ETIOLOGY OF MENINGITIS FROM PATIENTS SUSPECTED OF MENINGITIS ATTENDING TRIBHUVAN UNIVERSITY TEACHING HOSPITAL, KATHMANDU, NEPAL

A Dissertation

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Master of Science in Microbiology

(Medical)

By

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RECOMMENDATION

This is to certify that **Ms. Pinky Pandey** has completed this dissertation work entitled **"Etiology of Meningitis from Patients Suspected of Meningitis Attending Tribhuvan University Teaching Hospital, Kathmandu, Nepal"** as a partial fulfillment of the requirements of M.Sc. degree in Microbiology (Medical) under our supervision. To our knowledge this work is original and has not been submitted for any other degree.

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CERTIFICATE OF APPROVAL

On the recommendation of **Prof. Dr. Bharat Jha** and **Mrs. Anima Shrestha,** this dissertation work of **Ms. Pinky Pandey,** entitled **"Etiology of Meningitis from Patients Suspected of Meningitis Attending Tribhuvan University Teaching Hospital, Kathmandu, Nepal"** has been approved for the examination and is submitted to the Tribhuvan University in the partial fulfillment of the requirements for M.Sc. degree in Microbiology (Medical).

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ABSTRACT

Meningitis is an inflammatory infection of the membranes surrounding the brain and spinal cord, which occurs as either a primary disease or secondarily to disease in some other part of the body. Its most frequent causes are *Neisseria meningitidis, Streptococcus pneumoniae,* and *Haemophilus influenzae*. The epidemiological trend of acute meningitis varies with time and geography. Information on the relative frequency of the isolation and antibiotic susceptibility patterns of these pathogens is scarce in Nepal. The broad objective of this research was to analyze the various etiological agents of meningitis in all age group patients and to know the antimicrobial susceptibility pattern of bacterial isolates from the cerebrospinal fluids (CSF) of suspected cases of meningitis.

In this cross sectional study, a total of 356 CSF specimens were collected from patients suspected of meningitis and processed macroscopically, microscopically and microbiologically by standard microbiological methods in Emergency Lab of TUTH in Kathmandu, Nepal over a period of six months, from March 2014 to August 2014 to determine cytological, biochemical and microbiological parameters.

Out of 356 CSF samples, bacterial and fungal culture positivity rate was found to be 16 (4.5%). Among the positive isolates, the most common bacterial isolate was *Staphylococcus aureus*, 4 (25%). Isolation rate of *Cryptococcus neoformans* was 3 (18.8%) the only fungal etiology of meningitis which was seen in elderly patients indicating increased susceptibility in immune-compromised status of patients. All bacterial isolates were found to be sensitive against Chloramphenicol. Thus it is concluded that the isolation rate of pathogens from cerebrospinal fluids causing meningitis is low. Chloramphenicol is effective for the treatment of bacterial meningitis.

Key words: Meningitis, cytological, biochemical and microbiological parameters, antimicrobial susceptibility.

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ABBREVIATIONS

AIDS : Acquired Immunodeficiency Syndrome

ATCC : American Type Culture Collection

BA : Blood agar

BBB : Blood Brain Barrier

CA : Chocolate agar

CDC : Center for Disease Control

CFR : Case Fatality Rate

CLSI : Clinical and Laboratory Standards Institute

CNS : Central Nervous System

DLC : Differential Leukocytes Count

Hib : *Haemophilus influenzae* type B

HIV : Human Immuno Deficiency Virus

IL : Interleukin

LAT : Latex Agglutination Test

MA : MacConkey Agar

MSA : Mannitol Salt Agar

NA : Nutrient Agar

PMN : Polymorphonuclear

SDA : Sabouraud Dextrose Agar

SPSS : Statistical Package for Social Sciences

TLC : Total Leukocytes Count

TNF : Tumour Necrosis Factor

TUTH : Tribhuvan University Teaching Hospital

CHAPTER I
INTRODUCTION AND OBJECTIVES

1.1 Background of the Study

Meningitis is one of the medical emergencies related to infectious diseases, which is potentially associated with a high rate of complications. The term Meningitis is used to describe an inflammation of the membranes that surround the brain or the spinal cord. However, this inflammation not only involves the meninges but also spreads to the subarachnoid space and to the cerebrospinal fluid (CSF) contained within it (Adhikary and Chatterjee, 2013).

Meningitis can be acute, with a quick onset of symptoms, or chronic, lasting a month or more, or can be mild or aseptic, but the emphasis should be on identification of cause so that appropriate interventions can be applied. Generally, the meningitidis are of infectious etiology that can be viral, bacterial, fungal, or parasitic in nature, yet bacteria remain the common etiological agent (Nagarathna *et al.*, 2012). However, some non-infectious causes of meningitis also exist.

Rudolf *et al.* (2011) mentions that meningitis, especially bacterial meningitis, is a potentially life-threatening condition that can rapidly progress to permanent brain damage, neurologic problems, and even death of children (as cited in Mahmoud and Abd-ElSadik, 2013). Meningitis can affect anyone in any age group, from the newborn to the elderly. The highest incidence of meningitis is between birth and two years, with the greatest risk immediately following birth and at 3-8 months of age. Increased exposure to infections and underlying immune system problems present at birth increase an infant's risk of meningitis (Tunkel *et al.*, 2004).

Bacterial or pyogenic meningitis has the highest incidence in the first year after birth. Adolescence also shows a higher incidence between 15-24 years of age which accounts for almost 30% of all the cases of bacterial meningitis (Ahmed, 2012). Over the past several years, there has been a striking shift in the demography of meningitis. In 1986, the median age of a patient with meningitis was 15 months, as compared with 25 years in 1995. Before the widespread use of the conjugated *Haemophilus*

influenzae type B (Hib) vaccine in 1990, *H. influenzae* type b meningitis developed in nearly 1 in 200 children less than 5 years old, and almost 70% of the cases of meningitis in children less than 5 years old were due to *H. influenzae* (Greenwood, 2006).

Globally, bacterial meningitis affects approximately 1.2 million people each year and causes almost 170,000 deaths. In the absence of proper treatment, the mortality rate associated with bacterial meningitis can be as high as 50%. For this reason bacterial meningitis is among the 10 leading causes of mortality due to infections worldwide (Ahmed, 2012). Even when the disease is diagnosed early and adequate treatment is started, 5% to 10% of patients die, typically within 24 to 48 hours of the onset of symptoms. Bacterial meningitis may result in brain damage, hearing loss or a learning disability in 10% to 20% of survivors (World Health Organization [WHO], 2014).

Cryptococcal meningitis is the commonest life threatening opportunistic fungal infection in patients afflicted by Human Immuno Deficiency Virus (HIV). The condition is also known to occur in other immune-compromised conditions and immune-competent individuals (Kapila *et al.*, 2003). It is the most common lethal fungal infection in patients with acquired immunodeficiency syndrome (AIDS) worldwide. Recent data indicates that the incidence of cryptococcal infection is high in the developing countries such as India (Baradkar *et al.*, 2009).

Epidemic rates of meningococcal disease varies from <1-3/100,000 in many developed nations to 10-25/100,000 in some developing countries. This difference in attack rates reflects the difference in pathogenic properties of *Neisseria meningitidis* strains prevalent and differences in socioeconomic and environmental conditions (Manchanda *et al.*, 2006). The first recorded major outbreak occurred in Geneva in 1805. Still in the 21st century, every year, bacterial meningitis epidemics affect a large region of sub-Saharan Africa known as the "meningitis belt" which comprises 22 countries from Senegal in the west to Ethiopia in the east, in which large epidemics of Meningococcal meningitides occur with annual incidence reaching 1000 cases per 100,000 population (WHO, 2013).

Asia has also been focus of some major epidemics of meningococcal disease in the last 30 years. During 1982-1984, a large meningococcal serogroup A outbreak

occurred in Kathmandu valley, Nepal, with highest mortality and morbidity in children less than one year of age. Incidence and mortality was highest in infants aged<1 year (221/100,000 and 26%, respectively) with an annual attack rate of 103 cases per 100,000 population (Vyse *et al.*, 2011).

The three most common bacterial pathogens causing bacterial meningitis are *H. influenzae* type b (Hib), *N. meningitidis*, and *Streptococcus pneumoniae*, which together account for about 80% of all cases. Over two-thirds of all cases of bacterial meningitis occur in children less than five years old. Meningococcus affects all ages, most cases occurring in children and adolescents. More than 80% of meningitis caused by *H. influenzae* occurs in children less than 5 years old. Group B hemolytic *streptococcus* (*Streptococcus agalactiae*) is the commonest cause of meningitis in neonates. *Escherichia coli* is a frequent cause of meningitis in neonates and is rarely a cause after infancy (Andargachew *et al.*, 2005).

The first three of the above organisms (*H. influenzae* type b (Hib), *N. meningitidis*, and *Strep. pneumoniae*) are respiratory pathogens. They are spread from person to person by close contact with respiratory secretions. Once acquired, each species can colonize the mucosa of the nasopharynx and oropharynx, which is known as pharyngeal carriage. From there, they may cross the mucosa and enter the blood. Once in the blood, they can reach the meninges, cause meningitis. Whereas, group B *streptococcus, E. coli* and *Listeria monocytogenes* are the most common causative agents of meningitis in neonates. The uncommon pathogens are *Staphylococcus aureus*, and *Pseudomonas aeruginosa. Mycobacterium tuberculosis* is the less frequent of bacterial meningitis. Many viruses have the ability of causing meningitis such as *Echovirus, Coxasackie virus* type A and B, *Herpes simplex virus* type 1 and 2, Epstein barr virus, HIV, Vricella-zoster virus and Cytomegalovirus (AL-Zubiery, 2001). Over 1.2 million cases of bacterial meningitis are estimated to occur worldwide each year (Center for Disease Control [CDC], 2014a).

In addition, to bacterial and viral agents, fungi may also cause meningitis like *Cryptococcus neoformans* and *Candida*, which are the most common fungi isolated from CSF. Parasite such as *Naegleria* has been identified as a causative agent of meningitis so far (AL-Zubiery, 2001).

Before the 20th century, acute bacterial meningitis was almost always fatal. The invention of antibiotics has drastically improved the outcome of bacterial meningitis. Initiation of proper treatment within 6 hours of presentations reduces the mortality rates by more than 8 times (Ahmed, 2012).

Conclusive diagnosis of meningitis requires lumbar puncture and cerebrospinal fluid analysis. During meningitis, the normal composition of CSF, the liquid that bathes brain and spinal cord in subarachnoid space, gets altered. Normally, CSF is clear, colorless fluid that contains little protein (15-40 mg %) and has a lower pH, and lower concentration of glucose (45-72 mg %), potassium, calcium, bicarbonate and amino acids than blood plasma with few WBCs ranging from 0-8 cells/ mm^3 (in infants) to 0-5 cells/mm^3 (in adults). But during meningitis, pleocytosis occurs. The number and types of leukocytes in the CSF is important in differentiating the pyogenic (bacterial) and aseptic (viral) meningitis. Bacterial meningitis is characterized by marked pleocytosis of CSF, consisting predominantly of polymorphonuclear (PMN) leukocytes or Neutrophils, while in case of aseptic meningitis moderate pleocytosis consisting mainly lymphocytes. In pyogenic meningitis, the glucose concentration is reduced markedly and protein concentration is increased. In aseptic meningitis, glucose concentration is normal and protein concentration is increased little (Collee, *et al.*, 1996). John *et al.* (1984) summarizes the CSF laboratory findings as being very low sugar, high protein, low chloride, and very high lymphocytes in immune-competent patients and variable laboratory findings in immune-compromised patients suffering from fungal meningitis (Peter, 1997).Thus, changes in the composition (increased protein) or in the appearance (cloudiness) of the CSF would suggest some neurological disease like meningitis.

Since the pathogens responsible for the bacterial meningitis vary with time, geography and patient age, there is a need for a periodic review of bacterial meningitis worldwide (Tang *et al.*, 1999). At the meantime, what is true in the western world may not be so in other parts of the world. Therefore the current study was undertaken to analyze the etiology of meningitis and determine different parameters (cytological, biochemical and microbiological) and perform antibiotic susceptibility test of bacterial isolates from CSF of the meningitis suspected patients attending TUTH, Kathmandu, Nepal.

In recent years, despite improvement in antimicrobial therapy and sophisticated intensive care units, bacterial meningitis continues to be a significant cause of morbidity and mortality. This is reflected in the fact that meningitis continues to be present among the ten leading causes of death (Tegene, 2011).

The World Health Organization indicates that each year registers nearly half a million new cases suffering from meningitis (Mahmoud and Abd-ElSadik, 2013). Whereas bacterial meningitis affects approximately 1.2 million people each year globally (Ahmed, 2012) and causes almost 170,000 deaths worldwide each year. Young children are particularly vulnerable to bacterial meningitis, and when exposed poor outcomes may occur due to the immunity of their immune systems. Two-thirds of meningitis deaths in low income countries occur among children under 15 years of age (Meenakshi *et al.*, 2009).

The burden of the meningitis in the African "meningitis belt" is considerable and intense recurring large-scale epidemics with annual incidence reaching 1000 cases per 100,000 populations. The most recent large-scale meningitis epidemic in the African meningitis belt occurred in 2009, where 88,199 suspected cases, including 5352 deaths were reported the largest number since a 1996 epidemic (WHO, 2013). Similarly, a large meningococcal outbreak was also experienced during 1982-1984 in Kathmandu Valley, Nepal resulting in 875 cases and 95 deaths. The annual attack rate was 103 cases per 100,000 populations, with a peak attack rate occurring in April. Similarly, several studies conducted in different times have shown presence of meningitis in Nepalese population. In two prospective hospital-based studies between 2001 and 2007, *N. meningitidis* accounted for 5.4% (2 out of 37 bacteriologically confirmed cases) of bacterial meningitis in all age groups and 1.3% (2 out of 151 culture-positive meningitis cases) in the <5 years age group, of whom 26.7% of the 151 individuals had received prior antibiotics (Vyse *et al.*, 2011).

Vyse *et al.* (2011) mentioned that meningococcal disease in Asia are incomplete, due to absence of surveillance in many countries, poor bacterial detection methods and social and healthcare barriers to disease reporting. This suggests that meningococcal disease in some Asian countries may be under-recognized, with a need to introduce/improve existing surveillance and case identification systems. Nevertheless,

in some developing Asian countries, the disease burden may be significant and Nepal may not be the exceptional case.

There is also paucity of objective data on the causative agents of meningitis and their susceptibility in Nepalese population (Ansari and Pokhrel, 2011). Due to the urgency associated with the treatment of meningitis, there is, therefore, the need to have knowledge of the causative agents and their susceptibility profiles to come to better decisions of the empirical treatment. The incidence and prevalence of the pathogens causing meningitis as well as their susceptibility profile is necessary to be assessed. Knowledge of bacteria causing meningitis and their antimicrobial susceptibility profile would be justifiable as prerequisite for correct treatment and infection control measures. Thus it is vital to conduct research on etiological agents of meningitis and their susceptibility test in Nepalese context to review and update the status of meningitis.

Thus, the result of this study provides information about the etiological agents of meningitis and its prevalence in the current situation. Thereby concerned health authorities will have a data about the current status of meningitis and be able to evaluate the outcomes of prevention and control measures which have been practiced in the past thereby decreasing the morbidity of this dread disease and strengthen ability to plan, prioritize, and implement effective interventions.

Antimicrobial susceptibility testing of isolated bacteria in the study population would help us determine the sensitivity patterns of these isolates, thereby reducing antibiotics misuse and the incidence of microbial drug resistance. Moreover, this study will also serve as an input for future meningitis related studies and reviews.

1.2 Objectives of the Study

General Objective

- To analyze the etiology of meningitis from patients suspected of meningitis attending TUTH.

Specific Objectives

- To isolate and identify the etiological agents of meningitis
- To assess the frequency of pathogens in different age groups and sex
- To study the association of incidence of meningitis with age groups and sex
- To determine the cytological (TLC/DLC), biochemical (glucose and protein) and microbiological parameters (staining and culture) from the CSF samples
- To correlate cytological, biochemical and microbiological parameters with types of meningitis
- To correlate the CSF staining and culture results
- To determine antibiotic susceptibility pattern of the bacterial isolates

CHAPTER II
LITERATURE REVIEW

2.1 Meningitis: An Overview

The word “meningitis” comes from the modern Latin word “meninga” and the Greek word “Menix” meaning “membrane”. The suffix “itis” comes from the Greek word “itis” meaning “pertaining to” (Medical News Today, 2014).

Three layers of fibrous tissue viz. duramater, arachnoid mater and pia mater from outside to inner cover the surfaces of the brain and spinal cord (Wilson, 1995). These layers known as meninges cushion and protect the Central Nervous System (CNS). When organisms, such as bacteria or viruses infect meninges, the body takes steps to defend itself from infection. White blood cells and other infection-fighting substances pour into the CSF. This process results in a set of symptoms known as meningitis. The inflammation and swelling may extend through the membranes of the pia mater, arachnoid or subarachnoid (Al Bekairy *et al.*, 2014). When the inflammation occurs in duramater, the disease is termed as pachymeningitis and when aracnoid and piamater are involved, it is called as leptomeningitis or meningitis proper (Brain and Walton, 1969).

Meningitis can be classified into infectious and noninfectious disease. Noninfectious meningitis can emerge from administration of certain drugs such as non-steroidal anti-inflammatory drugs, immunoglobulins or some antibiotics. It can also develop from diseases like sarcoidosis and neoplastic meningitis. Infectious meningitis can be further sub-divided to non-bacterial and bacterial (pyogenic) meningitis. Non-bacterial meningitis is typically caused by viral or fungal infections (Al Bekairy *et al.*, 2014). Viral meningitis is sometimes called aseptic meningitis to indicate it is not the result of bacterial infection and cannot be treated with antibiotics (American Academy of Neurology, 2014). Also Aseptic meningitis refers to meningitis where an infective agent cannot be identified. Bacterial meningitis is characterized by the significant polymorph nuclear changes in the CSF.

Meningitis may be acute bacterial meningitis, subacute meningitis and chronic meningitis. Subacute meningitis is a meningeal inflammation that lasts >2 weeks and chronic meningitis last >1 month, may have infectious or noninfectious causes (Jacewicz, 2009).

