CHAPTER-I
INTRODUCTION

1.1 Background

The genus *Staphylococcus* contains more than 40 species and subspecies (Prescott 1996). The most virulent species for man in the genus include *Staphylococcus aureus* (Holt *et al.,* 1994). Staphylococci were formerly classified in a common genus with *Micrococcus* spp. (Baird-Parker, 1971) until recently when it was grouped with *Bacillus* spp. on the basis of ribosomal RNA sequences (Ahmad *et al.,* 2000). Subsequently, about 50% of the *S. aureus* genome shares homology with non-pathogenic *Bacillus subtilis*, indicating that the two organisms are quite close and have evolved from a common ancestor (Kuroda *et al.,* 2001).

Staphylococci are Gram positive, facultative anaerobes, spherical bacteria in cluster with diameter ranging from 0.5 to 1.5 µm (Adejuwon *et al.,* 2010). *S. aureus* has emerged as one of the most important human pathogens and has over the past several decades, been a leading cause of hospital and community acquired infections (Lowy 1998). It is associated with a variety of clinical infections including septicemia, pneumonia, wound sepsis, septic arthritis, osteomyelitis and post-surgical toxic shock syndrome with substantial rates of morbidity and mortality (Engemann *et al.,* 2003). One of the reasons for the success of this human pathogen is its great variability, occurring at different periods and places with diverse clonal types and antibiotic resistance patterns within regions and countries. Although infections caused by antibiotic resistant *S. aureus* bring about serious problems in the general population, such infections can be particularly devastating for the very young, the elderly and the immunocompromised individuals (Adebayo *et al.,* 2006).

Coagulase negative staphylococci (CONS) are common colonizers of the skin, anterior nares and ear canals of human beings. They have long been considered as non-pathogenic and were rarely reported to cause severe infections. However, as a result of the combination of increased use of intravascular devices and an increase in the number of hospitalized

immunocompromised patients, CONS have become the major cause of nosocomial bloodstream infections (Silvia *et al.*, 1992) and they account for 9% of nosocomial infections (Kloose and Bannerman, 1994). *S. epidermidis* is the predominant clinical isolate; however, *S. hemolyticus, S. hominis, S. lugdunensis*, and *S. warneri* also have been implicated in sepsis and *S. haemolyticus* in endocarditis and osteomyletis (Ruhe *et al.,* 2004). Patients at risk include those with intravascular catheters, or other foreign bodies in place, prosthetic devices, postoperative sternal wound infections and immunocompromised hosts (Sherif *et al.,* 1999). These infections are difficult to treat because of the risk factors and the multiple drug resistant nature of the organism (Roth and James, 1988).

Prolonged hospitalization and antibiotic therapy especially with β-lactam antibiotics predispose patients to the acquisition of MRSA and oxacillin resistant *S. aureus* (ORSA) (Hackbarth and Chambers, 1989; Mattner *et al.,* 2010). Hospital-acquired MRSA and ORSA are usually associated with increased expression of multiple antibiotic resistance genes, including those coding for aminoglycoside resistance (Deurenberg *et al.,* 2007). Staphylococci are inherently susceptible to most of the antibiotics in use except those with purely antibiogram negative spectrum. The organism, adept at developing resistance both by mutation and by DNA transfer is difficult to treat and remains a frequent cause of morbidity and mortality (Livermore 2000). A concurrent growth in resistance among coagulase negative staphylococci (CONS) is partly due to the increasing use of broad-spectrum antibiotics that promote selection of multidrug resistant strains (Raad *et al.,* 1998).

Development of resistance to antimicrobial agents by staphylococci is a major concern primarily because they are still frequently associated with hospital and community acquired infections (Locksley *et al.,* 1982). The organisms exhibit remarkable versatility in their behaviour towards antibiotics (Grassi 1988), with some strains having overcome most commonly used drugs. Exposure to new antibiotics often results in further selection of homologous resistant strains (Haley *et al.,* 1982), a phenomenon particularly favored by irrational antibiotic administration. Infection with such resistant strains is likely to be

more severe and require longer hospitalization with incumbent increased costs, than infection with susceptible strains (Baron 1992).

Antimicrobial resistance could be either due to biochemical factor or genetic factor. The major biochemical factor of antibiotic resistance includes inactivation of the antibiotic by bacterial enzymes. For instance, β-lactamase splits amide bond of β-lactam ring in antibiotic like penicillins and cephalosporins. Genetic acquisition of antibiotic resistance is mediated by gene transfer mechanism between bacterial species, successful genetic mutations and a combination of mutational and gene transfer events (Forbes *et al*., 2002). Genetic exchange is likely to arise in soil and the general environment as well as the gut of humans and animals. Due to misuse and overuse of antibiotics, most clinically relevant bacterial pathogens have acquired a selection process to adapt to the pressures of antimicrobial attack, so that certain strains are now no longer susceptible to one or more of these antimicrobial agents (Levi 2001).

The best known mechanism of bacterial resistance is resistance to β-lactam, which may be chromosomally or plasmid mediated and they may be constitutive or inductive. β-lactamase (also known as penicillinase) is an enzyme that cleaves the β-lactam ring and inactivates the antibiotic. A variety of β-lactamase detection techniques have been developed, including acidometric, iodometric and chromogenic cephalosporin-based assays. β-lactamases comprise the most widespread means by which bacteria resist β-lactam antibiotics, including penicillins, cephalosporins, and monobactams (Frere 1995). These enzymes can be categorized into four classes (termed A through D) based on their sequence similarities and substrate profiles (Joris *et al.*, 1991). Class A, C and D enzymes are serine hydrolases while the class B β-lactamases are metalloenzymes (Frere *et al.*, 1999). The serine β-lactamases and the D-Ala-D-Alatranspeptidases (DD-transpeptidases), which are responsible for the biosynthesis of the bacterial cell wall and are targets of the β-lactam antibiotics, are thought to have a common evolutionary history (Gordon *et al.,* 2000).

Incidence of β-lactamase production in *S. aureus* has consistently been reported to be over 80% in all parts of the world (Parker and Collier, 1990). Most developed countries have reported an increase in colonization and infection in hospitalized patients by CONS while there are scanty data on infections caused by CONS in developing countries. The levels of antibiotic resistant infections in the developing world have increased steadily in the last few decades as a result of combination of microbial characteristics and the selective pressure of antimicrobial use (Blondeau and Tillotson, 2002). Microorganisms' mechanisms of overcoming the activities of antimicrobial agents include the production of structure altering or inactivating enzymes (e.g. β-lactamase or amino glycoside-modifying enzymes), alteration of penicillin binding proteins or other cell-wall target sites, altered DNA gyrase targets, permeability mutations, active efflux and ribosomal modification (Paterson 2001; Levy 2002).

The morbidity and the mortality of infectious diseases have increased proportionately with the acquisition of antibiotic resistance by organisms, especially in regards to the strains that are completely resistant to antibiotics. With respect to the cost containment pressures of today's healthcare environment, antibacterial drug resistance places an added burden on healthcare costs, although its full economic impact remains to be determined. The present study focused on the evaluation of antibiogram of clinical staphylococcal isolates and detection of β-lactamase in the multidrug resistant *Staphylococcus* species isolated from various clinical specimens from Bir hospital. This study also compares the resistant pattern of clinical staphylococci to Cloxacillin of HI-MEDIA, OXOID and MAST Company. This study could be noteworthy for clinicians in selecting empiric antimicrobial therapy and providing useful information on the surveillance of this pathogen. The result might serve as a foundation for establishing empiric therapeutic approaches for the management of infections in Bir Hospital and elsewhere.

1.2 Objectives

1.2.1 General objective

a. To determine the antibiogram and prevalence of β-lactamase producing MDR *Staphylococcus* species from different clinical specimens in Bir Hospital.

1.2.2 Specific objectives

a. To isolate and identify the *Staphylococcus* species from different clinical specimens.
b. To analyze the antibiotic sensitivity pattern of *Staphylococcus* species isolated from different clinical specimens.
c. To screen the multi-drug resistant *Staphylococcus* species from different clinical specimens.
d. To test the incidence of β-lactamase enzyme in various clinical specimens.
e. To compare the sensitivity pattern of *Staphylococcus* to cloxacillin of three different manufacturing companies.
f. To evaluate the significant association between the age, gender, origin, clinical specimen, MDR and β-lactamase production in *Staphylococcus* species.

CHAPTER II
LITERATURE REVIEW

2.1 History

Staphylococci were first observed in human pyogenic lesions by Von Recklinghausen in 1871. Louis Pasteur in 1880 obtained liquid cultures of the cocci from the pus and produced abscesses by inoculating them into Rabbits thus demonstrated their pathogenicity. Alexander Ogston in 1880 established the causative role of Staphylococci in abscess and other pyogenic lesions and named them as "Staphylococci" from the typical occurrence of the cocci in grape like clusters (Staphylo-means bunches and kokos-mean berry).

Rosenbach in 1884 named the organism as *S. aureus* and *S. albus* taking golden yellow and white pigmentation as criteria. Based on Coagulase test staphylococci were divided into Coagulase positive and coagulase negative strains. Baird-Parker in 1965 described for the first time, the heterogenicity of *Staphylococcus* and divided the family into 6 subgroups, based on coagulase reaction, mannitol fermentation with acid production both aerobically and anaerobically and phosphatase activity.

A systemic classification using as many as 13 characters was introduced by Kloose and Schleifer in 1975. In 1965 a final report on classification of *Staphylococcus* and *Micrococcus* was produced by International Association of Microbiological Society. Later in mid 1960s it was concluded that members of genus *Staphylococcus* have a G+C content in DNA within the range of 30-39 mol% and that members of genus *Micrococcus* have within a range of 63-73 mol%.

Staphylococus that are pathogenic in humans are *S. aureus, S. epidermidis, S. haemolyticus, S. saprophyticus, S. lugdunensis* and *S. schleiferi*. A simplified method that will help to identify most Staphylococci is based on the reactions like colonial pigment, haemolysis, nitrate reduction, arginine utilization, urease, maltose, trehalose, mannitol, xylose, cellobiose, sucrose, xylitol, raffinose, mannose and novobiocin resistance (Kloose and Schleifer, 1975).

2.2 *Staphylococcus aureus*

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

S. aureus is one of the most important etiological agents of many hospital-acquired infections as well as community-acquired infections and poses a constant therapeutic problem to clinicians (Klein *et al*., 2007). Methicillin and its derivatives became the drugs of choice for the treatment of infections caused by *S. aureus*. Over time, treatment of serious *S. aureus* infections can be challenging as the widespread use of antibiotics has led some *S. aureus* becoming more resistant to antibiotics (Akinjogunla and Enabulele, 2010; Archer 1998). The appearance of methicillin resistant *S. aureus* (MRSA) was followed by various patterns of resistance to antibiotics (Goto *et al*., 2009).

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

2.3 Coagulase negative staphylococci (CONS)

Coagulase negative staphylococci (CONS) are inhabitants of the skin and mucous membranes of humans and represent a major part of the normal aerobic flora (Heilmann and Peters, 2006). Each species of the CONS has a predominance of specific parts of the body (Otto 2008). Several species of the CONS group, *S. epidermidis*, *S. capitis*, *S. hominis*, *S. haemolyticus*, *S. saccharolyticus*, *S. warneri*, *S. lugdunensis*, *S. saprophyticus* and *S. cohnii*, have been characterized as residents of the human body.

