ANTIBIOTIC SUSCEPTIBILITY PATTERN OF BACTERIAL ISOLATES FROM WOUND INFECTION IN PATIENT VISITING A TERTIARY CARE HOSPITAL, BIRATNAGAR, NEPAL

A

Dissertation Submitted to the Department of Microbiology, Central Campus of Technology, Tribhuvan University, Dharan, Nepal, in Partial Fulfillment of the Requirements for the Award of Degree of Master of Science in Microbiology (Medical).

By:
Amit Bhattarai
Department of Microbiology
Central Campus of Technology, Dharan Nepal
Roll. No: 25673
Reg. No: 5-2-8-142-2008
2018

RECOMMENDATION

This is to certify that **AMIT BHATTARAI** has completed this dissertation work entitled "**ANTIBIOTIC SUSCEPTIBILITY PATTERN OF BACTERIAL ISOLATES FROM WOUND INFECTION IN PATIENTS VISITING A TERTIARY CARE HOSPITAL, BIRATNAGAR, NEPAL**" as a partial fulfillment of the requirements of M.Sc. degree in Microbiology (Medical) under my supervision. To our knowledge, this work has not been for any other degree.

.....
Mr. Prince Subba
(Supervisor)
Teaching Assistant
Department of Microbiology
Central Campus of Technology
Tribhuvan University
Dharan

.....
Mr. Hemanta Khanal
(Supervisor)
Assistant Professor
Department of Microbiology
Central Campus of Technology
Tribhuvan University
Dharan

Date: .....

CERTIFICATE OF APPROVAL

On the recommendation of **Mr. Hemanta Khanal** and **Mr.Prince Subba** this dissertation work of **Mr. Amit Bhattarai** entitled "**ANTIBIOTIC SUSCEPTIBILITY PATTERN OF BACTERIAL ISOLATES FROM WOUND INFECTION IN PATIENTS VISITING A TERTIARY CARE HOSPITAL, BIRATNAGAR, NEPAL**" has been approved for the examination and is submitted to the Tribhuvan University in Partial fulfillment of the requirements for M.Sc. degree in Microbiology (Medical).

.....
Mr. Shiv Nandan Sah
Assitant Professor
Head of Department
Department of Microbiology
Central Campus of Technology,
Tribhuvan University,
Dharan

.....
Mr. Hemanta Khanal
Assitant Professor
M. Sc. Microbiology Coordinator
Department of Microbiology
Central Campus of Technology,
Tribhuvan University,
Dharan

Date:

BOARD OF EXAMINER

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Date of submission: **Amit Bhattarai**

ABSTRACT

Infection due to antibiotic resistant bacteria creates more alarming situation in both develop and developing country. Wound infection responsible for significant human mortality and morbidity worldwide. Present study was carried out in Nobel medical college and teaching Hospital Biratnagar. Aim of the study was to know about the different causative microorganism of wound infection and their antibiotic susceptibility pattern. Out of total 335 sample 225 sample were found positive and 110 sample were found negative Out of total 225 positive samples 115 (50.67%) were found to be gram positive and 109 (48.44%) were found to be gram negative and single *candida spp*. In total 225 positive samples *Staphylococcus aureus* was found as predominant occurring total 114 (50.67%) and then respectively followed by *Escherichia coli* total 71 (31.56%), *Enterococcus faecalis* 15 (6.67%), *pseudomonas aerogenosa* 13 (5.78%), *Enterobacter aerogenes* 4 (1.78%), *proteus vulgaris* 2 (0.89%), *Acenetobacter anitratus* 2(0.89%), *Klebsella oxytoca* 1 (0.44%), *Klebsella pneumonia*e 1 (0.44%), *diptheroids* 1 (0.44%) and *candida* 1 (0.44%). The diversity of isolated bacteria and their susceptibility patterns signify a need to implement a proper infection control strategy, which can be achieved by carrying out antibiotic sensitivity tests of the isolates.

Key words: Antibiotic Resistant, Morbidity, Mortality, Antibiotic sensitivity test.

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ABBREVIATION

ATCC	American Type Culture Collection
BA	Blood Agar
CFU	Colony Forming Unit
CONS	Coagulase Negative *Staphylococci*
CRP	C-reactive protein
GNB	Gram negative bacilli
GPC	Gram positive cocci
ICU	Intensive Care Unit
MA	Mac Conkey Agar
MHA	Mueller Hinton Agar
MRSA	Methicillin Resistant *Staphylococcus aureus*
MR/VP	Methyl Red /Voges Proskauer
NA	Nutrient Agar
NB	Nutrient Broth
NCCLS	National Committee for Clinical Laboratory Standard
PMNs	Polymorphonuclear Leukocytes
SSI	Surgical Site Infection
TSIA	Triple Sugar Iron Agar
WHO	World Health Organization
CDC	U.S. Centre for disease control and Prevention

CHAPTER- I

INTRODUCTION

1.1 Background

Wound is a breach in the skin, and exposure of subcutaneous tissue following loss of skin integrity providing a moist, warm and nutritive environment that is conducive for colonization and proliferation of opportunistic and pathogenic microorganisms (Bowler et al). Wound can be classified into two types, mainly open and closed wound (Alexander et al). Open wounds include incisions, lacerations puncture wounds, gunshot wounds and abrasions. Closed wounds include contusions more commonly known as bruises; hematomas crush injury (Alexander et al). Most times contaminating microbes are eliminated by the host immune system and do not persist, but species that grow and divide may become established, causing wound colonization and infection(Motayo B. O. et al) .When infectious bacteria are invading a host, toxic substances are produced by the microorganisms that cause damage to the host tissues. These substances are called virulence factors and allow the bacteria to establish in the host. The host responds to the bacterial invasion with attack of inflammatory cells such as neutrophils which release cytotoxic enzymes, oxygen radicals and inflammatory mediators which cause further damage to host tissue. This host response mechanism is also contributing to the nonhealing stage of the infected wound. (Bjarnsholt et al 2006). One of the most important strategies to keep the process of healing ongoing is to sterilize damaged tissue from any microbial infection (Al-Waili NS et al). Continued use of systemic and topical antimicrobial agents has provided the selective pressure that has led to the emergence of antibiotic resistant strains which in turn, has driven the continued search for new agents. Unfortunately, the increased cost of searching for effective antimicrobial agents and the decreased rate of new drug discovery has made the situation increasingly worrisome (Cooper RA et al).

Wound healing needs a good healthy environment so that the normal physiological process will result in a normal healing process with minimal

scar formation. One of the most important strategies to keep the process of healing ongoing is to sterilize damaged tissue from any microbial infection .However, the abundance and diversity of microorganisms in any wound will be influenced by factors such as wound type, depth, location, and quality, the level of tissue perfusion, and the antimicrobial efficacy of the host immune response. Wound can be infected by a variety of microorganisms ranging from bacteria to fungi and parasites as well as virus (Church et al. 2006). The most common organisms are *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Klebsiella* species and *Acinetobacter* species (Gupta et al 2002; Eselbelahie et al 2013).

The prevalent organisms that have been associated with wound infection include *Staphylococcus aureus* (*S. aureus*) which from various studies have been found to account for 20-40% and *Pseudomonas aeruginosa* (*P. aeruginosa*) 5-15% of the nosocomial infection, with infection mainly following surgery and burns. Other pathogens such as Enterococci and members of the Enterobacteriaceae have been implicated, especially in immune compromised patients and following abdominal surgery (Taiwo S et al 2002). The control of wound infections has become more challenging due to widespread bacterial resistance to antibiotics, and to a greater incidence of infections caused by methicillin-resistance *S. aureus,*, polymicrobic flora (Shittu et al. 2002).

Antibiotics, although, have been of great value in treatment and in prophylaxis to prevent infections, the timing of administration, choice of antimicrobial agent, durations of administration have clearly defined the value of antibiotics in reducing wound infections.

Advance in control of infection have not completely eradicated the problem of the wound infection because of development of drug resistant. Wound infection results in sepsis, limb loss, long hospital stays, higher costs and is responsible for significant human mortality and morbidity worldwide (Taiwo S et al). It is one of the most common hospital acquired infections (Gottrup et al 2005; Wilson et al 2004). It remains an ongoing problem which although,

cannot be completely eradicated however, by taking prompt control measures against the most commonly isolated organism and proper care of wound may lead to the minimum of wound infection (Mahat et al).

Wound infection has been regarded as the most common nosocomial infection especially in patients undergoing surgery (Dionigi et al 2001). It was found that wound infections remain a major source of postoperative morbidity. accounting for about a quarter of the total number of nosocomial infection. Surgical wound infection comprise 12% to 24% 0f all nosocomial infection and are the 3rd most commonly reported nosocomial infection (Trumbore and Kaye, 1984). The risk of surgical wound infection is based on the susceptibility of a surgical wound to microbial contamination (Raahave et al 1986). Surgical site infections (SSIs) are real risks associated with any surgical procedure and represent a significant burden contributing to morbidity and mortality, and increased cost to health services around the world (National Audit Office, 2000).

Postoperative wound infections caused more commonly by Gram negative bacilli and predominant pathogens involved were *E. coli*, followed by *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Proteus vulgaris* and *Staphylococcus epidermidis*. All isolates were sensitive to Imipenem and Chloramphenicol. A high degree of multidrug resistance was observed with *Pseudomonas aeruginosa* and *Proteus vulgaris* (Reddy KR et al).Continued use of systemic and topical antimicrobial agents has provided the selective pressure that has led to the emergence of antibiotic resistant strains which in turn, has driven the continued search for new agents. Unfortunately, the increased costs of searching for effective antimicrobial agents and the decreased rate of new drug discovery have made the situation increasingly worrisome. The control of wound infections has become more challenging due to widespread bacterial resistance to antibiotics, and to greater incidence of infection caused by methicillin-resistance *S. aureus,* polymicrobic flora (Shittu et al 2002).

In developing countries like Nepal, wound infection is very common due to the socioeconomic condition of people, illiteracy, lack of well managed

hospital, equipment, their knowledge on hygiene and sanitation. Specifically the major factors associated with burn injuries among children are low socio-economic status, inadequate time of mother's for their children and lack of proper attention to the children by the caregivers. People generally sick for medical services only after the development of chronic wound infection which leads to serious complication. Neonates and elderly patients, obese individuals, severe malnutrition, diabetic patients and burn patients are vulnerable to the wound infection (Wilson and Treasure, 1990) .Also unsterile surgical instruments, cotton unsterile hands of medical personnel also increase the incidence of wound infection. Hence the importance of wound infection in both economic and human terms should not be underestimated. A large number of complications arise as a result of poor initial management of wound. In this context, the present study was carried out to know about the incidence of wound infection among patient visiting at Nobel medical teaching Hospital, which is the leading and referral hospital of eastern Nepal. The present study` was also focused to know the pattern of bacterial isolates from infected wound and their antimicrobial susceptibility pattern. Therefore the acquired data of the causative agents of infected wounds has proved to be helpful in the selection of empiric antimicrobial therapy and infection control measures in Nobel medical Teaching Hospital. The next goal of this study was to isolate the bacteria from infected wound at different wards of the hospital.

1.2 Objectives

1.2.1 General Objectives

To identify the bacteriological Spectrum of Wound infection and its antibiotic sensitivity Profile among the patients visiting Nobel Medical Collage and Teaching Hospital.

1.2.2 Specific Objectives

i. To determine the prevalence of wound infection taken from different age group and gender.
ii. To isolate and identify the bacterial Pathogen of infected wound from pus and swab samples.
iii. To study the distribution pattern of organisms in different wards of Nobel Medical Collage and Teaching Hospital.
iv. To determine the antibiotic susceptibility pattern of bacterial isolates.

CHAPTER II

LITERATURE REVIEW

2.1 Definition of Wound infection

Wound is a breach in the skin and the exposure of subcutaneous tissue following loss of skin integrity provides a moist, warm and nutritive environment that is conductive to microbial colonization and proliferation (Bowler et al 2001). Exposure of subcutaneous tissue following a loss of skin integrity (i.e. wound) provides a moist, warm, and nutritious environment that is conducive to microbial colonization and proliferation. Since wound colonization most frequently poly-microbial, involving numerous microorganisms that are potentially pathogenic, any wound is at some risk of becoming infected. In general, a wound can be considered infected if purulent material drains from it, even without the confirmation of a positive culture. Wound results in a variety of cellular and molecular sequel. A wound may be caused by an act, such as a gunshot, fall, or surgical procedure; by an infectious disease; or by an underlying condition.

Wound infection is defined as the invasion of organisms through tissues following a breakdown of local and systemic host defenses (Russel et al 2000). According to Robson (1997), wound infection results when bacteria endogenous to the patient or exogenous to the wound achieve dominance over the systemic and local factors of host resistance. The development of a wound infection depends on the complex interplay of many factors. If the integrity and protective function of the skin is breached, large quantities of different cell types will enter the wound and initiate an inflammatory response (Collier et al 1998). This may be characterized by the classic signs of redness, pain, swelling, raised temperature and fever. Generally wound infection is defined as the discharge of pus which is a thick whitish fluid that formed in areas of infection.

Infection of wound and other lesions are liable to contamination with a multiplicity of organisms from the body surface and the environment. There is

plentiful supply of moisture and nutrients in the physical environment and the temperature gaseous requirements are ideal for microbial growth (Edward and Greenwood, 2003). Infection occurs when one or more of the contaminants evades the clearing effect of the host's defense, replicates in large numbers and attacks and harms the host's tissue (Collee et al 1999). The development of an infection will be influenced largely by the virulence of the organism and immunological status of the patient; for example, patients considered most at risk are those being treated with long-term steroids and those receiving chemotherapy (Collier et al 1998). Virulence describes both the pathogenicity and invasiveness of the relevant microorganism.