2.2 Historical Perspective of Meningitis

The earliest documentation of patients with meningitis can be found in the writings of Hippocrates. Bacterial meningitis was first described by Hippocrates in the 5th century B.C. in his volume *Diseases II* of the *Corpus Hippocraticum* as cited by Brouwer (2010):

> …chills, pain and fever throughout the head, especially in the ear, temples and bregma. The patient feels pain in the sockets of his eyes, his eyebrows seem to press down on him, and heaviness befalls his head. If anyone moves him, he vomits copiously and easily; his teeth are set on edge, and he is numb. The vessels in his head are raised up and throb, and he cannot bear to be still, but is beside himself and frenzied from pain. If, in this patient, a watery discharge breaks out through the nostrils or ears, it runs out mixed with pus, and he recovers; if not, he usually dies in seven days (p.9).

It took however many centuries, before the Englishman T. Willis in 1684 described a clinical picture similar to meningococcal disease. The first description of the clinical and pathological features of bacterial meningitis was only recorded in the early 19th century. Epidemic cerebrospinal meningitis was first described by Gaspard Vieusseux of Geneva in 1806. Vieusseux observed that many of his patients had purple spots on the skin and marked engorgement of the brain at autopsy. Most victims were infants and children. He noted that the patients complained of violent headache, stiffness of the spine and convulsions. Elisha North of Goshem, Connecticut, observed a similar epidemic in 1807 which he described in a monograph published in 1811, the first published book on this disease. The puzzling late discovery of meningitis as a clinical entity did not delay a further rapid development of knowledge on its etiology (Kornelisse, 1996).

Several other epidemics in Europe and the United States were described shortly afterward. In Africa the first outbreak was described in 1840. African epidemics became much more common in the 20th century. The first major one was reported in Nigeria and Ghana in 1905-1908 (Mandal, 2012).

The sporadic nature of outbreaks and the apparent absence of transmission of infection by victims led many nineteenth-century writers to believe that the disease was not contagious and was somehow linked to climatic or environmental conditions such as crowding. However by about 1860, it was widely assumed that some sort of poison or agent was involved in the pathogenesis of meningitis. In 1887 an Australian pathologist, Anton Weichselbaum, described the meningococcus under the name *Diplococcus intracellularis meningitidis*. This was followed by the first in vivo diagnosis of meningitis by lumbar puncture performed by Quincke in 1891 and the first identification of the pathogen in a living patient by Heubner in 1896. Heubner found typical "biscuit shaped" diplococci in pus cells. By 1910 it was recognized that the meningococcus was responsible for epidemics of meningitis and that other bacteria could cause sporadic cases (Kornelisse, 1996).

Organisms causing meningitis were identified in the late 19th century including *Strep. pneumoniae, N. meningitidis* and *H. influenzae*. Vladimir Kernig (1840-1917) and Josef Brudzinski (1874-1917) described their eponymous signs in 1882 and 1909 (Tyler, 2009).

H. influenzae was initially identified in 1892, when Pfeiffer described a pleomorphic gram-negative rod and erroneously identified it as the causative agent of influenza. After 1918, when this microorganism was only infrequently isolated from the lungs of patients who died during an influenza pandemic, it was reduced to the role of secondary invader. The organism was given its name "Haemophilus" by Winslow *et al.* in 1920. Prior to that time, *H. influenzae* became recognized as the major pathogen responsible for bacteremia and meningitis in children. In 1931, Pittman first reported the presence of encapsulated and unencapsulated forms of *H. influenzae*. Encapsulated forms were classified into six serological types. Invasive disease was subsequently documented to be mainly caused by type b organisms (Kornelisse, 1996).

Human cryptococcal infection was first described during the last decade of the 19th century. Busse (1894), a pathologist, and Buschke (1895), a surgeon, separately reported the isolation of yeast from the tibia of a young woman (Berger, n.d.). In 1905 the yeast was identified as a CNS pathogen when von Hansemann (1905) described the first case of cryptococcal meningitis (Berger, n.d.).

Successful treatment of meningitis began with the introduction of serum therapy for meningococcal meningitis by Georg Joachmann (1874-1915) in Germany and Simon Flexner (1863-1946) in America. Antibiotic therapy began in the 20th century with the use of sulfonamides by Francois Schwentker (1904-1954) and penicillin by Chester Keefer (1897-1972). Vaccination against meningitis debuted in the early 20th century, and progressed to the development of vaccines against *N. meningitidis* and *H. influenzae*, which remain mainstays of modern medicine (Tyler, 2009). In 2002, evidence emerged that treatment with steroids could improve the prognosis of bacterial meningitis. This also revolutionized therapy of meningitis and improved long term outcome of the condition (Mandal, 2012).

2.3 Anatomical and Physiological Considerations in Meningitis

2.3.1 Anatomy of Meninges

Meningitis is inflammation of the meninges, the thin anatomical structure (three layers or "membranes") that intimately and delicately covers the brain and spinal cord. Specifically, meningitis is an infection within the subarachnoid space, a space between the middle and innermost layers. The three layers of the meninges are *the dura mater*, the *arachnoid mater*, and the *pia mater*. The primary function of these membranes is protection.

The dura mater (Latin: dura, "hard"; mater, "mother") the outermost layer, is composed of tough, nonelastic, dense connective tissue and adheres to the skull and vertebral column. It consists of two fused layers: an inner dura mater that is continuous with the spinal dura mater and an outer dura mater, which is actually the periosteal layer of the skull bones. The dura mater is covered on its innermost surface by squamous epithelial cells (Gray and Fedorko, 1992).

Sheaths of dura mater extend outwards for a short distance as covering for cranial nerves as they pass through their respective foramina. The spinal dura forms a lining to the vertebral canal from which it is separated by epidural space containing fatty tissues and venous plexus. The dura extends to the second or third sacral vertebra. The dura mater is also known as pachymeninx, so the infection of dura matter is called pachy meningitis (Gajipara, 2008)

The arachnoid (Greek: arachnoeides; "like a cobweb"), the middle layer, is composed of dense collagenous and elastic connective tissue, adheres to the dura mater, and has delicate spiderweb like projections (trabeculae) which connect it to the third layer, the pia mater. The arachnoid and its trabeculae are covered with squamous epithelial cells (Gray and Fedorko, 1992). Arachnoid is a delicate avascular membrane (i.e. no blood vessels of its own) but blood vessels are present in the arachnoid space. It extends over the spinal cord up to the second sacral vertebra (Gajipara, 2008).

The pia mater (Latin: pia, "tender"; mater, "mother"), the innermost layer, is composed of delicate collagenous and elastic connective tissue and is covered by squamous epithelial cells. The pia mater is the only meningeal layer which contacts the central nervous system; specifically, the pia mater (and, thus, the meninges) covers the surfaces of the brain and spinal cord (Gray & Fedorko, 1992). The arachnoid and pia mater is also known as the leptomeninges. So the infection of arachnoid and pia is called as leptomeningitis or simply as meningitis (Gajipara, 2008).

Highly vascularized villi of the pia mater project into four ventricles (cavities) within the brain and are covered with ependymal epithelial cells. These projections are known as the choroid plexuses and are the sites at which the fluid component of the blood is modified (by secretion and absorption of certain solutes) and secreted into the ventricles. This modified and secreted fluid is CSF. CSF circulates in the ventricles and the subarachnoid space around the brain and spinal cord and returns to the blood circulatory system through subarachnoid villi that project into the superior sagittal sinus, which traverses the inner roof of the skull (Gray and Fedorko, 1992).

Three anatomical spaces in the central nervous system, which are sites of distinct bacterial infections are epidural space (between the vertebrae and the dura mater),

subdural space (between the dura mater and the arachnoid), subarachnoid space (between the arachnoid and the pia mater). Subdural space is devoid of CSF (Gajipara, 2008). The subarachnoid space is the largest of the three spaces and is the main reservoir of CSF. Meningitis occurs in the subarachnoid space (Gray and Fedorko, 1992).

2.3.2 Blood Brain Barrier

The blood brain barrier (BBB) is a diffusion barrier essential for the normal function of the central nervous system. Three cellular elements of the brain microvasculature compose the BBB - endothelial cells, astrocyte end-feet, and pericytes (PCs). Tight junctions (TJ), present between the cerebral endothelial cells, form a diffusion barrier, which selectively excludes most blood-borne substances from entering the brain with a low rate of pinocytosis, making it different from other endothelia in the body. Astrocytic end-feet tightly ensheath the vessel wall and appear to be critical for the induction and maintenance of the TJ barrier, but astrocytes are not believed to have a barrier function in the mammalian brain. Pericytes are the least studied cellular component of the BBB but appear to play a key role in angiogenesis, structural integrity and differentiation of the vessel, and formation of endothelial TJ (Ballabh *et al.*, 2004; Bergman *et al.*, 2006).

The BBB is a monolayer of cells that regulates the passage of solutes between the CNS and the blood. The BBB not only restricts the entry of serum proteins into the CNS, but it also controls the passage of nutrients, electrolytes, vitamins, minerals, free fatty acids, peptides, and regulatory proteins in both the brain to blood and blood to brain direction (Banks, 1999).

The BBB performs these functions through a number of saturable and non-saturable mechanisms. For example, efflux (CNS to blood) mechanisms regulate the levels of nutrients and minerals in the CSF, detoxify the CNS, reinforce the impermeability of the BBB against circulating toxins and many drugs, secrete CNS-originating substances into the blood, and drain substances directly into the cervical lymphatic nodes. Influx mechanisms control the homeostatic environment of the CNS, supply the brain with nutrients, and help to integrate CNS and peripheral functions (Banks, 1999).

The BBB is essentially impermeable for large and hydrophilic molecules as well as for most bacteria and viruses (Bergman *et al.*, 2006). In contrast, small lipophilic substances such as O_2 and CO_2 diffuse freely across plasma membranes along their concentration gradient. Nutrients including glucose and amino acids enter the brain via transporters, whereas receptor-mediated endocytosis mediates the uptake of larger molecules including insulin, leptin, and iron transferrin (Ballabh *et al.*, 2004). However, certain bacterial species have developed strategies to translocate across the BBB and enter the subarachnoid space, initiates a massive inflammatory response involving the release of cytokines and chemokines, ultimately leading to meningitis (Bergman *et al.*, 2006).

The subarachnoid space and its CSF are relatively defenseless while infection in stopping invasion by bacterial pathogens because of the CSF's paucity of phagocytic cells and low concentrations of complement and immunoglobulin. Unchecked invasion and multiplication of bacteria in the CSF result in meningitis. Inflammation of the meninges is initiated by the presence of bacterial lipopolysaccharide, teichoic acid, and/or other bacterial cell wall components in the subarachnoid space. The bacterial antigens stimulate monocytes to produce the cytokine interleukin-1 and stimulate macrophages, astrocytes, microglial cells, ependymal cells, and endothelial cells in the central nervous system to produce the cytokine tumor necrosis factor (cachectin). Tumor necrosis factor and interleukin-1 probably act synergistically to elicit inflammatory responses which manifest clinically as meningitis (Gray and Fedorko, 1992).

2.3.3 Cellular Anatomy of Choroid Plexus

Choroid plexuses (CP) are highly vascularized structures that consist of a single layer of epithelial cells surrounding a core of fenestrated capillaries and venules. They are located in the lateral, third and fourth ventricles of the brain, and they are continuous with and branch out of the ependymal lining of these cavities (Morrison, 2009).

The choroid plexuses are outpouchings of blood vessels that are covered by an epithelium and float in the CSF. The choroidal epithelial cells are joined together on the CSF side by tight junctions. This constitutes the site of the blood-CSF barrier in the choroid plexus. Unlike the majority of the cerebral vasculature, the choroidal

capillaries are fenestrated and freely permeable to small molecules. The epithelial cells of the choroid plexus feature many mitochondria within the cytoplasm, microvilli and cilia on the CSF side, and complex intracellular clefts on the vascular side of the cell, suggesting this epithelium is involved extensively in active transport (Venkatesh *et al.*, 2000).

2.3.4 Anatomical Structure of Cerebrospinal Fluid Spaces

The cerebrospinal fluid is divided into an internal space, i.e., the ventricular space and the central canal, and an external one called the subarachnoid space. The thin epithelial membrane that lines the inner wall of the cavity is sealed, forming a biological barrier between the liquid and nervous tissues.

Ventricles

The ventricular space consists of a series of cavities within the brain, lined by cuboidal epithelial cells known as ependyma and filled with cerebrospinal fluid. A network of blood vessels called a choroid plexus is formed in several places where the ependymal contacts the pia mater. It includes four cerebral ventricles, namely, the two lateral ventricles, the third ventricle and the fourth ventricle (Lebret, 2013).

Subarachnoid space

The pia mater inside and the arachnoid mater outside, delimit the subarachnoid space. The space is narrow over the brain convexity and it has several wider regions, called cisterns, at the base of the brain. This cistern drains the cerebrospinal fluid from the fourth ventricle through the median aperture. The pontocerebellar cistern is on the ventral aspect of the pons and receives cerebrospinal fluid from the fourth ventricle through both lateral apertures (Lebret, 2013).

2.3.5 Cerebrospinal Fluid

Cerebrospinal fluid is a clear colorless solution that bathes the central nervous system, that is, the brain and the spinal cord. It fills the cavities within the brain called ventricles and the peripheral area of the brain and spinal cord called the subarachnoid space. The fluid circulates from ventricles where it is produced to the subarachnoid

space where it is resorbed into the circulatory system. Cerebrospinal fluid plays an active role in the homeostasis of central nervous system (Lebret, 2013).

CSF formation

CSF is predominantly, but not exclusively, secreted by the choroid plexuses. Brain interstitial fluid, ependyma and capillaries may also play a poorly defined role in CSF secretion (Sakka *et al.*, 2011). Less than 10% of the CSF is produced by extrachoroidal sources, mostly arising from the interstitial fluid (ISF) of the brain (Morrison, 2009).

Choroidal secretion of cerebrospinal fluid comprises two steps. The first step consists of passive filtration of plasma from choroidal capillaries to the choroidal interstitial compartment according to a pressure gradient. The second step consists of active transport from the interstitial compartment to the ventricular lumen across the choroidal epithelium, involving carbonic anhydrase and membrane ion carrier proteins.

Cytoplasmic carbonic anhydrase catalyses the formation of H^+ and HCO_3^- ions from water and CO_2. The carrier proteins of basolateral membranes of choroidal cells exchange H^+ and HCO_3^- ions for Na^+ and Cl^- ions. ATP-dependent ion pumps of the apical membrane expel Na^+, Cl^-, HCO_3^- and K^+ ions towards the ventricular lumen. Water transport, facilitated by aquaporins I of the apical membrane, follows the osmotic gradients generated by these pumps. The NaK2Cl cotransporter of the apical membrane generates ion transport in both directions and participates in regulation of CSF secretion and composition (Sakka *et al.*, 2011).

Extrachoroidal secretion is derived from extracellular fluid and cerebral capillaries across the blood-brain barrier. This pathway appears to play a minimal role under physiological conditions. CSF can also be derived from the ependymal epithelium, the target of regulations mediated by neuropeptides and growth factors, which can be altered by ependymal changes induced, in particular, by ventricular dilatation (Sakka *et al.*, 2011).

Cerebrospinal fluid circulation and absorption

CSF circulation is a dynamic phenomenon and regulation of CSF circulation is responsible for cerebral homeostasis. CSF circulates from the sites of secretion to the sites of absorption according to a unidirectional rostrocaudal flow in ventricular cavities and a multidirectional flow in subarachnoid spaces. CSF flow is pulsatile, corresponding to the systolic pulse wave in choroidal arteries. CSF produced by the choroid plexuses in the lateral ventricles travels through interventricular foramina to the third ventricle, and then the fourth ventricle via the cerebral aqueduct and finally to the subarachnoid spaces via the median aperture (foramen of Magendie) and two lateral formaina of Luschka of the fourth ventricle. In the cranial subarachnoid space, CSF circulates rostrally to the villous sites of absorption or caudally to the spinal subarachnoid space (Sakka *et al.*, 2011; Jozefowich, n.d.).

CSF is mainly absorbed by the arachnoid villi embedded in the dura mater of super sagittal sinus and spinal nerves. Each arachnoid villus is thought to have a one-way (CSF outward) valve-like mechanism that opens in response to a positive hydrostatic pressure gradient between CSF and dural venous blood (Johanson *et al.*, 2008).

A significant fraction of CSF drains via the spinal nerve roots into the local lymphatic networks. The normal CSF pressure varies between 5-18cm H_2O. When CSF pressure is greater than venous pressure, fluid drains from the CSF into the blood. If the pressure is greater in the veins, the arachnoid villi collapse and no flow occurs. The absorption rate increases linearly with CSF pressure. At a CSF pressure of 11 cm H_2O, the formation and absorption rates are equal (Venkatesh *et al.*, 2000).

Function of CSF

The functions of CSF include, provision of buoyant physical support to the brain (e.g. the effective brain weight is reduced from 1500g to as little as 50g), maintenance of constant intracranial pressure, defense against bacterial invasion, intracerebral transport of biomolecules, and a drainage pathway for waste products, electrolytes and excess neurotransmitters (i.e. the ‘sink action’ of CSF) (Venkatesh *et al.*, 2000).

Composition of CSF

CSF is a clear aqueous solution that, compared with plasma, contains higher concentration of sodium, chloride, and magnesium and lower concentration of glucose, proteins, amino acids, uric acid, potassium, bicarbonate, calcium and phosphate (Artru, 2010). The protein content is lower in the lateral ventricles than in the subarachnoid space (Mathayya, 2002). CSF contains 99.13% water and only 0.87% solids. Since is a part of extracellular fluid (ESF), it contains more amount of sodium than potassium. CSF also contains some lymphocytes (6/mm^3). CSF secreted by ventricles doesn't contain any cell. Lymphocytes are added when CSF flows in the spinal cord (Sembulingam and Sembulingam, 2012).