Infection is the major complication associated with the use of foreign bodies such as catheters. Based on the type of device and its insertion site, dissimilar infection syndromes create with CONS for example: Peritonitis, Septicemia, endocarditis, and Ventriculitis (Heilmann and Peters, 2006). These bacteria usually infect immunocompromised patients, such as premature newborns and patients with leukemia or other malignant diseases who acquired neutropenia after receiving cytotoxic agents (Souvenir *et al*., 1998).

Among the CONS, *S. epidermidis* principal cause of infection, chiefly in hospitalized patients with indwelling foreign bodies and in immunocompromised patients (Piettet and Verschraegen, 2009). *S. epidermidis* has caused some cases of bacteremia, surgical wound infections, conjunctivitis and keratitis, (Kamalarajah and Best, 2002) also, osteomyelitis, wound infection, otitis media, endophthalmitis, UTI, was reported (Heilmann and Peters, 2006). *S. saprophyticus* was often regarded as a more important opportunistic pathogen than *S. epidermidis* in human urinary tract infections (UTIs), particularly in young sexually active females. It was considered to be the second most common cause of acute cystitis or pyelonephritis in these patients (Raz *et al*., 2005).

The most important virulence factor of the *S. epidermidis* isolates is their ability to adhere, aggregate and form multilayered biofilms, embedded in an extracellular matrix, on medical devices used in the hospital setting (Otto 2008). Due to lack of toxins and aggressive virulence factors, CONS usually cause sub-acute or even chronic infections without fulminate signs of

infections (Heilmann and Peters, 2006). A biofilm develops when microorganisms adhere to a surface and produce extracellular matrix that facilitates adhesion of the microorganisms to each other and provide them with a structural matrix. The surface may be living tissue, non-living materials in the environment or plastics, catheters, prosthesis and other medical devices used in the medical centres (Donlan 2001). With the increased use of these devices it has become clear that biofilm formation is a trait that plays a significant role in nosocomial infections (Darouiche 2001).

2.4 Antibiotic resistance

2.4.1 Definition

When the organism is expected not to respond to a given drug irrespectives of the doses and location of infection (Bartoloni *et al.,* 2006; WHO 2004).

Antibiotic are the agent that has the biological activity against living organism, which was originally developed to treat human infectious diseases. The broad use of antibiotics had created a strong selective pressure, which consistently had resulted in the survival and spread of resistance that has evolved with the increased number, volume and diversity of antimicrobial applications. (Baquero *et al.,* 2003). Bacteria are able to inherit antibiotic resistant genes to provide protection against most antibiotics. The dissemination of antibiotic genes by horizontal gene transfer has led to the rapid emergence of antibiotic resistance among bacteria (Barlow *et al*., 2004).

2.4.2 Risk factors

Different numbers of factors are responsible for the development of resistance but exact factors due to which the organisms acquire resistance is to be still defined (Levi 2001; Oteo *et al*., 2001). Levi in 2001 and WHO in 2004 have enlisted some risk factors which are as follows:

i. Excessive and irrational over utilization of antibiotics by outpatient practice and in hospitalized patients, either therapeutically or prophylactically.
ii. Use of antibiotics in agricultural industries, particularly in the production of food.

iii. Longer survival of severely ill patients.
iv. Longer life expectancy with increased use of antibiotics in elderly.
v. Advances in medical sciences that have resulted in the survival of many patients with severe illness and at risk for infections such as critically ill patients, immunosuppressed patients etc.
vi. Increased use of invasive procedures.
vii. Increased use of prosthetic devices and foreign bodies amenable to super infection with resistant bacteria.
viii. Lack of use of proven and effective preventive infection control measures such as hand washing, antibiotics usage restrictions and proper isolation of patients with resistant infections.

2.4.3 Trends of resistance development

History of development of antibiotic resistance is quite colorful. Alexander Fleming, the discoverer of penicillin cautioned: "the greatest possibility of evil in self- medication is the use of too small doses so that instead of clearing up infection, the microbes are educated to resist penicillin" (Levy 2002). Unfortunately, Fleming's words proved correct and within a few decades the world is shouldering a huge burden of emerging and re-emerging infectious diseases caused by multidrug resistant organisms.

By 1944 some strains of *S. aureus* were capable of destroying penicillin V by secreting β-lactamase. Later in 1960s MRSA firstly isolated in UK. These days MRSA is resistant to almost all β-lactam antibiotics and to some other antibiotics like erythromycin, fusidic acid, tetracycline, monocycline, streptomycin and sulphonamides. In 2002, vancomycin resistant *S. aureus* (VRSA) also isolated.

The emergence of antimicrobial resistance pathogens now treats the discovery of potent antimicrobial agents. Antimicrobial resistance has resulted in increased morbidity and mortality as well as health care costs. Yearly expenditures arising from drug resistance in the United States are $4 billion and are rising (Archibald and Marinho, 2005).

The tremendous therapeutic advantage afforded by antibiotics is being treated by the emergence of increasing the resistance strains of microbes. Selective pressure favoring resistant strains arises from misuse and over use of antibiotics (Thomson and Moland, 1999). The emergence of antibiotic resistance is an evolutionary process that is based on selection for organism that has enhanced ability to survive (Cownley *et al*., 2008).

Antimicrobial resistance in staphylococci is a growing problem worldwide. Seriously ill patients admitted to intensive care units are highly susceptible to infections and are exposed to high antibiotic pressure (Hanberger *et al*., 2004). Consequently, strategies for the prevention of emerging resistance have mainly focused on intensive care units and other hospital wards (Fridkin *et al*., 1999). In spite of these efforts, new cases of resistant Gram positive cocci are reported from many hospitals (Simonsen *et al*., 2003). Several studies have also reported high and increasing rates of resistance to antimicrobial agents among Gram positive cocci isolated from outpatients (Barisic and Pund-Polic, 2000).

2.5 Multidrug resistance

2.5.1 Definition

Multidrug resistance is defined as resistance to two or more than two classes of antibiotics (Bartoloni *et al*., 2006; Wright *et al.*, 2000).

2.5.2 Multidrug resistance in global context

The spread of microbial multiple drug resistance is a global public health challenge, which impairs the efficacy of antimicrobial agents and results in substantial increased illness and death rate and health care associated costs. In low-resources countries, the extent and the impact of phenomenon tend to be even larger than in industrialized countries. Moreover, in low-resource countries the impact of multiple antimicrobial drug resistance on illness and death rates tend to be greater because of the high prevalence of bacterial infections and the major role of antimicrobial agents in combating infectious diseases (Bartoloni *et al*., 2006).

Among the MDR organisms, MRSA (Methicillin resistant *S. aureus*), VRE (vancomycin resistant Enterococci), ESBL (extended spectrum β-lactamase) producing Gram negative bacteria warrants special attention because of their limited therapeutic options. Until recently, only vancomycin provided effective therapy for MRSA infections (Chang *et al.*, 2003). Nevertheless, it is obvious that percentage of MDR strains are higher in hospitalized patients, device used patients and other complications associated with the patients e.g. diabetes, microalbuminuria (Daniel *et al*., 2001).

2.5.3 Multidrg resistance in Nepal

Multiple drug resistance study in Nepal does not underscore the magnitude of the problem of antimicrobial drug resistance in low-resource settings and the urgent need for surveillance and control of this phenomenon. Inexpensive, sensitive and simple methods to monitor antimicrobial drug resistance among different clinical isolates could be valuable tools for large scale surveillance studies and to improve the efficacy of resistance control intervention. However, resistance trend of commonly used drugs in Nepal also depend upon the localities. The small differences observed among localities, although sometimes statistically significant, are probably of limited clinical and epidemiological relevance (Pokhrel *et al*., 2006; Paterson *et al*., 2005). In a study conducted by Wagle *et al*. (2004) at Tribhuvan University Teaching Hospital (TUTH), 30% of MDR isolates were found.

2.6 β-lactamase

2.6.1 Definition

β-lactamase is the enzyme that inactivates the β-lactam antibiotics by cleaving the bond in β-lactam ring. β-lactamase production is the principle mechanism for acquisition of resistance to β-lactam antibiotics. The ability of a β-lactamase to cause resistance vary upon its activity, quantity and cellular location (Bush *et al*., 1995).

Methicillin was introduced in the early 1960s to combat hospital strains of pencillinase producing *S. aureus* (Woodford 2005). Oxacillin and methicillin

are modified penicillin effective against staphylococci. These penicillins have a bulky 6´phenylacetyl group that sterically hinder attack on the β-lactam ring which make these compounds stable against the β-lactamase or pencillinase enzymes produced by staphylococci (Livermore 2000). β-lactamase, an enzyme that may be produced by certain bacteria, functions to hydrolyse the β-lactam ring converting the antimicrobial containing this ring into an innocuous form (Dyke and Gregory, 1997).

β-lactamases are the commonest cause of bacterial resistance to β-lactam antimicrobial agents. Their spread destroyed the utility of benzylpenicillin against staphylococci and has hugely undermined that of ampicillin against enterobacteria and *Haemophilus* and *Neisseria* spp. New enzymes and new modes of production of old enzymes now threaten the value of extended-spectrum cephalosporins against enterobacteria. Staphylococci are the only common Gram positive pathogens in which β-lactamases have caused major resistance problems. Penicillinases occurred in only about 5% of *S. aureus* isolates when benzylpenicillin was introduced but have since spread, through plasmid transfer and strain selection, to 80 to 90% of isolates, both of *S. aureus* and of the coagulase negative isolates (Lacey 1984).

2.6.2 Classification

β-lactamases (EC 3.5.2.6) have been designated by Nomenclature Committee of the International Union of Biochemistry as enzymes hydrolyzing amides, amidines and other CON bonds.

There are great varieties of name for β-lactamases. However, the enzymes can be classified on the basis of their primary structure into four main classes (A through D), (Ambler and Levy, 1980). Bush *et al.* (1995) classified β-lactamases on the basis of the substrate specificities.

2.6.3 Action of β-lactamase

A few β-lactamases utilize zinc ions to disrupt the β-lactam ring, but a far greater number operate via the serine ester mechanism. Penicillin-binding proteins (PBPs) also react with β-lactams to give serine esters, but, unlike the

similar esters formed by β-lactamases, these do not hydrolyze readily (Waley 1992).

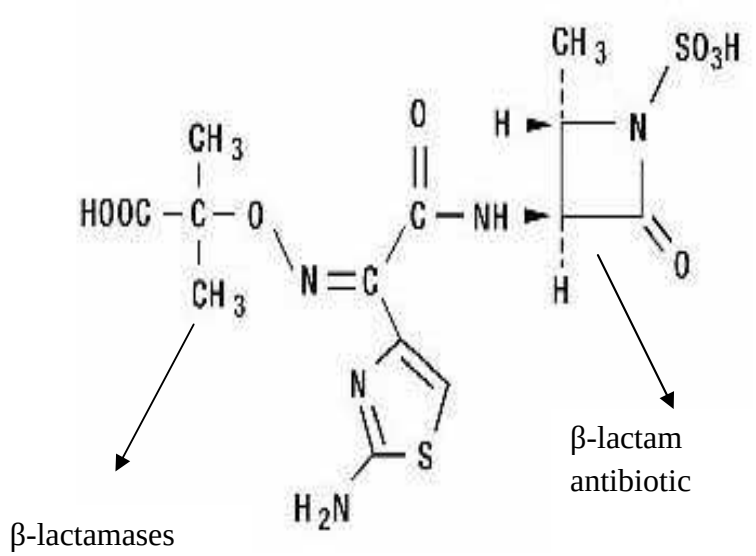

Fig. Action of β-lactamases

2.6.4 Tests for detection of β-lactamases

β-lactamases are the main cause of bacterial resistance to penicillins and cephalosporins. Definitive identification of these enzyme is only possible by gene or protein sequencing; (Livermore and Williams, 1996) aspects beyond the scope of diagnostic laboratories. Nevertheless, simple β-lactamase detection and typing tests can be valuable in the clinical laboratory. These include: (i) direct tests for β-lactamase activity in fastidious Gram-negative species; (ii) tests for extended β-lactamases (ESBLs); and (iii) tests for inducibility of chromosomal β-lactamases. Tests for metallo-carbapenemases are being developed and may become increasingly useful if these enzymes spread in the future. In the longer term, gene chip technology may allow precise routine identification of β-lactamases (Felmingham and Brown, 2001).