Surgical wound infections are the second most common cause of nosocomial infections (Motayo B. O. et al, Bowler et al). The high rate of surgical wound infections is associated with higher morbidity, mortality and increased medical expenses (Bowler et al, Alexander M.F et al). In spite of the new antibiotics available today, surgical wound infection still remains a threat due to secondary bacterial contamination and widespread use of prophylactic antibiotics that lead to emergence of multi-drug resistant bacteria (Bowler et al). There are a number of ways microorganisms can get into wounds.

- Direct contact – transfer from surgical equipment or the hands of the surgeons or nurses
- Airborne dispersal – surrounding air contaminated with micro-organisms that deposit onto the wound
- Self-contamination – physical migration of the patient's own endogenous flora which are present on the skin, mucous membranes or gastrointestinal tract to the surgical site.

2.2 Signs of wound infection

Signs of wound infection are closely associated with the wound type. Generally purulent discharge and spreading erythema are recognized as diagnostic. However these features are not always present in the early stages when diagnosis is important for treatment and the avoidance of complicating

sequel (Cutting and Richard, 2005).The signs of wound infection have been described by some authors as:

- Pus or cloudy fluid draining from the wound
- Pimple or yellow crust formed on the wound
- Scab has increased in size
- Increasing redness around the wound
- Red streak is spreading from the wound toward the heart
- Wound has become extremely tender
- Pain or swelling increasing after 48 hours since the wound occurred
- Wound has developed blisters or black dead tissue
- Lymph node draining that area of skin may become large and tender
- Onset of widespread bright red sunburn-like rash
- Onset of fever
- Wound hasn't healed within 10 days after the injury

2.3 Wound colonization

Wound colonization is most frequently polymicrobial involving microorganisms that are potentially pathogenic; any wound is at some risk of becoming infected. Chamberlain (2004) defines wound colonization as the presence of replicating microorganism's adherent to the wound in the absence of injury to the host. Polymicrobial colonization and the presence of antibiotic resistant bacteria may impede the healing of delayed closure surgical wounds, pressure ulcers and diabetic foot ulcers (Motta et al 2004).

2.4 Wound contamination

Collier et al 1998 defines wound contamination as the presence of bacteria within a wound without any host reaction. According to Chamberlain (2004) wound contamination is the presence of non-replicating microorganisms in wound. Wound contamination, as shown by intra-operative culture, is associated with later wound infection. Garibaldi et al (1991) found that 30 or more colony forming units (CFU) of bacteria cultured from a wound are

predictive of wound infection, regardless of wound class. Heavy microbial contamination has a negative effect on wound healing.

2.5 Wound types

There are a number of classifications of wound related to their position, their depth and the amount of tissue damage. Russell et al (2000) give the general classification of wounds as:

i) Major wound: It is defined as a wound which discharges pus and may need a secondary procedure to be sure of adequate drainage. There may be systemic signs of tachycardia, pyrexia and a raised white cell count.

ii) Minor wound: It may discharge pus or infected serous fluid but should not be associated with excessive discomfort, systemic signs or delay in returning home.

Wounds can be broadly categorized as having either an acute or chronic etiology.

2.5.1 Acute wounds

Acute wounds are caused by external damage to intact skin and include surgical wounds, bites, minor cuts and abrasions, and more severe traumatic wounds such as lacerations and those caused by crush or gunshot injuries (Davis et al 1992). According to Bowler et al (2001), acute wounds are expected to heal within a predictable time frame, although the treatment required to facilitate healing will vary according to the type, site, and depth of a wound. Wounds may also be referred to as open, in which the skin has been compromised and underlying tissues are exposed, or closed, in which the skin hasn't been compromised, but trauma to underlying structures has occurred.

2.5.1.1 Closed wounds

Closed wounds result from blunt trauma and usual causes are falls, sporting injuries with a blunt weapon. A blunt injury may result in a bruise or contusion and there is danger of secondary infection (Russell et al 2000).

a) Contusion: These are the result of a forceful trauma that injures an internal structure without breaking the skin. The skin surface remains intact but small blood vessels may be injured or torn and bleeding may discolor the skin producing a bruise. Blows to the chest, abdomen, or head with a blunt instrument (e.g. a football or a fist) can cause contusions.

b) Hematoma: This result from rather more severe injury, particularly to the vessels, allowing the escape of larger volumes of blood which collect in the tissue or tissue planes. A subcutaneous hematoma may become infected, particularly if the overlying skin is damaged, and the resulting abscess will require incision.

c) Crushing injuries: These are caused by a great or extreme amount of force applied over a long period of time.

2.5.1.2 Open wounds

Open wounds can be classified according to the object that caused the wound. The types of open wound are:

a) Abrasion and friction burn: An abrasion is a shearing injury of skin in which the surface is rubbed off. Abrasion occurs when the skin is rubbed away by friction against another rough surface. A friction burn is similar but there will be an element of thermal damage as well as abrasion (Russell et al 2000).

b) Bites: Animal bite infections develop in humans when an animal's teeth break the skin and introduce saliva containing disease organisms below the skin surface. The saliva of dogs, cats, ferrets, and rabbits is known to contain a wide variety of bacteria. These microorganisms may grow within the wound and cause an infection. Due to complex nature of the oral micro flora in humans and animals, the majority of bite wounds harbor potential pathogens, many of which are anaerobes as well as the common anaerobes in both human and animal bite wounds, such as *Bacteroides, Prevotella, Porphyromonas* and *Peptosterptococcus spp*, less common potential pathogens such as *Pasteurella multocida* and *Eikenella corrodens* may also be involved (Bowler et al 2001).

c) Laceration: A laceration or cut is the result of contact with a sharp object (the surgical equivalent is an incised wound). Frequently these wounds are grossly contaminated by clothing material or dirt forced into the tissues at the time of injury.

d) Puncture wounds: They are deep, narrow wounds produced by sharp objects such as nails, knives, and broken glass. A puncture wound is an open injury in which foreign material and organisms are likely to be carried deeply into the underlying tissue (Pintu and Ahmed, 2001).

e) Traction and avulsion: Avulsion injuries are open injuries where there has been a severe degree of tissue damage producing a deglazing injury. Deglazing is caused by shearing forces that separate tissue planes, rupturing their vascular interconnection and causing tissue ischemia.

f) Gunshot injuries: Gunshot injuries are caused by a bullet or similar projectile driving into or through the body. There may be two wounds, one at the site of entry and one at the site of exit. Low velocity injuries such as from a hand gun result in an entry and exit wound, the latter being the larger, and damage along the tract of the missiles. High velocity injuries cause exposure pressure and decompression effect such that there is widespread tissue damage.

2.5.2 Chronic wounds

Chronic wounds are mostly caused by endogenous mechanisms associated with a predisposing condition that ultimately compromise the integrity of dermal and epidermal tissue (Davis et al 1992). Pathophysiological abnormalities that may predispose to the formation of chronic wounds include compromised tissue perfusion as a consequence of impaired arterial supply or impaired venous drainage and metabolic disease such as diabetes mellitus (Bowler et al 2001).

2.6 Classification of wound infection

2.6.1 Superficial wound infection

These infections mainly involve the skin and can vary in severity from barely noticeable (e.g. erythrism) to life threatening (e.g. secondary infection of burn).The outcome of the resulting infection depends upon the characteristics of the organisms those of the host and the effectiveness of therapy (Rytel and Mogabgab, 1984). According to Rytel and Mogabgab (1984), the superficial infections are of three types.

2.6.1.1 Primary cutaneous infection

I. Superficial fungal infection

The causative organisms are filamentous fungi of genera *Trichophyton, Epidemophyton* and *Microsporum*. Superficial candidiosis produced by *Candida albicans*

II. Streptococcal skin infection

A) Impetigo: Impetigo is the most common infection in children together with folliculitis. Chakraborty (1995) defined impetigo as a superficial discrete crusted spot, especially in children, usually less than one inch in diameter. The etiological agent is usually Group A Streptococci and in some cases it may be caused by *S. aureus.* Patients report skin lesions, often with associated adenopathy, but have minimal systemic signs and symptoms. Impetigo may present in two forms: small vesicles with a honey-colored crust or purulent-appearing bullae (O'Dell, 1998)

b) Erysipelas: Erysipelas is a serious cutaneous streptococcal infection that is characterized by an advancing raised border sharply demarcated from the normal skin. The erythrotoxins produced by the *Streptococcus* make the infected area red, hot, tender, and edematous. Edema of the reddened skin gives the involved area raised border diagnostic clinical appearance.

c) Cellulitis: Cellulitis is characterized by the acute localized inflammation and edema with pain or sensitivity without a well-defined border. Cellulitis

may be caused by a variety of organisms, including group A *streptococci*, *S. aureus, Haemophilus influenzae* and, in coastal areas, halophilic *Vibrio* species. Cellulitis is a relatively deep infection, generally resulting from a break in the skin and involves subcutaneous spaces in addition to the dermis (O'Dell, 1998).

III. Staphylococcal skin infection

a) Staphylococcal carriage: *S. aureus* has its primary reservoir in the anterior nares and the perineum of healthy human carrier. Staphylococcal carriers are generally asymptomatic and immunological evaluation dose reveals any defects.

b) Staphylococcal cellulitis: Staphylococci may also produce deeper cellulitis with lymphangitis similar to that produced by Streptococci.

c) Abscesses: Abscess is a localized collection of pus and contains live and dead PMNs, lymphocytes and macrophages, as well as bacteria and damaged tissue (Mclatchie and Leaper, 2002). An abscess is the last stage of a tissue infection that begins with a process called inflammation which is characterized by heat, swelling, redness, and pain (Bennett et al 1993). Many different agents cause abscesses. The most common are the pyogenic or pus-forming bacteria, such as *Staphylococcus aureus*, which is nearly always the cause of abscesses directly under the skin. Abscesses are usually caused by organisms that normally inhabit nearby structures or that infect them. Shenoy (2001) classifies abscess into three types; Pyogenic, Pyretic and Cold abscess.

d) Boil/ Furuncle: It is a hair follicle infection caused by *S. aureus*. It starts with painful indurated swelling with surrounding edema. After 1-2 days, softening occurs in the center and pustules develop which bursts spontaneously discharging pus (Shenoy, 2001). Furuncles, or boils, are more aggressive forms of folliculitis. Patients present with a painful, often fluctuant swelling in a non-weight bearing area, most commonly areas of friction, the nasal area or the external ear.

e) Carbuncle: A carbuncle is a deep-seated mass of fistulous tracts between infected hair follicles. Carbuncles are more extensive infection that develops

when the organisms extend along the tissue plan and have many sources. It usually occurs at the nape of neck, back and shoulder region. .The lesions have many interconnecting sinuses and tend to recur despite drainage and antibiotics. Surgical drainage and resection of the lesions is often necessary.

f) Miscellaneous primary infection: Several other types of bacteria can also cause cutaneous infection. For e.g. Pseudomonas can produce hot-tub folliculitis. It is generally a self-limited condition, although the infection can progress to a serious illness in immune-compromised persons. It occurs when patients bathe in poorly maintained hot tubs (O'Dell, 1998).

2.6.1.2 Secondary infection

I. Infection from bites

Infection from bites is the major risk due to mixed mouth organisms being deeply implanted into the tissues. According to one study, bacteria or other pathogens show up in about 85 percent of animal bites. These microorganisms may grow within the wound and cause an infection. The consequences of infection from these bites range from mild discomfort to life-threatening complications. Animal bites, particularly dog, cat or primate bites, may result in serious infection. These bites allow entry of *Pasteurella multocida, Eikenella corrodens*, *Bacteroides* species or other organisms into subcutaneous tissue, potentially resulting in rapidly spreading and destructive cellulitis (O'Dell, 1998). Brook (1987) reported that 74% of 39 human and animals bite wounds contained a polymicrobial aerobic-anaerobic micro flora, with *S. aureus, Peptostreptococcus spp,* and *Bacteriodes* spp being the predominant isolates in both wound types. Goldstein et al (1989) studied bacteria involved in infection of bite wounds and observed *S. aureus, Pasteurella multocida, S. intermedus*, alpha hemolytic streptococci, *Capnocytophaga canimorsus* and other members of oral flora.

II. Infection from burn

According to the International Society for Burn Injuries, a burn or thermal injury of the skin occurs when some or all the different layers of cells in the skin are destroyed by a hot liquid (scalds), a hot solid (contact burns), or a

flame (flame burns). Burn injuries are the most devastating of all childhood injuries and have the potential to cause death, lifelong disfigurement and dysfunction (Morgan et al 2000).

Loss of functional skin barrier after thermal injury results in increase susceptibility to infection, which is the major cause of morbidity and mortality following burn. The burn wound surface (in deep partial-thickness and in all full-thickness burns) is a protein-rich environment consisting of avascular necrotic tissue (Escher) that provides a favorable niche for microbial colonization and proliferation (Nasser et al 2003). Many studies have reported that the prevalence of aerobes such as *S. aureus, Pseudomonas aerogenosa, E. coli, Klebsiella spp, Enterococcus spp and Candida spp* (Vindenes and Bjerknes, 1995). Ones were *Peptostreptococcus spp, Bacteroides spp* and *Propionibacterium acnes* (Brook and Randolph, 1981). It is estimated that up to 75% of death following burn injury are related to infection (Robson, 1997). The risk of burn wound infection is directly correlated to the extent of the burn and is related to impaired resistance resulting from disruption of the skin's mechanical integrity and generalized immune suppression (Schwarz and Dulchavsky, 2005).