Table 1: Normal CSF Values

Volume	Adults: 90-150 mL; Child: 60-100 mL	
Appearance	Crystal clear, colorless	
Pressure	Adults: 90-180 mm H_2O ; Child: 10-100 mm H_2O	
Total Cell Count	Essentially free cells	
Specific Gravity	1.006-1.008	
Osmolality	280-300 mmol/kg	
pH	Lumber: 7.28-7.32 ; Cisternal: 7.32-7.34	
	Adults	**Newborn (0-14 d)**
WBCs	0-5 cells	0-30 cells
Differential		
Lymphocytes	40%-80% (0.40-0.80)	5%-35% (0.05-0.35)
Monocytes	15%-45% (0.15-0.45)	50%-90% (0.50-0.90)
Polys	0%-6% (0-0.06)	0%-8% (0-0.80)
Glucose	40-70 mg/dL	60-80 mg/dL
	(2.2-3.9 mmol/L)	(3.3-4.4 mmol/L)
Protein(Lumber)		
Adults	15-45 mg/dL	
Neonates	15-100 mg/dL	
Elderly(> 60 y)	15-60 mg/dL	

(Source: Fischbach and Dunning, 2009)

Changes in appearance, cellular and chemical compositions of CSF during meningitis

The inflammation by various pathogens induces anatomical and physiological changes in the meninges which are responsible for characteristic changes in the laboratory values of CSF from patients with meningitis. The loss of integrity of cerebral capillaries and thus, the loss of integrity of the blood-brain barrier results in leakage of protein into the CSF and increased migration of PMN leukocytes into the CSF (Gray and Fedorko, 1992).

Table 2: Laboratory Values of CSF Components during Meningitis

Adult patients with:	Protein (mg/dL)	Glucose (mg/dL)	Leukocytes (per µL)	Predominant cell type (%)
Bacterial meningitis	>100	<40	>1000	PMN (>50)
Fungal meningitis	Increased	<30	Increased	Lymphocytes
Viral or aseptic meningitis	<100	Normal	<500	PMN (early) and Lymphocytes (late)

(Source: Gray and Fedorko, 1992)

Gross appearance of CSF

CSF is normally a crystal-clear fluid. Cloudy, purulent, bloody, or pigmented CSF is associated with many disease states (Watson and Scott, 1995). Under pathological conditions, the CSF may become turbid or discoloured or both. Turbidity may be caused by elevated numbers of RBC or WBC in the CSF (Counts of >200 WBC per mm^3or > 400 RBC per mm^3), high bacterial or fungal count in the CSF even in the absence of a raised cell count and may be due to epidural fat aspirated at the time of the lumbar puncture (Venkatesh *et al.*, 2000). The breakdown of haemoglobin first to oxyhaemoglobin (pink) and later to bilirubin (yellow), leads to discolouration of the CSF known as xanthochromia (McGing and O'Kelly, 2009). Because visual determination is unreliable, xanthochromia should be determined by examination of the supernatant of centrifuged CSF by spectrophotometry to seek macroscopically invisible haematin or bilirubin (Public Health England, 2014).

CSF pleocytosis

Normal CSF contains 0-5 lecukocytes/mm^3, mainly lymphocytes, though in neonates cell count is up to 30/mm^3 (Collee *et al.*, 1996). CSF pleocytosis refers to an increase in the CSF WBC count. WBC count of >500/$mm^3$with a preponderance of neutrophils is characteristic of a bacterial meningitis, and a WBC count of >100/$mm^3$with a preponderance of monocytes is characteristic of a viral meningitis a considerable pattern overlap is often found. As significant neutrophil lysis occurs in the CSF within 1-2 hours of collection, delay in analysis may also lead to an artificially low CSF cell count (Venkatesh *et al.*, 2000).

It is important to note that a false-positive elevation of the CSF WBC can be found after traumatic lumbar puncture, or in patients with intracerebral or subarachnoid hemorrhage in which both red blood cells and white blood cells are introduced into the subarachnoid space. In these instances, the following formula can be used as a correction factor for the true WBC count in the presence of CSF red blood cells (RBC) (Nagarathna *et al.*, 2012).

$$\text{True WBC in CSF} = \text{Actual WBC in CSF} - \frac{\text{WBC in blood x RBC in CSF}}{\text{RBC in blood}}$$

Also, CSF pleocytosis should not be ascribed to seizure activity alone unless the fluid is clear and colorless, the opening pressure and CSF glucose are normal, the CSF Gram stain is negative, and the patient has no clinical evidence of bacterial meningitis.

Alterations in CSF glucose

During CSF storage glucose is degraded. Therefore, glucose determination must be performed immediately after CSF collection. CSF glucose levels are used to distinguish bacterial meningitis (where it is usually decreased, usually <40 mg/dl) from aseptic meningitis (where the glucose levels are usually unaltered) (Venkatesh *et al.*, 2000). Decreased CSF glucose results from changes in the physiological functioning of the choroid epithelium as well as from consumption by bacterial pathogens and leukocytes (Watson and Scott, 1995).

CSF glucose concentrations depend on the serum concentration, and measurement of the former without the latter can be uninterpretable. A CSF/serum ratio cut-off of <0.4 is helpful in distinguishing between bacterial and aseptic meningitis with a sensitivity and specificity of 80% and 98% respectively (Venkatesh *et al.*, 2000). Chemically meningitis can be differentiated from bacterial meningitis by CSF glucose levels (< 10 mg/dL) and CSF WBC values (>7500 cells/μL) (Forgacs *et al.*, 2001).

Alterations in CSF protein

Normal CSF protein level is 15-45 mg/dl. Protein level greater than 200 mg/dL (Mace, 2008), is highly significant for bacterial meningitis indicating disruption of the blood-brain or the blood-CSF barrier. CSF protein concentrations are usually higher in bacterial meningitis compared with aseptic meningitis.

In case of aseptic Meningitis protein level of CSF may be 50-200mg/dl (Cheesbrough, 2000). At a 'cut off' value of 1000 mg/L, the sensitivity and specificity for distinguishing bacterial from aseptic meningitis are 82% and 98%, respectively (Venkatesh *et al.*, 2000).

2.4 Etiological Agents of Meningitis

Bacterial meningitis

Although most of the environmentally acquired bacteria have the potential to cause meningitis, the majority of the bacterial meningitis cases are due to *Strep. pneumoniae, N. meningitidis* and *H. influenzae* type b (Hib). Different age groups show high susceptibility to certain organisms. Gram negative enteric rods such as *E. coli, Kl. pneumoniae, Proteus mirabilis* and some other organisms such as *L. monocytogenes* are more common during the neonatal period, while *Strep. Pneumonia* and *N. meningitidis* are more common in the children and young adults (Agrawal and Nadel, 2011).

The etiological agents of bacterial meningitis depend on the season of year, the age, ethnic background and geographic location of the patients. Unusual and rare bacteria that have been reported to cause meningitis include *Bacteroids fragilis, Achromobacter xylosoxidans, Gordona aurantiaca (Rhodococcus aurantiacus),*

Lactobacillus spp., *Corynebacterium aquaticum*, *Streptococcus mitis*, *Pasteurella multocida*, *H. influenzae* type f, *Psychrobacter immobilis*, *Pasteurella multocida* (Gray and Fedorko, 1992).

Other uncommon causes of bacterial meningitis include *Staph. aureus*, *Ps. aeruginosa* and some other *enterococci*. They are usually associated with nosocomial infections and may be acquired after trauma or some surgical interventions (Tunkel *et al.*, 2004).

Viral meningitis

Viral meningitis is often less severe than bacterial meningitis and usually resolves without specific treatment. But it can be severe or fatal depending on the virus causing the infection, the person's age, or whether a person has a weakened immune system.

Viral infections that can lead to meningitis include *Mumps*, *Herpes virus*, including Epstein-barr virus (EBV), Herpes simplex viruses (HSV), and Varicella-zoster virus *(VZV)*, *Measles*, *Influenza*, *Arboviruses* and in rare cases *Lymphocytic choriomeningitis virus (LCMV)* (CDC, 2014c).

Fungal meningitis

Fungal meningitis is rare and usually the result of spread of a fungus through blood to the spinal cord. The most common cause of fungal meningitis for people with weakened immune systems is *Cryptococcus* (CDC, 2014b). The fungi causing meningitis includes: *Aspergilllus fumigatus*, *Candida* spp., *Coccidioides immitis*, *C. neoformans*, *Histoplasma capsulatum*, and *Blastomyces dermatitis* (Cheesbrough, 2000).

Parasitic meningitis

Wide range of parasites is known to cause meningitis. They are *Acanthaemoeba* spp. *Echinococcus granulosus*, *Cysticercus* (*Taenia*spp.), Malarial parasites, *Nagleria fowleri*, *Pargonimus westermani*, *Schistosoma* spp., *Strongyloides stercolis*, *Toxoplasma gondii*, and *Trichinella spiralis* (Deisenhammer *et al.*, 2006).

2.5 Features of Some Basic Pathogens Causing Meningitis

N. meningitidis

Meningococcal disease was described by Vieusseux in 1805 during an outbreak with 33 deaths in the vicinity of Geneva, Switzerland. The Italian pathologists Marchiafava and Celli first described intracellular oval micrococci in a sample of CSF in 1887. Anton Weichselbaum in 1887 first identified bacterium causing meningococcal disease in the CSF of six of eight patients of bacterial meningitis and the bacterium was named *Neisseria intracellularies* (Manchanda *et al.*, 2006).

N. meningitidis are Gram-negative diplococci, with flattened or concave opposing edges (kidney shaped). They are non-motile, and typically seen in large number inside PMN leukocytes (Doern and Morse, 1980).

Strep. pneumoniae

Strep. pneumoniae has been one of the most extensively studied microorganisms since its first isolation in 1881. It was isolated from human saliva in 1881 in independent studies by Sterberg and Pasteur (Finegold and Baron, 1986; Alonsodevelasco *et al.*, 1995).

Strep. pneumoniae are Gram positive, lancet shaped with narrow distal ends, non-motile, non-sporing and encapsulated diplococcic (0.5 to 1.25) µm in diameter. They can occur in pairs, singly or in short chain. Three major surface layers can be distinguished in their surface: plasma membrane, cell wall, and capsule (Alonsodevelasco *et al.*, 1995).

H. influenzae

H. influenzae is pleomorphic, small Gram's negative rods with fastidious growth requirements. The name of genus comes from the requirement by these organisms for accessory growth factors found in blood, that is *haemo* (Greek for blood) and *philos* (Greek for loving) (Habiba, 2012).

The organism was first isolated by Pfeiffer during the 1892 influenza pandemic. The frequency of its presence in the nasopharynx of patients with influenza and in

postmortem lung cultures led to the erroneous assumption that it was the etiologic agent of influenza-thus the designation the *influenzae bacillus*. As was later shown, influenza is caused by virus. The role of *H. influenzae* during the pandemics of 1890 and 1918 was apparently that of secondary invader (Habiba, 2012).

E. coli

E. coli originally were known as *Bacterium coli* and were identified in 1885 by the German pediatrician, Theodor Escherich. *E. coli* is widely distributed in the intestine of humans and warm-blooded animals and is the predominant facultative anaerobe in the bowel and part of the essential intestinal flora that maintains the physiology of the healthy host (Feng *et al.*, 2007).

E. coli is Gram-negative, facultative anaerobic and non-sporulating. Cells are typically rod-shaped and are about 2 µm long and 0.5 µm in diameter, with a cell volume of 0.6 - 0.7 $(\mu m)^3$ (Feng *et al.*, 2013; Obata-Yasuoka *et al.*, 2002).

C. neoformans

C. neoformans is encapsulated yeast that is present in the environment worldwide and can cause disease in both immune-competent and immune-compromised hosts. Patients with a T- cell deficiency are most susceptible (Prince, 2010). *C. neoformans* is a basidiomycetous, yeast-like fungus that, following inhalation from an environmental source, causes respiratory and neurological infection in humans and animals (Zarrin *et al.*, 2010).

This fungus has five serotypes (A, B, C, D, and AD), and recently was subdivided into three varieties known as *C. neoformans* var. *grubii* (serotype A), *C. neoformans* var. *neoformans* (serotype D), and *C. neoformans* var. *gattii* (serotype B and C). According to this classification, the correct designations for serotype AD isolates were not yet resolved (Zarrin *et al.*, 2010). *C. neoformans* has a number of virulence factors that can contribute to progression of the disease. These virulence factors include the capsule, cryptococcal products, melanin production, mannitol production, and proteases, amongst others. The polysaccharide capsule and the soluble extra cellular constituents of *C. neoformans* are the dominant virulence factors (Prince, 2010).

2.6 Pathogenesis and Pathophysiology

Bacterial Meningitis

As Quagliarello and Sheld (1992), describes in the Table 3 that the neurotropic potential of the most common bacterial causes of meningitis (*Strep. pneumoniae, H. influenzae, N. meningitidis* and *E. coli*) relates to their ability to evade several host defenses.

Table 3: Pathogenic Sequence of Bacterial Neurotropism

Neurotropic Stage	Host Defense	Strategy of Pathogen
Colonization or mucosal invasion	Secretory Ig A Ciliary activity Mucosal epithelium	Ig A protease secretion Ciliostasis Adhesive pili
Intravascular survival	Complement	Polysaccharide capsule
Crossing of blood brain barrier	Cerebral endothelium	Adhesive pili
Survival within CSF	Poor opsonic activity	Bacterial replication

Bacteria enter the CNS either by haematogenous spread or by direct extension from a contiguous site (Figure 1). In the neonatal period, bacteria are acquired mainly during birth from maternal genital secretions (Chavez-Bueno and McCracken, 2005). In infants and children, the organisms that cause meningitis colonize the upper respiratory tract by attaching to the nasopharyngeal mucosal epithelium. Pneumococci, meningococci and Hib all secrete IgA proteases that cleave the proline rich hinge region of IgA rendering it nonfunctional facilitating bacterial attachment to the epithelium. Choline binding protein A (Cbp A) and neuraminidase NanA are other pneumococcal proteins that aid the colonization of the nasopharynx (Koedel *et al.*, 2002). Binding to the epithelium is dependent on the presence of pili on the surface of the bacteria. *N. meningitidis* uses fimbria or pili to adhere to the host's mucosal epithelial cells (Rosenstein *et al.*, 2001). If the ciliated epithelial cells are damaged, as in viral infection or with smoking, their ability to prevent mucosal adhesion of invading bacteria are weakened (Mace, 2008).

Strep. pneumoniae uses Cbp A that binds to the polymeric immunoglobulin receptor to traverse the mucosal barrier (Koedel *et al.*, 2002). The bacteria traverse the

endothelium by endocytosis (meningococci) or by separating tight junctions (*H. influenzae*) to invade intravascular space. In the bloodstream, encapsulated bacteria (e.g. *H. influenzae*, *Strep. pneumoniae*, and *N. meningitidis)* avoid the host defense mechanisms, because their polysaccharide capsule inhibits neutrophil phagocytosis and complement-mediated bactericidal activity (Mace, 2008). The organisms then cross the vulnerable sites of the BBB (e.g. cerebral capillaries and choroid plexus) and reach the subarachnoid space (Leib and Tauber, 1999). In the CSF, the organisms multiply rapidly because the host lacks defense mechanisms (Tunkel and Scheld, 1993).

Meningitis can also develop by direct spread of bacteria from a paranasal sinus or from the middle ear through the mastoid to the meninges. Severe head trauma with a skull fracture or penetrating wounds can also lead to meningitis. Congenital dural defects such as dermal sinuses or meningomyelocele, or neurosurgical procedures can directly inoculate bacteria into the CSF (Saez-Llorens and McCracken, 2003).

Once bacteria reach the CSF, they are likely to survive because humoral defenses such as immunoglobulin, complement activity and opsonic activity are undetectable in the CSF (Quagliarello and Sheld, 1992).

When the pathogens have entered the CSF, they replicate rapidly and release active cell wall or membrane-associated components; gram-positive bacteria liberate lipoteichoic acid and peptidoglycan, and gram-negative bacteria liberate lipopolysaccharide. β-lactam antibiotics that act on cell walls and cause rapid lysis of bacteria, can in the beginning of the treatment, cause a burst of release of these active bacterial products into the CSF. The interaction of bacterial products with host pattern recognition receptors, such as Toll-like receptors, initiates the inflammatory reaction (Koedel *et al.*, 2010). As a consequence, CNS macrophages, astrocytes, ependymal, glial and endothelial cells release pro inflammatory cytokines: interleukin (IL)-1β, IL-6, and tumour necrosis factor-α (TNF-α) (Chavez-Bueno and McCracken, 2005). These cytokines stimulate chemotactic cytokines called chemokines (IL-8, macrophage inflammatory proteins), other chemotactic substances (complement factor C5a, platelet-activating factor), and adhesion molecules (intercellular adhesion molecule-1) which facilitate the passage of leukocytes from the circulation into the subarachnoid space (Koedel *et al.*, 2002). Plasma and cerebrospinal concentrations of

endotoxin and IL-1β, but not of IL-6 and TNFα, correlate with severity or adverse outcome of bacterial meningitis. Thus Tumor necrosis factor and interleukin-1 probably act synergistically to elicit inflammatory responses which manifest clinically as meningitis (Pelkonen, 2011).

Consequently, leukocytes release nitric oxide, toxic oxygen metabolites, matrix metalloproteinases, and prostaglandins, which results in injury to the vascular endothelium and alteration of the BBB permeability (Figure 1). Penetration of low molecular-weight serum proteins into the CSF leads to vasogenicoedema. Bacteria and neutrophils release cytotoxic substances causing cytotoxic oedema. As a result of high protein and cell content, and increased CSF volume due to a blockade of CSF resorption across the inflamed arachnoid villi causes interstitial oedema. The accumulation of inflammatory cells, protein, and other material within the CSF interferes with functioning of the arachnoid villi and blocks the resorption of CSF from the subarachnoid space. Similarly, the ventriculitis that often accompanies meningitis may occlude the cerebral aqueduct and lead to hydrocephalus, further exacerbating interstitial edema and elevating intracranial pressure (Chavez-Bueno and McCracken, 2005). Cerebral oedema and intracranial hypertension contribute to neuronal damage. Cerebral blood flow increases early in meningitis, but decreases later, which can aggravate the neurological damage.

Hence a logical temporal sequence of inflammatory responses of meningitis is as follows: chemotaxis and adherence of PMN leukocytes to cerebral capillaries; damage to capillary endothelial cells; structural changes in the BBB; cytotoxic parenchymal edema; increased intracranial pressure; decreased intracranial perfusion; cerebral infarction; and focal or diffuse brain damage (Gray and Fedorko, 1992).