2.6.4.1 Direct tests for β-lactamase activity

Direct β-lactamase tests are mostly used for *Haemophilus influenzae*, *Moraxella catarrhalis* and *Neisseria* spp., where few different enzyme types occur, and where enzyme production has clear implications for therapy. Most

use chromogenic cephalosporins, or link the hydrolysis of penicillin to a colour change mediated by iodine or a pH indicator.

i. Nitrocephin test

Nitrocephin is a chromogenic cephalosporin that changes from yellow to red on hydrolysis. It provides the most sensitive test for most β-lactamases, exceptions being staphylococcal penicillinase and ROB-1, an uncommon plasmid-mediated enzyme of haemophili (O'Callaghan *et al*., 1972). Nitrocefin is available as pure powder from Becton Dickinson (Oxford, UK). Various commercial devices based on nitrocefin are also available (e.g. from Oxoid and Becton Dickinson).

ii. Iodometric tests

Hydrolysis of penicillin yields penicillinic acid, which reduces iodine, decolourising starch-iodine complex. This reaction can be exploited to detect β-lactamase activity in tubes or on paper strips. These tests are particularly sensitive for staphylococcal penicillinase, but are less sensitive than nitrocefin for most of the β-lactamases from Gram-negative bacteria (Oberhofer and Towle, 1982; Rosenblatt and Neumann, 1978).

iii. Acidimetric tests

Hydrolysis of the β-lactam ring generates a carboxyl group, acidifying un-buffered systems. The resulting acidity can be tested in tubes or on filter papers. The method is useful for tests on *H. influenzae* and *Neisseria gonorrhoeae* (Sindhu and Shanmugam 1998; Tu *et al.*, 1981).

2.6.4.2 Microbiological tests of β-lactamase activity

β-lactamase activity can be detected biologically by demonstrating the loss of activity of β-lactam agent against a susceptible indicator organism. There are several variations, including the cloverleaf (Hodge) method, which is highly sensitive for staphylococci, and the Masuda double disc method, which can be

used with whole cells or cell extracts of test strains. While the use of such methods has declined, they remain very sensitive (Watts and Salmon 1997).

2.7 Epidemiology

2.7.1 Antibiotic resistance and β-lactamase production in *Staphylococcus* spp.

The first antibiotic, the β-lactam ring containing penicillin was also introduced to treat staphylococcal infections. Few years later the first β-lactamase producing penicillin resistant strains appeared, so new antibiotics had to be found or developed. The infections caused by such strains were treated with penicillin applied in combination with β-lactamase inhibitors (clavulanic acid, sulbactam) or with semisynthetic penicillinase-resistant penicillins (PRPs) like methicillin, oxacillin, nafcillin, cloxacillin. With the spread of nosocomial and lately community associated strains becoming methicillin or oxacillin resistant not because of β-lactamase production, the application of other antibiotics (like glycopeptides) were favoured, but these antibiotics are the last defense lines against multiresistant strains.

In several publications, β-lactamase production rates vary from 55.7% to 92.6% for staphylococci (Shanmugam and Beena, 1996). Umolu *et al.* (2002) reported that 84.1% of methicillin sensitive (MSSA) isolates were β-lactamase positive.

In the study conducted by Oncel *et al*. (2004), β-lactamase production rate of *Staphylococcus* isolates determined by nitrocefin containing identification sticks (OXOID BR66A) was found to be 62(33.8 %). In the retrospective study, β-lactamase production was found among 77-84% of the *S. aureus* and 61-75% of the CONS isolates (Hope *et al*., 2001; Fritsche *et al.,* 2005).

In another study by Ekrem and Meltem (2006), a total of 251 isolates from various clinical sites was included of which 141 were β-lactamase positive (~55%) and *S. aureus* was the highest (79.4%). Eighty-three of these isolates were coagulase positive and 240 were coagulase negative. In β-lactamase test,

85.5% and 83.3% of coagulase positive and coagulase negative isolates were β-lactamase positive, respectively.

Of the *S. aureus* isolates which showed methicillin resistance (MR), 100% were also resistant to penicillin G, ampicillin, amoxycillin, cloxacillin, and gentamicin. MR-CONS isolates were also 100% resistant to penicillin G, ampicillin, amoxycillin and 96.8% resistant to cloxacillin. Out of 68 *S. aureus* isolates, 38 (55.9%) produced β-lactamase. All β-lactamase-producing isolates were susceptible to amoxycillin/clavulanic acid (Hulya *et al*., 2006).

Two hundred and forty (74.3%) of 323 staphylococcal isolates were coagulase negative and 83 (25.7%) were coagulase positive. One hundred and fifty-three (63.8%) of coagulase negative and 54 (65.1%) of coagulase positive isolates were resistant to penicillin and 42 (50.6%) of coagulase positive and 124 (50.5%) of coagulase negative isolates were resistant to oxacillin. All of the isolates were susceptible to vancomycin. Using chromogenic cephalosporin method, 71 (85.5%) of coagulase positive and 200 (83.3%) of coagulase negative isolates were β-lactamase positive (Ekrem and Meltem, 2006).
In addition, 128 clinical isolates were tested for β-lactamase activity. Of the 26 *S. aureus* isolates, 88.4% (23) were β-lactamase positive and 57.1 % coagulase negative staphylococci were positive (Pal and Marissa, 2010).

Also, the results showed that 14 (33.3%) *S. aureus* are β-lactamase producer, while only 9 (42.9%) of CONS produced β-lactamase (Akinjogunla and Enabulele, 2010).

The recent study in Nigeria showed that 14 (33.3%) *S. aureus* and 9 (42.9%) of CONS are β-lactamase producer. The antibiotics susceptibility testing showed that 29 (69.0%), 26 (61.9%), 27 (64.3%), 28 (66.7%) and 29 (69.0%) of *S. aureus* were sensitive to penicillin, ceftriazidime, cefoxitin, ciprofloxacin and levofloxacin, respectively. About 12 (28.6%) of *S. aureus* were resistant to streptomycin and iminipen, while about 45.2%-50.0% were resistant to cephalothin and amoxicillin. CONS also showed varied percentages of sensitivity ranging between 42.9% for streptomycin and 71.4% for

moxifloxacin. The result also showed that 19.2 % of *S. aureus* and 9.6% of CONS were resistant to more than eight antibiotics (Akinjogunla and Enabulele, 2010).

2.7.2 *S. aureus* and drug resistance and β-lactamase production

The antibiogram and β-lactamase test of 73 isolates of *S. aureus* from 235 different human clinical specimens were determined and the results of the antibiogram showed 100% susceptibiity to vancomycin, 78.1% to gentamicin, 71.3% to chloramphenicol, 69.8% to erythromycin and 61.6% to cloxacillin. The results of the β-lactamase detection showed that 84.1% of the isolates were penicillinase positive, which probably accounted for the 100% resistance obtained for both ampicillin and penicillin (Umolu *et al*., 2002).

In the study conducted by Chigbu *et al.* (2003), *S. aureus* isolates varied in their antibiotic susceptibility pattern when tested for their sensitivity to 16 antibiotics. Eighty percent of the isolates were resistant to more than one antimicrobial agent. All the isolates showed resistance to nalidixic acid and 100% sensitivity to rifampicin.

A survey of antibiotic resistant *S. aureus* strains from clinical specimens was carried out by Uwaezuoke and Aririatu (2004) of which 100 different clinical specimens were investigated with a yield of 48 *S. aureus* isolates. A high resistance of 95.8% to penicillin, 89.6% to ampicillin, 87.5% to tetracycline, and 75.0% to chloramphenicol by *S. aureus* strains were recorded. A high susceptibility of 91.7% to gentamicin and 85.4% to cloxacillin were also recorded.

S. aureus was the most prevalent species isolated from inpatient specimens (18.7% of all bacterial isolates) and the second most prevalent (14.7%) from outpatient specimens. Multidrug resistance phenotypes (resistance to ≥ 3 non-β-lactams) were common among both inpatient MRSA (59.9%) and outpatient MRSA (40.8%). Greater than 90% of multi drug resistant MRSA were susceptible to trimethoprim-sulfamethoxazole, linezolid, and vancomycin (David *et al*., 2005).

Out of the total Gram positive cocci, 56% were resistant to penicillin group, 27% were resistant to cephalosporin group, 22% were resistant to aminoglycoside group, 15% were resistant to quinolone group and 31% were resistant to other antibiotics (cotrimaxazole, erythromycin, aztreonam, vancomycin, nitrofurantoin and meropenam). The rate of resistance to most of the antibiotics was higher when tested against the isolates collected from pus as compared to those from blood and urine. Antibiotic resistant strains were more prevalent in pus samples than the other clinical isolates (blood and urine). The randomly selected 155 strains of *S. aureus* when tested against five groups of antibiotics showed resistance rate against ampicillin (92%), cephradine (60%), and gentamicin (58%). However intermediate resistance was found in case of vancomicin (38%), in hospitalized and non-hospitalized patients (Kalsoom and Abdul, 2006).

Also, in study of the prevalence of β-lactamase producing *S. aureus* infections and their antimicrobial susceptibility pattern in Nigeria, out of total 100 strains of *S. aureus*, 80% were found to be β-lactamase producer, which probably accounted for 100% and 96% resistant rate obtained for penicillin and ampicillin respectively. Among the β-Lactamase producing organisms, susceptibility to antibiotics were: erythromycin (82.5%), cephalexin (71%) ceftriaxone (70%), cloxacillin (66%), others were chloramphenicol, gentamicin, tetracycline and streptomycin with 62.5%, 61%, 30% and 53.8% susceptible respectively (Akindele *et al*., 2010).

2.7.3 CONS and drug resistance and β-lactamase production

The group of Gram positive bacteria identified as coagulase negative staphylococci (CNS), usually harmless commensals, have become important, commonly isolated pathogens in clinical microbiology laboratories around the world (Bayram and Balci, 2006; Widerstrom *et al.*, 2006). CONS have historically been more resistant to antimicrobials, including the β-lactam antibiotics, than *S. aureus* and some hospitals reveal rates of oxacillin resistance in CONS approaching 90%. Cross resistance to non β-lactam agents has been a recurrent theme over the past 40 years in the CONS. Recently use

of broad-spectrum antibiotic for treatment infections lead to CONS bacteria increasing the development of antibiotic resistance. In resulting large amount of nosocomial isolates of CONS became resistant to various antibiotics (Shubhra *et al*., 2008)

In a report, an antibiotic resistance pattern of the CONS (106), maximum resistance was seen against penicillin 82 (77.3%) and minimum was to gentamicin 23 (21.6%). In the study, the incidence of human infections and resistance pattern of 106 strains of CONS from different clinical specimens, maximum incidence was observed in wound infection (21.6%) followed by UTI (18.8%) and post operative pelvic infections (13.2%). *S. epidermidis* was found predominant in UTI (Phatak *et al*., 1994).