III. Leg and decubitus (pressure) ulcer infection

Decubitus ulcers develop as a consequence of continued skin pressure over bony prominences; they lead to skin erosion, local tissue ischemia, and necrosis, and those in the sacral region are particularly susceptible to fecal contamination. *S. aureus*, *Peptostreptococcus spp*., *Bacteroides spp*. and *P. aeruginosa* were the predominant isolates. Septicemia occurs quite frequently from severely infected decubitus ulcers. Osteomyelitis of underlying bones can develop and gas gangrene can result (Rytel and Mogabgab, 1984).

IV. Acute soft tissue infection

Infections of skin and soft tissue are common in community and hospital settings. Acute soft tissue infections include cutaneous abscesses, traumatic wounds and necrotizing infection. Microbiological investigations have shown

that *S. aureus* is the single causative bacterium in approximately 25 to 30% of cutaneous abscesses (Brook and Finegold, 1981), and the same organism has also been recognized as being the most frequent isolate in superficial infections seen in hospital Accident and Emergency Departments (Page and Bohnen, 1993).

However, other studies have demonstrated that approximately 30 to 50% of cutaneous abscesses, 50% of traumatic injuries of varied etiology and 47% of necrotizing soft tissue infections have a polymicrobial aerobic and anaerobic micro flora (Bowler et al 2001). Necrotizing fasciitis is a significant and life-threatening illness which most commonly develops in episiotomy sites or abdominal incision sites (O'Dell, 1998).

V. Infection of surgical wound

Surgical wound infection is a common postoperative complication and causes significant postoperative morbidity and mortality, prolongs hospital stay, and adds between 10-20% to hospital costs (Haley et al 1985). It has been estimated that each patient with a surgical site infection will require an additional 6.5 days in hospital, which results in the doubling of hospital costs associated with that patient (Collier, 2002). A wound infection is defined by the US Centre for Disease Control and Prevention (CDC) as surgical site infection (SSI). This is further defined as:

- Superficial incisional SSI – infection involves only skin and subcutaneous tissue of incision.
- Deep incisional SSI – infection involves deep tissues, such as facial and muscle layers.
- Organ/space SSI – infection involves any part of the anatomy in organs and spaces other than the incision, which was opened or manipulated during the operation.

A surgical wound/site infection is defined by the following criteria:

- Infection must occur within 30 days of the surgical operation.
- And at least one of the following is present:

- Purulent discharge from the surgical site
- Purulent discharge from wound or drain placed in wound
- Organisms isolated from aseptically obtained wound culture

At least one of the signs and symptoms of infection pain or tenderness, localized swelling, or redness/heat must be present.

Other signs of wound infection include:

- Delayed healing not previously anticipated.
- Discolorations of tissues both within and at the wound margins.
- Abnormal smell coming from wound site.
- Friable, bleeding granulation tissue despite appropriate care and management.
- Lymphadenitis, a red line originating from the wound and leading to swollen tender lymph glands draining the affected area.

In 1992 The Surgical Wound Infection Task Force replaced the term „surgical wound infection' with „surgical site infection, to include infection of organs or spaces deep in the skin and soft tissues, such as peritoneum and bone. These infections complicate illness, anxiety, increase patient discomfort and can lead to death. Surgical site infections are the third most commonly reported nosocomial infection and they account for approximately a quarter of all nosocomial infections (Mangram et al 1999). Surgical site infection has varied from a low of 2.5% to a high of 41.9% (Berard and Gandon, 1996). The rate of infection varies depending on the type of surgery undertaken. According to Bowler et al (2001), the risk of infection generally is based on the susceptibility of a surgical wound to microbial contamination. Clean surgery carries 1-5% risk of postoperative wound infection, and in dirty procedures that are significantly more susceptibility to endogenous contamination, a 27% risk of infection has been estimated.

2.6.1.3 Cutaneous manifestation of systematic infection

Several bacteraemia can produce cutaneous manifestation of systemic infections. *S. aureus* bacteraemia may result in postural skin lesions,

sometimes surrounded by a purpuric area. Certain systemic fungal infections are associated with cutaneous lesions.

2.6.3 Deep wound

They are associated with visceral damage which include subcutaneous and sub mucous abscesses example; breast abscess, abdominal abscess etc. (Cruickshank et al 1974)

2.7 Origin of wound infection

Infection of a wound occurs when one or more of the contaminants evades the clearing effect of the host's defense, replicates in large numbers and attacks the host's tissue (Collee et al 1999). Wounds acquire infection through the exogenous or endogenous route.

a) Exogenous infection

This infection is due to some microbial species mainly or exclusively from source outside the body of the person becoming infected. Hospital acquired infection are mostly exogenous, from other people or immediate objects in the environment (Chakraborty, 1995). Exogenous source for contamination may come from the hospital environment or from any healthcare personnel. Stokes et al (1993) stated that airborne contamination is still an important factor in wound infection in operating room as well as in post-operative care even if standard ventilation equipment is used. Various types of equipments come to vicinity of the wound time to time, bacteria laden particles may fall from them into the wound. Epidemics due to *S. aureus* and group A streptococci suggest personnel carriers as a source.

b) Endogenous infection

Endogenous infection is caused by the patients' own floras which are non-pathogenic under normal condition (Chakraborty, 1995). Source of contamination include the gastrointestinal and genitourinary tract, sites of active infection remote from the wound, the skin and anterior nares, According to Walter and Israel (1979), the normal flora of various body

surfaces are not pathogenic in their normal habit but may behave as pathogens if they escape or are implanted elsewhere. For e.g. abdominal surgical wound may become infected with organisms from large bowel after an operation, involving incision of colon. Thus, Cruickshank et al (1974) has stated that the source of endogenous infection is the site of the patients' body where organisms grow harmlessly as commensals.

2.8 Pathophysiology of wound infection

Bowler et al (2001) stated that infection occurs when virulence factors expressed by one or more microorganisms in a wound out-compete the host natural immune system and subsequent invasion and dissemination of microorganism in viable tissue provokes a series of local and systemic host responses. In order to cause infection, a pathogen must accomplish the following.

a) Entry of pathogen into the host: The most frequent portals of entry of pathogens into the body are the sites where mucous membrane meets with the skin. Abnormal areas of mucous membrane and skin (cuts, burns, insect's bite, accidental wounds, surgical incision) are also the frequent sites of infection. Normal skin provides the primary defense against infection (Brook and Frazier, 2000). At the point of entry, usually at small breaks or lesions in the skin or in mucosal surface, growth is often established in the sub mucosa (Madigan et al 2000).

b) Spread and multiplication: The term spread has two shades of meanings. It suggests direct, lateral propagation of organism from original site of entry, but it can also refer to dissemination to distant sites. If the pathogen gains access to tissues, it may multiply, a process called colonization. Cellular damage to the skin and soft tissues may be mediated by toxins, degrades enzymes and the induction of the host cellular response that destroy tissues usually by immune mediated mechanisms (Schaechter et al 1989).

c) Host defenses: When microorganisms cross the protective epidermis of skin, it encountered defense mechanisms that are constitutive, in the sense that they do not require previous contact with the invading microorganisms.

Inflammatory response is a protective mechanism that aims to neutralize and destroy any toxic agents at the site of an injury and restore tissue homeostasis (Collier et al 1998). This characteristic response results in redness, swelling, pain and heat which are localized at the site of infection (Madigan et al 2000).when neutrophils die, they release powerful hydrolases from their lysosomal granules. These enzymes damage surrounding tissues, extending the lesion to adjacent areas (Schaechter et al 1989).

2.9 Fate of wound infection

- Spontaneous resolution
- Wound sepsis
- Abscess formation
- Wound rupture
- Septicemia and pyemia
- Metastatic abscess formation
- Osteomyelitis and septic arthritis
- Delayed healing
- Incisional hernia (Pintu and Ahmed, 2001)

2.10 Wound healing

Wound healing is a biological process that begins with trauma and ends with scar formation (Hess and Cathy Thomas, 2002). Infection in a wound delays healing and may cause wound breakdown, herniation of the wound and complete wound dehiscence (Alexander, 1994).

The three phases of wound healing or repair are lag/ inflammatory, proliferative, and remodeling/maturation. Directly after injury, homeostasis is achieved with clot formation. The fibrin clot acts like a highway for the migration of cells into the wound site. Within the first four hours of injury, neutrophils begin to appear. These inflammatory cells kill microbes, and prevent the colonization of the wound. Next the monocyte, or macrophage, appears. Functions of these cells include the killing of microbes, the breakdown of wound debris, and the secretion of cytokines that initiate the proliferative phase of repair. Synthetic cells, or fibroblasts, proliferate and synthesize new connective tissue, replacing the transitional fibrin matrix. At this time, an efficient nutrient supply develops through the barbarization

(terminal branching) of adjacent blood vessels. This ingrowth of new blood vessels is called angiogenesis.

This new and very vascular connective tissue is referred to as granulation tissue. The first phase of repair is called the lag or inflammatory phase. The inflammatory response is dependent on the depth and volume of tissue loss from the injury. Characteristics of the lag phase include acute inflammation and the initial appearance and infiltration of neutrophils (Dipietro et al 2003). Neutrophils protect the host from microorganisms and infection. If inflammation is delayed or stopped, the wound becomes susceptible to infection and closure is delayed.

The proliferative phase is the second phase of repair and is anabolic in nature. The lag and remodeling phase are both catabolic processes. The proliferative phase generates granulation tissue. In this process, acute inflammation releases cytokines, promoting fibroblast infiltration of the wound site, and then creating a high density of cells. Collagen is the major connective tissue protein produced and released by fibroblasts (Fernig and Gallagher, 1994). The connective tissue physically supports the new blood vessels that form and endothelial cells promote ingrowth of new vessels. These new blood vessels are necessary to meet the nutritional needs of the wound healing process. The mark of wound closure is when a new epidermal cover seals the defect. The process of wound healing continues beneath the new surface. This is the remodeling or maturation phase and is the third phase in healing. The wound continuously undergoes remodeling to try to achieve a state similar to that prior to injury.

2.11 Etiological agent of wound infection

A number of aerobic and anaerobic bacterial species may be present, either singly or in combination in wounds and other soft tissue infections. The nature of the infecting flora will depend on the underlying problem and the location of the process.

The potential pathogens commonly encountered in wound infections:

Gram positive cocci	*S. aureus, S. pyogenes, S. faecalis, S. epidermidis, S. pneumoniae*, CONS
Gram negative aerobic rods	*Pseudomonas spp*
Gram negative facultative rods	*Escherichia coli*, *Klebsiella spp, , Enterobacter spp, Proteusspp,Citrobacter spp, Acinetobacter spp, Morganella spp* and other *Enterobacteriaceace*
Anaerobes	*Peptostreptococcus spp*, *Bacteroides fragilis*, *Bacteroides melaninogenicus, Clostridium spp*
Fungi	*Candida spp*, *Aspergillus spp*

(Forbes et al 2002)

2.12 Research on wound infection

To find out the aerobic and anaerobic microbiology of wound infections following spinal fusion in children, Brook and Frazier, 2000 carried out a study which showed anaerobic bacteria in 3 (17%), aerobic bacteria only in 3 (17%) and mixed aerobic and anaerobic bacteria in 12 (67%). The predominant anaerobes were *Bacteroides spp*. (9 isolates, including 8 *Bacteroides fragilis* group) and 5 *Peptostreptococcus spp*. The predominant aerobes were *Escherichia coli* (6) and *Proteus spp*. An increase in recovery of *E. coli* and *B. fragilis* was noted in children with bowel or bladder incontinence.

In USA, Brook and Finegold (1981) performed a study in cutaneous abscesses in children and found that 4% of total 209 specimens were sterile, 24% yielded pure culture and the rest yielded mixed growth. In the study, *S. aureus* (45.17%) was th most common bacteria followed by non-haemolytic *streptococci* (14.7%), β-hemolytic *streptococci* (8.12%), *Enterobacter spp* (5.07%) and *Escherichia coli* (4.06%).

In a retrospective study, to determine the bacterial profile and antibiotic susceptibility pattern of burn isolates at the Queen Elizabeth Central Hospital (QECH), Blantyre Malawi, Komolafe et al 2003, showed Gram positive bacteria as the predominant isolates over Gram negative. Among the Gram-

negatives, *P. aeruginosa, Proteus mirabilis, Escherichia coli* and *Klebsiella pneumoniae* were the most common accounting for 94.1% while in the Gram-positive *Staphylococcal spp*. and *Streptococci spp*. predominated.

Ang and Lee, 1997 in Singapore, conducted a retrospective study on the pattern of infection in burn patients for a period of 15 months from January 1993 to March 1994. Organisms like methicillin-resistant *S. aureus* (MRSA) and multidrug resistant *Acinetobacter baumannii* constituted a problem in the patients. Other organisms that were isolated from burn patients included *P. aeruginosa*, *Enterobacter* spp. and *Klebsiella* spp. Mousa (1999) studied on fungal infection of burn wound and found that predominant fungi were *Aspergillus spp* and *Candida spp.* In a study done by Brook (1995) on microbiology of gastrostomy site wound infection in children, polymicrobial flora was found in 21 of 22 wounds. A total of 102 bacterial isolates (57 aerobic and 45 anaerobic) and 7 cultures of *Candida albicans* were obtained. The most frequent isolates were *E.coli* (16 isolates), *Peptostreptococcus spp* (14 isolate), *Enterococcus* (14 isolate), *Bacteroides spp* (12 isolate) and *S. aureus* (6 isolate).