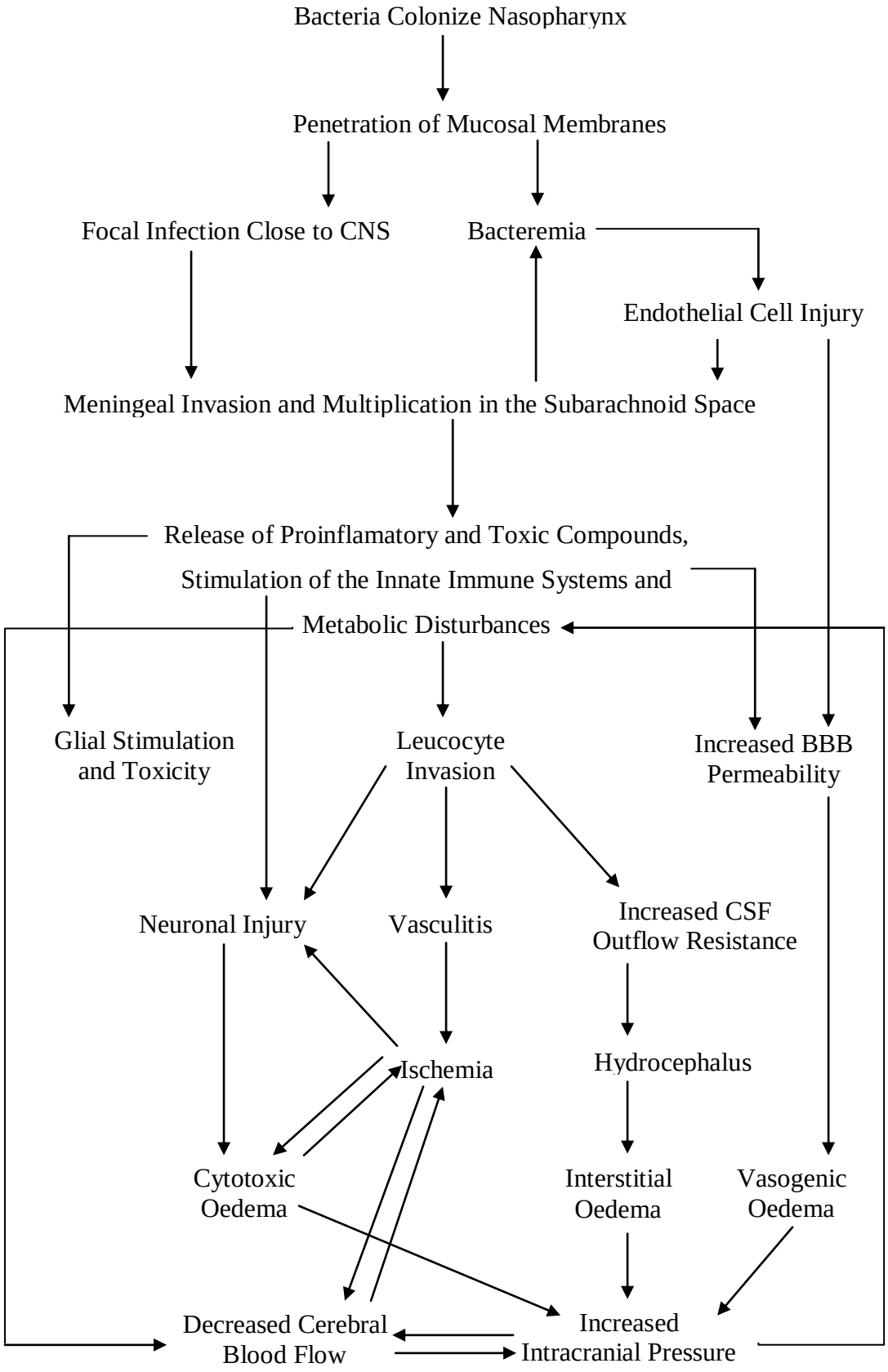


Figure 1: Scheme Depicting Pathogenesis and Pathophysiology of Bacterial Meningitis (Adapted from Tunkel & Scheld, 1993)

Cryptococcal Meningitis

The pathogenesis of cryptococosis is determined by three factors: (1) the status of the host's defenses, (2) the virulence of the strain of *C. neoformans*, and (3) the size of the inoculum. In the alveoli, the yeast makes contact with the alveolar macrophages, which recruit other inflammatory cells through cytokines and a proper Th1 response with granulomatous inflammation is elicited (Prince, 2010). The infection can follow one of three pathways:

1. In an immune suppressed host, the yeast continues to proliferate and disseminate, causing clinical disease.

2. The effective immune response completely eliminates the yeast from the host.

3. The yeast produces a small lung/lymph-node complex and remains dormant in the tissues. The infection is clinically asymptomatic until loss of local immunity occurs, for example corticosteriod use or progression of an HIV infection. The yeast may then begin to replicate in the pulmonary lymph-node complex and eventually disseminate to the organs outside the lung (Prince, 2010).

Peter (1997) states that cryptococcus exhibits no known toxins. Most of the pathophysiology observed is due to tissue invasion by multiplying organisms but no necrosis or organ dysfunction has been observed until late in the course of the disease when the fungal burden is very heavy.

Cellular Host Defence Mechanism

There does seem to have accumulated evidence that an integration of a number of cells such as neutrophils, lymphocytes and macrophages is responsible for strong host resistance to *Cryptococcus*. Human neutrophils and monocytes can ingest and kill *Cryptococcus*, while activated macrophages also ingest the fungus. Besides the above cells, sensitized T cells and natural killer cells also play a very important role. While these cells seem to be important in clearing the fungi, there is no proven evidence that defects in the functioning of these cells result in increased cases. Cryptococcal cell wall is capable of activating the alternate complement pathway (Peter, 1997).

Cryptococcal Polysaccharide versus Host Immunity

This capsule has been shown to be immunosuppressive in the host. Long after being cured of *cryptococcosis*, patients may exhibit prolonged unresponsiveness to the antigen. This polysaccharide capsule also seems to inhibit phagocytosis by binding to the yeast surface and blocking phagocytosis. The capsule may impair leukocytes kinesis and may activate alternative complement pathway (Peter, 1997).

Human Oral Host Defence

Though anti-cryptococcal antibody and complement do not lyse the organism directly, there are critical factors in the functioning of the cellular immune response. There are other factors present in serum which are not present in the CNS and may explain why we tend to get predominant CNS manifestation (Peter, 1997).

2.7 Epidemiology and Incidence of Meningitis: Global Review

In spite of the introduction and widespread use of antibiotics and other advances in medical care, meningitis still has a high morbidity and mortality (Durand *et al.*, 1993). In developed countries the mortality rate is decreased gradually in the last 3 decades (Salwen *et al.*, 1987). On the other hand, in developing countries the mortality rate remained higher, it was estimated that children die from acute BM under the age of 5 years (Salih *et al.*, 1991).

Bacterial meningitis is more significant problem in developing countries. Despite the fact that many microbes that are pathogenic to humans have the potential to cause meningitis, relatively few of them are responsible for most cases of bacterial meningitis; these include *Strep. pneumoniae, N. meningitidis,* group B *streptococcus*, Hib, *E. coli*, *My. tuberculosis,* and *L. monocytogenes* (Thigpen *et al.*, 2011).

Several early studies revealed very varying pattern of BM with respect to age specificity, the incidence rate and endemicity, type of bacterial agents incriminated, morbidity, and mortality rates and other relevant epidemiological parameters which all vary from study to study and from country to country (Salih *et al.*, 1991).

The age group with highest prevalence of meningitis is that of newborn with mortality rate as high as 20% (Finegold and Baron, 1986). The high incidence rate of BM

among this age group is due to the immature immune system of neonates, colonization of the organisms in the female genital tract and the increase of the permeability of the BBB of the newborn (Finegold and Baron, 1986). Franco *et al.* (1992) stated that the neonatal meningitis caused by Gram-positive bacteria was twice of that caused by Gram-negative bacteria, whereas the mortality rate due to Gram-negative microorganisms was almost three times higher than that of gram positive. It was reported that meningitis caused by *H. influezae* was the most common seen at the age 2-6 months and *N. meningitidis* is predominant at the age 7-12 months, while group B *streptococci* occur in infants up to 6 months of age with the mortality rate of 25%. The high rate of mortality among these age groups is due to low birth weight, which is a significant risk factor in both neonates and post-neonatal infants (Franco *et al.*, 1992).

The exact incidence of bacterial meningitis worldwide remains difficult to estimate due to the variation in the surveillance mechanisms present in the different parts of the world. While surveillance is well established in the industrialized world, the incidence of bacterial meningitis is underreported in many developing countries. In the past decade a sharp decline in the incidence of bacterial meningitis in the developed countries has been witnessed, where the incidence now lies between 1-3 per 100,000 populations (Segretti and Harris, 1996). But still in some developing countries the incidence may be as high as 800 cases per 100,000 populations (Harrison *et al.*, 2009). Similarly In developing countries the incidence of meningitis in children is about 10 times as great as in industrialized countries (Greenwood, 1987).

In the United States, the overall incidence of BM is about 2 to 10 cases per 100,000 populations per year. The incidence is greatest in neonates with about 400 per 100,000; this is followed by children younger than 3 years with 20 per 100,000, and is lowest in adults with 1 to 2 per 100,000 (Mace, 2008). Between 1998 and 2007, more than 3000 patients with bacterial meningitis were identified in eight surveillance area in the United States (about 17.4 million persons). Of the 1670 cases reported in the same study during 2003-2007, *Strep. Pneumoniae* was the predominant infective species (58.0%), followed by group B *streptococci* (18.1%), *N. meningitidis* (13.9%), *H. influenzae* (6.7%), and *L. monocytogenes* (3.4%) (Thigpen *et al.*, 2011). Between 1986 and 2001 in Kaohsiung-Taiwan, *H. influenzae*, Salmonella species, *Strep.*

agalactiae, and *E. coli* were identified as the most causative agents of bacterial meningitis (about 59%) in 80 cases of reported bacterial meningitis in children of one month to one year of age (Chang *et al.*, 2004).

Recently, in a study performed in Turkey, 408 CSF samples were collected for a one year period (2005-2006) from children aged 1-17 months. Bacterial meningitis was identified in 243 samples. *N. meningitidis* was the most common causative bacterial agent (56.5%) followed by *Strep. pneumoniae* (22.5%) then Hib (20.5%) (Ceyhan *et al.*, 2008).

In a surveillance study between 2002 and 2005 conducted in the children between 2 months to 5 years of age in Ulaanbaatar, Mongolia found, the average annual incidence rate for confirmed and probable bacterial meningitis was 68 cases per 100,000 children aged 2 months to 5 years. The average annual incidence rate of confirmed cases was 28 cases per 100,000 children for Hib meningitis, 11 cases per 100,000 children for pneumococcal meningitis, and 13 cases per 100,000 children for meningococcal meningitis (Mendsaikhan *et al.*, 2009).

In Dakar, Senegal, from 1970 through 1979, the average incidence was 50 cases per 100,000 populations, with approximately 1 in 250 children developing bacterial meningitis during the first year of life. In African countries with high rates of HIV infection, the majority of meningitis cases are caused by *Strep. pneumoniae*, and this has been associated with high mortality rates. Sub-Saharan Africa, also referred to as the meningitis belt, is known for epidemics of meningococcal meningitis, with incidence rates of 101 cases per 100,000 population in the period of 1981 to 1996 (Brouwer *et al.*, 2010). During 1993-2012, nearly 1 million (947 000) suspected meningitis cases were reported, including an estimated 100 000 deaths; 80% of epidemics were caused by *N. meningitidis* serogroup A (WHO, 2013).

In Sudanese children 38% of acute bacterial meningitis cases were due to *H. influenzae* and *N. meningitidis*, followed by *Strep. pneumoniae*, which account for 23% of cases, and the fatality rate was 29%. The proportion of causative organisms differs during endemic and post epidemic periods where the incidence of *N. meningitidis* is higher in post epidemic. In Saudi Arabia the rate of incidence of bacterial meningitis in childhood (up to 11 years old) was recorded as follow, Hib

66%, *Strep. pneumoniae* 24%, *N. meningitidis* 4%, Group B *streptococcus* 4% and 2% for *Staph. aureus* (Almuneef *et al.*, 1998).

The majority of the bacterial meningitis cases are due to *Strep. pneumoniae, N. meningitidis* and *H. influenzae* type b. In recent years, due to the decline in the cases of *H. influenzae, Strep. Pneumoniae* and *N. meningitidis* have become the most common causes of bacterial meningitis. The etiology of bacterial meningitis varies with the population under study, the geographical conditions and the season of the year. Different age groups show high susceptibility to certain organisms. Gram negative enteric rods such as *E. coli, Kl. pneumoniae, Pr. Mirabilis* and some other organisms such as *L. monocytogenes* are more common during the neonatal period, while *Strep. Pneumoniae* and *N. meningitidis* are more common in the children and young adults (Agrawal and Nadel, 2011).

Development of vaccine against *H. influenzae* type b and its routine use in childhood immunization schedules has nearly eradicated the corresponding organism from developed countries. Similarly, a substantial reduction in childhood pneumococcal meningitis has been observed following the introduction of the conjugate vaccine covering seven different serotypes of *Strep. Pneumoniae* (Patel *et al.*, 2010).

The following sections review the epidemiology of the common etiological agents of meningitis.

Epidemiology of *N. meningitidis*

N. meningitidis is an obligate commensal residing in the human nasopharynx. The highest incidence of nasopharyngeal carriage of *N. meningitidis* is in adolescents especially those residing in overcrowded spaces (Yazdankhah and Caugant, 2004).

Meningococcal infections are common in both temperate and subtropical climates with sporadic cases throughout the year (Moor, Schwartz, Reeves, Gellin, Broome, 1989). Recent estimates show that the global incidence of meningococcal disease is 500,000 per annum with a worldwide mortality rate of 10% (CDC, 2012).

Serogroups A, B, C, W135, X and Y are isolated in almost 90% of the infections (Harrison *et al.*, 2009). Serogroups A, B, and C are responsible for most cases of

meningococcal disease and meningitis globally, with serogroups B and C predominating in Europe and the Americas and serogroups A and C in Asia and Africa (Rosenstein *et al.*, 2001).

In 2008 in the United States, disease caused by serogroup B (32% of cases), serogroup C (32% of cases), and serogroup Y (24% of cases) accounted for most of the endemic disease, causing meningitis in 53% of cases (Brouwer *et al.*, 2010). In the USA the incidence of endemic meningococcal disease has been 0.9 to 1.5 cases per 100,000 per year. The rates are highest during infancy, adolescence, and early adulthood (Rosenstein *et al.*, 1999).

Major epidemics of meningococcal meningitis caused primarily by serogroup A have been reported for a number of developing countries (including Brazil, Nepal, China, and several sub-Saharan African nations); attack rates during these epidemics can approach 1% of the population (Brouwer *et al.*, 2010).

In the African meningitis belt, from Ethiopia to Senegal, the incidence of endemic meningococcal disease is much higher. During 1993-2012, nearly 1 million (947000) suspected meningitis cases were reported, including an estimated 100,000 deaths; 80% of epidemics were caused by *N. meningitidis* serogroup A. The most recent large-scale meningitis epidemic in the African meningitis belt occurred in 2009, when nearly 80 000 cases were reported. During the 2012 epidemic season (1 January to 1 July 2012), 19 African countries using enhanced surveillance reported a total of 22,673 suspected meningitis cases including 1931 deaths (case-fatality rate [CFR], 8.5% (WHO, 2013)

Epidemic meningococcal disease was reported in Nepal during 1982 when a large meningococcal serogroup A outbreak occurred in the Kathmandu Valley with an overall attack rate of 103/100,000. India has also experienced repeated meningococcal serogroup A epidemics, most recently in 2005 in Delhi and surrounding districts. Between March and July 2005, 444 cases and 62 deaths [CFR, 16.9%] were recorded. The majority of cases (44%) and deaths (62%) were in adolescents and young adults aged between 15 and 29 years. Large meningococcal serogroup A epidemics originating in China spread to Mongolia in 1973-1974 and 1994-1995. During the 1994-1995 epidemic, the overall attack rate ranged between 80 and >90/100 000. A

meningococcal serogroup A epidemic occurred in Karachi, Pakistan, in 1988 with 112 cases of which 20% had septicaemia and 80% had meningococcal meningitis (Vyse *et al.*, 2011).

Epidemiology of *Strep. pneumoniae*

Strep. pneumoniae is one of the most common causes of bacterial meningitis worldwide. It is a capsulated bacterium which has 93 serotypes based on the different polysaccharide characteristics of the capsule. Most of the serotypes are capable of causing disease but majority of the infections in the developing countries are caused predominantly by serotypes 1 and 5 (Johnson *et al.*, 2010).

Strep. pneumoniae causes sporadic infection. It is an endemic disease with a high incidence of carrier. The incidence of disease mostly occurs during cold months of year, where the carriage appears to be highest during coolest months of the year (fall, winter and early spring). The carrier rates of *Strep. pneumoniae* appear to be higher in children particularly those of a preschool age than in adults (Al-Zubiery, 2001).

Based on surveillance studies and laboratory confirmed cases the estimate for incidence of pneumococcal meningitis in children younger than 5 years of age in Europe in 2000 was 6, in Africa 38, and globally 17 per 100,000, causing respectively 3,300, 43,100, and 103,000 cases, which resulted in 1,300, 31,700, and 60,500 deaths (mortality 29-73%) (O'Brien *et al.*, 2009).

Brouwer *et al.* (2010) mentioned that *Strep. pneumoniae* is now the most common etiological agent of bacterial meningitis in the United States and Europe, accounting for 61% of total cases in the United States.

Kornelisse *et al.* (1995) reported in their study, epidemiologic studies in the Netherlands have estimated that the annual incidence of pneumococcal meningitis is 1.5 cases per 100,000 populations and the incidence of pneumococcal meningitis in children younger than 5 years of age is substantially higher that is 7 cases per 100,000 populations.

Epidemiology of *H. influenzae*

Before the advent of *H. influenzae* type b (Hib) conjugate vaccines, *H. influenzae* type b meningitis was the leading cause of bacterial meningitis in childhood (Howie *et al.*, 2007; Mendsaikhan *et al.*, 2009).