It was showed that 80 to 90% of the CONS strains isolated from human specimens create β-lactamase (Diekema *et al*., 2001; Peters *et al*., 1995). *S. epidermidis*, *S. haemolyticus*, *S. warneri*, *S. hominis* and *S. saprophyticus* among the CONS species were found to have elevated levels of resistance to a variety of antibiotics (York *et al*., 1996).

Cercenado *et al.* (1996) found that 32 isolates of CONS exhibited decrease levels of susceptibility or true resistance to teicoplanin. Twenty nine strains were also methicillin resistant and all were susceptible to vancomycin, 24 strains were *S. epidermidis,* 4 were *S. haemolyticus*, 4 were *S. hominis.*

Marry *et al.* (1996) eported methicillin resistant coagulase negative staphylococci (49%). Also Sloos and Dijkshoorn, (2000) reported that CONS, which caused septicemias, were resistant to both β-lactam antibiotics and aminoglycosides. The empirical therapy of choice is a glycopeptides, is either vancomycin or teicoplanin. The prevalence of CONS isolate with reduced susceptibility to glycopeptides the prevalence was 28.8% (Lallemound *et al*., 2002).

In another study, multi drug resistance CONS were found with more than 96% resistance to penicillin, more than 50% to cephalexin and ciproflxacin and

more than 20% to methicillin (Aggarwal *et al.*,2002). CONS isolated showed that neonatal intensive care units had decreased susceptibilities to vancomycin (Larsen *et al.*, 2007).

Of 134(66.7%) CONS isolated, the antimicrobial susceptibility patterns showed that the most resistant of the CONS belonged to oxacillin (94.02%) and least resistant belong to vancomycin (20.89%). The majority of CONS were isolated from urine specimens, the higher resistant of CONS belonged to oxacilin and the lowest to vancomycin (Ahmad and Manijeh, 2012).

CHAPTER-III
MATERIALS AND METHODS

3.1 Materials

The materials required in this study are listed in Appendix I.

3.2 Methods

The present research work was a descriptive cross sectional study conducted in the laboratory of Department of Pathology, Bir Hospital, Mahabauddha, Kathmandu in collaboration with Central Department of Microbiology. The study was carried out from September 2011 to February 2012. During this period, a total of 205 significant growth *Staphylococcus* species were reported (urine-75, sputum-9, blood-10, pleural fluid-6, pus-105).

Staphylococci obtained from different clinical specimens were studied for antimicrobial sensitivity pattern, multidrug resistance pattern, and the strain showing resistance to two or more than two different classes of antibiotics are tested for β-lactamase production. Further the sensitivity pattern against cloxacillin of Oxide, Mast and HI-Media were compared. The identification of the organism was based on standard laboratory criteria (growth in blood agar and McConkey agar media, for 24- 48 hrs at 37°C, colonial morphology, Gram staining, catalase test, oxidase test and coagulase test) (Collee *et al.*, 2006). After differentiation based on coagulase, the isolates were examined for AST pattern. The organisms were screened for (MDR) pattern. MDR isolates then were tested for β-lactamase production. β-lactamase production was assessed by standard nitrocefin stick test method.

3.2.1 Study design

This study is a cross-sectional study. It includes all the clinical culture specimens of all patients admitted in Bir Hospital and the outdoor patients from September 2011 to February 2012. All indoor and outdoor patients admitted during this period were included in the study. Patient charts were stored in a computerized database for statistical evaluation.

3.2.2 Study population

The study comprises 4550 clinically ill, both indoor and outdoor patients which include children, adult and old of both sexes, aged between one month to ninety years who visited at National Academy of Medical science, Bir Hospital, Mahabauddha, Kathmandu.

3.2.3 Inclusion and exclusion criteria

All specimens from patients attending Bir Hospital during the study period were included in the study. Those specimens which do not meet standard acceptance criteria were excluded.

3.2.4 Data collection

Each patient with culture specimens were asked for their medical history. The information of patient including name, sex, age, site of infection, origin of the patient and clinical history were collected.

3.2.5 Collection of clinical samples

Samples were collected from indoor and outdoor patients. Sample taken for this study were sputum, blood, urine, pus swab. All these clinical specimens were collected in strictly sterile, leak proof, dry containers which were free from all traces of disinfectants. The sample specimens were collected by medical officer or as per the instruction by the medical officers before the use of antibiotics, and then immediately transferred to microbiology laboratory. The samples taken to the laboratory were processed as quickly as possible. Generally two samples taken from each patient, one was used for Gram stain and other for culture (Collee *et al.*, 2006). Clinical sample collection procedures are described in Appendix-I.

3.2.6 Sample Evaluation

Before processing of the samples, the quality of each was evaluated. If the samples do not meet the acceptance criteria then rejected and requested for another specimen. Consideration included the improper labeling, watery, non-purulent sputum, leaked container, visible signs of contamination and any delay in transportation.

3.2.7 Sample processing

3.2.7.1 Macroscopic examination

Specimen obtained in the laboratory were observed for its color, odour, consistency and appearance and reported accordingly.

3.2.7.2 Microscopic examination

Sample meeting the acceptance criteria were gram stained for examining the gram positive cocci in clusters. The organisms from samples after overnight incubation were also microscopically examined for staphylococci via gram staining technique.

3.2.7.3 Culture of the specimen

The received specimens were streaked onto plates of MacConkey agar (MA), Blood agar (BA) and Mannitol salt agar (MSA) and incubated aerobically at 37°C for 24 hours. Preliminary identification of bacterial isolates was done using colony morphology and colony characteristics such as haemolysis pattern on blood agar and also by Gram-staining whenever necessary. After incubation, the colonies were sub-cultured onto petri dishes containing nutrient agar and incubated as described above. Routine conventional laboratory techniques including gram staining, motility, catalase, oxidase, slide and tube coagulase tests were carried out (Cheesbrough, 2006). The preparation and composition media is mentioned in the Appendix-II.

3.2.8 Identification of *Staphylococcus* species

In the identification of staphylococci standard microbiological procedures were followed as described in Bergey's manual of systematic bacteriology. This involves morphological appearance of the isolated colony, staining reactions and biochemical properties. Each of the organisms was first isolated in the pure form. Then after colony morphology was noted, which was followed by Gram's staining and biochemical tests.

3.2.8.1 Colony characteristics

The colony morphology of staphylococci on culture media is mentioned in Appendix-III.

3.2.8.2 Biochemical test used for identification of *Staphylococcus*

Different sets of biochemical tests were performed to identify the isolates as *Staphylococcus* species. Firstly the pure form of culture was obtained from primary culture and then it was used aseptically to perform the biochemical tests detail of which is described in the appendix. Routine conventional laboratory techniques including gram staining, catalase, oxidase and slide and tube coagulase tests were carried out. The procedure of different biochemical tests are given in Appendix-III.

3.2.9Antibiotic sensitivity test

The detail of the antibiotic disc used and its interpretative chart is mentioned in the Appendix-III.

3.2.10 Screening of Multidrug resistant *Staphylococcus*

After the antibiotic sensitivity testing of each of staphylococcal isolates by Kirby-Baur method, the organisms were screened for MDR. The organisms resistant to two or more antibiotic of the different classes were reported as the MDR isolates (Bartoloni *et al.,* 2006; Wright *et al.,* 2000).

3.2.11 Detection of β-lactamase enzyme production (Chromogenic Nitrocephin stick test)

The production of β-lactamase enzymes was determined by using commercially prepared nitrocephin impregnated touch sticks (BR0066A, Oxoid, UK) according to the manufacturer's instructions. Briefly, sticks were touched to colony material from a 24 hours culture of test bacteria kept up to 24 hrs in an incubator at 37°C with moisture. A change in the color of the sticks indicated β-lactamase production. The tips of the sticks are impregnated with Nitrocefin, a chromogenic cephalosporin (Glaxo Reasearch 87/312). The sticks are convenient to use and overcome the necessity for preparing fresh reagents daily.

Techniques of nitrocefin stick test

i. The container was removed from the refrigerator and allowed to bring at room temperature.
ii. The well separated representative colony of the test Staphylococcus was picked from the preserved culture and inoculated into sterile NA plate incubated aerobically at 37°C for 24 hours.
iii. The β-lactamase sticks were removed (color coded black) from the container by holding at colored end.
iv. The representing colony was touched with the impregnated tip of the stick, the stick rotated, picking up a small mass of cells.
v. The inoculated tip of the stick is placed on a stand.
vi. The reaction requires moisture, so the tip of the stick was placed in the moisture condensate in the lid. On the moisture unavailable case, one or two drops of distilled water was added in the lid and moistened the tip of the stick.
vii. The reagent impregnated and colony picked tip of the stick was examined for upto five minutes and in negative case re-examined after fifteen minutes to one hour.
viii. A positive reaction was shown by the development of pink to red color. If no color change is observed within one hour, the isolate was reported as β-lactamase negative. To ensure correct reading, the stick should be compared with the fresh and unused stick.
ix. Some staphylococci may give pigmented colonies. Such colonies may give false positive result. So stick test was not used for such isolates.
x. Reference strains (positive and negative stains of β-lactamase prodution) were used. (OXOID BR66A)

3.3 Quality control for test

Quality of each test was maintained by employing a standard technique. The quality of each agar plate was tested by incubating one plate in each lot on the incubator. For sensitivity test, MHA was prepared carefully maintain 4 mm thickness. The pH of the media was also checked. Disc containing the correct amount of antimicrobial were used. The antibacterial quality of the antibiotics was also checked. For the quality control of β-lactamase test reference strains

of (positive control and negative control) were also used. Strict aseptic conditions were maintained while carrying out all the procedures.

3.4 Purity plate

The purity plate was used to ensure that the inoculation used for biochemical tests and AST were pure culture. This also notify whether the biochemical tests were performed in the aseptic condition or not. Thus, while performing biochemical tests, the same inoculums was sub-cultured in respective medium and incubated. The media were then checked for the pure growth of organisms.

3.5 Statistical analysis of result

Frequencies and percentage were calculated for study variables. The data were analysed using statistical package for the social sciences (SPSS) 19.0 statistical software. Chi-square ($\chi 2$) test was used to calculate probabilities and determine significance. A p-value of less than or equal to 0.5 was considered to be statistically significant ($p<0.5$), while p-value more than 0.05 was considered to be statistically non significant (NS).

CHAPTER IV
RESULTS

In this study different clinical samples were taken from out-patients and various wards of the National Academy of Medical Science, Bir Hospital, Mahabauddha, Kathmandu. Among all the clinical specimens processed during the study period, 1280 showed positive growth and 205 were isolated and identified as staphylococci by standard microbiological technique. The staphylococcal isolates were screened for MDR and the MDR were tested for β-lactamase production by standard nitrocefin stick test method.

4.1 Clinical pattern of isolates

4.1.1 Staphylococcal growth in clinical specimens

Out of 1280 different bacterial isolates from various clinical samples, 16% was Staphylococci. More than half of the skin and soft tissue infections were due to the staphylococcal infection. Around one fifth of respiratory tract infection and UTI was contributed by staphylococci. In the clinical specimens *S. aureus* (10.7%) were more prevalent compared to the CONS (5.3%). *S. aureus* were more frequent cause of skin and soft tissue infection and respiratory tract infection compared to CONS, whereas CONS were more common in UTI and bloodstream infection than *S. aureus*. It was found that staphylococcal isolation and clinical specimens was statistically significant.