To determine the incidence, pathogens and risk factors associated with development of sternal wound and other infections in children undergoing cardiac surgery, a retrospective chart review was carried out for all cardiac surgeries performed on children <18 years of age (Mehta et al 2000). In the study, sternal wound infection developed in 10 of 202 (5%) children after median sternotomy. Superficial sternal wound infection developed in 6 (3%) children, and 4 (2%) had deep infection. Causative agents for sternal wound infection were *Staphylococcus aureus* (6), *Pseudomonas aeruginosa* (1) and *Haemophilus influenzae* non-type b (1).

.According to The National Nosocomial Infection Surveillance (NNIS) program (CDC,1996) pathogens commonly associated with wound infections and frequency of occurrence are *S. aureus* (20%), Coagulase negative *staphylococci* (14%).*enterococci* (12%), *E. coli* (8%), *P. aeruginosa* (8%), *Enterobacter spp* (7%), *P. mirabilis* (3%), *K. pneumoniae* (3%), other

streptococci (3%), *Candida albicans* (2%), Group D *streptococci* (2%), other gram positive aerobes (2%) and *Bacteroides fragilis* (2%).

Goldstein (1992) studied bacteria involved in infection of bite wounds and observed that *S. aureus, S. epidermidis,* alpha haemolytic *Streptococci*, *Capnocytophaga canimorsus* and other members of oral flora. Anaerobic bacteria were present in approximately one third of bite wound and were associated with the formation of abscess and with relatively serious infections.

Brook (1987) studied microbiology of human and animal bite wounds in children and found that a total of 59 isolates were recovered from animal bites (37 aerobes and 22 anaerobes) while 97 isolates were from human bites (44 aerobes and 53 anaerobes). The most frequent isolates were *S. aureus,* anaerobic cocci and *Bacteroides spp*. Group A *streptococci* was only present in human bites while *Pasteurella multocida* and *Pseudomonas florescens* were only present in animal bites.

2.14 Microbiological analysis of wound

Analysis of wound specimen

A] Macroscopic observation: Direct observation of the pus to detect color, consistency, odor and other physical characteristics is often of great diagnosis of wound infection. The color of pus varies from green-yellow to brown-red. Pseudomonas infection may have characteristic blue green exudates and fruity odor and that of Proteus infection has a fishy smell. The consistency of pus may vary from a turbid liquid to one that is very thick and sticky. Pus containing anaerobic organisms often has an offensive putrid smell. In some fungal infections such as mycetoma, black or brown granules may be present (Collee et al 1999).

B] Microscopic examination: The microscopic examination is very important and should never be omitted. The gram's stain together with knowledge of the patient's history and symptoms may provide a presumptive diagnosis of the etiological agent of the disease. Staining for fungal elements can be obtained at the same time. Gram's stain is still the most important stain in microbiology

(Popescu and Doyle, 1996).However the value of Gram stain as a diagnostic tool is debatable. When clinical material is stained, it is important to evaluate also the presence of the other types of cells. For e.g. Presence of polymorphonuclear leukocytes (PMNL's) indicates a bacterial infection.

C] Culture of wound specimen: Routine analysis of wound specimens normally involves the use of selective and nonselective agar media to culture aerobic bacteria and yeasts and, if a specimen is purulent and/or malodorous, anaerobic bacteria also (Bowler et al 2001). Blood agar for aerobes and anaerobes, macConkey agar for the differentiation of coliforms, staphylococci and enterococci, cooked meat broth for the enrichment of exacting aerobes and anaerobes, and potato dextrose agar for fungi are mainly used in the culture of specimens. Isolation of single colonies allows further growth and identification of the specific organism. Sensitivity testing then follows mainly for aerobic organisms.

Newer techniques

- Tests for antigens from the organism through enzyme-linked immunoassay (ELISA) or radioimmunoassay
- Detection of antibody response to the organism in the host sera
- Detection of RNA or DNA sequences or protein from the infective organism by Northern, Southern, or Western blotting, respectively
- Polymerase chain reaction (PCR) is a sensitive assay to detect small amounts of microbe DNA.

Imaging studies

- Ultrasound can be applied to the infected wound area to assess whether any collection needs drainage.

Further investigations include:

Serum investigations: These involve small amounts of blood being obtained from the patient to identify elevated white cell counts and elevated levels of serum C-reactive protein (CRP), a protein normally not found in the serum, but present in many acute inflammatory conditions and with necrosis.

However, it should be remembered that the latter is not diagnostic of a chronic wound infection.

Quantitative analysis: (e.g. through wound biopsies). This can assist with the recognition of an increased bacterial burden; however, this is not regularly undertaken in the UK and previous studies have shown that wounds can heal despite high bacterial count. (Woolfrey et al 1991)

Histological analysis: Histological diagnosis of burn wound infection is based on the observation of microorganisms invading viable tissue beneath the Escher surface (Deirdre et al 2006).

The removal of devitalized and contaminated tissue from wounds to expose healthier tissue and facilitate wound healing (Vowden and Vowden, 2002). Devitalized tissue provides a favorable environment for microbial growth, and thus its removal will also reduce the microbial load. If the wound has necrotic material present, a debriding dressing should be chosen while a protective dressing is best for clean, healing wounds (Sharma et al 2005). If an invasive infection is present, surgical excision of the infected wound is usually required, as well as appropriate systemic antimicrobial therapy.

Prior to the advent of antibiotic therapy, the use of larvae (maggots) as an effective method of wound debridement (Mumcuoglu et al 1999) was routine. Larval therapy is currently being used in the treatment of a variety of infected acute and chronic wounds, including those colonized by resistant bacteria such as methicillin resistant *S. aureus (*Thomas et al 1999).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

Materials used in this study are enlisted in Appendix II.

3.2 Methods

The research study was carried out in the microbiology laboratory of Nobel teaching hospital Biratnagar morang. The study was performed from ashwin 2074 to Falgun 2074. To know about the incidence and causative agent of different infected wounds, information was obtained by asking to each patient, which is presented in Appendix-I. Within the study period, three hundred and thirty five samples were examined from infected wound infected patient.

3.2.1 Collection of sample and transport

The sample taken for this study were pus, pus aspirates and swab. the wounds were assumed as infected by the presence of purulent material. The purulent material was aspirated with the help sterile syringe. Where the aspiration was not possible, sterile cotton swabs were used. (For each sample two consecutive swabs were taken). The samples were collected trained health practitioner as directed by medical officer before the application of antiseptic dressing. The samples were immediately taken to the laboratory as soon as possible.

3.2.2 Processing of the sample

In the laboratory the samples were immediately processed. During each sample processing one swab was used for microscopic examination and the other swab for culture (Collee et al 1999).

3.2.3 Macroscopic examination

The color, odor and other characteristics of the pus were noted.

3.2.4 Microscopic examination

The smear of the specimen was made on a sterile slide. The smear was heat fixed and stained by Gram stain method as described in Appendix-IV The stained smear was examined under the microscope using 40X and then 100X objectives for the presence of pus cells, morphology of bacteria.

3.2.5 Culture of the sample

The inoculation of the collected specimens was done on Blood agar (BA), Mac Conkey agar (MA) and Nutrient agar (NA). Blood agar plate was incubated in microaerophilic condition using carbon dioxide enriched candle jar. Mac Conkey agar (MA) and Nutrient agar (NA) plates were incubated at 37^0C for 24 hours aerobically.

The composition and preparation of the media were described in Appendix-III

3.2.6 Identification of isolated organisms

The standard microbiological technique, which involved colony morphology, staining reaction, biochemical properties was followed for the identification of organisms (Cheesbrough, 2000).The identification procedure was followed after 24 hours. Gram positive cocci were identified following catalase, O/F, coagulase, bacitracin and optochin sensitivity test.

For Gram negative organisms, biochemical tests (Oxidase, Catalase, Methyl red, Voges Proskaeur test, Citrate utilization test, Indole production test, Triple sugar iron agar test, Urease test) were performed by inoculating a single isolated culture from media on to the respective biochemical media. Hemolysis in blood agar, morphological and cultural characteristic on Mac Conkey agar was observed.

3.2.7 Antibiotic sensitivity tests of isolated organisms

After the identification of isolated organisms, the sensitivity tests were performed. The medium used for this test was Mueller Hinton Agar. The antibiotic susceptibility test was performed by Kirby-Bauer sensitivity testing

method, according to guidelines given by the formerly known as National Committee for Clinical Laboratory Standard (NCCLS).

1) Mueller Hinton agar was prepared and sterilized as instructed by the manufacturer.
2) The p^H of the medium 7.2-7.4 and depth of the medium at 4mm (about 25ml plates) was maintained in 90mm Petri dish.
3) Using a sterile wire loop, a single isolated colony of which the sensitivity pattern is to be determined was touched and inoculated into a nutrient broth tube and was incubated for 2-4 hours.
4) After incubation in a good light source, the turbidity of the suspension was matched with the turbidity of standard of Mac Farland 0.5(Prepared by adding 0.6ml of 1% w/v barium chloride solution to 99.4ml of 1% v/v solution of sulphuric acid (Cheesebrough, 2000).
5) Using a sterile swab, a plate of Mueller Hinton agar was inoculated with bacterial suspension using carpet culture technique. The plate was left for about 5 minutes to let the agar surface dry.
6) Using sterile forceps, appropriate antimicrobial discs (6mm diameter) was placed, evenly distributed on the inoculated plates, not more than 7 discs were placed on 90mm diameter Petri dishes.
7) Within 30 minutes of applying the discs, the plates were taken for incubation at 37^OC for 18-24 hours.
8) After overnight incubation, the plates were examined to ensure confluent growth. Using a measuring scale, the diameter of each zone of inhibition in mm was measured and result interpreted accordingly.

List of various types of antibiotics disc used according to the nature of organism are as follows

Chloramphenicol (30mcg)
Levofloxaci (5mcg)

Co-trimoxazole (25mcg)
Tobramycin (10mcg)

Clindamycin (2mcg)
Aztronam (30mcg)

Erythromycin(15mcg)
Cefepime (30mcg)

Linezolid (30mcg)

Oxacillin (1mcg)

Penicillin (10mcg)

Teicoplanin (30mcg)

Vancomycin (30mcg)

Amikacin (30 mcg)

Ampicillin (10mcg)

Ceftazidime (30mcg)

Cefotaxime (30mcg)

Cefuroxime (30mcg)

Gentamycin (10mcg)

Ciprofloxacin (5mcg)

Meropenem (10mcg)

Cetriaxone (30mcg)

Piperacillin+Tazobactum
(100/10mcg)

High level Gentamycin (120mcg)

Streptomycin (10mcg)

Penicillin (10unit)

Teicoplanin (30mcg)

The result was interpreted as whether the organism was sensitive or intermediate or resistant to the tested antimicrobial agents. All the antibiotics disc used are of Himedia.

3.2.8 Purity plate

The purity plate was used to ensure that the inoculum used for the biochemical test was pure culture and also to see whether the biochemical tests were performed in an aseptic condition or not. So while performing biochemical test, the same inoculum was subcultured in respective medium and incubated. The development of pure culture in the medium would confirm the purity of the inoculum.

3.2.9 Quality control for the tests

Quality control is considered as one of the important factor for the correct result interpretation (Cheesebrough 2000). According to Vandepite et al (2004), quality control is absolutely essential for good operating procedure. So during this study, quality control was applied in various areas. During sample collection and processing, aseptic method was followed in order to avoid contamination.

The quality of the media were assured by testing each batch of medium, using control strain of bacteria and also 10% of uninoculated media were kept in an incubator to ensure the contamination during preparation.

Stains and reagents, for a new batch was tested by preparing, a control smears to ensure correct staining reactions.

Quality of susceptibility tests was maintained by maintaining the thickness of Mueller Hinton agar at 4mm and p^H at 7.2-7.4. Similarly, antibiotic discs containing the correct amount as indicated were used.

3.2.10 Statistical analysis

Three hypotheses were analyzed using Chi-square at 5% level of significance. Significant tests of present work are shown in Appendix VIII.