H. influenzae is endemic throughout the world and usually occurs as sporadic cases. Nasopharangeal carriage of *H. influenzae* is more common in developing countries and occurs in younger children. The incidence of Hib meningitis is greater in developing countries than in developed countries and the disease occurs in younger children with cases occurring before 12 months of age and almost half before the age of 6 months. In developed world as the US, the mortality rate due to invasive Hib in children less than 5 years ranged between 3% to 6% but the permanent neurological sequelae affect 20-30 % of meningitis survivors (AL-Zubiery, 2001).

In another review, based on surveillance studies and laboratory-confirmed cases the estimate for the incidence of Hib meningitis in children younger than 5 years in Europe in 2000 was 16, in Africa 46, and globally 31 per 100,000, causing a respective 5,200, 51,300, and 173,000 cases, which resulted in 2,000, 34,600, and 78,300 deaths (mortality 22-67%) (Watt *et al.*, 2009).

Epidemiology of *C. neoformans*

C. neoformans is not part of the normal microbial flora of humans. Their main habitats include debris around pigeon roosts and soil contaminated with decaying pigeon or chicken droppings.

The organism causes disease in immune competent, as well as immune compromised, hosts. The yeast enters the host by the respiratory route in the form of dehydrated haploid yeast or as basidiospores. After some time in the lungs, the organism spreads to extra pulmonary tissues, and, since it has a predilection for the brain, infected persons usually contract meningoencephalitis (Kent *et al.*, 1998, as cited in Prince, 2010). The vast majority of patients with symptomatic disseminated *cryptococcosis* have an identified underlying immune compromised condition. The most common underlying conditions worldwide include AIDS, prolonged treatment with corticosteriods, and organ transplantation. In less well-developed countries with major

epidemics of HIV, such as sub18 Saharan Africa, cryptococcal disease appears to reach very high prevalence (Bicanic and Harrison, 2004).

C. neoformans is the leading cause of meningitis in central and southern Africa, accounting for 26.5% of cases in a series from Malawi, 31% in a series from the Central African Republic and 45% from Zimbabwe (Jarvis and Harrison, 2007). Access to antifungal therapy is limited in developing countries. Whereas in sub-Saharan Africa reported mortality rates with antifungal treatment are very high, median survival time of 19 days in a Zambian study and 64% in hospital mortality in a South African study (Jarvis and Harrison, 2007) have been reported.

2.8 Epidemiology and Incidence of Meningitis: Nepalese Context

Mishra *et al.* (2013) carried a hospital based prospective cross-sectional study at Kanti Children's Hospital during December 2004 to August 2005. Cerebrospinal fluid from 150 consecutive clinically suspected cases of acute bacterial meningitis between the age group of 2 months to 14 years were analyzed. Of the 150 cerebrospinal fluid samples analyzed bacterial culture identified only 4 meningitis cases giving isolation rate of 1.3% whereas latex agglutination test (LAT) identified 29 cases giving an isolation rate of 19.3% from 150 samples. *Strep. pneumoniae*, *H. influenzae* type b and Group B *Streptococcus* were the most common causative organism.

A hospital based study by Chhetri *et al.* (2011) on "Clinical profile of invasive pneumococcal disease in Patan Hospital, Nepal", out of 42 cases of invasive pneumococcal diseases studied admitted at the hospital, six (14%) as cases were found of having meningitis. Out of 6 meningitis cases 4 grew *Strep. pneumonae* both in blood and CSF. A similar study entitled "Hospital-based surveillance of invasive pneumococcal disease among young children in urban Nepal" was conducted by Williams *et al.* (2009) at Patan Hospital, Kathmandu. The study included all children aged 2 months to 5 years who were admitted to hospital with fever and/or suspected pneumonia, meningitis, or bacteremia. A total of 885 children were recruited during the 21-month study period. Of these, 76 (9%) had meningitis on the basis of clinical criteria. But later on probable or definite meningitis occurred in 47 (5%).

In a prospective observational study conducted in paediatric ward of Patan Hospital, out of 7,751 children, 296 (3.8%) had meningitis. This was a group ranging from

neonates to adolescents aged 18 years. The organisms found, were *pneumococcus* (6), *H. influenzae 'b'* (3), β-hemolytic *Streptococcus* (1), α-hemolytic *Streptococcus* (1), *N. meningitidis* (1) and *Pseudomonas* (1). Whereas *H. influenzae 'b'* was isolated from young infants, pneumococci were found in the young as well as the old (Ansari and Pokhrel, 2011).

Mishra *et al.* (2010) mentioned that recent data of Nepal shows that as many as 47,315 cases of tuberculosis are registered annually with 21,245 new smear positive cases. Similarly, tuberculosis in children represents 5 to 15% of all tuberculosis cases in Nepal. It has been seen that tubercular meningitis occurs in approximately 7-12% of patients of tuberculosis. Hence, the prevalence of the disease tubercular meningitis is supposed to be high in Nepal.

During the 28-month study period (November 2004 to March 2007) at Kanti Children Hospital, Kathmandu conducted by Shah *et al.* (2009); 2528 children aged <5 years who had suspected invasive bacterial disease were studied. Meningitis was diagnosed in 243 (9.6%). Of 243 meningitis cases, 92 (37.9%) were suspected, 90 (37.0.1%) were probable, and 61 (25%) were definite meningitis. Two out of 151 (1.3%) culture-positive meningitis cases in the <5 years age group, was found, of whom 26.7% of the 151 individuals had received prior antibiotics.

According to Ministry of Health and Population (MoHP) (2007/08) meningitis is a communicable disease which is kept under the class "other communicable disease" in Nepal. Nepal planned to introduce pentavalent (DPT-HepBHib) vaccine from April 2009. Among the five leading causes of morbidity among patient as reported by central hospitals, meningitis is the 4th cause found in Kanti Children's Hospital. Five leading causes of mortality among patients by central hospitals, meningitis is 3rd cause recorded in Kanti Children's hospital. A total of 5,082 confirmed cases of meningitis were recorded in that period.

In a hospital based study conducted among the children suspected of meningitis attending Kanti Childern's Hospital from June 2006 to September 2006 by Tuladhar (2007), reported only 21 (4.87%) culture positive isolates of bacteria from 431 CSF samples. Among 21 bacterial isolates, 11 (52.38%) were Gram negative organisms whereas 10 (47.62%) were Gram positive organisms.

A prospective study of 42 children admitted to Kanti Children's Hospital, Katmandu by Tiwari (2003) from the June 1993 to February 1994 suggested bacterial meningitis positive in 33 out of the 41 (80.5%) cases by LAT. 18 (54.5%) of the LAT positive results revealed *H. influenzae*, 10 (30.3%) revealed *N. meningitidis*, 4 (12.1%) revealed *Strep. pneumoniae*, and 1 (3.0%) revealed group B *Streptococcus*. LAT and positive result of Gram stain corresponded in 71.87% cases.

Cryptococcal meningitis that occurred in a patient with no obvious predisposing factor was reported by Khanal *et al.* (2002) at BP Koirala Institute of Health and Service Hospital, Nepal.

During 1983, an epidemic of serogroup A meningococcal meningitis occurred in the Kathmandu valley of Nepal, resulting in 875 cases and 95 deaths. The annual attack rate was 103 cases per 100,000 populations, with a peak attack rate occurring in April. Epidemic meningococcal disease had not been recognized previously in Nepal. Early in 1984, a review of hospital-based data on pyogenic meningitis in Kathmandu showed three times as many cases per month compared with the same period the previous year, suggesting that a recurrent epidemic was unfolding (Cochi *et al.*, 1987).

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

All the materials required for the present work are listed in the Appendix II.

3.2 Methods

3.2.1 Study Design

This study was a cross sectional study conducted at the Emergency laboratory of Tribhuvan University Teaching Hospital (TUTH) over six months from March 2014 to August 2014.

3.2.2 Study Site

This study was accomplished at the Emergency laboratory of TUTH which is located in Kathmandu, the capital city of Nepal. TUTH is a premier medical institution of Nepal. This is one of the largest referral hospitals in Nepal.

3.2.3 Source Population

The source population for the study was all patients who attended Emergency laboratory of TUTH for pathological diagnosis within the specified period (March 2014 to August 2014) of study.

3.2.4 Study Population

During the study, all patients suspected of meningitis who had attended the Emergency laboratory at TUTH within the specified period (March 2014 to August 2014) were included as sample of the study.

3.2.5 Inclusion/Exclusion Criteria

All CSF samples of patients suspected of meningitis, based on clinical diagnosis only, by a medical specialist at TUTH during the specified period (March 2014 to August 2014) were included in the study.
Samples other than CSF for diagnosis of meningitis were excluded from this study. Repeated samples from same person were also excluded from this study.

3.2.6 Variables

Isolated pathogens and antibiotic susceptibility pattern were the dependent variables, whereas the socio-demographic characteristics (variables) such as sex and age of patients included in the study were the independent variables.

3.2.7 Sample Size

No predefined sample size was set. A total of 356 patient CSF samples suspected of meningitis at TUTH between the periods of March 2014 to August 2014, were included in the study.

3.3 Laboratory Diagnosis

3.3.1 Specimen Collection

CSF samples were collected in the Hospital by experienced medical officer by lumbar puncture on immediate arrival of the cases with clinical suspicion of meningitis. About 3 ml of CSF was collected in sterile container with all aseptic precautions. A sterile wide-bore needle was inserted between the L4-L5 lumbar vertebrae and the CSF was allowed to drip into a dry sterile container.

- CSF was collected into two sterile, dry, screw-capped containers one for culture (1ml) and another one for biochemical and cytological investigation (2-3 ml).
- Sample was immediately delivered to the laboratory after proper labeling (Cheesbrough, 2000).

3.3.2 Transport

Sample was immediately (<1 hour) sent to the laboratory and processed without delay on arrival. Samples collected during night hours were kept in the incubator (37^0C) and processed without delay the following day. The specimen was neither refrigerated nor exposed to sunlight/heat.

3.3.3 Sample Processing

Each CSF sample was processed macroscopically, microscopically and microbiologically. After macroscopic observation, 1 ml CSF sample was centrifuged in clean sterile dry test tube for 10 minute at 1000x g. The sediment was thoroughly mixed and used for Gram's stain and India ink preparation.

CSF procedures performed, included observation of appearance and color, total leukocytes count (TLC) and differential leukocytes count (DLC), protein and glucose concentration, Gram's stain, culture, and antibiotic sensitivity tests.

CSF Specimens were designated normal on initial examination if no organisms were seen on Gram's stained film, the white blood cells count was less than 5 per cubic milliliter, the glucose concentration between 40-80 mg/dL and the protein concentration between 15-45 mg/dL.

3.3.4 Macroscopic Observation

During macroscopic observation, gross appearance and color of CSF i.e. clear, slightly turbid, cloudy, purulent, and bloody was recorded. Normal CSF appears clear and colorless.

3.3.5 Cytological Examination

Total leukocytes count (TLC)

The leukocytes were counted by Neubauer counting chamber. Clear or slightly turbid CSF samples were examined undiluted, but highly turbid CSF was diluted for cell counting by using Turk solution.

Four drops and nine drops of Turk's Solution were added to obtain 1:5 and 1:10 dilutions. To obtain 1:100 dilutions, a drop of 1:10 diluted CSF sample was taken in

another clean test tube and then nine drops of Turk's Solution was added. The diluted samples were gently shaken.

A Neubauer Counting Chamber with 0.1mm depth of counting surface and 9 squares with $1mm^2$ areas each on each counting side was used. The slide was placed safely on the horizontal bench plane and a cover slip was placed on the slide. A well washed micropipette was used to charge the counting chamber. The charged counting chamber was allowed to stand for about 2-3 minute and observed in the microscope under 10X objective. The leukocytes were counted on each corner square. Total number of leukocytes per mm^3 was obtained by using following calculation:

$$\text{Total Leukocyte (Count per cube mm)} = \frac{\text{Total cell X Dilution Factor X Depth Factor}}{\text{Area Counted}}$$

Where, Depth Factor = 10, and

Area counted = 4

The CSF specimens contaminated with blood, the leukocytes and erythrocytes were counted separately.

Wright's staining for differential leukocytes count (DLC)

The smear was prepared from the centrifuged deposits of CSF and then the smear was stained with Wright's stain. After air dry of the smear it was examined under microscope using high power and oil immersion lens (Cheesbrough, 2000).

A drop of centrifuged CSF was transferred to a slide and a smear was made. The smear of CSF was covered with Wright's stain. Equal volume of buffered water (pH 6.8) as stain was added. The diluted stain should not overflow. It should be ensured that the water is well mixed with the stain by blowing on the diluted or mixing the stain and water using a plastic bulb pipette. It was allowed to stain for 5 minutes. The stain was washed off with tap water. The back of the slide was wiped clean and stranded it in a draining rack for the smear to dry (Chessbrough, 2000).

3.3.6 Glucose and Protein Determination

The CSF glucose and protein were determined by using Biochemistry Automatic Analyzer (XL-200) machine, Erba Company, Germany by application of CSF sample type program according to the method described by the manufacturer.

3.3.7 Microscopic Examination

Gram's staining

The CSF samples were centrifuged at 1000x g for 10 minutes. Those samples which were received in a volume less than 2 ml were not centrifuged.

A smear was prepared by placing 1 or 2 drops of centrifuged samples on a clean glass slide. The smear was allowed to air dry in a safe place and was heat fixed. Then it was stained with Gram's technique. The composition, preparation of the reagents and the staining method are given in the Appendix III & IV. Then the Gram's stain was observed for the presence of microorganism for its cell morphology and number. Any bacteria seen were considered significant. However, low numbers only seen in one or two fields were confirmed with a second smear.

India ink preparation

India ink preparation was performed for *C. neoformans*. Smear was prepared by a drop of centrifuged CSF sediment on a clean glass slide and was stained with India ink. The detail procedure of India ink preparation is given in Appendix IV.

Culture

The CSF samples were inoculated onto the Chocolate agar (CA), Blood agar (BA), MacConkey agar (MA), Nutrient agar (NA), Mannitol salt agar (MSA), and Sabouraud dextrose agar (SDA). For bacterial isolates, BA and CA plates were incubated in candle jar (5-10% CO_2) at 37^0C for overnight. MA plates were incubated at 37^0 C in incubator for overnight. SDA plates were used for culture of fungal isolates and incubated at 37^0C for 2-3 days.

All plates were read daily up to four days and plates with no visible growth were reincubated. Specimens with positive Gram's stain and negative culture were held for

one week. Control strain American Type Culture Collection (ATCC) were used parallel as a part of quality control of test system. Both positive and negative control was included during test.

3.3.8 Identification of the Isolates

The bacterial culture plates were examined after overnight incubation and the organism showing the growth on the streaked line were identified with the use of standard microbiological techniques as described in the Bergey's manual of Systematic Bacteriology (2005) which includes observation of colony morphologies, staining reactions, and biochemical properties. Standard protocol provided by Cheesbrough (2000); Gray and Fedorko (1992) were followed for identification of bacteria isolated from CSF specimens.

Biochemical tests

Appropriate biochemical tests were performed for the confident identification of the bacterial and fungal isolates. For the growth of the bacteria from the primary culture, the plates were subcultured onto the different agar plates as required to obtain the pure culture which were inoculated onto different biochemical media.

Gram-positive organisms were identified primarily on the basis of their response to Gram's staining, Catalase, Oxidase and Coagulase tests. Optochin disc and Bacitracin discs were also placed on the subcultured plate in order to differentiate *pneumococcus* from other gram positive cocci. Bile solubility test was also done for the identification of *Strep. pneumoniae* (Appendix V).

The biochemical tests used for the identification of Gram-negative bacterial isolates included Catalase test, Oxidase test, Indole test, Methyl red test, Voges Proskauer test, Citrate utilization test, Triple Sugar Iron (TSI) test, Urease test, Motility test, Oxidation and fermentation (OF) test, and Gas production tests (Appendix V).

'X' and 'V' factor was used to identify the colony of *H. influenzae.* This was done in disc test; the presence or absence of growth was observed around the paper disc impregnated with 'X' factor alone, 'V' factor alone or 'X+V' factor, placed on a nutrient agar (Appendix IV).

Morphological characteristics and biochemical tests were used for the identification of *C. neoformans.* Seventy-two hours after incubation, the isolates were subcultured to obtain single colonies on SDA plate. All isolates were identified by colony morphology and microscopic morphology of yeast cells. Presence of a capsule on India ink preparation, and urease test positive were also used to confirm *C. neoformans.*

Purity plate

Purity plate was performed during each biochemical test to observe whether the tests were performed aseptically. One half of the NA plate was inoculated from the four hour broth culture prepared for biochemical testing just before the test was put up and the other half of the same NA plate was inoculated immediately after completing the biochemical tests. The plate was incubated overnight at 37^0C along with the other tests. The growth of same organism in pure form in both the pre and post inoculated portion of the plated was taken as an indication of maintenance of aseptic condition throughout the experiment.

3.3.9 Antibiotics Susceptibility Patterns

Antibiotic susceptibility testing was done using standard Kirby-Bauer disk diffusion method following Clinical and Laboratory Standards Institute (CLSI) guidelines. The pure culture of the isolates were inoculated into Nutrient broth and incubated for about four hours at 37^0C and then using a sterile swab the organism was swabbed on Mueller Hinton agar (MHA) surface plate. For the isolates like *Strep. pneumoniae* MHA containing 10% of sheep blood was used for testing its antibiotic susceptibility testing. Commercially available antibiotic discs were used (Appendix II). The antibiotics discs were taken out from refrigerator and after bringing to room temperature. The surface was lightly and uniformly inoculated by cotton swab. Prior to inoculation, the swab stick was dipped into bacterial suspension having visually equivalent turbidity to 0.5 McFarland standards (Appendix III). The swab stick was then took out and squeezed on the wall of the test tube to discard extra suspension. The plates were then incubated at 37^0C for overnight. Similarly, MHA containing 10% BA plates and CA plates were incubated anaerobically in candle jar incubator for overnight.

On the next day, plates were read by taking measurement of zone of inhibition. Results were recorded and graded as Resistant (R), Intermediate (I) and Sensitive (S) according to the standard zone of inhibition of particular antibiotic.

3.4 Quality Control for Tests

All tests were performed with regular quality control. During the study, the sterility of each batch of the test medium was confirmed by incubating un-inoculated plates and tubes overnight at 37^0C. The incubated plates and tubes of the batch of the medium were not used if those plates and tubes showed the evidence of bacterial growth and other visual reactions after incubation.