Table 1: Staphylococcal growth in clinical specimen

Clinical specimen	**No. of cases**		**Growth positive cases**		***Staphylococcus* isolated**				**p-value**
					S. aureus		**CONS**		
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	
Urine	2090	45.5	607	47.4	31	22.6	44	64.7	
Sputum	85	1.9	17	1.3	9	6.6	-	-	
Blood	1415	30.8	102	8.0	2	1.5	8	11.8	**0.000****
Pleural fluid	56	1.2	12	0.9	3	2.2	3	4.4	
Pus	944	20.6	542	42.3	92	67.1	13	19.1	
Total	**4590**	**100**	**1280**	**100**	**137**	**100**	**68**	**100**	

****Significant at 1% level of significance

4.1.2 Gender wise distribution of *Staphylococcus* species

More than half of the staphylococcal isolates were recovered from female. Both *S. aureus* and CONS were more frequently isolated from female with 35.6% and 20.5% respectively. However on application of $\chi 2$, it was found that gender and isolation of staphylococci was statistically insignificant. (p=0.249).

Table 2: Gender wise distribution of *Staphylococcus* species

***Staphylococcus* Isolated**	**Male**		**Female**		**p-value**
	No.	**%**	**No.**	**%**	
S. aureus	64	31.2	73	35.6	**0.249**
CONS	26	12.7	42	20.5	
Total	**90**	**43.9**	**115**	**56.1**	

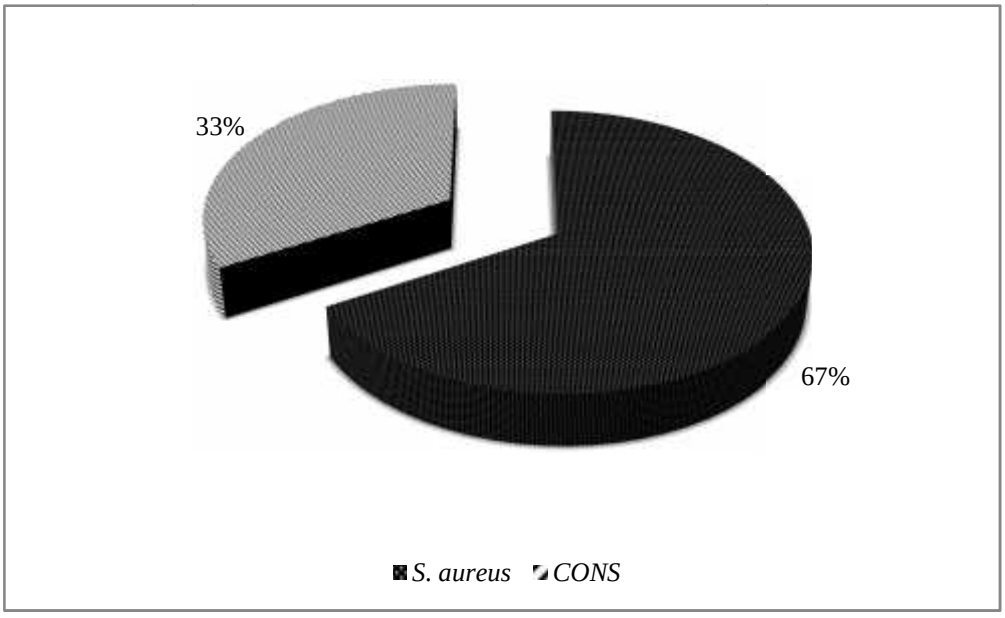

Figure 2: Distribution of *Staphylococcus* species

4.1.3 Origin (Ward) based distribution of staphylococci

On the basis of origin, most staphylococcal infections were community acquired, which accounted for more than half (61%). Among this, *S. aureus* infections were more frequent compared to CONS with 40.5% and 20.5% incidence respectively. Among the nosocomial infections, *Staphylococcus*

infections are more frequent in the burn ward followed by FMW and MSW. In MSW, POST-OP, NSTA, CTVS, BURN, NSICU, SKIN *S. aureus* infection were predominant than CONS. On statistical analysis, it was found that the origin of the patient and staphylococcal isolation was significant (p=0.01).

Table 3: Origin (Ward) based distribution of staphylococci

Origin	**Staphylococcal isolates**				**p-value**
	S. aureus		**CONS**		
	No.	**%**	**No.**	**%**	
OPD	83	40.5	42	20.5	
MSW	5	2.4	3	1.5	
FSW	2	1	4	2	
MMW	1	0.5	4	2	
FMW	1	0.5	8	3.5	
NSW	0	0	1	0.5	**0.01***
POST-OP	2	1	0	0	
NSICU	5	2.4	1	0.5	
SKIN	3	1.5	0	0	
ENT	6	2.9	0	0	
NSTA	2	1	0	0	
CTVS	4	2	0	0	
BURN	23	11.2	5	2.4	

*significant at 5% level of significance

4.1.4 Age wise *Staphylococcus* species distribution

Out of 205 staphylococcal infection 66.8% was due to *S. aureus*. Higher frequency of *S. aureus* and CONS infection was seen among age group 20-29 with 21% and 12.7% of incidence respectively. No *S. aureus* infection was found in above 80 years and no CONS infection was found below 10 years. The age wise distribution of staphylococci species was statistically insignificant, when $\chi 2$ was applied (p-value=0.54).

Table 4: Age wise *Staphylococcus* species distribution

Age	*S. aureus*		CONS		p-value
	No.	%	No.	%	
<10	6	2.9	0	0	
10-19	27	13.2	2	1	
20-29	43	21	26	12.7	
30-39	17	8.3	15	7.3	
40-49	24	11.7	6	2.9	**0.54**
50-59	8	3.9	10	4.9	
60-69	5	2.4	5	2.4	
70-79	7	3.4	2	1	
80<	0	0	2	1	
Total	**137**	**66.8**	**68**	**33.2**	

4.2 Antibiotic sensitivity pattern of the isolates

4.2.1 Antibiotic sensitivity pattern of *Staphylococcus* species

S. aureus were comparatively more resistant than CONS to many antibiotics such as ampicillin, amoxicillin, amikacin, vancomycin, cefotaxime etc. However, CONS were comparatively more resistant than *S. aureus* to

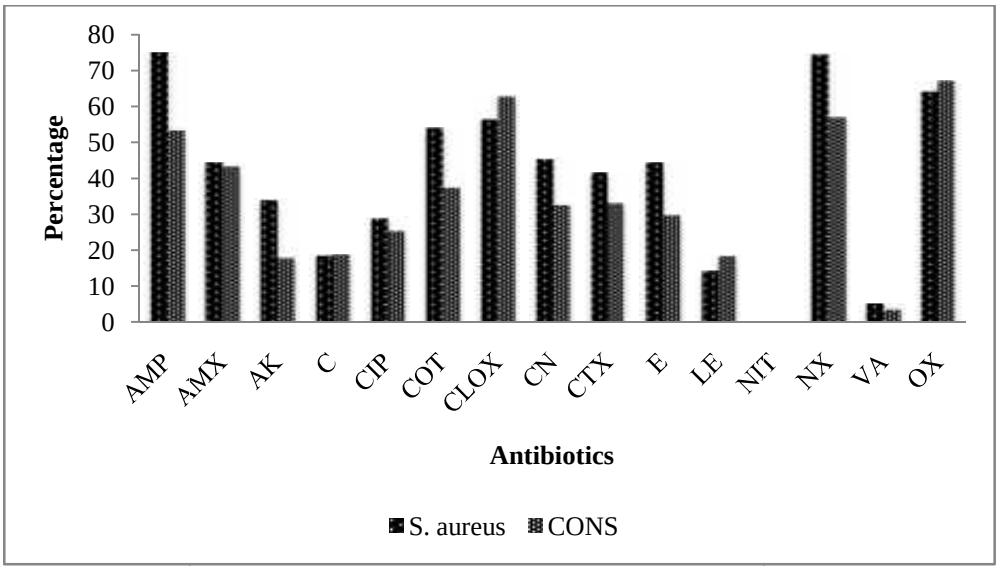

Figure 3: Antibiotic resistance pattern of *Staphylococcus* species

chloraphenicol, cloxacillin, levofloxacin and oxacillin. Antibiotic resistance and staphylococcus species were statistically significant only for amicillin

(p=0.002), amikacin (p=0.017) and cotrimoxazole (p=0.026). For ampicillin, amikacin and cotrimoxazole *S. aureus* were more resistant than CONS with ampicillin (*S. aureus*-74.5% and CONS-52.9%), amikacin (*S. aureus*-33.6% and CONS-17.6%) and cotrimoxazole (*S. aureus*-53.3% and CONS-36.8%).

4.2.2 Antibiotic susceptibility pattern of *S. aureus*

About 75% of *S. aureus* were resistant to ampicillin and norfloxacin. More than 50% were resistant toward cloxacillin, cotrimoxazole and oxacillin. However, ampicillin, erythromycin, and cefotaxime were about 50% sensitive. Nitrofurantoin and vancomycin were drug of choice as they were 100% and 95.6% sensitive respectively.

Table 5: Antibiotic susceptibility pattern of *S. aureus*

Antibiotics	Sensitive		Resistant		Total
	No.	%	No.	%	
AMP	35	25.5	102	74.5	**137**
AMX	77	56.2	60	43.8	**137**
AK	91	66.4	46	33.6	**137**
C	37	82.2	8	17.8	**45**
CIP	98	71.5	39	28.5	**137**
CLOX	42	43.8	54	56.2	**96**
CN	17	54.8	14	45.2	**31**
COT	64	46.7	73	53.3	**137**
CTX	81	59.1	56	40.9	**137**
E	54	56.2	42	43.8	**96**
LE	92	86.0	15	14.0	**107**
NIT	31	100.0	0	0	**31**
NX	8	25.8	23	74.2	**31**
VA	131	95.6	6	4.4	**137**
OX	44	38.3	71	61.7	**115**

4.2.3 Antibiotics resistant pattern of *Staphylococcus aureus* in different clinical specimens

Among various clinical specimens, the isolates from blood were 100% sensitive to the chloramphenicol, ciprofloxacin, cloxacillin, cotrimoxazole,

cefotaxime, erythromycin vancomycin, and levofloxacin whereas 50% resistant to ampicillin and amoxicillin. Pus isolates were about 50% or more resistant to β-lactam antibiotics, aminoglycoside, macrolides and phenicols. They showed 5.4%, 14.3% and 27.2% resistance to vancomycin, ciprofloxacin and levofloxacin respectively. The isolates from urine were 100% sensitive toward amikacin, levofloxacin, vancomycin and nitrofurantoin. They were highly resistant toward norfloxacin (74.2%) and ampicillin (61.3%). Plueral fluid isolates were 66.6% resistant to chloramphenicol, ciprofloxacin, cloxacilin, cotrimoxazole, levofloxacin and oxacillin. They were 33.3% resistant to ampicillin, amoxicillin, amikacin, cefotaxime and vancomycin. Sputum isolates were highly resistant to ampicillin (77.8%) and cotrimoxazole (77.8%). They were 55.6%, 44.4% and 33.3% resistant to chloramphenicol, amoxicillin and cefotaxime respectively. They were 100% sensitive toward amikacin, levofloxacin and vancomycin.