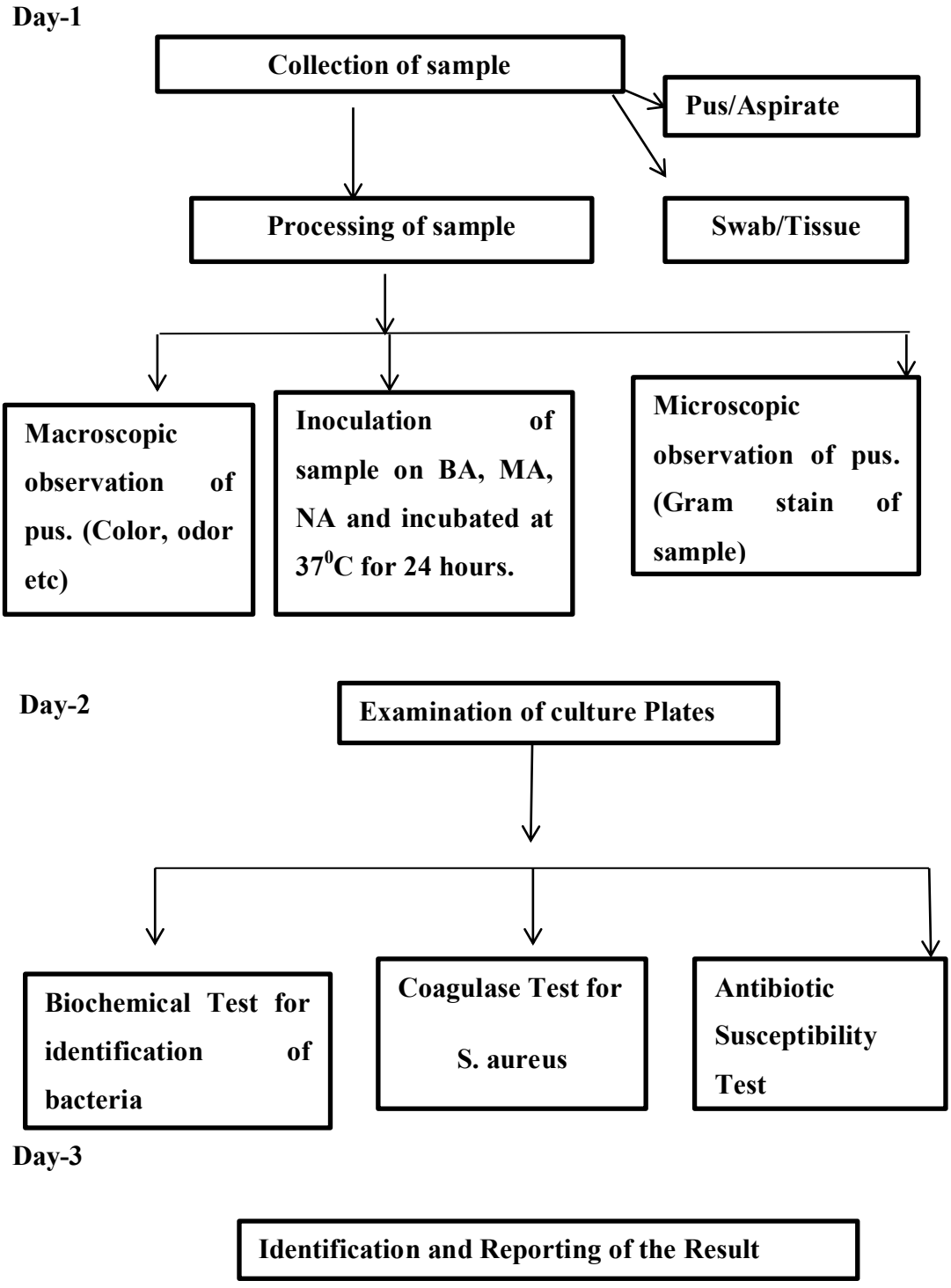

Fig 1: Scheme for the isolation and identification of organisms from pus samples

CHAPTER IV

RESULT

Study was conducted for 6 month starting from 2074 ashwin 14 to 2074 falgun 17 and total 335 samples were collected. Out of total 335 sample 225 saple were found positive and 110 sample were found negative. In this study sample taken include pus, swab, pus aspirate and tissue. Type of wound for sample collection were boils, lesions, abscesses, trauma wounds, burns, bite wounds, accidental wounds etc.

4.1 Gender wise distribution of total patients

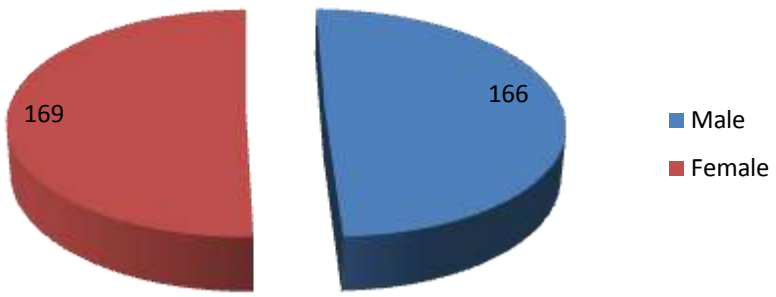

Fig 2: Gender wise distribution of total patients

Out of total 335 samples 169 were male and 166 were female.

4.2 Age wise distribution of sample

Out of total 335 patient below 10 years patient were 23 (6.86%), 10-20 year age group patient were 64 (19.10%), 20-30 year age group patient were 87 (25.97%), 30-

40 year age group patient were 59 (17.61%),40-50 year age group patient were 32 (9.55%), 50-60 year age group patient were 36 (10.74%), 60-70 year age group patient were 21(6.26%), 70-80 year age group patient were 7 (2.08%) and in the age group above 80 years includes 6 (1.79%) patients. maximum number of patient were fall on 20-30 years age group followed by 10-20 year category and least number patient were found in above 80 year age group.

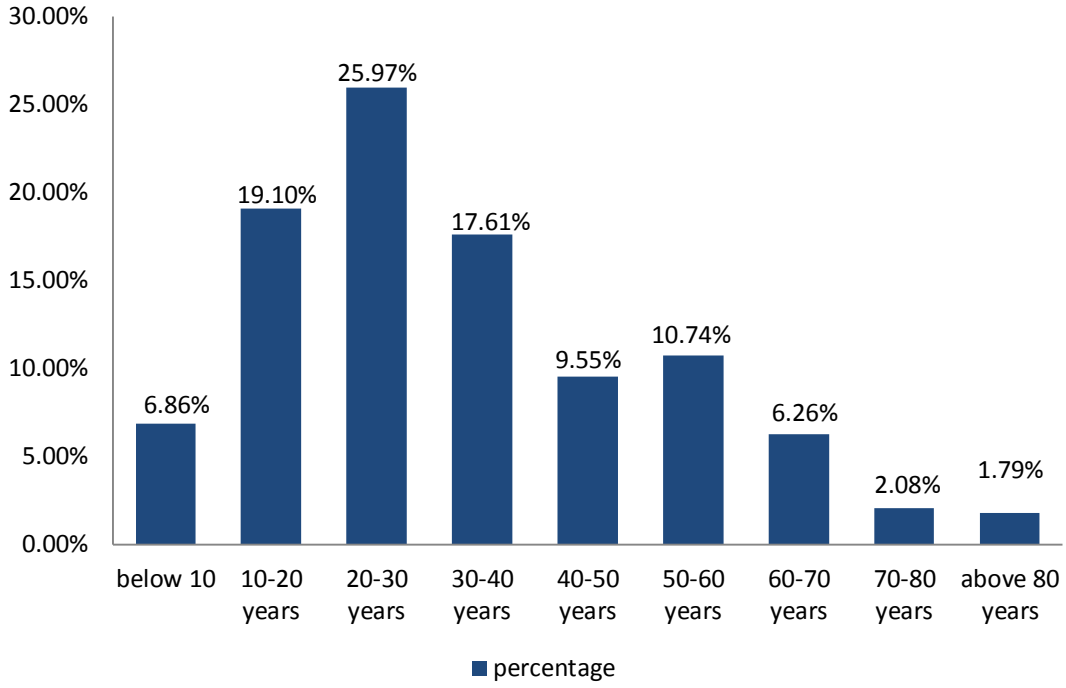

Fig 3: Age wise distribution of sample

4.3 Gender wise distribution of sample

In total 335 samples 225 samples were found to be positive and 110 were negative samples. In total 166 male population 108 samples were positive and 58 samples were negative And in total 169 female population 117 sample were found positive and 52 samples were negative samples.

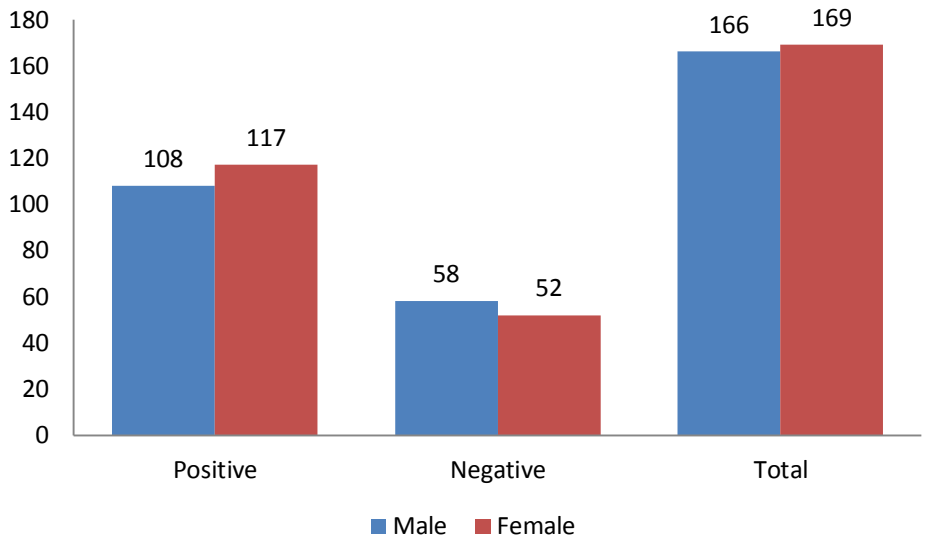

Fig 4: Gender wise distribution of sample

4.4 Microorganisms distribution in the total population

Out of total 225 positive samples 115 (50.67%) were found to be gram positive and 109 (48.44%) were found to be gram Negative and single fungi. In total 225 positive samples *Staphylococcus aureus* was found as predominant occurring total 114 (50.67%) and then respectively followed by *Escherichia coli* total 71 (31.56%), *Enterococcus faecalis* 15 (6.67%), *pseudomonas aerogenosa* 13 (5.78%), *Enterobacter aerogenes* 4(1.78%), *proteus vulgaris* 2(0.89%), *Acenetobacter anitratus* 2 (0.89%), *Klebsella oxytoca* 1 (0.44%), *Klebsella pneumonia* 1 (0.44%), *diptheroids* 1 (0.44%) and *candida* 1 (0.44%).

Table 4.1 Microorganism distribution in the total population

Name	Number	Percentage
Staphylococcus aureus	114	50.67%
Escherechia coli	71	31.56%
Enterococcus faecalis	15	6.67%
Pseudomonas aerogenosa	13	5.78%
Enterobacter aerogenes	4	1.78%
Proteus vulgaris	2	0.89%
Acenetobacter anitratus	2	0.89%
Klebsella oxytoca	1	0.44%
Klebsella pneumonia	1	0.44%
Diptheroids	1	0.44%
Candida	1	0.44%

4.5 Antibiotic susceptibility pattern of *Staphylococcus aureus*

Out of total 114 isolates of *Staphylococcus aureus*, 112 (98.25%) isolates were found to be susceptible to chloramphenicol, 71 (62.28%) were sensitive to co-trimoxazole, 46 (40.35%) were sensitive to clindamycin, 39 (34.21%) were isolates to Erythromycin, 81 (71.05%) were sensitive to oxacillin, and all 114 (100%) isolates were sensitive to Vancomycin, Linezolid and Teicoplanin. 108 (94.74%) isolates of *Staphylococcus aureus* were resistant to penicillin and 6 (5.26%) isolates were found to be intermediate sensitive to Cotromoxazole followed by 1 (0.88%) to Erythromycin. 33 (28.29%) isolate were found to be Methicillin Resistant *Stahylococcus aureus* (MRSA).

Table 4.2 Antibiotic susceptibility pattern of *Staphylococcus aureus*

Antibiotic	*Staphylococcus aureus*					
	Sensitive		Resistant		Intermediate	
	N	percentage	N	Percentage	N	Percentage
Chloramphenicol	112	98.25%	2	1.75%	-	-
Cotrimoxazole	71	62.28%	37	32.46%	6	5.26%
Clindamycin	46	40.35%	68	59.65%	-	-
Erythromycin	39	34.21%	74	64.91%	1	0.88%
Linezolid	114	100.00%	-	-	-	-
Oxacillin	81	71.05%	33	28.95%	-	-
Penicillin	6	5.26%	108	94.74%	-	-
Teicoplanin	114	100.00%	-	-	-	-
Vancomycin	114	100.00%	-	-	-	-

4.6 Antibiotic susceptibility pattern of *Escherichia coli*

The most effective antibiotic was found to be Piperacillin+Tazobactum (94.37%) followed by Gentamycin (80.28%), Meropenem (80.28%), Amikacin (77.46%), Levofloxacin (50.70%), Cefotaxime (21.13%), Ceftazidime (19.72%), Cefuroxime (16.90%), Ampicillin (7.04%) and Intermediate resistance towards levofloxacin (15.49%), Gentamycin (7.04%), Meropenem (7.04%). Out of total 71 E.coli Positive sample 37 sample was found to be ESBL.

Table 4.3 Antibiotic susceptibility pattern of *Escherichia coli*

Antibiotic	*Escherichia coli*					
	Sensitive		Resistant		Intermediate	
	N	Percentage	N	Percentage	N	Percentage
Amikacin	55	77.46%	16	22.54%	-	-
Ampicillin	5	7.04%	66	92.96%	-	-
Ceftazidime	14	19.72%	57	80.28%	-	-
Cefotaxime	15	21.13%	56	78.87%	-	-
Gentamycin	57	80.28%	9	12.68%	5	7.04%
Levofloxacin	36	50.70%	24	33.80%	11	15.49%
Meropenem	57	80.28%	9	12.68%	5	7.04%
Piperacillin+Tazobactum	67	94.37%	4	5.63%	-	-
Cefuroxime	12	16.90%	59	83.10%	-	-

4.7 Antibiotic susceptibility pattern of *Enterococcus faecalis*

Out of total 14 positive isolates of *enterococcus faecalis* teicoplanin (100%), vancomycin (100%) and Linezolid (100%) was found as most effective drugs followed by Chloramphenicol (93.33%), Streptomycin (93.33%), High level Gentamycin (86.67%), erythromycin (73.33%) and Penecillin (53.33%).