Control test were also made to confirm that test medium has been made correctly or not. For this, from each batch, one test medium was inoculated with a standard culture of bacterium known to give a positive reaction. Control strains of *E. coli* (ATCC 25922), *Staph. aureus* (ATCC 25923) and *Ps. aeruginosa* (ATCC 27853) were used to check the quality of the medium from each batch. During identification of the organism, for each test ATCC control positives and control negatives was taken simultaneously. Quality of sensitivity tests was maintained by maintaining the thickness of MHA and Mueller Hinton blood agar (MHBA) at 4 mm and the pH 7.2-7.4. Similarly antibiotics discs containing the correct amount as indicated were used. Strict aseptic conditions were maintained while carrying out all the procedures.

3.5 Validity and Reliability

For the valid test results, CLSI recommended antibiotic discs were used. The expiry date of discs, media, and reagents were carefully noted. Standard microbiological protocols were followed throughout this study.

The reliability of this study was maintained by considering the opinion of subject experts, supervisors, examiners and reviewing relevant literatures.

3.6 Data Analysis

The program, Statistical Package for the Social Sciences (SPSS) version 20 for windows was used to analyze the results. Descriptive statistics were used to summarize the frequencies and percentages and distribution of microbial isolates and

their sensitivity to various antibiotics. The variables were also calculated for mean, median, range and standard deviation as per requirement. As inferential statistics, Chi square test was performed in order to identify the association between the age group and sex with cases of meningitis if any relationship between the variables could be identified. All tests of significance were two-tailed and a P-value less than 0.05 was considered to be significant. Comparison of antimicrobial resistance patterns of the isolates were analyzed using a standard interpretative chart.

3.7 Ethical Considerations and Confidentiality

No consent was obtained from the parents or guardians since the study was a part of routine admissions. However, official consent and clearance was obtained from the Institutional Review Board (IRB) of the hospital.

The name of the patients and any clues leading to the identification of the patients were not recorded in the record book. The data was identified only by the code number that could not be linked to any identifiable data to maintain the strict confidentiality. The results were presented as overall data. No individual data was presented.

3.8 Limitations of the Study

- Only CSF samples were processed for diagnosis of meningitis
- Samples were not processed for viral etiology of meningitis
- Antifungal susceptibility test was not performed for fungal meningitis cases
- Time framework for the study was only six months
- This study is a hospital population-based so the result may not represent the general population

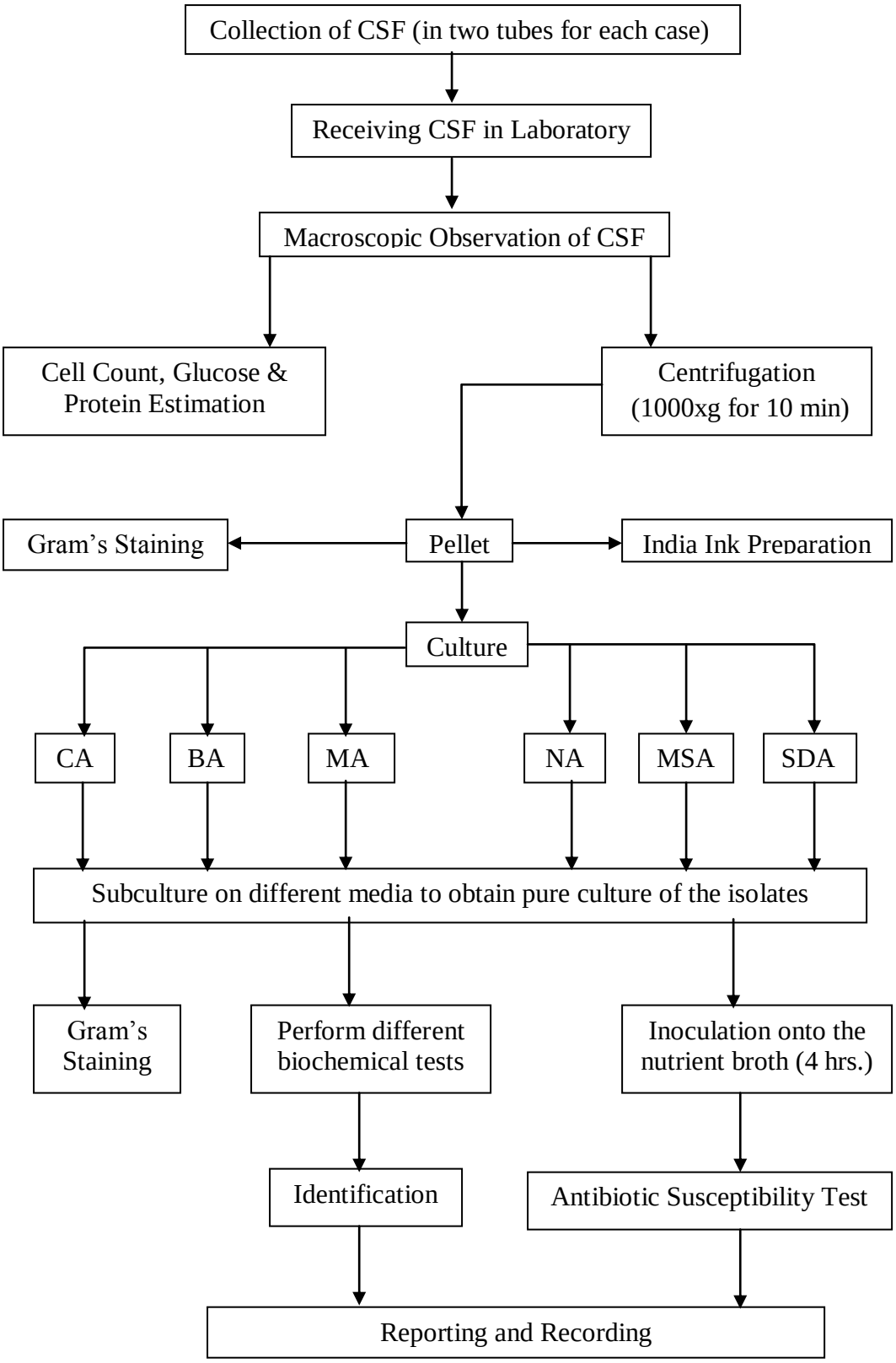


Figure 2: Flow Chart Showing Processing of CSF Sample

CHAPTER IV
RESULTS

During the six months' time period considered for the study, 356 CSF samples from patients suspected of meningitis were examined macroscopically, microscopically and microbiologically for the laboratory diagnosis in the Emergency laboratory at TUTH. After CSF examination 16 cases were diagnosed to have meningitis. Thus the hospital frequency of meningitis was 4.5%.

This chapter describes the etiological profile of meningitis and their macroscopic characteristics, cytological, biochemical and microbiological parameters from the CSF samples. The chapter consists of several sections, more specifically; the first section describes the demographic characteristics of suspected cases of meningitis under investigation. The second section presents the microbial pattern of the CSF culture results. The third section illustrates the gross appearances of all CSF samples and the next sections followed cytological profile, biochemical profile, CSF staining and culture results. The final section examines the antibiotic susceptibility pattern of bacterial isolates from the CSF samples.

4.1 Demographic Characteristics of Suspected Cases of Meningitis

Three hundred fifty six patients clinically diagnosed (suspected cases) to have meningitis constituted the study samples and were taken for laboratory diagnosis. The age and sex of all study samples were recorded from the laboratory record file and their frequency distributions were found as in Table 4.

There was a slight male predominance in the sex ratio (1.3:1) with males contributing 201 (56.5%) and females 155 (43.5%) respectively. The youngest and oldest patients in the study were 1 month and 85 years old respectively. The mean age of the patients was 27.8 years with standard deviation of 22.4 years.

Table 4: Age and Sexwise Distribution of Suspected Cases of Meningitis

Age Group (Years)	Sex				Total	
	Male		Female			
	N	%	N	%	N	%
<1	21	5.9	16	4.5	**37**	**10.4**
1 - 14	56	15.7	45	12.6	**101**	**28.4**
14 - 30	47	13.2	37	10.4	**84**	**23.6**
30 - 60	49	13.8	42	11.8	**91**	**25.6**
> 60	28	7.9	15	4.2	**43**	**12.1**
Total	**201**	**56.5**	**155**	**43.5**	**356**	**100**

The age group with the highest number of the patients 101 (28.4%) suspected of meningitis was 1 - 14 years and the lowest number of patients suspected of meningitis was from the age group <1 years old 37 (10.4%) and >60 years old 43 (12.1%) involved in this study (Table 4).

4.2 Microbial Pattern of the CSF Culture Results

Among the total processed CSF specimen (n=356) only 16 (4.5%) cases were known to have laboratory confirmed cases of meningitis with the help of CSF culture results. The remaining 340 (95.5%) cases showed no growth during CSF culture (Figure 3).

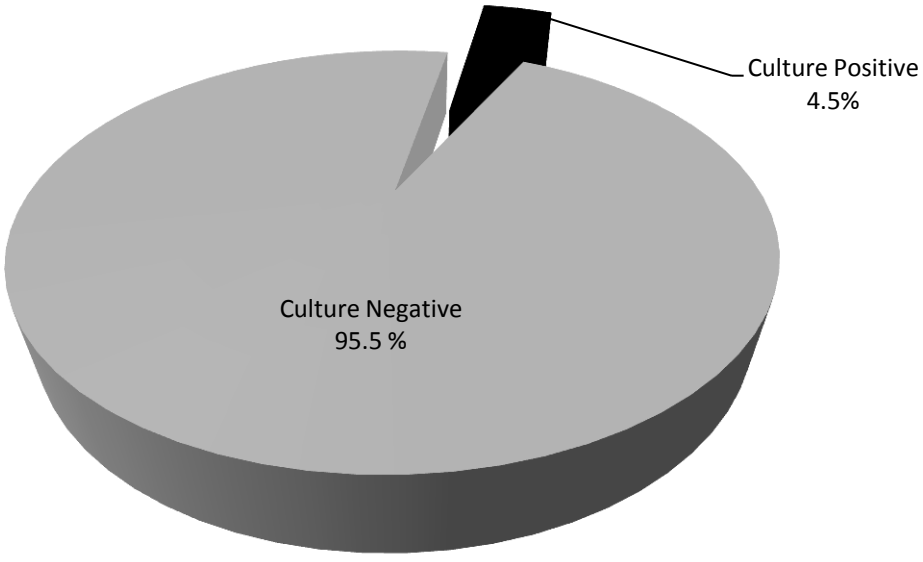


Figure 3: Proportion of Culture Growth Results

Table 5: Age and Sexwise Distribution of Isolated Pathogens from CSF

Age Group (Years)	Sex				Total	
	Male		Female			
	N	%	N	%	N	%
< 1	3	18.8	2	12.5	**5**	**31.3**
1 - 14	1	6.3	2	12.5	**3**	**18.8**
14 - 30	1	6.3	2	12.5	**3**	**18.8**
30 - 60	2	12.5	0	0	**2**	**12.5**
> 60	2	12.5	1	6.3	**3**	**18.8**
Total	**9**	**56.3**	**7**	**43.8**	**16**	**100**

Out of total positive cases (n=16) the number of isolates in male CSF samples 9 (56.3%) was slightly greater than the isolates in female CSF samples 7 (43.8%). The highest number of isolates was obtained from the patients aged <1 year old which accounts to 5 (31.3%) positive cases. This was followed by the children of age group 1 - 14 years and adult patients of age group 14 - 30 years which equally constituted 3 (18.8%) positive cases. Another peak was observed in the older aged patients (>60 years) which also made up 3 (18.8%) of the positive cases. The patients of this age group (>60 years) may be supposed to have immune-compromised or immune-competent status. Among the female positive cases CSF samples, from the age group of 30 - 60 years, no any pathogens were isolated, whereas among the male positive cases at least one pathogen was isolated from each of the age group mentioned in this study (Table 5).

The frequency of isolates in each of the six age category (Table 5) was less than 5, so, some age categories were merged and reframed into only two age groups (≤14 years and >14 years) to safeguard the assumption of Chi square test (Table 6).

The frequency distribution of meningitis in relation to the age groups (≤14 years and >14 years) showed no any statistically significant association ($p>0.05$). Thus it may be concluded that the cases of meningitis is not dependent on the ages of the patients considered in this study. Similarly it was observed that statistically, there is no significant difference in the incidence of meningitis across different sex of the patient in this study (Table 6).

Table 6: Association of Meningitis with Age Groups and Sex

	Patients with Meningitis	Patients without Meningitis	Total
Age Groups			
χ^2 = 0.891, df=1, p=0.345, ns			
0 - 14 Year	8	130	**138**
> 14 Year	8	210	**218**
Total	**16**	**340**	**356**
Sex			
χ^2 =0.000, df=1, p=0.986, ns			
Male	9	192	**201**
Female	7	148	**155**
Total	**16**	**340**	**356**

(df = degree of freedom, ns = not significant)

CSF culture results identified two types of meningitis; bacterial meningitis and fungal meningitis. Bacterial meningitis was confirmed in 13 patients (81.3%) with six types of etiological agents and *Staph. aureus* having the highest incidence 4 (25%) among the common agents of bacterial meningitis. *E. coli* and *Strep. pneumoniae*, each was the second commonest bacterial agents which constituted 3 (18.8%) positive cases. *H. influenzae, Kl. pneumoniae* and *Ps. aeruginosa,* each was isolated in only 1 (6.3%) positive case. The fungal meningitis was confirmed in only 3 (18.8%) positive cases with *C. neoformans* being the only culture isolate, identified (Table 7).

Table 7: Laboratory Confirmed Cases of Meningitis and Their Etiology

Types of Meningitis and Their Etiologies	Frequency (%)
Bacterial Meningitis (N = 13)	
Staph. aureus	4 (25.0)
E. coli	3 (18.8)
Strep. pneumoniae	3 (18.8)
H. influenzae	1 (6.3)
Kl. pneumoniae	1 (6.3)
Ps. aeruginosa	1 (6.3)
Fungal Meningitis (N = 3)	
C. neoformans	3 (18.8)
Total	**16 (100)**

(Figures in parentheses indicate percent)

Table 8: Distribution of Isolates among different Sex and Age Groups

Etiological Agents	Sex/Age Groups (Years)									
	< 1		1 - 14		14 - 30		30 - 60		> 60	
	M	F	M	F	M	F	M	F	M	F
Bacterial Etiology										
Staph. aureus (N=4)			1	2		1				
E. coli (N=3)	1	2								
Strep. pneumoniae (N=3)	1						1			1
H. influenzae (N=1)	1									
Kl. pneumoniae (N=1)						1				
Ps. aeruginosa (N=1)					1					
Fungal Etiology										
C. neoformans (N=3)							1		2	
Total (N=16)	**3**	**2**	**1**	**2**	**1**	**2**	**2**		**2**	**1**

(M = Male, F = Female)

A variation in the incidence of pathogens was observed in various age groups. Most marked variation was observed with *Strep. pneumoniae*, and some less common organisms of bacterial meningitis such as *E. coli* were more common in infants. Among three identified cases of *Strep. pneumoniae*, one of them was present in infants, one in age group 30 - 60 years and one in patient aged above 60 years old. *H. influenzae* was observed in only one child <1 year old. The incidence of *Ps. aeruginosa*, and *Kl. pneumoniae* were predominant among male and female patients of age group 14 - 30 years respectively. *Staph. aureus* was obtained from children age group 1 - 14 years and adults of age group 14 - 30 years.

The *C. neoformans* was the pathogen most commonly observed in the male patients of age group 30 - 60 years and older aged patients (>60 years).

4.3 Gross Appearance of CSF Specimen

Table 9: Distribution of Appearance of Total CSF Specimen and Culture Positive Isolates

Appearances of CSF	Total CSF Samples (N=356)		Culture Positive Isolates (N=16)	
	Frequency (N)	Percentage (%)	Frequency (N)	Isolation Rate (%)
Clear	243	68.3	1	0.4
Slightly Turbid	39	11.0	3	7.7
Cloudy	13	3.7	7	53.8
Purulent	4	1.1	3	75
Bloody	57	16.0	2	3.5

Majority of the CSF (n=243, 68.3%) appeared clear in color. However, one pathogen was isolated from that clear CSF sample which was later identified to be *C. neoformans.* Purulent color of CSF was observed in very few (n=4, 1.1%) cases. But the isolation rate of pathogens from such purulent samples was highest 75% (3 out of 4), followed by cloudy samples 53.8% (7out of 13) and slightly turbid samples 7.7% (3 out of 39). Bloody color was observed among 57(16%) CSF samples from which only 3.5% (2 out of 57) isolates were isolated (Table 9).

4.4 Cytological Profile of CSF Specimen

Table 10: TLC of Total CSF Specimen and Culture Positive Isolates

TLC (cells/mm^3)	Total CSF Specimen (N=356)		Culture Positive Isolates (N=16)	
	Frequency (N)	Percentage (%)	Frequency (N)	Isolation Rate (%)
(Normal) 0 - 5	153	43.0	0	0
5 - 100	129	36.2	6	4.7
> 100	74	20.8	10	13.5

Among most of the CSF samples (n=153, 43.0%), TLC was most commonly found in the normal range from which no any pathogen was isolated. Among 129 (36.2%) cases TLC were in the range of 5 - 100 cells/mm^3, where 4.7% (6 out of 129) pathogens were isolated and in 74 (20.8%) cases TLC was found to be greater than 100 cells/mm^3 where the isolation rate of the pathogens was highest 13.5% (10 out of 74) (Table 10). Thus it could be inferred that greater the number of TLC, more the prevalence of pathogens.

Table 11: Cytological Parameters in Different Types of Meningitis

Types of Meningitis/Cytological Parameters	Range	Mean	Standard Deviation
Bacterial Meningitis (N=13)			
TLC (cells/mm^3)	10 - 2000	337.3	523.8
DLC (%)			
Lymphocytes	5 - 46	26.2	12.9
Neutrophils	54 - 95	73.8	12.9
Fungal Meningitis (N=3)			
TLC (cells/mm^3)	10 - 15	11.7	2.9
DLC (%)			
Lymphocytes	100	100	0.0
Neutrophils	0	0	0.0
Total CSF Samples (N=356)			
TLC (cells/mm^3)	3 - 4860	104.5	334.1
DLC (%)			
Lymphocytes	0 - 100	80.4	31.3
Neutrophils	0 - 100	19.6	31.3

Among the total CSF samples, TLC varied a wide range from 3 - 4860 cells/mm^3 with mean value 104.5 cells/mm^3 and standard deviation 334.1 cells/mm^3. This shows that the number of TLC was hugely scattered among the CSF samples. Lymphocytes (80.4%) were found to be predominant in the total CSF sample.