Table 6: Antibiotics resistant pattern of *Staphylococcus aureus* in different clinical specimens

Antibiotics	**Clinical Specimen - No. (%)**					**p-value**
	Urine	**Sputum**	**Blood**	**Pleural fluid**	**Pus**	
	No (%)	**No (%)**	**No (%)**	**No (%)**	**No (%)**	
AMP	19(61.3)	7(77.8)	1(50)	1(33.3)	74(80.4)	**0.095**
AMX	10(32.3)	4(44.4)	1(50)	1(33.3)	44(47.8)	**0.654**
AK	0(0)	0(0)	1(50)	1(33.3)	44(47.8)	**0.000**
C	1(3.2)	5(55.6)	0(0)	2(66.7)	-	**0.000**
CIP	12(38.7)	0(0)	0(0)	2(66.7)	25(27.2)	**0.085**
CLOX	-	-	0(0)	2(66.7)	52(57.1)	**0.255**
CN	14(45.2)	-	-	-	-	**-**
COT	17(54.8)	7(77.8)	0(0)	2(66.7)	47(51.1)	**0.109**
CTX	10(32.3)	3(33.3)	0(0)	1(33.3)	42(45.7)	**0.480**
E	-	-	0(0)	1(33.3)	41(45.1)	**0.417**
LE	0(0)	0(0)	0(0)	2(66.7)	13(14.3)	**0.061**
NIT	0(0)	-	-	-	-	**-**
NX	23(74.2)	-	-	-	-	**-**
VA	0(0)	0(0)	0(0)	1(33.3)	5(5.4)	**0.085**
OX	20(64.5)	3(33.3)	2(100)	1(50)	45(61.7)	**0.329**

4.2.4 Antibiotic susceptibility pattern of CONS

CONS are highly resistant to ampicillin (52.9), cloxacillin (62.5%), cephalexin (50%), norfloxacin (56.8) and oxacillin (66.7%). They are least resistant to nitrofurantoin (0%), vancomycin (2.9%), amikacin (17.6%), chloramphenicol (18.2%) and levofloxacin (18.9%).

Table 7: Antibiotic susceptibility pattern of CONS

Antibiotics	Sensitive		Resistant		Total
	No.	%	No.	%	
AMP	32	47.1	36	52.9	**68**
AMC	39	57.4	29	42.6	**68**
AK	56	82.4	12	17.6	**68**
C	45	81.8	10	18.2	**55**
CIP	51	75.0	17	25.0	**68**
CLOX	9	37.5	15	62.5	**68**
CN	22	50.0	22	50.0	**44**
COT	43	63.2	25	36.8	**68**
CTX	46	67.6	22	32.4	**68**
E	17	70.8	7	29.2	**24**
LE	30	81.1	7	18.9	**37**
NIT	44	100.0	0	0	**44**
NX	19	43.2	25	56.8	**44**
VA	66	97.1	2	2.9	**68**
OX	18	26.5	50	66.7	**68**

4.2.5 Antibiotic resistant pattern of CONS in different clinical specimens

In clinical specimens, most of the CONS isolates from the pus are highly resistant antibiotics except vancomycin (0%), and levofloxacin (23.1%). They were 100% resistant to cloxacillin and around 70% resistant to amikacin and oxacillin. Isolates from blood were least resistance to antibiotics used. CONS isolated from blood were 100% sensitive to amoxicillin, ampicillin, ciprofloxacin, cefotaxime, levofloxacin and vancomycin. One fourth of the blood isolated CONS were resistant to amikacin, chloramphenicol, cephalexin and cotrimoxazole. Urine isolates are highly resistant to ampicillin (59.1%) and norfloxacin (56.8%) and least resistant to amikacin (0%) and

nitrofurantoin (0%). Pleural fluid isolates were 100% sensitive ciprofloxacin, cefotaxime, erythromycin and vancomycin. They were 66.7% resistant to amoxicillin, chloramphenicol and cephalexin.

Table 8: Antibiotic resistant pattern of CONS in different clinical specimens

Antibiotics	Clinical Specimen				
	Urine No (%)	**Sputum No (%)**	**Blood No (%)**	**Pleural fluid No (%)**	**Pus No (%)**
AMP	26(59.1)	-	0(0)	2(22.7)	**8(61.5)**
AMX	20(45.5)	-	0(0)	2(66.7)	**7(53.8)**
AK	0(0)	-	2(25)	1(33.3)	**9(69.2)**
C	6(13.6)	-	2(25)	2(66.7)	**-**
CIP	10(22.7)	-	0(0)	0(0)	**7(53.8)**
CLOX	-	-	1(12.5)	1(33.3)	**13(100)**
CN	22(50)	-	2(25)	2(66.7)	**-**
COT	15(34.1)	-	2(25)	1(33.3)	**7(53.8)**
CTX	16(36.4)	-	0(0)	0(0)	**6(46.2)**
E	-	-	0(0)	0(0)	**7(53.8)**
LE	3(23.1)	-	0(0)	1(33.3)	**3(23.1)**
NIT	0(0)	-	-	-	**-**
NX	25(56.8)	-	-	-	**-**
VA	2(4.5)	-	0(0)	0(0)	**0(0)**
OX	33(75)	-	4(50)	2(66.7)	**11(84.6)**

4.2.6 Antibiotic resistance pattern of β-lactamase producing *Staphylococcus* species

β-lactamase producers showed highest (>70%) resistance towards amoxicillin, cephalexin, norfloxacillin, cloxacilin, cotrimoxazole, ampicillin, cefotaxime and oxacillin. β-lactamase producers were least resistant to nitrofurantoin and vancomycin with 0% and 2.1% respectively. β-lactamase producing *S. aureus* were highly resistant to cephalexin, norfloxacin, ampicillin, cloxacillin, cotrimoxazole, cefotaxime with 100%, 100%, 96.9%, 3.8%, 91.3%, 81.2%, and 75% resistance respectively. They were least resistant to vancomycin and nitrofurantoin with 0% for each. For CONS β-lactamase producers were considerably resistant to amoxicillin (93.8%), cephalexin (90%), norfloxacin

(90%), ampicillin (87.5%), cloxacillin (83.3%), and cefotaxime (75%). They were least resistant to nitrofurantoin (0%), vancomycin (6.2%).

Table 9: Antibiotic resistance pattern of β-lactamase producing *Staphylococcus* species

Antibiotics	*S. aureus*		CONS		Total	
	NO.	%	No.	%	No	%
AMX	21	65.6	15	93.8	36	**75**
AMP	31	96.9	14	87.5	45	**93.8**
AK	18	56.2	4	25	23	**47.9**
C	2	20	3	27.3	5	**23.8**
CIP	18	56.2	4	25	22	**45.8**
CLOX	21	91.3	5	83.3	26	**89.7**
CN	6	100	9	90	15	**93.8**
COT	26	81.2	11	68.8	37	**77.1**
CTX	24	75	12	75	36	**75**
E	15	65.2	2	33.2	17	**58.6**
LE	5	19.2	4	44.4	9	**25.7**
NIT	0	0	0	0	0	**0**
NX	6	100	9	90	15	**93.8**
VA	0	0	1	6.2	1	**2.1**
OX	18	69.2	13	81.2	31	**73.8**

4.3 Multidrug resistance pattern

4.3.1 Multidrug resistance pattern among *Staphylococcus* species

Altogether 98 (47.8%) of MDR isolates were found in different clinical specimen. Of the total *Staphylococcus* species isolated, 48.9% and 45.6% were MDR in *S. aureus* and CONS respectively. Multidrug resistance pattern and staphylococcal isolation was statistically insignificant (p-value=0.654).

Table 10: Multidrug resistance pattern among *Staphylococcus* species

Staphylcoccus isolated	Total	MDR	%	p-value
S. aureus	137	67	48.9	**0.654**
CONS	68	31	45.6	
Total	**205**	**98**	**47.8**	

4.3.2 Multidrug resistance pattern on gender

Of the 90 *Staphylococcus* isolates in male 51.1% were found to be MDR whereas only 45.2% *Staphylococcus* isolates in female were MDR. Gender wise distribution of MDR was statistically insignificant (p=0.402).

Table 11: Multidrug resistance pattern on gender

Gender	Total	MDR	%	p-value
Male	90	46	51.1	**0.402**
Female	115	52	45.2	
Total	**205**	**98**	**47.8**	

4.3.3 Age wise multidrug resistance pattern

Out of the total isolates in each group, percentage of multidrug resistant *Staphylococcus* spp. were high among age group 70-79 (66.7%) followed by age group 10-19 (65.5%) and 20-29 (53.6%). No MDR incidence was found above 80 years age. However, below 10 years age MDR incidence was 33.3%. The age wise distribution of MDR was statistically insignificant (p-value=0.086).

Table 12: Age wise multidrug resistance pattern

Age	Total	MDR	%	p-value
<10	6	2	33.3	
10-19	29	19	65.5	
20-29	69	37	53.6	
30-39	32	14	43.8	
40-49	30	10	33.3	**0.086**
50-59	18	5	27.8	
60-69	10	5	50	
70-79	9	6	66.7	
>80	2	0	0	
Total	**205**	**98**	**100**	

4.3.4 Multidrug resistance pattern on origin

Out of 125 staphylococcal isolates from OPD, 46.4% were found to be MDR. All the isolates from NSW, POST-OP and CTVS were MDR. More than fifty

percent of isolates were MDR from NSICU and ENT. The ward-based distribution of MDR was statistically insignificant (P=0.579).

Table 13: Multidrug resistance pattern on origin

Origin	Total	MDR	%	P-value
OPD	125	58	46.4	
MMW	8	2	40	
FMW	6	2	22.2	
MSW	5	3	37.5	
FSW	9	3	50	
NSW	1	1	100	
NSICU	6	4	66.7	**0.579**
POST-OP	2	2	100	
BURN	28	14	50	
SKIN	3	1	33.3	
ENT	6	4	66.7	
CTVS	4	2	100	
NSTA	2	2	50	

4.3.5 Multidrug resistance pattern of *S. aureus* isolated from different clinical specimens

Equal percentage (66.7%) of the total isolates were MDR from sputum and pleural fluid. Lower incidence of MDR was found in urine (45.2%) and pus (48.9%). However, no MDR *S. aureus* was isolated from blood specimen.

Table 14: Multidrug resistance pattern of *S. aureus* isolated from different clinical specimens

Clinical specimen	Total	MDR	%	p-value
Urine	31	14	45.2	
Sputum	9	6	66.7	
Blood	2	0	0	**0.462**
Pleural fluid	3	2	66.7	
Pus	92	45	48.9	
Total	**137**	**67**	**48.9**	

4.3.6 Multidrug resistance pattern of CONS isolated from different clinical specimens

Higher incidence of multidrug resistant CONS were isolated in pus (76.9%) in contrast, no multidrug resistant CONS was isolated from blood specimen. About half of the CONS from urine were multidrug resistant.

Table 15: Multi-drug resistance pattern of CONS isolated from different clinical specimens

Clinical specimen	Total	MDR	%	p-value
Urine	44	20	45.5	
Sputum	-	-	-	
Blood	8	0	0	**0.007***
Pleural fluid	3	1	33.33	
Pus	13	10	76.9	
Total	**68**	**31**	**45.6**	

***Significant at 5% level of significance

4.4 β-lactamase production pattern

4.4.1 β-lactamase stick test in MDR *Staphylococccus* species

Of 98 MDR staphylococcus spp. isolated 47 (47.9%) were found β-lactamase positive. Higher percent of CONS (51.6%) isolates were β-lactamase positive in comparison to *S. aureus* (46.3%). When $\chi 2$ test was applied, it was found that staphylococcal species isolate and β-lactamase production was statistically insignificant (P=0.978).

Table 16: β-lactamase stick test in MDR *Staphylococccus* species

Staphylococcus spp.	Total	Positive	%	p-value
S. aureus	67	31	46.3	**0.978**
CONS	31	16	51.6	
Total	**98**	**47**	**47.9**	

4.4.2 Gender wise distribution pattern of β-lactamase

Male showed the higher prevalence of β-lactamase producing staphylococci with 50% incidence than female with 48% incidence. However, this was found statistically insignificant (p-value=0.704).