Table 4.4 Antibiotic susceptibility pattern of *Enterococcus faecalis*

Antibiotic	*Enterococcus faecalis*			
	Sensitive		Resistant	
	N	Percentage	N	Percentage
Chloramhenicol	14	93.33%	1	6.67%
Erythromycin	11	73.33%	4	26.67%
High level gentamycin	13	86.67%	2	13.33%
Linezolid	15	100.00%	-	-
Vancomycin	15	100.00%	-	-
Streptomycin	14	93.33%	1	6.67%
Penicillin	8	53.33%	7	46.67%
Teicoplanin	15	100.00%	-	-

4.8 Antibiotic susceptibility pattern of *Pseudomonas aerogenosa*

Table 4.5 Antibiotic susceptibility patterns of *Pseudomonas aerogenosa*

Antibiotic	*Pseudomonas aerogenosa*					
	Sensitive		Resistant		Intermediate	
	N	Percentage	N	Percentage	N	Percentage
Amikacin	11	84.62%	2	15.38%	-	-
Aztronam	11	84.62%	1	7.69%	1	7.69
Ceftazidine	6	46.15%	7	53.85	-	-
Ciprofloxacin	10	76.92%	2	15.38	1	7.69
Cefepime	5	38.46%	7	53.85	1	7.69
Gentamycin	9	69.23%	2	15.38	2	15.38
Levofloxacin	11	84.62%	2	15.38	-	-
Meropenem	11	84.62%	2	15.38	-	-
Tobramycin	11	84.62%	2	15.38%	-	-

Out of total 13 isolates amikacin (84.62%), Aztronam (84.62%), Levofloxacin (84.62%), Meropenem (84.62%) And Tobramycin (84.62%) were found as most effective drugs followed by Ciprofloxacin (76.92%), Gentamycin (69.23%), Ceftazidine(46.15%) and Cefepime(38.46%) was found to be least sensitive. Levofloxacin (15.38%), Aztronam (7.69%), Ciprofloxacin (7.69%), Cefepime (7.69%) were found to be intermediate sensitive.

4.9 Antibiotic Susceptibility Pattern Of *Enterobacter Aerogenes*

Out of total four positive isolates of *Enterobacter aerogenes* Amikacin, Gentamycin, Meropenem was found as the most effective drugs followed by Ciprofloxacin And the isolates were resistant to Ampicillin, Ceftazidine, Cefotaxime and Cefuroxime.

4.10 Antibiotic susceptibility pattern of *proteus vulgaris*

Proteus vulgaris were found to be sensitive toward the Ampicillin, Ceftazidime, cefotaxime, Ciprofloxacin, Gentamycin and Amikacin.

4.11 Antibiotic susceptibility pattern of *Diptheroids*

Out of total 1 isolates of *Diptheroids* was found to be sensitive towards the Cetriaxone, Gentamycin, Imipenem, linezolid and resistant towards Chloramphenicol, Clindamycin, Erythromycin.

4.12 Antibiotic susceptibility pattern of *Acenetobacter anitratus*

Acenitobacter anitratus was found to be sensitive towards Amikacin, Gentamycin, Tobramycin, levofloxacin and resistants towards Ceftazidime , Cefotaxime, Ciprofloxacin, Cetriaxome and Meropenem.

4.13 Antibiotic susceptibility patterns of *klebsella oxytoca*

The most effective antibiotic for *Klebsella oxytoca* was Ceftazidime, Ciprofloxacin, Gentamycin, Levofloxacin, Meropenem and resistant towards Ampicillin and Cefotaxime.

4.14 Antibiotic susceptibility patters of *klebsella pneumoniae*

Klebsella Pneumoniae was found to be sensitive towards Ampicillin, Cefotaxime, Ciprofloxacin, Gentamycin, Levofloxacin, Meropenem and resistant towards Ceftazidime.

CHAPTER V

DISCUSSION AND CONCLUSION

5.1 Discussion

Wound is a breach in the skin and the exposure of subcutaneous tissue following loss of skin integrity provides a moist, warm and nutritive environment that is conductive to microbial colonization and proliferation (Bowler et al 2001). Most of the time the host immune defense mechanism eliminate the foreign pathogen but when host defense mechanism fail to stop the foreign pathogen they enter into the body of the host and tends to colonize making the suitable environment for their growth and multiplication.

Wound infection is an important cause of illness that results in a prolongation of hospital stay, increased trauma care, treatment costs and the increasing requirement for cost- effective management within the health care system. Here, wound cultures represent a general category for a group of extremely diverse anatomic samples that range from superficial specimens of cutaneous structures (folliculitis, cellulitis) to specimens revealing invasive infections involving deep facial planes and muscles.

This study was carried out with an objective to find out the causative microorganisms of different types of wound infection and their antibiotic susceptibility patterns. The study was performed in Nobel medical college and Teaching Hospital Biratnagar, Morang which is the one of the most referred hospital in eastern Nepal. Total 335 samples were subjected under study where 110 samples were negative samples and 225 samples were positive samples. Out of total 335 samples 167 (49.8%) were female samples and 168 (50.2%) were male samples.

Out of toal 335 patient below 10 years patient were 23(6.86%), 10-20 year age group patient were 64(19.10%), 20-30 year age group patient were 87(25.97%), 30-40 year age group patient were 59(17.61%),40-50 year age group patient were 32(9.55%), 50-60 year age group patient were 36(10.74%), 60-70 year age group patients were 21(6.26%), 70-80 year age group patient

were 7(2.08%) and in the age group above 80 years includes 6(1.79%) patients. Maximum numbers of patient were fall on 20-30 years age group followed by 10-20 year category and least number patient were found in above 80 year age group.

The most frequent Microorganism associated with wound infection was found to be *Staphylococcus aureus* (50.67%) followed by *Escherichia coli* (31.56%), *Enterococcus faecalis* (6.67%) and *Pseudomonas aerogenosa* (5.78%). Similar type of study conducted by Roy.s et al 2017 *Staphylococcus aureus* was found to be the most frequent isolate (55.7%), followed by *Escherichia coli* (23.7%), *Pseudomonas spp*. (8.2%). According to Brook and Frazier (1990), *S. aureus* was the most common in abscess from all body sites, but predominant in abscess of legs. Brook's study on aerobic and anaerobic microbiology of infections after trauma in children reported 51 *S. aureus* from 175 specimens.

E. coli was found to be the second common isolates (31.56%) in total samples and predominant among Gram negative bacteria. Higher prevalence of *E. coli* among Gram negative isolates seen in this study coincides with the reports given by (Bhattacharyya and Kosloske, (1990); Nasser *et al* 2003; Brook *et al*., 1998). However, in a study conducted by Brook *et al* (1998) on gastrostomy site wound infection in children, it was found that the most frequent bacterial isolate was *E.coli* (28.07%).

Third most common isolate was found to be *Enterococcus faecalis* (6.67%) this results coinside with the study done by *Trupti B. Naik et al. 1016 where the bacteriological profile of wound sample found to be Enterococcus fecalis (8.16%). Other most common bacterial isolate was found to be Pseudomonas aerogenosa(5.78%) similar type of study conducted by K.C.R et al 2013 Pseudomonas aeruginosa* (3.33%) was found.

In this study *Enterobacter aerogenes* was found to be associated with the wound infection occurring 1.78% of the total positive isolates. Levy et al (2003) reported (17%) of *Enterobacter spp*, Zhang and Zhao, 2003 at China showed (10.4%) of *Enterobacter spp* from the burn wound. The normal habitat of *Enterobacter spp.* is believed to be soil and water, but the organism

is occasionally found in the feces and the respiratory tract of humans. In recent years, infection of hospital patients with *Enterobacter spp* has been reported more often than previously (Collier et al 1998). Other gram negative bacteria associated with wound infection were found to be *Proteus vulgaris* (0.89%) and *Acenetobacter anitratus* (0.89%). Brook and Frazier, 2000 carried out a study on wound infections following spinal fusion in children which showed 11.9% of *Proteus spp*. Similarly in a study carried out by Wei-Jen et al (2005) in Taiwan showed (5.06%) of *Proteus spp. Proteus mirabilis* causes 90% of *Proteus* infections. Similarly, Song et al 2001 isolated (13.4%) of *Acinetobater spp.* Agnihotri et al (2004) reported (7.2%) of *Acinetobacter spp*. from burn wound. The species belonging to the *Acinetobacter* genus are widely distributed in nature since they are found frequently in soil, water, and dry environments. The least common isolates in my study was found to be *Klebsella oxytoca* (0.44%), *Klebsella pneumonia* (0.44%), *Diptheroids* (0.44%) and *candida* (0.44%).

Out of total 114 isolates of *Staphylococcus aureus*, 112 (98.25%) isolates were found to be susceptible to chloramphenicol, 71 (62.28%) were sensitive to co-trimoxazole, 46 (40.35%) were sensitive to clindamycin, 39 (34.21%) were isolates to Erythromycin, 81 (71.05%) were sensitive to oxacillin, and all 114 (100%) isolates were sensitive to Vancomycin, Linezolid and Teicoplanin. 108 (94.74%) isolates of *Staphylococcus aureus* were resistant to penicillin and 6 (5.26%) isolates were found to be intermediate sensitive to Cotromoxazole followed by 1 (0.88%) to Erythromycin. 33 (28.29%) isolate were found to be Methicillin Resistant *Stahylococcus aureus* (MRSA). Chen and Zhang 2007, at China showed (82.5%) resistance of S. *aureus* to methicillin. According to Voss and Doebbeling (1995) the increasing prevalence of MRSA is a worldwide problem, affective both affluent and poor countries and accounts for substantial hospital morbidity, mortality and cost

Second most predominant isolates was *E.coli* (31.56%) and The most effective antibiotic was found to be Piperacillin+Tazobactum (94.37%) followed by Gentamycin (80.28%), Meropenem (80.28%), Amikacin (77.46%), Levofloxacin (50.70%), Cefotaxime (21.13%), Ceftazidime (19.72%), Cefuroxime (16.90%), Ampicillin (7.04%) and Intermediate resistance

towards Levofloxacin (15.49%), Gentamycin (7.04%), Meropenem (7.04%). Out of total 71 *E.coli* Positive sample 37 sample was found to be ESBL. Similar study done by Mulugeta k. et al *E. coli* isolates had resistant rates of 85.0% to amoxicillin and resistance rates to Ciprofloxacin and gentamicin were 7.7% and 14.4%, respectively.

In this study after *Staphylococcus aureus* and *Escherichia coli* third most isolated pathogen was *enterococcus faecalis* which is most susceptible to Teicoplanin (100%), vancomycin (100%) and Linezolid (100%) was found as most effective drugs followed by Chloramphenicol (93.33%), Streptomycin (93.33%), High level Gentamycin (86.67%), erythromycin (73.335) and Penecillin (53.33%). Most resistant towards penicillin (46.67%) followed by Erythromycin (26.67%), High level Gentamycin (13.33%), streptomycin (6.67%) and Chloramphenicol (6.67%). For *Pseudomonas aerogenosa* Amikacin (84.62%), Aztronam (84.62%), Levofloxacin (84.62%), Meropenem (84.62%) And Tobramycin (84.62%) were found as most effective drugs followed by Ciprofloxacin (76.92%), Gentamycin (69.23%), Ceftazidine (46.15%) and Cefepime (38.46%) was found to be least sensitive. Levofloxacin (15.38%), Aztronam (7.69%), Ciprofloxacin (7.69%), Cefepime (7.69%) were found to be intermediate sensitive. For *Enterobacter aerogenes* most sensitive drugs found was Amikacin (100%) followed by Gentamycin (100%), Ciprofloxacin (75%) and resistant towars Ampicillin (100%), Ceftazidine (100%), Cefotaxime (100%) and Cefuroxime (100%).

The other isolates *Proteus vulgaris* were found to be 100% sensitive toward the Ampicillin, Ceftazidime, cefotaxime, Ciprofloxacin, Gentamycin and Amikacin. Two species of *klebsella* were isolate from the wound sample. The most effective antibiotic for *Klebsella oxytoca* was Ceftazidime, Ciprofloxacin, Gentamycin, Levofloxacin, Meropenem and resistant towards Ampicillin and Cefotaxime. And the drug of choice for *klebsella pneumonia* was Ampicillin, Cefotaxime, Ciprofloxacin, Gentamycin, Levofloxacin, Meropenem and resistant towards Ceftazidime. The isolates *Acenitobacter anitratus* was found to be sensitive towards Amikacin, Gentamycin, Tobramycin, levofloxacin and resistants towards Ceftazidime , Cefotaxime, Ciprofloxacin, Cetriaxome and Meropenem. From the total 225 positive

samples 1 isolates of *Diptheroids* was found which is sensitive towards the Cetriaxone, Gentamycin, Imipenem, linezolid and resistant towards Chloramphenicol, Clindamycin, Erythromycin.

The presence of enteric organisms probably resulted in subsequent sepsis (J.O.Isibor et al and R.L.Nicols et al). *E. coli* 71 (31.56%) *was* the commonest gram negative bacteria isolated. *E. coli* invasion of the wound is a clear case of poor hospital hygiene, just like other implicated organ-isms which are frequent agents of nosocomial infections (S.O. Samuel et al). *S. aureus* 114 (50.67%) was the single predominant gram positive bacterial isolate obtained. Several reports have cited *S. aureus* as the predominant isolate involved in causing SSIs (J.O.Isibor et al,S.O.Samuel et al, F. Biadglegneet et al and S. Malik et al).