Among 13 cases of bacterial meningitis TLC showed a large range of 10 - 2000 cells/mm^3 and average number of TLC was found to be 337.3 cells/mm^3 with large

standard deviation (523.8 cells/mm^3). Thus among the bacterial cases, the CSF samples showed an uneven distribution of TLC. In the differential leucocyte count, mean percentage of neutrophils was 73.8% with standard deviation 12.9%. The range of the lymphocytes was less (5 - 46 %) than the neutrophils (54 - 95 %) in bacterial meningitis. Thus the cases of bacterial meningitis sample showed predominance of neutrophils (Table 11).

Fungal meningitis was identified in only 3 cases where the range of TLC was found to be 10 - 15 cells/mm^3 which is extremely lower unlike in bacterial meningitis cases (10 - 2000 cells/mm^3). Similarly the mean number (11.7 cells/mm^3) of TLC in fungal meningitis was lower than in bacterial meningitis (337.3 cells/mm^3). The TLC may be supposed to be evenly distributed (standard deviation 2.9 cells/mm^3) among the CSF sample of fungal meningitis cases. Lymphocyte was found to be 100% in all 3 cases of fungal meningitis showing a complete absence of neutrophil. Thus the cases of fungal meningitis sample showed predominance of lymphocytes (Table 11).

4.5 Biochemical Profile of CSF Specimen

Table 12: Glucose Level of Total CSF Specimen and Culture Positive Isolates

Glucose Level (mg/dL)	Total CSF Samples (N=356)		Culture Positive Isolates (N=16)	
	Frequency (N)	Percentage (%)	Frequency (N)	Isolation Rate (%)
<40	72	20.2	13	18.1
(Normal) 40 - 80	193	54.2	3	1.6
>80	91	25.6	0	0

More than half (n=193, 54.2%) of the CSF samples were in normal range of glucose from which 1.6% (3 out of 193) pathogens were isolated. About a quarter (n=91, 25.6%) of the total CSF samples had glucose level greater than 80 mg/dL, from which no pathogens were isolated. In 72 cases out of 356 CSF samples (20.2%), the glucose level was below normal range (<40 mg/dL) from which highest proportion 18.1% (13 out of 72) pathogens were isolated (Table 12).

Table 13: Protein Level of Total CSF Specimen and Culture Positive Isolates

Protein Level (mg/dL)	Total CSF Samples (N=356)		Culture Positive Isolates (N=16)	
	Frequency (N)	Percentage (%)	Frequency (N)	Isolation Rate (%)
< 15	72	20.2	0	0
(Normal) 15 - 45	134	37.6	3	2.2
> 45	150	42.1	13	8.7

No any pathogens were isolated from the 72 (20.2%) CSF samples which were found to have protein below the normal range (<15 mg/dL). The isolation rate of pathogen was highest 8.7% (13 out of 150) among those CSF samples which had protein level above the normal range (>45 mg/dL). The protein level above 45 mg/dL was found in 150 (42.1%) cases. In 134 (37.6%) CSF samples protein level was found to be normal from which 3 (2.2%) isolates were isolated (Table 13).

Table 14: Biochemical Parameters in Different Types of Meningitis

Types of Meningitis/ Biochemical Parameters	Range	Mean	Standard Deviation
Bacterial Meningitis (N=13)			
Glucose (mg/dL)	1.8-68.4	28.8	20.0
Protein (mg/dL)	46.9 - 175.6	89.4	42.6
Fungal Meningitis (N=3)			
Glucose (mg/dL)	30.6 - 72	45.0	23.4
Protein (mg/dL)	39.8 - 57.3	48.7	8.8
Total CSF Samples (N=356)			
Glucose (mg/dL)	1.8 - 1674	81.0	129.6
Protein (mg/dL)	0.2 - 380	48.0	42.2

The measures of biochemical parameters (protein and glucose level) among the total (n=356) CSF samples, revealed that glucose level was in the range of 1.8 - 1674 mg/dL with the mean value 81 mg/dL and standard deviation 129.6 mg/dL, and the protein level ranged from 0.2 - 380 mg/dL with mean value 48.0 mg/dL and standard

deviation 42.2 mg/dL (Table 14). Among the 13 cases identified as bacterial meningitis, the mean value of glucose was 28.8mg/dL which is less than in cases of fungal meningitis (45mg/dL). Unlike the glucose level, the mean value of protein in bacterial meningitis (89.4 mg/dL) was found to be higher than in cases of fungal meningitis (48.7 mg/dL). The range of protein level in bacterial meningitis also varied hugely (46.9 - 175.6 mg/dL) than in comparison to fungal meningitis (39.8 - 57.3 mg/dL) (Table 14).

4.6 CSF Staining and Culture Results

Seven types of pathogens were identified as causative agents of bacterial meningitis whereas *C. neoformans* was the only causative agent of fungal meningitis. Total 19 pathogens were isolated on Gram's staining whereas the CSF culture results showed growth of only 16 pathogens.

Table 15: Correlation between Isolated Pathogens on CSF Staining and Culture

Organism Observed on	Number of Isolates on Staining	Number of Isolates on Culture
Gram's Staining		
Staph. aureus	5	4
E. coli	3	3
Strep. pneumoniae	3	3
Kl. pneumoniae	2	1
H. influenzae	1	1
Ps. aeruginosa	1	1
N. meningitidis	1	0
India Ink Staining		
C. neoformans	3	3
Total	**19**	**16**

Staph. aureus was the commonest organism isolated (n=5, 26.3%) on Gram's staining followed by *Strep. pneumoniae* and *E. coli* in 3 (15.8%) cases each, *Kl. pneumoniae* in 2 (10.5%) cases. *Ps. aeruginosa*, *N. meningitidis*, and *H. influenzae* were isolated in 1 (5.3%) case each on Gram's staining (Table 15). The CSF culture didn't showed growth of *N. meningitidis* which was isolated on Gram's staining. Similarly one of *Staph. aureus*, and *Kl. pneumoniae* each, was not identified on CSF culture. However a high degree of positive correlation (r = 0.933) was found in between the organisms isolated in Gram staining of fresh CSF sample and growth in culture medium.

4.7 Antibiotic Susceptibility Pattern of Bacterial Isolates

Antibiotic susceptibility tests were carried out to determine the sensitivity of bacterial isolates to the different antibiotics. The bacterial isolates showed varied susceptibility to the twelve antibiotics (Table 16, Table 17). But, the antifungal susceptibility test of fungal isolate (*C. neoformans*) was not performed due to lack of access and facility in the lab where the study was carried out.

Table 16: Antibiotic Susceptibility Test of Gram Negative Isolates

Organisms/Antibiotics Used	Susceptibility Pattern		
	Susceptible	Intermediate	Resistant
***H. influenzae* (N = 1)**			
Ampicillin	0	0	1
Ceftriaxone	1	0	0
Chloramphenicol	1	0	0
Ciprofloxacin	1	0	0
Cotrimoxazole	1	0	0
Erythromycin	1	0	0
***E. coli* (N = 3)**			
Amikacin	1	0	2
Cefotaxime	1	1	1
Ceftriaxone	2	0	1
Chloramphenicol	3	0	0
Cotrimoxazole	1	0	2
Ofloxacin	1	1	1
***Kl. pneumoniae* (N = 1)**			
Amikacin	0	0	1
Cefotaxime	1	0	0
Chloramphenicol	1	0	0
Ciprofloxacin	0	0	1
Cotrimoxazole	1	0	0
Ofloxacin	1	0	0
***Ps. aeruginosa* (N = 1)**			
Amikacin	1	0	0
Chloramphenicol	1	0	0
Ciprofloxacin	1	0	0
Gentamicin	0	0	1
Meropenem	1	0	0
Ofloxacin	1	0	0

H. influenzae was found to be resistant to Ampicillin but susceptible to Ceftriaxone, Chloramphenicol, Ciprofloxacin, Cotrimoxazole, and Erythromycin.

Three isolates of *E. coli* were isolated from different patients suspected of meningitis. *E. coli* was most susceptible towards Chloramphenicol 3 (100%), followed by Ceftriaxone 2 (66.7%), Ampicillin, Cefotaxime, Cotrimoxazole and Ofloxacin

1(33.3%) each. *E coli* was comparatively more resistant to Amikacin and Cotrimoxazole 2 (66.7%) each. *E. coli* was equally susceptible (1), intermediate susceptible (1) and resistant (1) against Cefotaxime and Ofloxacin.

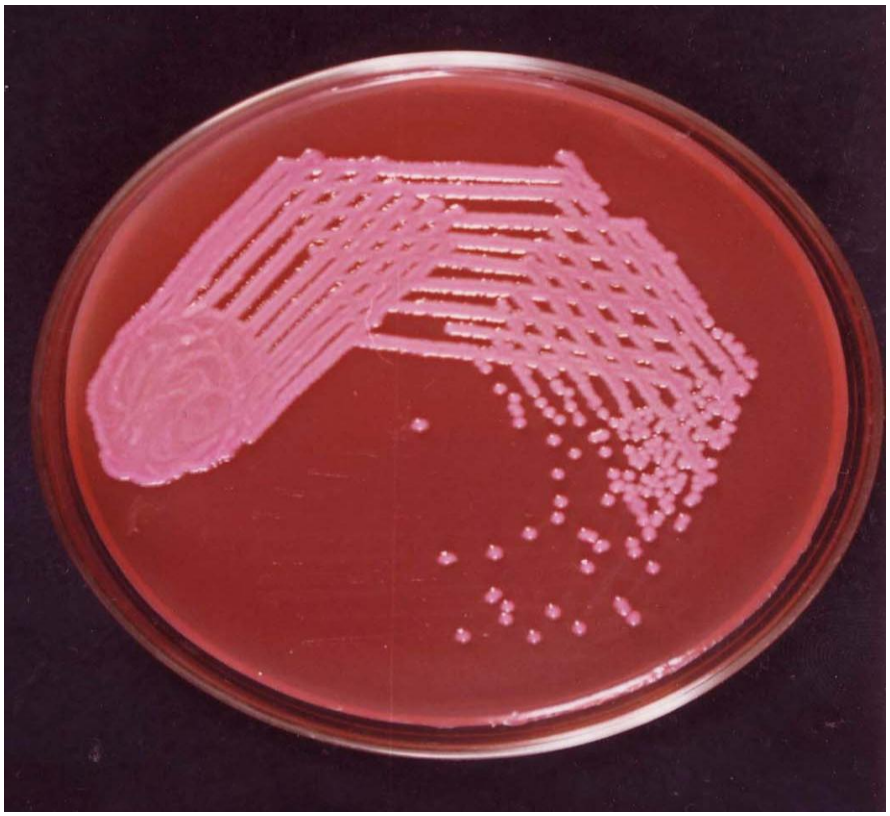
Cefotaxime, Chloramphenicol, Cotrimoxazole, and Ofloxacin were 100% effective against *Kl. pneumoniae*, whereas *Kl. pneumoniae* was found to be resistant against Amikacin and Ciprofloxacin. *Ps. aeruginosa* was found to be susceptible towards Amikacin, Chloramphenicol, Ciprofloxacin, Meropenem and Ofloxacin but resistant against Gentamicin.

Table 17: Antibiotic Susceptibility Test of Gram Positive Isolates

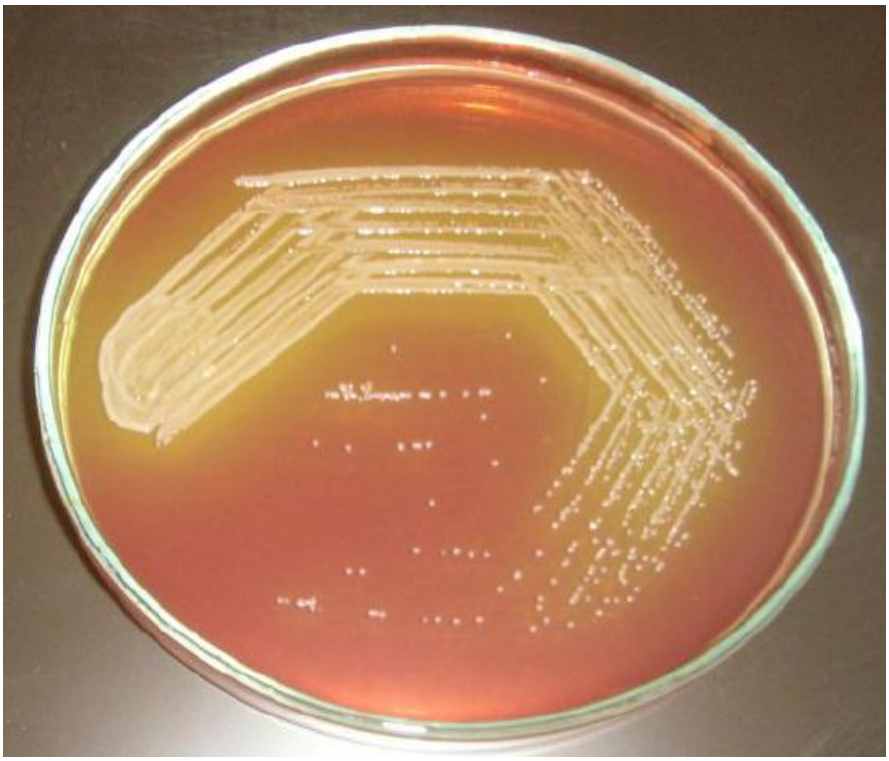
Organisms/Antibiotics Used	Susceptibility Pattern		
	Susceptible	**Intermediate**	**Resistant**
***Staph. aureus* (N = 4)**			
Amikacin	4	0	0
Cefotaxime	3	0	1
Ceftazidime	4	0	0
Chloramphenicol	4	0	0
Ciprofloxacin	3	1	0
Ofloxacin	3	0	1
***Strep. pneumoniae*(N = 3)**			
Ampicillin	3	0	0
Cefotaxime	2	1	0
Chloramphenicol	3	0	0
Ciprofloxacin	3	0	0
Cotrimoxazole	0	1	2
Erythromycin	3	0	0

There were four isolates of *Staph. aureus*. Amikacin, Ceftazidime and Chloramphenicol were 100% effective against *Staph. aureus*, followed by Cefotaxime, Ciprofloxacin and Ofloxacin 3 (75%) each. In one case of *Staph. aureus*, it was found to be resistant against Cefotaxime and intermediate susceptible against Ciprofloxacin.

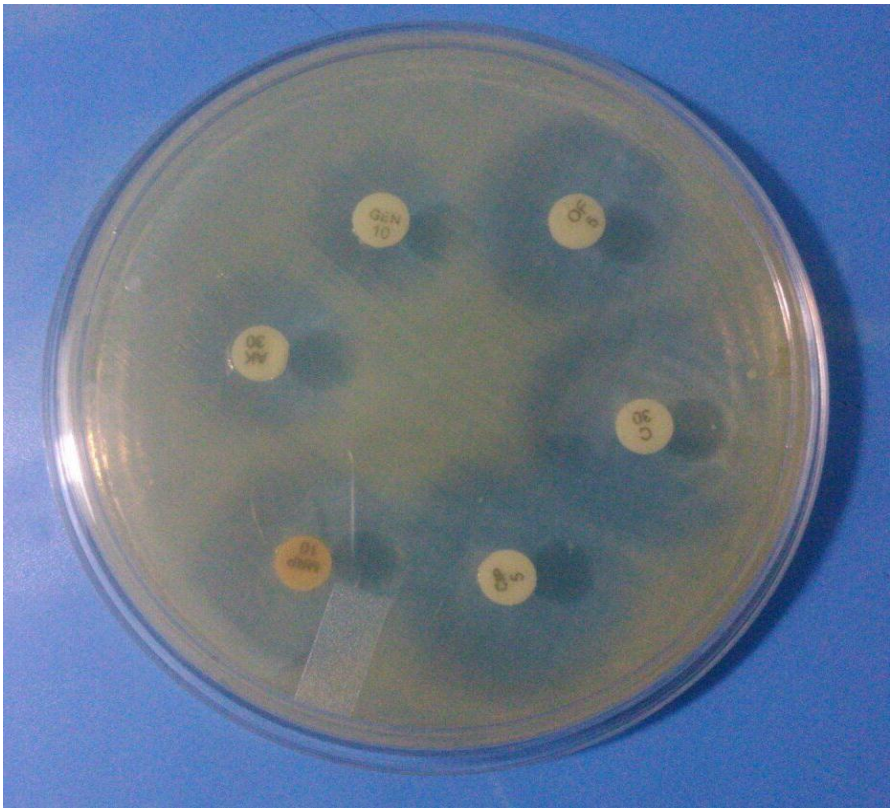
Strep. pneumoniae was isolated in three cases. Ampicillin, Chloramphenicol, Ciprofloxacin, and Erythromycin were found to be highly effective (100%) against *Strep. pneumoniae*, followed by Cefotaxime 2 (66.7%). But Cotrimoxazole was found to be ineffective in 2 (66.7%) cases against *Strep. pneumoniae* and was intermediate susceptible in one case.



Photograph 1: Pure Culture of *Kl. pneumoniae* on MA



Photograph 2: Golden Yellow Colonies of *Staph. aureus* on MSA



Photograph 3: Antimicrobial Susceptibility Test *Ps. aeruginosa* on MHA

Photograph 4: Biochemistry Automatic Analyzer (Erba XL- 200)

CHAPTER V
DISCUSSION

There is a need for periodic review of meningitis worldwide since the pathogens responsible for infection vary with time, geography, and patient's age. Increase in awareness availability of vaccines may also reflect a change in the epidemiological pattern of these pathogens.

This study was conducted among the patients suspected of meningitis attending TUTH, Kathmandu, Nepal with an aim to analyze the etiological agents of meningitis and to test the efficacy of antibiotics being used against isolated bacteria. A total 356 CSF samples were collected for the study during six months period and were subjected for the cytological, biochemical and microbiological examination.