Table 17: Gender wise distribution pattern of β-lactamase

Gender	Total	Positive	%	p-value
Male	46	23	50	**0.704**
Female	52	25	48	
Total	**98**	**48**	**49**	

4.4.3 Age wise distribution pattern of β-lactamase

Age wise distribution of β-lactamase producing *Staphylococcus* spp. showed higher incidence in age group 10-19 (31%) followed by age group 60-69 (30%) and 50-59 (27.8%). Distribution β-lactamase producing staphylococci on age group was statistically insignificant (p-value=0.746).

Table 18: Age wise distribution pattern of β-lactamase

Age	Total	Positive	%	p-value
<10	6	1	16.7	
10-19	29	9	31	
20-29	69	15	21.7	
30-39	32	7	21.9	
40-49	30	6	20	**0.746**
50-59	18	5	27.8	
60-69	10	3	30	
70-79	9	2	22.2	
>80	0	0	0	
Total	**98**	**48**	**49**	

4.4.4 Clinical specimen wise distribution pattern of β-lactamase

Of the total MDR isolates from each clinical specimen, pleural fluid accounted for the highest incidence (66.7%) of β-lactamase producing *Staphylococcus* spp. followed by sputum (50%) and pus (49.1%). Specimen wise distribution of β-lactamase producing staphylococci was statistically insignificant (p-value=0.929).

Table 19: Clinical specimen wise distribution pattern of β-lactamase

Clinical specimen	Total	Positive	%	p-value
Urine	34	16	47.1	
Sputum	6	3	50	**0.929**
Pleural fluid	3	2	66.7	
Pus	55	27	49.1	
Total	**98**	**48**	**49**	

4.5 Resistance pattern of *Staphylococcus* to cloxacillin of three different manufacturing companies

For Cloxacillin of HI-MEDIA both *S. aureus* and CONS showed almost 100% resistance whereas for that of OXOID and MAST *S. aureus* and CONS showed equal resistance of 56.25% and 61.7% respectively. In the study action of cloxacillin of OXOID and MAST were comparable to each other but that of HI-MEDIA was too high. This shows the cloxacillin disc of HI-MEDIA were of poor quality and were unsuitable for using in antibiotic susceptibility testing.

Table 20: Resistance pattern of *Staphylococcus* to cloxacillin of three different manufacturing companies

Antibiotics	Staphylococcal isolate			
	S. aureus		CONS	
	No.	%	No.	%
HI-MEDIA	133	97.13	65	**96**
OXOID	77	56.25	42	**61.7**
MAST	77	56.25	42	**61.7**

CHAPTER V
DISCUSSION

This study was designed to emphasize the current antimicrobial susceptibility pattern of *Staphylococcus* and to test β-lactamase production in the MDR strains in order to guide clinicians to apt the appropriate antimicrobial agents. This type of study could help to estimate to employ effective antimicrobial strategy so that the emergence of resistant strains could be reduced. The specimens collected for this study were from outpatients and various wards of the hospital, which included both male and female from one month to 90 years old.

Out of total samples, major portion were contributed by urine sample with 2090 (45.5%), which was followed by blood specimen 1415 (30.8%). In a similar study conducted by Baral (2008) showed the similar pattern of sample distribution. In another studies conducted by Chhetri *et al.* (2001), Obi *et al.* (1996) showed lower growth rate.

Among total samples 1280 (27%) showed the positive growth and *Staphylococcus* accounted for 16% of infections. This finding was also compatible with the study by Dhakal (1999) with growth positivity of 25.16% and Baral (2008) with 22.35%. In contrast to this finding, Bomjan in 2005 and Khan *et al.* (2008) showed 35.4% and 34.55*% S. aureus* incidence respectively. Another study by GadGamal *et al.* (2009) showed the 39.8% staphylococcal incidence. This lower prevalence of growth could be due pre-administration of antibiotic before sample collection or the infection due to viruses, fungi, protozoans, mucoplasma, anaerobes, chlamydia, legionellas or fastidious bacteria (Smith and Easmon, 1990).

Of the total *Staphylococcus* isolated, 137 (66.8%) *S. aureus* and 68 (33.2%) CONS were identified. In a similar study by Khadri and Alzohariy (2010) showed out of the 235 isolates, 164 (69.8%) were *S. aureus* and the remaining 71 (30.2%) were coagulase negative staphylococci (CONS).

Out of 205 staphylococi isolated 90(43.9%) were from male and 115 (56.1%) were from female. This gender wise distribution pattern of staphylococci was statistically insignificant (p=0.706). Similarly, 125 (61%) samples were from outdoor patients whereas 80 (39%) were from various wards of the hospital. *S. aureus* (40.5%) was predominating *Staphylococcus* isolated from OPD whereas CONS (20.5%) accounted for half of the *S. aureus.* Among hospital wards higher incidence of staphylococcal infection was found in burn ward 28 (35%), which was followed by FMW 9(11.25%). The staphylococcal distribution in different wards of the hospital was statistically significant (p=0.01). In a study conducted in TUTH, Nepal by Shrestha *et al.* (2009) showed higher staphylococal infection in FSW.

Higher prevalence of staphylococci in burn ward may be due to lack of employment of hygiene practice among the hospital staff or due to the patient's own nasal or body flora which might had transferred to burnt area. The compliance of the staff to hand disinfection could be poor in burn wards, which further exposes the vulnerable patients to colonization of resistant organisms prone to cause infection. Cross-transmission between patients could also be possible via hospital devices (Warren and Fraser, 2001).

Colonization with potentially pathogenic multidrug resistant *Staphylococcus* spp. is common among patients in the ICU due to the high antibiotic pressure in these wards (Agvald-Ohman *et al.*, 2003; Drakulovic *et al*., 2001). It has previously been shown that cross-transmission of potential pathogens in severely ill patients is associated with nosocomial infections (Weist *et al.*, 2002) and that improved hygiene prophylaxis leads to a decrease of cross-transmissions and, subsequently, a decrease of nosocomial infections of *Staphylococcus* spp. (Bonten, 2002; Pittet 2000).

On age wise distribution higher incidence was found in 20-29 years group with 69 (33.7%). This finding was statistically insignificant (p=0.54). The study done by Shrestha *et al.* (2007) showed staphylococcal distribution was indifferent to age. The higher incidence observed for staphylococcal infection in the particular age group may be due to frequent visit by that group. Among

the total staphylococcal isolates 90 (43.9%) were from male, while 115 (56.1%) were female. This gender wise distribution of isolate was statistically insignificant (p=0.249).

The isolated staphylococci were highly resistant to commonly prescribed antibiotics. In this study, resistance pattern of staphylococci for β-lactam antibiotics, amoxicillin, ampicillin, cloxacillin, oxacillin, cephalexin and cefotaxime respectively were 43.41%, 67.31%, 53.17%, 66.12%, 48% and 38%. Similarly for floroquinolones such as levofloxacin and ciprofloxacin showed 15.27% and 27.31% resistance respectively. For glycopeptides (vancomycin), staphylococci were found to be 3.9% resistant. Likewise, staphylococci showed 28.29%, 18%, 47.8%, 40.83%, 0% and 64% resistance to amikacin, chloramphenicol, cotrimoxazole, erythromycin, nitrofurantoin and norfloxacin respectively.

It is worrisome that the present study reports an alarmingly high proclivity of antibiotic resistant staphylococcal infection. Further the uncontrolled growing trend of methicillin resistant staphylococci has posed a serious consequence in community acquired infections (CDC, 1999). Other studies have also shown such a high prevalence in various parts of the country. But prevalence reported varied place to place. This variation might be because of several factors like efficacy of infection control practices, healthcare facilities and antibiotic prescription and usage that vary from hospital to hospital.

Shortly after the penicillin became available in late 1940s for treatment of serious staphylococcal infections, resistance to these antibiotics emerged at augmenting rate in almost every part of the world. Staphylococci learned to inactivate the antibiotics and hence were emerged as resistant and posing devastating condition in the treatment by augmenting the cost, duration and complexity of the treatment. (Fridkin *et al.*, 1999).

One hundred thirty seven *S. aureus* after antibiotic sensitivity assay showed higher degree of resistance to β-lactam antibiotics, ampicillin (74.5%), oxacilin (61.7%), cloxacillin (56.2%), cephalexin (45.2%), amoxicillin

(43.8%) and cefotaxime (40.9%). Lower degree of resistance was shown to chloramphenicol, ciprofloxacin, levofloxacin, and vancomycin with 17.8%, 28.5%, 14% and 4.4% respectively. In a study done by Shrestha *et al.* (2009) among 149 *S. aureus* isolates, highest resistance was observed against penicillin (91.94%) and 44.96 % of the isolates were methicillin resistant *S. aureus* (MRSA) for fluoroquinolone (61.74%), and chloramphenicol (94.85%). None of the isolates were resistant to vancomycin and teicoplanin. On a similar study by Adegoke and Komolafe (2009), 105 isolates of *S. aureus* were harvested. The resistance pattern to ampicillin, amoxicillin, cefotaxime, methicillin and cloxacillin were 68%, 54%, 25%, 50% and 20% respectively. The susceptibility of the bacterial isolates to antibiotics indicated 71.5%-85% sensitivity to ciprofloxacin and levofloxacin. This was also compatible to the study of Adegoke and Komolafe (2009). In next study by Akinjogunla and Enabulele (2010) 100% resistant rate obtained for oxacillin and 93.8% ampicillin and cephalexin.

The isolates were sensitive to the commonly used antibiotics, namely vancomycin and a floroquinolones. However other drugs such as erythromycin, and the amino-glycosides, cotrimoxazole and have been rendered practically useless as resistance has become increasingly common (Brumfitt and Hamilton-Miller, 1989). The higher prevalence of drug resistant isolates to such antibiotics suggests possible abuse of these drugs, poor hospital attendance, lack of public awareness and the need for better enlightenment campaign against the use of drug without prescription. In our study for the third generation cephalosporin, cefotaxime staphylococci showed 40.9% resistant. This result was less than our finding. Since parenteral drugs are not easily abused by individuals, the observation of higher prevalence of resistance can be attributed to abuse of antibiotics by illegal hospitals, medical centers or imprudent use of antibiotics by medical practitioners of various part of the country as the study area was a referral hospital of the country. Inadequate doses of antibiotics results moreover in emergence of resistant strains, when low doses of antibiotics are used against bacteria, they inhibit the growth of susceptible bacteria, leaving the smaller number of already resistant bacteria to thrive and grow. These bacteria spread their resistance traits to

other previously non-resistant cells then eventually affecting other cells (Craig 1998).

Although the sensitivity of the organism isolated to the chloramphenicol, glycopeptides and fluoroquinolone were generally excellent and that of third generation cephalosporin (cefotaxime) is considerable in the present study, the high cost of this group of drugs precludes their use as first choice in the treatment, usage policy that would be made applicable to the different tiers of our health care providers at the primary, secondary and tertiary levels. This can be done concurrently focusing attention on the dangers of high incidence of bacterial resistance to antibacterial agents in general and the ultimate consequences. The idea of vaccine against staphylococcal infection would be a most appreciated development (Yukiko *et al.,* 2006) since vaccines are not generally available for abuse. Since resistance to almost all antibiotics has observed, it is the time to embrace the use of local plant extract with proven therapeutic and prophylactic potency (Adegoke and Adebayo-tayo, 2009; Oloke *et al.,* 1988).