5.2 Conclusion

This study revealed the presence of wound infection causing bacteria, those are capable of causing various human illness. The commonest isolates of Wound infection are *Staphylococcus aureus* followed by *Escherichia coli*. Hence, Knowledge of the most common causative agents of infection and their antimicrobial susceptibility pattern is very essential for the judicious administration of empirical therapy before the culture results are available. Antimicrobial susceptibility of microorganisms varies from time to time and from place to place. Hence regular monitoring of bacterial susceptibility to antibiotics is essential. antibiogram should be prepared regularly and made readily available to the clinicians to guide them in therapy. In conclusion, wound infections are one of the most common hospitals acquired infections and are an important cause of morbidity and mortality. Depending on the site of wound infection and clinical symptoms, the role of the microbiology laboratory is to determine the clinically significant isolates, perform antimicrobial susceptibility testing, and subsequently provide guidance on the most appropriate treatment. This will help in successful wound management and will also assist in the control of antibiotic usage and hence curtail the spread of antibiotic resistant bacteria.

CHAPTER VI

RECOMMENDATION

6. Recommendations

1) Since there is Significant presence of anaerobic bacteria in different types of wounds, routine anaerobic culture of the specimens should be performed.
2) As different types of wound infection were studied here, in the similar manner different types of samples from wound infection like wound biopsy, body fluid should also be studied for etiological agents.
3) To decrease the risk of wound infection, all operating and dressing rooms should be closed and the health care personnel should focus on adoption of aseptic technique during handling of patients.
4) The antibiotic policies should be formulated and implemented to resist and overcome the problem of antimicrobial resistance because organisms are gaining more and more resistance to even newer drugs due to indiscriminate and injudicious use of these antibiotics even in the hand of expertise.
5) Increase in wound infection rates should be evaluated. If an outbreak is confirmed, appropriate epidemiological studies should be initiated.
6) The nature of microbial wound colonization, flora changes and antimicrobial sensitivity profile should be taken into consideration in using empirical antimicrobial therapy of infected patients

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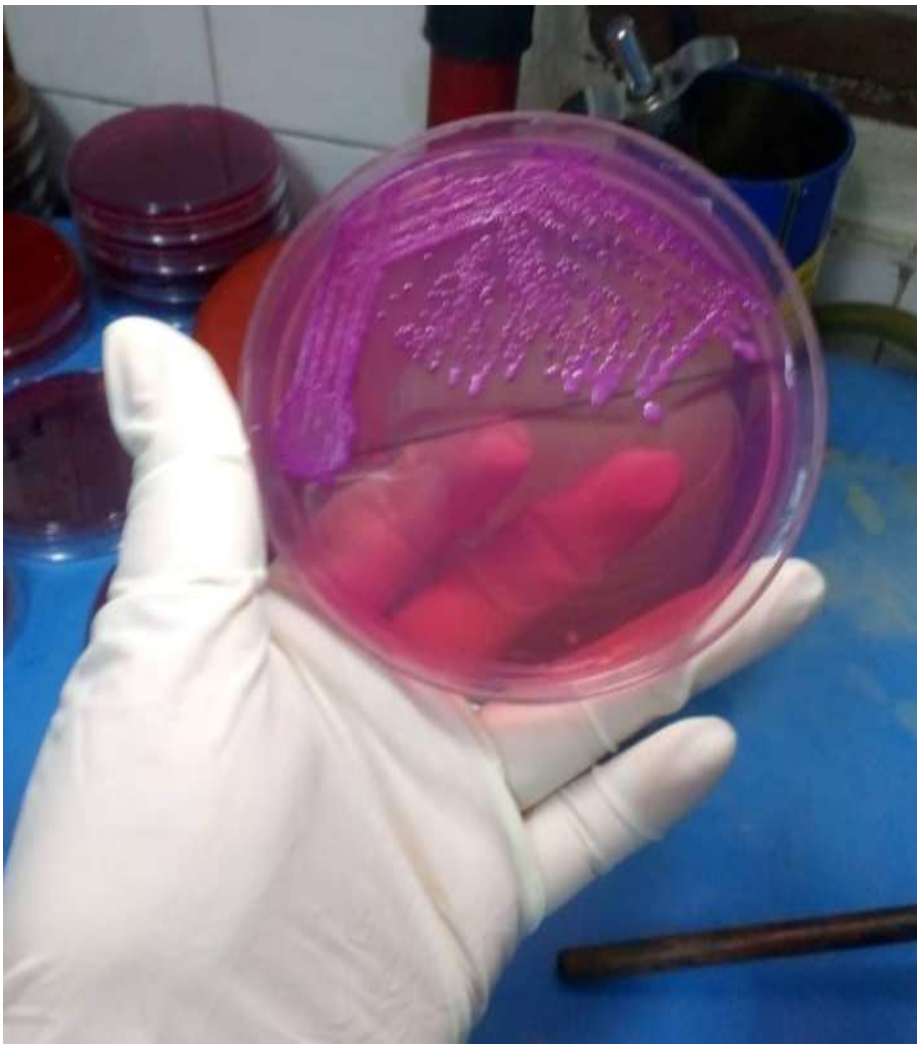
PHOTOGRAPHS



Photograph 1: Isolated colonies of E.coli on MacConkey Agar

Photograph 2: Antibiotic sensitivity pattern of *S. aureus*

Photograph 3: Antibiotic sensitivity pattern of *E. coli*

APPENDICES

APPENDIX-I: Proforma

Clinical and microbiological profile of attaint

A] Clinical profile

Name: ..... Lab no:……..

Age / Sex: ..... Date:…….

Address ..... Ward:……

Consent: Yes/No Bed no: … … … …

B] Microbiological Profile

Day 1 (… …/… …/… … ...)

Direct Microscopic Observation:

1)… … … 2)… … … 3)… … …

Culture on: 1)… … … 2)… … … 3)… … …

Incubation: 1) Aerobic 2) Microaerophilic

Day 2 (… …/… …/… … …)

Reading of culture Plates:

Media used	Shape	Size	Color	Elevation	Opacity	Consistency

Gram staining results: … … … … … … …

Catalase:… … … … … Oxidase: … … … … ….

Coagulase: … … … … … Others:

Provisional Identification of Organisms: … … … … … …

Inoculation on biochemical media

Result of biochemical test

TSI: … … … … .. SIM: … … … … …

Citrate: … … … … Urea: … … … … …

Organisms Identified as: … … … … … …

Antibiotic susceptibility test method: Kirby-Bauer Method

Antibiotic used	Zone of inhibition (mm)	Interpretation

Note:

… … … … … … … … … … … … … … …

Performed by Checked by

APPENDIX II: Equipment and Materials

A] List of equipments and materials used during the study

1. Equipments

1. Oven: Oven	Ambassadors, Laboratory Electronics
2. Incubator: Pvt. Ltd	Universal, Narang Scientific Works
3. Autoclave:	Made in USA (DAK) product, DA. KADALCompany Inc.mt Vermony
4. Refrigerator:	Sanyo
5. Microscope:	Humanscope Human D-65205 Wiesbaden, Germany

2. Microbiological media (Hi-Media)

i) Nutrient agar	vi) Simmon's Citrate agar
ii) Nutrient broth	vii) TSI agar
iii) MacConkey agar	viii) MRVP broth
iv) Blood agar	ix) Urease broth
v) Mueller Hinton agar	x) SIM media

xi) Hugh and Leifson (OF) media

3. Chemicals/ Reagents

i) Catalase reagent
ii) Oxidase reagent
iii) Kovac's reagent
iv) Barritt's reagent
v) Methyl red
vi) Crystal violet
vii) Gram's iodine
viii) Acetone-alcohol
ix) Safranin

4. Antibiotic Discs

All the antibiotic discs used for the susceptibility test were from Hi-Media Laboratories Pvt. Limited, Mumbai, India. The antibiotics used were as follows

i) Amikacin (30mcg)
ii) Ceftriaxone (30mcg)
iii) Chloramphenicol (30mcg)
iv) Ciprofloxacin (5mcg)
v) Ofloxacin (5mcg)
vi) Teicoplanin (25mcg)
vii) Gentamicin (30mcg)
viii) Cotrimoxazole (25mcg)
ix) Cefotaxime (30mcg)
x) Azithromycin (30mcg)
xi) Erythromycin (15mcg)
xii) Vancomycin (10mcg)
xiii Linezolid (10mcg)
xiv) Tigecycline (15mcg)

5. Miscellaneous

Inoculating loops, straight wires, cotton swabs, distilled water, immersion oil, dropper

APPENDIX-III : Bacteriological Media and Reagents

A. Composition and preparation of different culture media

The culture media used were from Hi-Media Laboratories Pvt. Limited, Mumbai, India. (All compositions are given in gram per litre and at 25^0 C temperatures)

1. Blood agar (BA)

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

Ingredients	gm/liter
Beef heart infusion	500.0
Tryptose	10.0
Sodium Chloride	5.0
Agar	15.0

Final pH (at 25^0C) 7.3$\pm$0.2

42.5 grams of the blood agar base medium was suspended in 1000 ml distilled water and sterilized by autoclaving at 121^0C (15lbs pressure) for 15 minutes. After cooling to 40-50^0C, 50 ml sterile defibrinated sheep blood was added aseptically and mixed well before pouring.

2. MacConkey Agar (MA)

Ingredients	gm/lit
Peptone	20.0
Lactose	10.0
Sodium taurocholate	5.0
Sodium chloride	5.0
Neutral Red	0.04
Agar	20.0

Final pH (at 25^0C) 7.4$\pm$ 0.2

55 grams of the medium was suspended in 1000ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121^0C (15lbs pressure) for 15 minutes.

3. Mueller Hinton Agar (MHA)

Ingredients	gm/liter
Beef infusion form	300.0
Casein Acid Hydrolysate	17.5
Starch	1.5
Agar	17.0

Final pH (at 25^0 C) 7.4$\pm$ 0.2

38 grams of the medium was suspended in 1000ml distilled water and the medium was warmed to dissolve. 10ml was distributed in test tubes and sterilized by boiling in water bath for 10 minutes.

4. Nutrient Agar (NA)

Ingredients	gm/liter
Peptone	10.0
Sodium Chloride	5
Beef Extract	10.0
Yeast Extract	1.5
Agar	12.0

Final pH (at 25^0C) 7.4$\pm$0.2

28 grams of the medium was suspended in 1000ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121^0C (15lbs pressure) for 15 minutes.

5. Nutrient Broth (NB)

Ingredients	gm/liter
Peptone	5.0
Sodium chloride	5.0
Beef Extract	1.5
Yeast Extract	1.5

Final pH (at 25^0C) 7.4$\pm$ 0.2

13 grams of the medium was dissolved in 1000ml distilled water and autoclaved at 121^0C for 15 minutes.

B. Biochemical Test Media

1. MR-VP Medium

Ingredients	gm/liter
Buffered peptone	7.0
Dextrose	5.0
Dipotassium Phosphate	5.0

Final pH (at 25^0C) 6.9±0.2

17 grams was dissolved in 1000ml distilled water. 3ml of medium was distributed in each test tube and autoclaved at 121^0C for 15 minutes.

2. Hugh and Leifson's Medium

Ingredients	gm/liter
Tryptone	2.0
Sodium Chloride	5.0
Dipotassium Phosphate	0.3
Bromothymol Blue	0.08
Agar	2.0

Final pH (at 25^0C) 6.8±0.2

9.4 grams of the medium was rehydrated in 1000ml cold distilled water and then heated to boiling to dissolve completely. The medium was distributed in 100ml amounts and sterilized in the autoclave for 15 minutes at 15lbs pressure (121^0C). To 100 ml sterile medium aseptically added 10ml of sterile Dextrose and mixed thoroughly and dispensed in 5ml quantities into sterile culture tubes.

3. Sulphide Indole Motility (SIM) medium

Ingredients	gm/liter
Beef Extract	3.0
Peptone	30.0

Peptonized Iron	0.2
Sodium Thiosulphate	0.025
Agar	3.0

Final pH (at 25^0C) 7.3±0.2

36 grams of the medium was suspended in 1000ml distilled water and dissolved completely. Then it was distributed in tubes to a depth of about 3 inches and sterilized.

4. Simmon's Citrate Agar

Ingredients	**gm/liter**
Magnesium Sulfate	0.2
Mono-ammonium Phosphate	1.0
Dipotassium Phosphate	1.0
Sodium Citrate	2.0
Sodium Chloride	5.0
Agar	15.0
Bromothymol Blue	0.08

Final pH (at 25^0C) 6.08±0.2

24.2 grams of the medium was dissolved in 1000ml distilled water. 3 ml medium was distributed in test tubes and sterilized by autoclaving at 121^0C for 15 minutes. After autoclaving tubes containing medium were tilted to form slant.

5. Triple Sugar Iron (TSI) Agar

Ingredients	**gm/litre**
Peptone	10.0
Tryptone	10.0
Yeast Extract	3.0
Beef Extract	3.0

Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous Sulphate	0.2
Sodium Chloride	5.0
Sodium Thiosulfate	0.3
Phenol Red	0.024
Agar	12.0

Final pH (at 25^0C) 7.4±0.2

65 grams of the medium was dissolved in 1000ml of distilled water and sterilized by autoclaving at 15lbs (121^0C) pressure for 15 minutes. The medium was allowed to set in slope form with a butt about 1 inch of thickness.

