony of test organism was emulsified in each drop of water tomake thick suspensions.

iii. To one of the suspension, a loopful of plasma was added and observedfrom clumping of organisms within 10 seconds.

II. Tube test method (detects free coagulase)

i. Three tubes were taken and labeled as:

T= test organism (18-24 hour broth culture)

P= Positive control (Staph aureus broth culture)

N= Negative control (sterile broth)

ii. To each test tube, 0.2 ml of plasma was added.

iii. 0.8 ml of each test broth culture, positive control, and sterile broth wasadded to tube labeled as ‘T’, ‘P’ and ‘N’ respectively.

iv. After mixing gently, all the tubes were incubated at 35-37°C for 1hour. Observation was done for appearance of clotting. For noclotting observed, the tubes were further incubated for 3 hours andobserved. If still there was no appearance of clotting, the tubeswere left at room temperature overnight.

5. Oxidative-fermentative (O/F) test

i. Using a sterile straight wire, the test organism was inoculated to thebottom of two tubes of sterile O/F media.

ii. Sterile paraffin oil was added over the surface of medium in one of theinoculated tube.

iii. The tubes were incubated at 35-37°C for 24 hours and then examined for carbohydrate utilization.

Observations for above mentioned test:

S.NO	Tests performed	Observations
1	Gram staining	Gram positive cocci of uniform size, grape-like clusters, may also occur singly, pairs, or tetrads.
2	Catalase	Positive: gives bubbles immediately
3	Oxidase	Negative: no development of blue-purple color within 10 seconds
4	Coagulase	Positive: gives clotting in slide and tube coagulase method.
5	O/F	Fermentative: carbohydrate utilized in both tubes, i.e. color of media change from green to yellow in both tubes

C. Antimicrobial susceptibility test (AST):

Requirements: Mueller-Hinton agar, antibiotic discs, forceps, 0.5 McFarland standard, test organism (suspension)

Procedure for Kirby Bauer disc diffusion antibiotic susceptibility technique:

A. Preparation of 0.5 Mc Farland Standard

Add 0.5ml of 0.048M $BaCl_2$ (1.17% w/v $BaCl_2$. 2 H_2O) to 99.5ml of 0.18M H_2SO_4 (1% v/v) with constant stirring.

B. Preparation of Inoculum

By touching 2-3 colonies with sterile loop, inoculate into MHB or NB and incubate at 37°C until turbidity matches with that of 0.5 Mc Farland Standard. Direct colony suspension method can also be used.

C. Inoculation of Agar plates

a) The agar plates, canister of the discs are brought to room temperature before use. It should be, made sure that the agar surface doe snoot haveany moisture, if so should be dried by keeping it in the incubator.

b) Using a sterile swab, a plate of Mueller-Hintonagar is inoculated with the bacteria suspension using carpet culture technique. The plate is leftfor about five minutes to let the agar surface dry.

c) Using sterile forceps, appropriate antimicrobialdiscs (6mm in diameter) is placed, evenly distributed on the inoculated plates, not more than6 discs placed on a 90mm diameter petri plates.

d) Within 30 minutes of applying the discs, theplates are incubated at 37°C for 16-18 hours.

e) After the overnight incubation, the plates areexamined to ensure confluent growth. Using a measuring scale, the diameter of each zone ofinhibition in mm is measured and resultsinterpreted accordingly.

D. Quality Control

- Monitoring Accuracy
- Running AST for QC strains side by side withpathogenic bacteria.
- comparing the zone size with CLSI QC table

Zone size interpretation chart:

Antibiotics	**Symbol**	**Disc content (µg)**	**Resistant (mm or less)**	**Intermediate(mm)**	**Sensitive (mm or more)**
Amikacin	AK	30	16	16-17	18
Amoxicillin	AMX	10	12	13-15	16
Ciprofloxacin	CIP	30	13	14-20	21
Ofloxacin	OF	5	14	15-17	18
Gentamycin	GEN	30	12	13-14	15
Cefotaxime	CTX	30	14	15-22	23
Nitrofurantion	NIT	300	14	15-16	17
Cefoxitin	CX	30	21	-	22
Chloramphenicol	C	30	12	13-17	18
Erythromycin	E	15	13	14-22	23
Clindamycin	CD	2	14	15-20	21
Linezolid	LZ	30	20		21
Azithromycin	AZM	30	13	14-17	18
Oxacillin	OX	5	17	-	18

Determination of MIC by E test:

1. Preparation of inoculum:

Direct colony suspension method was performed for inoculums preparation. 2- 3 colonies of *S. aureus* were taken from plate by sterile loop and inoculate into NB and was matched with 0.5 MacFarland solution.

2.Inoculation:

Agar plates and vancomycin strip for E test were brought to room temperature. Then, in MHA bacterial suspension were inoculated by carpet culture technique. The plate was left for 5 minutes. Using sterile forcep, single vancomycin strip were properly fitted in the plate and left for few minutes.

3. Incubation:

The plates were incubated at 35-37˚C for 18 – 24 hours.

4. MIC interpretation:

MIC is defined as the lowest concentration of antibiotic at which there is no visible growth of the organism.The resulting elliptical zone of bacterial inhibition is read at the point of intersection of the ellipse with an MIC scale on the strip