Out of total 205 *Staphylococcus* spp. isolated, the MDR incidence was found to be 98 (47.8%). In this, 48.9% and 45.6% of *S. aureus* and CONS were MDR respectively. The MDR pattern and staphylococcal isolation were statistically insignificant (p=0.054). MDR pattern was higher in male (51.1%) than female (45.2%). This outcome was comparable with the study by Mahmood *et al.* (2010) which showed the relative MDR predominance was observed in males 155 (58.5 %) cases, 110 (41.5 %) in female patients. MDR incidence was highest in age group 70-79 (66.7%). This was also comparable with the Mahmood *et al.* (2010) in which 170 (64.1%) MDR were in age group 41-80 year group. In a study done in Nepal, MDR MRSA was reported to be 40.1%. High rates of MDR MRSA reports could lead to the possibility of exploitation of vancomycin by clinicians. Only 100% sensitivity of MRSA to vancomycin suggests its prudent use and continuous monitoring of MIC levels should be done. Haphazard use of antibiotics bring a stage at which organism will be resistant to all antibiotics and we fall back into pre-antibiotic era. Glycopeptides seems to be the only antimicrobial agents that may be used as

the drug of choice to treat MDR MRSA infections. The high prevalence of MRSA and glycopeptide use, both thought to be risk factors for VRSA, make the widespread dissemination of these organisms an alarming and realistic possibility once it happens to emerge. So, glycopeptides must be kept reserved for life-threatening infections caused by MDR MRSA.

Our studies showed a 66.12% prevalence of MRSA in the tested clinical samples which was almost similar to that reported by Tiwari *et al.* (2009) in Bhairahawa with 69.1%. But in contrast to our result Rahman *et al.* (2002) reported 27%. Such high rates of MRSA have also been reported in India (Anupurba *et al*., 2003; Vidhani *et al.,* 2001). The prevalence of MRSA seems to be higher in Bangladesh, India and Nepal as compared to other parts of the world (Herwaldt and Wenzel, 1996; Mansouri 1997; Mulligan *et al.,* 1993; Udo *et al.,* 1993) except in Africa (Olukoya *et al.,* 1995). This might be due frequent and unnescssary prescription, self medication by people or incomplete dose in these countries. For correction of these practices national therapeutic guidelines must be forwarded and public awareness programs should be practiced via different means.

In this study more than 80% of isolates were resistant to two or more antibiotics. Most of them were resistant to β-lactam antibiotics. In a similar study of the multiple drug resistance among the isolates by Murugan *et al.* (2012) observed the resistance against the regularly used common fifteen antibiotics was very high. In addition, more number of isolates was being resistant to two or more antimicrobials. Probable major contributing factor may be the self-medication and attending medical care only at the advanced level. This also highlights the tendency of clinicians to prescribe antibiotics without pre-antibiotic sensitivity testing.

Out of total MDR, incidence in male was higher than female with 51.1% and 45.2% respectively. This gender wise prevalence was statistically insignificant. On Age wise distribution MDR incidence was higher in (70-79) year group. This might be due to the high dose of medication and difficulties in infection treatment in that group.

Out of 98 MDR isolated 58 (59.18%) were community acquired and 40 (40.82%) were hospital acquired. In a study by Tiwari *et al.* (2009), 37 (33.1%) were community acquired and 75 (66.9%) were hospital acquired. Inappropriate and haphazard medication among community people could be responsible for this outcome. More or less people in communities intake antibiotics by their own volition without any prescription by clinicians. This trend should be stopped by bringing strict rules so that no medicine will be used without prescription. This practice can reduce emergence of resistance and we can preserve some antibiotics for our next generation.

This study might provide a platform for physicians to choose and prescribe rational antibiotics in the treatment of MDR in hospital and community infections. This piece of work has demonstrated vividly the urgent need for management strategies designed for specific groups of patients with infections in order to maximize therapeutic benefits, cost reduction and possible reduction in the incidence of adverse drug reactions. If effective action is not putforth then there is no doubt that physicians will eventually need the next generation of novel agents to prevent and treat infection. As resistance to old antibiotics spreads, the development of new antimicrobial agents has to be expedited if the problem is to be contained. However, the past record of rapid, widespread and emergence of resistance to newly introduced antimicrobial agents indicates that even new families of antimicrobial agents will have a short life expectancy (Coates *et al.,* 2002).

At least one-third of all hospitalized patients receive a course of antimicrobial therapy during their hospital stay and studies have suggested that a large portion of this use is unnecessary or inappropriate. This pattern of use increases the cost of health care and contributes to the emergence and spread of resistant microorganisms within the healthcare environment. When a culture is performed, it often shows colonizing flora, which should not usually be treated. Antibiotic therapy should be given at the correct dose for an appropriate duration. An inadequate dose, duration, or both may make evolution of resistance in an infecting organism more likely. An excessive

duration of antibiotic consumption may result resistance development among colonizing flora in gastrointestinal tract. In interior and remote regions of Nepal where availability and use of antibiotics is limited, the prevalence of MRSA is low [Subedi and Brahmadath, 2005]. Hence, to preclude such situation, national, state and hospital level programs of surveillance and intervention must be strengthened to prevent the continued emergence of multi drug resistant pathogens and to limit their spread into other communities or other institutions (Mathai *et al.*, 2002; Sasidharan *et al*., 2011).

Higher MDR incidence was found in hospital wards like POST-OP, NSW, CTVS, NSICU and ENT. This high incidence might be due to the patients in those wards acquired infection from the hospital environment and equipments isolates. Hospital isolates generally grow and survive in high antibiotic pressure and hence they are more prone to be MDR.

Multidrug resistant *S. aureus* isolates were more in sputum and plural fluid. Blood isolated *S. aureus* were resistant to most of the antibiotics. Whereas MDR CONS was high in pus isolate followed by urine isolate with 76.9% and 45.5% incident respectively. None of the CONS isolated from blood were MDR. The high incidence of MDR in sputum and pus specimen may be due to the easy access of pathogen to injured area and respiratory tract compared to blood circulation.

Among 98, 46.3% of total *S. aureus* accounted for β-lactamase producing *S. aureus* and 51.61% of total CONS were accounted for β-lactamase producing CONS. In the various reports by authors, the burden of such strains has ranged from 23.2% to 63.6%. On a similar type of study by Ekrem and Meltem (2006) observed that out of 251 staphylococcal isolates 141 (55%) were β-lactamase. This result was congruent with our result. Similarly in the study of Pal and Marissa (2010) showed that out of 128 isolates 88.4% and 57.1% of *S. aureus* and CONS produced β-lactamase respectively. In a study by Hope *et al.* (2001) and Fritsche *et al.* (2005) showed 77.84% of the *S. aureus* and 61.75% of the CONS produced β-lactamase. This difference could be due to the result of variation in susceptibility patterns between different geographical

locations, or even in different communities in the same location. Our findings together with the results of previously conducted studies inside Nepal and neighboring country India suggest that the existence of β-lactamase positive *S. aureus* strains could pose a problem to practitioners in the treatment. These findings highlight the importance of local data on susceptibility patterns of antimicrobials.

Out of 98 MDR tested, 47 (48%) staphylococci were β-lactamase producers. Among them 46.3% and 51.6% respectively were *S. aureus* and CONS. This result was in agreement with the study by Akinjogunla and Enabulele, (2010**)** which showed 29 (34.5%) and 20 (47.6%) of *S. aureus* and CONS respectively. This β-lactamase positivity is comparable with the results showed that 14 (33.3%) *S. aureus* are β-lactamase producer, while only 9 (42.9%) of CONS produced β-lactamase (Akinjogunla and Enabulele, 2010).

In this study, β-lactamase producing staphylococci were distributed more in male (50%) than in female (48%). But in contrast to this Shrestha (2007) showed the incidence of β-lactamase producing *S. aureus* was greater among female (27.0%) than among male (17.0%). β-lactamase producers were 31% prevalent among age group 10-19 years which was followed by 60-69 years with 30% and 50-59 with 27.8%.

Out of 47 β-lactamase producing *S.aureus* 65.6%-100% was resistant to β-lactam antibiotics. But for β-lactamase negative isolates resistance pattern was 37.1%-67.6%. In a research by Oncel *et al.* (2004) of the tested *S. aureus* strains, 33.8 % (62 strains) were positive for β-lactamase production. In disk diffusion tests, β-lactamase positive strains showed high resistance against penicillin G and ampicillin. In contrast, the activitiy of β-lactam was higher against β-lactamase negative strains in comparison to β-lactamase positive strains. β-lactamase plasmids found in methicillin-resistant *S. aureus* may carry a gene or genes that regulate the production of both PBP 2' and β-lactamase. Thus, plasmid-mediated repressors of the genes that determine PBP 2' may result in heterogeneous resistance to β-lactam antibiotics by inhibiting production of PBP 2'. Conversely, β-lactamase-negative strains, lacking these

repressor genes, may produce PBP 2' constitutively (Boyce and Medeioer, 1987). But cloxacillin was highly resistant against both β-lactamase positive and negative strains. It might be due to its penicillinase stability and the resistance to this antibiotic is not governed by β-lactamase enzyme.

In a study by Pitkala *et al.* (2007) indicated that the sensitivities and specificities of the different tests vary for *S. aureus* and CONS. The β-Test MW980 strip, nitrocefin disks, sticks and Patho Proof Mastitis PCR assay were the only methods that did not produce any false-positive results.

Some researchers stated that the exposure of isolated bacteria to penicillin before testing for β-lactamase production resulted in higher β-lactamase rate. This indicates induction of β-lactamase by penicillin. As penicillin induction was not performed before β-lactamase testing in this study, the possible effect of these phenomena on the β-lactamase production of isolates is not yet known. The role of β-lactamases in the resistance development of *S. aureus* against lactam antibiotics has been described (Byongkyu *et al.,* 1995; Nazer and Tavakoli, 1994; Oliviera 2000; Watts and Salmon, 1997).

Finally, our study also compared the efficacy of cloxacillin by three different manufacturing companies, HI-media, Oxoid and MAST companies. Among three staphylococci showed resistivity of 56.25% for Oxoid and MAST companies. But for HI-media they showed 97.13% resistance. This was too much high compared to the Oxiod and MAST. This indicates the cloxacillin disc by HI-media was inappropriate to use for antibiotic susceptibility pattern as this may lead false interpretation to physicians.

CHAPTER-VI
CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

The present study was done on 205 clinical isolates of *Staphylococcus* species (*S. aureus* and CONS), which were studied, based on various biochemical tests and antibiogram with special reference to β-lactamase test. Staphylococcal infections are prevalent in various communities and hospital environments as opportunistic pathogens. Staphylococci are known to have a remarkable genetic versatility which allows for adaptation to the presence of antibiotics, such that many strains can be multi drug resistant to several classes of drugs. The results concluded that multiple drug resistant *S. aureus* and CONS are commonly present among diseased and there is the need for surveillance in order to monitor antimicrobial resistance pattern. These results also concluded that *Staphylococcus* spp. isolated from patients with infection should be considered as a possible etiological agent of the infection. In light of these findings, diagnostic medical microbiology laboratories should perform antibiotic susceptibility tests in addition to tests for the β-lactamase production.

6.2 RECOMMENDATION

i. The practice of empiric therapy when an infectious syndrome has been identified should be discouraged, only pathogen directed antibiotic therapy should be practiced.
ii. Hospital should maintain clean and hygienic environment.
iii. Staphylococci are highly susceptible to vancomycin and nitrofurantoin so such antibiotics should not be abused and kept for serious and life threatening staphylococcal infection.
iv. Self medication, inadequate or excessive medication should be discouraged.
v. Monitering of MDR and public awareness program in antibiotic usage must be conducted.

vi. Unecessary prescription of β-lactam antibiotics can be preventd by β-lactamase test as it is rapid test it can be helpful to determine sensitivity to β-lactam prior to routine sensitivity test.

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