6. Christen Urea Agar

Ingredients	**gm/liter**
Peptone	1.0
Dextrose	1.0
Sodium Chloride	5.0
Dipotassium Phosphate	1.2
Mono-potassium Phosphate	0.8
Phenol Red	0.012
Agar	15.0

Final pH (at 25^0C) 7.4±0.2

24 grams of the medium was suspended in 950ml distilled water and sterilized by autoclaving at 121^0C for 15 minutes. After cooling to about 45^0C, 50 ml of 40% urea was added and mixed well. Then 5ml was dispensed in test tube and set at slant position.

C. Staining and Test Reagents

1. **For Gram's Stain**

a) Crystal Violet Stain

Crystal Violet	20.0g
Ammonium Oxalate	9.0g
Ethanol or Methanol	95ml
Distilled water (D/W) to make	1000ml

Preparation: In a clean piece of paper, 20gm of crystal violet was weighed and transferred to a clean brown bottle. Then, 95ml of ethanol was added and mixed until the dye was completely dissolved. To the mixture, 9 gm of ammonium oxalate dissolved in 200ml of distilled water was added. Finally the volume was made 1000ml by adding distilled water.

(b) Lugol's Iodine

Potassium Iodide	20.0g
Iodine	10.0
Distilled water	1000ml

Preparation: To 250ml of distilled water, 20gm of potassium iodide was dissolved. Then 10gm of iodine was mixed to it until it was dissolved completely. Finally the volume was made 1000ml by adding distilled water.

(c) Acetone-Alcohol Decoloriser

Acetone	500ml
Ethanol (Absolute)	475ml
Distilled Water	25ml

Preparation: To 25ml distilled water, 475ml of absolute alcohol was added, mixed and transferred into a clean bottle. Then immediately, 500ml acetone was added to the bottle and mixed well.

(d) Safranin (Counter Stain)

Safranin	10.0g
Distilled Water	1000ml

Preparation: In a clear piece of paper, 10gm of safranin was weighed and transferred to a clean bottle. Then 1000ml distilled water was added to the bottle and mixed well until safranin dissolved completely.

2. Normal Saline

Sodium Chloride	0.85g
Distilled Water	100ml

Preparation: The sodium chloride was weighed and transferred to a leak-proof bottle premarked to hold 100ml. Distilled water was added to the 100ml mark, and mixed until the salt completely dissolved. The bottle was labeled and stored at room temperature.

Test reagents

a) For Catalase Test

Catalase Reagent (3% H_2O_2)

Hydrogen peroxide	3ml
Distilled Water	97ml

Preparation: To 97ml of distilled water, 3ml of hydrogen peroxide was added and mixed well.

b) For Oxidase Test

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

Tetramethyl *p*-phenylene diamine dihydrochloride (TPD)	1gm
Distilled Water	100ml

Preparation: This reagent solution was made by dissolving 1gm of TPD in 100ml distilled water. To that solution strips of Whatman's No.1 filter paper were soaked and drained for about 30 seconds. Then these strips were freeze dried and stored in a dark bottle tightly sealed with a screw cap.

c) For Indole Test

Kovac's Indole Reagent

Isoamyl alcohol	30ml
p-dimethyl aminobenzaldehyde	2.0g

Hydrochloric acid	10ml

Preparation: In 30ml of isoamylalcohol, 2g of *p*-dimethyl aminobenzaldehyde was dissolved and transferred to a clean brown bottle. Then to that, 10 ml of concentrated HCL was added and mixed well.

d) For Methyl Red Test

Methyl Red Solution

Methyl Red	0.05g
Ethyl alcohol (absolute)	28ml
Distilled water	22ml

Preparation: To 28ml ethanol, 0.05 gm of methyl red was dissolved and transferred to a clean brown bottle. Then 22ml distilled water was added to that bottle and mixed well

e) Mc Farlands' Nephelometer Standards

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

f) For Voges- Proskauer Test (Barrit's Reagent)

Solution A

Alpha-Naphthol	5.0g
Ethyl alcohol (absolute)	100ml

Preparation: To 25ml distilled water, 5g of alpha-naphthol was dissolved and transferred into a clean brown bottle. Then the final volume was made to 100ml by adding distilled water.

Solution B

Potassium hydroxide	40.0g
Distilled Water	1000ml

Preparation: To 25ml distilled water, 40gm of KOH was dissolved and transferred into a clean brown bottle. Then the final volume was made 100ml by adding distilled water.

APPENDIX IV: Gram Staining and Ast Procedure

A. Gram-staining procedure

First devised by Hans Christian Gram during the late 19th century, the Gram-stain ca be used effectively to divide all bacterial species into two large groups: those that take up the basic dye, crystal violet (Gram positive) and those that allow the crystal dye to wash out easily with the alcohol or acetone (Gram negative). The following steps are involved in Gram stain:

1. A thin film of the material to be examined was prepared and dried.
2. The material on the slide was heat fixed and allowed to cool before staining.
3. The slide was flooded with crystal violet stain and allowed to remain without drying for 10-30 seconds.
4. The slide was rinsed with tap water, shaking off excess.
5. The slide was flooded with iodine solution and allowed to remain on the surface without drying for twice as long as crystal violet was in contact with the slide surface.
6. The slide was rinsed with tap water, shaking off excess.
7. The slide was flooded with alcohol acetone decolorizer for 10 seconds and rinsed immediately with tap water until no further color flows from the slide with the decolorizer. Thicker smear requires more aggressive decolorizing.
8. The slide was flooded with counter stain (safranin) for 30 seconds and washed off with tap water.
9. The slide was blotted between two clean sheets of bibulous paper and examined microscopically under oil immersion at 100X.

B. Antibiotic sensitivity tests of isolated organisms

After the identification of isolated organisms, the sensitivity tests were performed. The medium used for this test was Mueller Hinton Agar. The antibiotic susceptibility test was performed by Kirby-Bauer sensitivity testing method, according to guidelines given by the formerly known as National Committee for Clinical Laboratory Standard (NCCLS).

1) Mueller Hinton agar was prepared and sterilized as instructed by the manufacturer.
2) The p^H of the medium 7.2-7.4 and depth of the medium at 4mm (about 25ml plates) was maintained in 90mm Petri dish.
3) Using a sterile wire loop, a single isolated colony of which the sensitivity pattern is to be determined was touched and inoculated into a nutrient broth tube and was incubated for 2-4 hours.
4) After incubation in a good light source, the turbidity of the suspension was matched with the turbidity of standard of Mac Farland 0.5(Prepared by adding 0.6ml of 1% w/v barium chloride solution to 99.4ml of 1% v/v solution of sulphuric acid (Cheesebrough, 2000).
5) 4) Using a sterile swab, a plate of Mueller Hinton agar was inoculated with bacterial suspension using carpet culture technique. The plate was left for about 5 minutes to let the agar surface dry.
6) Using sterile forceps, appropriate antimicrobial discs, (6mm diameter) was placed, evenly distributed on the inoculated plates, not more than 7 discs were placed on a 90mm diameter Petri dishes.
7) Within 30 minutes of applying the discs, the plates were taken for incubation at 37^OC for 18-24 hours.

After overnight incubation, the plates were examined to ensure confluent growth. Using a measuring scale, the diameter of each zone of inhibition in mm was measured and result interpreted accordingly

APPENDIX V: Biochemical Test For Identification of Bacteria

A. Catalase test

A small amount of a culture from Nutrient Agar plate was taken in a clean glass slide and about 2-3 drops of 3% H_2O_2 was put on the surface of the slide. The positive test is indicated by the formation of active bubbling of the oxygen gas. A false positive reaction may be obtained if the culture medium contains catalase (e.g; Blood Agar) or if an iron wire loop is used.

B. Oxidase test

A piece of filter paper was soaked with few drops of oxidase reagent. Then the colony of the test organism was smeared on the filter paper. The positive test is indicated by the appearance of blue-purple color within 10 seconds.

C. Oxidation-Fermentation test

The test organism was stabbed into bottom of two sets of tubes with Hugh and Leifson's media, bromothymol blue being pH indicator. The inoculated medium in one of the tubes was covered with a 10mm deep layer of sterile paraffin oil. The tubes were then incubated at 37^0C for 24 hourrs. After incubation the tubes were examined for carbohydrates utilization as shown by acid production.

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

D. Indole Production test

A smooth bacterial colony was stabbed on SIM (Sulphide Indole Motility) medium by a sterile stab wire and inoculated media was incubated at 37^0C for 24 hours. After 24 hours incubation, 0.5ml of Kovac's reagent was added. Appearance of red color on the top of media indicated indole positive. Indole if present combines with the aldehyde present in the reagent to give a red color in the alcohol layer. Color reaction is based on the presence of the pyrrole structure present in

E. Methyl Red test

A pure colony of the test organisms was inoculated into 2ml of MRVP medium and incubated at 37^0C for 24 hours. After incubation, about 5 drops of methyl red reagent was added and mixed well. The positive test was indicated by the development of bright red color, indicating acidity.

F. Voges-Proskauer (VP) test

A pure colony of the test organisms was inoculated, into 2ml MRVP medium and was incubated at 37^0C for 24 hours. After incubation, about 5 drops of Barrit's reagent was added and shaken well for maximum aeration and kept for 15 minutes, positive test is indicated by the development of pink red color.

G. Citrate Utilization test

A loopful of test organism was streaked on the slant area of Simmon's Citrate Agar medium and incubated at 37^0C for 24 hrs. A positive test was indicated by the growth of organism and change of media by green to blue, due to alkaline reaction. The pH indicator bromothymol blue has a pH range of 6.0-7.6, i.e.above pH7.6; a blue color develops due to alkalinity of the medium.

H. Motility test

The motility media used for motility test are semisolid, making motility interpretations macroscopic. Motile organisms migrate from the stab line and diffuse into the medium causing turbidity. Whereas non-motile bacteria show the growth along the stab line, and the surrounding media remains colorless and clear.

I. Triple Sugar Iron (TSI) Agar

The test organism was streaked and stabbed on the surface of TSI and incubated at 37^0C for 24 hours. Acid production limited only to the butt region of the tube is indicative of glucose utilization, while acid production in slant and butt indicates sucrose or lactose fermentation. Phenol red is the pH indicator which gives yellow reaction at acidic pH, and red reaction to indicate an alkaline surrounding.

J. Urea Hydrolysis test:

The test organism was inoculated in a medium containing urea and the indicator phenol red. The inoculated medium was incubated at 37^0C overnight. Positive organisms show pink red color due to the breakdown of urea to ammonia. With the release of ammonia the medium becomes alkaline as shown by a change in color of indicator to pink.

k. Coagulase test:

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

Slide Coagulase Test:

For slide coagulase test, a drop of physiological saline was placed on three and then a colony of the test organism was emulsified in two of the drops to make thick suspensions. Later drop plasma was added to one of the suspensions and mixed gently. Then a clumping was observed within 10 seconds for the positive coagulase test. No plasma was added in second suspension. This was used for the differentiation of any granular appearance of the organism from true coagulase clumping. The third drop of saline was used for a known strain of coagulase positive staphylococci.

Tube Coagulase Test

Tube test is performed when negative or doubtful are obtained in slide coagualse test.

In the tube coagulase test, plasma was diluted 1 in 10 in physiological saline. Four small tubes were taken, one for test organism, one for positive control, one for ngetive control, and one to observe self-clotting of plasma. Then 0.5ml of the diluted plasma was pipetted into each tube and 0.5ml of test organism, 0.5ml of positive control (*Staphylococcus aureus* culture), and 0.5ml negative control (*Staphyloccoccus epidermis* culture) was added to three tubes, to the

fourth tube, 0.5ml sterile broth was added. After mixing gently, all tubes were incubated at 37^0C on a water bath for 6 hours and observed for gel formation in every 30 minutes.

APPENDIX- VI Zone Size Interpretation Chart For Antibiotic Sensitivity Test

Antimicrobial Agent	Symbol	Disc Content	Resistant (mm or less)	Intermediate (mm)	Sensitive (mm or more)
Amikacin	Ak	30mcg	14	15-16	17
Ceftriaxone	Ci	30mcg	13	14-20	21
Chloramphenicol	C	30mcg	12	13-17	18
Ciprofloxacin	Cf	5mcg	15	16-20	21
Ofloxacin	Of	5mcg	12	13-15	16
Cotrimoxazole	Co	25mcg	10	11-15	16
Cefotaxime	Ce	30mcg	14	15-22	23
Cloxacillin	Cx	5mcg	11	12-13	14
Erythromycin	E	15mcg	15	16-20	21
Vancomycin	VA	30mcg	17	15-16	14
Tigecycline	TGC	15mcg	18	15-17	15
Teicoplanin	TEI	30mcg	14	11-13	10
Piperacillin	PI	30mcg	20	17-19	17
Piperacillin/Tazobactam	PIT	30mcg	21	18-20	17
Linezolid	LZ	30mcg	23	21-22	20

(Source: Product Information Guide, Hi-Media Laboratories Pvt. Limited, Mumbai,India).

APPENDIX VII: Chi-Square Test

Association between positive case and negative case among gender.

Gender	**Male**	**Female**	**Total**
Positive case	108	117	225
Negative case	58	52	110
Total	166	169	335

Test statistics is χ^2

H_0: There is no significant association between positive case and negative case among gender.

H_1: There is significant association between positive case and negative case among gender.

From $\chi^2 = \sum (O-E)^2/E$ we find $\chi^2 = 0.65$

Thus χ^2_{cal} (0.65) $< \chi^2_{tab}$ at α 0.05 and d.f = 1 i.e.3.841

Hence, H_0 is accepted i.e. there is no significant association between positive case and negative case among gender.