Out of total 356 suspected cases of meningitis, 201 (56.6%) were male and 155 (43.5%) were female. In a similar study carried out by Mengistu *et al.* (2011) in Ethiopia, 53.5% males and 46.5% females' cases were investigated. Similar cases were observed in a two year hospital based study by Joardar *et al.* (2012) in Kolkata, India where majority of the cases were male (60.43%). A similar pattern of the cases among male and females were observed in a study conducted by Farag *et al.* (2005) during a period of 2002-2003 Alexandria, Egypt where 195 (62.9%) male and 115 (37.1%) females were reported.

Out of 356 CSF samples processed, only 16 (4.5%) samples showed growth on CSF culture. The findings of culture positive result (4.5%) is in agreement with several similar studied conducted in Nepal. A study conducted by Shaw *et al.* l (2007) in Manipal Teaching Hospital Nepal from 2000 to 2005 demonstrated 4.58% growth on CSF culture. A similar study done by Rijal *et al.* (2010) from 2004 to 2008, showed 4.4% growth on CSF culture. But in contrary to these findings Bhagawati *et al.* (2014) isolated 16.14% fungal and bacterial pathogens from CSF culture. Ataee (2011) showed a very high proportion of culture positive result (63.3%). A study conducted by Adhikari and Wiejesinghe (2008-2009) in Kanti Children Hospital and TUTH, Nepal under South Asian Pneumoccocal Alliance Network showed 8.2% CSF culture

growth positive. Wang *et al.* (2014) demonstrated a rate of bacterial isolation of 9% in china. Mulu *et al.* (2005) carried out a study in Ethiopia from 2002 to 2003 and found 5.6% growth in CSF culture. Al-Rawazq (2010) in a study carried out in Bagdad found 10% CSF culture positive result. Habiba (2012), in his study carried out in Bangladesh, 7(20.0%) cases yielded positive by culture, Ceyhan *et al.* (2008) in Turkey had observed, cultures were positive in 10.0% of cases in their study. Alam *et al.* (2007) in Bangladesh reported in their research 13.7% bacterial isolation among bacterial meningitis cases which is contradictory with this study. The difference in isolation rate of pathogens in various studies may be due to sampling technique, laboratory procedures, and technical appropriateness during CSF culture and culture media being used. The isolation rate of pathogens was found to be comparatively low in this study. This may be due to fastidious nature of the organism and or intake of antibiotics before presentation for treatment at the hospital.

In this study statistically no significant difference between the age groups (<14 years and ≥14 years) and the incidence of meningitis was reported. This might be due to low number of positive cases encountered in this study. But in contrary, Farag *et al.* (2005) showed that acute bacterial meningitis has a significant association with age group 1 - 9 years and also asceptic meningitis showed significant association with age group 3 - 15 months. AL-Zubiery (2001) reported a statistically significant difference in the occurrence of bacterial meningitis with age group <2 years. Similarly a no significant association between the sex of the patients and incidence of meningitis was established in this study. This finding is in agreement with the findings of AL-Zubiery (2001) where no significant association was observed between sex of the children and bacterial meningitis infection occurrence.

In this study, maximum numbers of children 5 (31.3%) were found to have meningitis in the age group of <1 year. This result is consistent with the findings of Rao *et al.* (1998) where majority (64.9%) cases of acute bacterial meningitis was in children of up to 1 year of age. Chowdhury *et al.* (1992) from Bangladesh also reported 49.6% cases of bacterial meningitis in the age range of 1 month to 1 year.

Whereas the age group with less number of pathogens isolated is 30 - 60 years where only 2 (12.5%) isolates were isolated in this study. The decline in the percentage of

cases in the age group of 30 - 60 years may be attributed to the development of protective antibodies.

Upon CSF examination, the culture results revealed only two types of etiologies of meningitis; bacterial and fungal. The bacterial isolates accounted for 13 (81.3%) and fungal meningitis only 3 (18.8%) and *C. neoformans* was the only fungal isolate identified in this study. This finding is in agreement with the findings of a similar hospital based study conducted by Mengistu *et al.* (2011) in Ethiopia where they found 84.6% bacterial isolates and 15.4% fungal isolates in which *C. neoformans* was the only fungal isolate. Similarly Selim *et al.* (2007) reported that out of 165 clinically diagnosed meningitis cases, 72 (43.6%) were due to bacterial causes, 10 (6.1%) due to viral causes, and in 1 (0.6%) case fungal meningitis. In a similar study conducted in Kenya by Honnas and Peterson (1998), 81.3% cases of bacterial meningitis were reported. In contrary to the proportion of bacterial meningitis (81.3%) cases reported in this study, a low percentage (44.8%) of bacterial isolates was reported by Ahmed *et al.* (1996) in Sudan. Similarly a lower isolation rate (20.9%) of bacterial pathogens was reported by Mindadou *et al.* (2006) in Niger. A much lower percentage of bacterial meningitis cases were reported by Dubos *et al.* (2006) in France, as among 166 meningitis patients 20 (12%) had bacterial meningitis and 146 had aseptic meningitis. In the present study, only three out of 16 cases were diagnosed as fungal meningitis (18.3%), whereas a very low percentage (1.6%) was reported by Bekondi *et al.* (2006), as out of 61 meningitis cases, only one case of cryptococcal meningitis was detected. Similarly a lower percentage (8%) of *C. neoformans* than in this study was reported in Colombia (Tique *et al.*, 2006; Bhagawati, *et al.*, 2014).

In this study the most common organisms responsible for bacterial meningitis were *Staph. aureus* (25%), followed by *Strep. pneumoniae* (18.8%) and *E. coli* (18.8%), *H. influenzae* (6.3%), *Ps. aeruginosa* (6.3%) and *Kl. pneumoniae* (6.3%) and the fungal pathogen identified was *C. neoformans* isolated in 3 (18.75%) cases only. Our finding also shows a clear difference in the etiological agents of meningitis between children and adults. For children, bacterial were more dominant whereas *C. neoformans* was dominant among patients aged >30 years old.

Staph. aureus remains the leading etiological agent isolated from CSF culture for bacterial meningitis over the study period. *Staph. aureus* was found mostly in the

children of the age group 1-14 years and adults of the age group 14-30 years. This finding is in agreement with the finding of a study conducted by Bhagawati *et al.* (2014) where the predominant organism isolated from CSF culture was found to be *Staph. aureus*15 (29.41%). Higher prevalence of meningitis due to *Staph. aureus* may be due to predisposing factors as well as co-morbidities associated with the patient. Similar isolation rate of *Staph. aureus,* 23.8% was observed by Chang and Lu (2009), and 13.1% reported by Rasoul *et al.* (2006). However, the finding of this study was dissimilar to the findings reported by Mani *et al.* (2007) where they found 1.8% *Staph. aureus*.

Strep. pneumoniae was found in 3 (18.8%) positive cases in almost all age categories, in children below 1 year of age (1 case), in the age group of 30-60 years (1 case) and in the old aged patients (1 case) in this study. But Prieto *et al.* (2009) states that *Strep. pneumoniae* is the cause of pediatric meningitis resulting in substantial morbidity and mortality in developing countries. In a similar study by Rao *et al.* (1998); Matee and Matre (2001); and Al-Binali and Al-Fifi (2002) isolated 26%, 21.8% and 16.6% *Strep. pneumoniae* respectively whereas Adhikari and Wiejesinghe (2008-2009) in a study at Kanti Children's Hospital and TUTH, *Strep. pneumoniae* was found to be only 5.15%. AL-Zubiery (2001), revealed slightly higher proportion (38.7%) of *Strep. pneumoniae*. Duma (1996) as cited in AL-Zubiery (2001), states that the increase in the rate of *Strep. pneumoniae* carriers among population and the socioeconomic status of population play a major role in spreading of pneumococcus infection, and carrier rates of *Strep. pneumoniae* are higher in preschool age children than adults.

E. coli was predominant (18.8%) among the children below 1 year of age in this study. Nwadioha *et al.* (2011), in a study carried out in Nigeria they isolated 16% *E. coli* and it was predominant in neonates. Similarly Aslam *et al.* (2006) in Pakistan isolated 16% *E. coli.* In contrary, Laving *et al.* (2003) in Kenya isolated very high percentage (46.7%) of *E. coli* whereas in a retrospective study by Abate (2011) in Ethiopia showed only 6.76% of *E. coli*. The prevalence of this pathogen in neonates is probably due to the immature immune system and permeability of the BBB. The predominance of *E. coli* in neonates in the study might suggest that these infections were acquired during passage through colonized mothers' vaginal vault.

In this study *H. influenzae* was isolated in only one case in the children of age below 1 year. Similarly AL-Zubiery (2001), found *H. influenzae* as the second most causative agent of bacterial meningitis in children, and it is the leading microorganism causing meninges infection among children with less than one year of age, which is also in agreement with that foundation by Bijlmer (1994), as cited in AL-Zubiery (2001) who reported that the most cases of Hib occurring before 12 months of age. Similarly, Ahmed *et al.* (1996), in Sudan; and Almuneef *et al.* (1998), in Saudi Arabia, reported that the Hib is the main causative agent of bacterial meningitis in children less than 2 years years of age. Susan and Robert (2008) also mentioned that *H. influenzae* is a disease of under-five children. Kristensen *et al.* (1990) showed that the failing of the immune system to protect the body with other factors such as inheritance, socioeconomic status, and inadequate breast feeding, influence the risk of acquiring Hib infection. And the low incidence of *H. influenzae* in this study may be due to the introduction of Hib vaccine in Nepal recently.

In this study *Ps. aerugonisa* was isolated in one male patients of adult age group (14 - 30 years). Similarly in 25 cases of CSF culture proven, Adult Bacterial Meningtis due to *Ps. aerugonisa* was reported in 17 men and 8 women by Huang *et al.* (2007).

Kl. pneumoniae was isolated in only one case in adult's age group (14 - 30 years) in this study and is supported by a study in Taiwan, conducted by Tang *et al.* (1999), where *Kl. pneumoniae* was the most important in both community acquired and nosocomial meningitis in adults. Bhagawati *et al.* (2014) found *Kl. pneumoniae* to be the most common isolate, 8 (15.7%) among the Gram negative isolates. In contrary, *Kl. pneumaniae* were found to be the most common (22%) causative agents for bacterial meningitis among neonates as reported from Ethiopia, Addis Ababa (Gebremariam, 1998), and in Iran 35.5% (Aletayeb, 2010). So the incidence of *Kl. pneumaniae* may be supposed to vary among the different age and geographic regions.

C. neoformans was confirmed in 3 (18.75%) cases of culture positive results, in two cases in the old aged patients (>60 years) and in one case in patient aged between 30 - 60 years of age in this study. This may not mean that this is the most vulnerable group but may actually be a reflection of the fact that most of the hospital based population in this study falls in that age category. So due to the low occurrence of *Cryptococcus*

in our sample series we cannot comment on its incidence by age group. Bhagawati *et al.* (2014) reported isolation rate of *C. neoformans* as 8% which seems to support this finding. But a very low isolation rate of *C. neoformans* (1 out of 78 cases) by CSF culture was reported by Prince (2010). Similarly, in a study between November 1999 to June 2000 conducted by Matee and Matre (2001), in Tanzania, they reported *C. neoformans* in 12(21.8%) cases in children (aged less than 15 years) whereas 163 (97.6%) cases in adults. Thus it could be agreed with the view of Bicanic, and Harrison (2004) that quotes that cryptococcal meningitis is a common opportunistic infection in AIDS patients, particularly in Southeast Asia and Africa. Such cases also occur in patients with other forms of immunesupression and in apparently immune-competent individuals.

The isolation rate of pathogens was recorded highest among the purulent (75%) and cloudy (53.8%) sample in this study. Cloudy CSF indicates higher levels of protein, white and red blood cells and/or bacteria, and therefore may suggest bacterial meningitis (Tunkel *et al.*, 2004). Also one pathogen was isolated from the clear colored sample which was later identified to be *C. neoformans*. Thus the initial appearance of the fluid may provide an indication of the nature of the infection.

Among the 13 bacterial meningitis cases it was observed that TLC were greater than normal count and occurred in the range of 10 - 2000 cells/mm^3 with predominant neutrophils (73.8%) in all cases, protein level were greater than normal value (89.4 mg/dL) and glucose contents were lower than normal range (28.8 mg/dL). Markedly decreased CSF glucose with markedly increased total protein, high WBC count with 89% Neutrophils, and the presence of a large number of PMN leukocytes and bacteria in the Gram-stained smear of the CSF sediment are the most striking laboratory results in bacterial meningitis (Harrington and Plenzler, 2004). Similarly Arditi *et al.* (1989) also mentioned that the examination of the CSF of a patient with acute bacterial meningitis characteristically reveals a cloudy fluid, consisting of an increased white blood cell count and predominance of PMN leukocytes, a low glucose concentration in relation to serum value, a raised concentration of protein, and positive Gram stained smear and culture for the causative microorganism. During the bacterial infection, due to microbial physiology, the protein is released and thus the level of protein is increased in CSF. The change in protein level than normal can be

used to get the idea to distinguish viral from bacterial meningitis, as in bacterial infection, the protein level is usually raised than normal and in case of viral infection, the level of protein remains almost normal. Thus this finding in this study goes well with the established medical knowledge.

Among the 3 fungal meningitis cases it was observed the TLC were 11.7±2.9 cells/mm^3 and occurred in the range of 10 - 15 cells/mm^3 with 100% predominant of lymphocytes in all cases, mean value of protein level was 48.7mg/dL, slightly increased than normal level and glucose contents (45 mg/dL) were in normal range. Our positive cases were too small to comment on the value of CSF cell count and biochemical value in the diagnosis of cryptococcal meningitis. However, there is enough evidence in the literature that points to the fact that this has limited value, especially in immune compromised patients (Bicanic and Harrison, 2004). However, the findings in this study compares favorably with other researcher's findings which also displayed typical changes with elevated lymphocytes, elevated proteins (0.82 g/L), and decreased glucose levels (1.3 mmol/L) in a study done by Prince (2010).

Gram's stained smears identified more (19 isolates, 5.3%) isolates than culture (16 isolates, 4.5%) of total clinically suspected cases of meningitis. This finding is similar to the finding of Rao *et al.* (1998) in which they found more isolates on Gram's staining 85.7% (66 out of 77 cases) than on CSF culture (48 isolates). Similar result was also obtained in a study done by Rojita (2007) where Gram stain (7.89%) detected more organism than culture (4.87%). The finding of this study also agrees with the finding of Adhikary and Chatterjee (2013) in which the CSF culture was positive only in 45.7% cases, whereas Gram stain of CSF produced positive results in 86.8% cases. The lower rate of the culture result of the pathogens in this study may be due to the fastidious and delicate nature of the organisms. Given the wide availability, inexpensive, high accuracy, and rapid diagnostic value, the value of Gram staining in the clinical workup of suspected meningitis cannot be understated (Wu *et al.*, 2013). However culture remains important in assessing the antibiotic susceptibility pattern of the causative organism. It is worth noting that *N. meningitidis* was an unidentified etiology on culture may be because of the fastidious nature of this organism. Similarly the pretreatment with antibiotics might be a reason for *H. influenzae* (1 case) and *Kl. pneumoniae* (1 case) to be unidentified on the CSF culture.

H. influenzae was found to be resistant to only Ampicillin in the present study. But in contrary to this finding Al-Binali and Al-Fifi (2002) showed that 35.7% of *H. influenzae* is also resistant to Penicillin and Cotrimoxazole. A high rate of resistance to Cotrimoxazole (33.3%) and Ampicillin (21.7%) was seen in a study carried out by Nag *et al.* (2001) in India against *H. influenzae*. These differences in antibiotic resistance pattern may be due to geographical variations, local antibiotics prescribing habits and selection of cases. But due to very low incidence of *H. influnzae* in this study, it is difficult to make firm conclusions on the antibiotic sensitivities against *H. influenzae*.

E. coli was only 33.3% susceptible to Ofloxacin in the present study but Ngwai *et al.* (2012) showed 58.75% sensitivity of Ofloxacin. Chloramphenicol was 100% effective for *E. coli* which is comparable to the finding of Mengistu *et al.* (2013); and Kibret and Abera (2011) where they found 100% and 63.2% effectiveness of Chloramphenicol respectively against *E. coli* but Nagwai *et al.* (2012) showed sensitivity of Chloramphenicol to only 20%. *E. coli* was found to be 33.3% resistant to Ceftriaxone and 66.7% susceptible to it in the present study which is exactly similar to the findings of Iregbu *et al.* (2013) in Nigeria. *E. coli* was resistance to Amikacin (66.7%) and Cotrimoxazole (66.7%) in this study. Kibret and Abera (2011) also reported the resistance (62.9%) of *E. coli.* against Cotrimoxazole. Because antimicrobial resistance patterns are continually evolving and *E. coli* invasive isolates undergo progressive antimicrobial resistance, continuously updated data on antimicrobial susceptibility profiles will continue to be essential to ensure the provision of safe and effective empirical therapies.

Kl. pneumoniae was found to be resistant against Amikacin and Ciprofloxacin in this study and other antibiotic such as Cefotaxime, Chloramphenicol, Cotrimoxazole and Ofloxacin were found to be effective against *Kl. pneumoniae.* In contrary to this result, Shaw *et al.* (2007) reported Amikacin (58.3%) and Ciprofloxacin (91.7%) sensitive against *Kl. pneumoniae.* At the mean time they also found Cefotaxime (83.3%) and Ofloxacin (91.7%) sensitive as comparable to the present study. Due to low incidence of *Kl. pneumoniae* in this study, we cannot comment on the contradictory result about the susceptibility pattern of this pathogen.

All the antibiotics used against *Ps. aeruginosa* in this study were found to be sensitive except Gentamicin. Taneja *et al.* (2009) also reported Meropenem (70%), Amikacin (70%) and Ofloxacin (40%) effective against *Ps. aeruginosa* which is comparable to this study. But in contrary to this study (Gentamicin, 100% resistant), Taneja *et al.* observed 50% of the *Ps. aeruginosa* to be resistant against Gentamicin. But due to very low frequency of *Ps. aeruginosa,* no firm conclusion can be reached on this contradictory result.

The all antibiotics used for *Staph. aureus* in this study were found to be sensitive in greater than 65% cases in the present study. This finding is almost comparable to the finding of Chugh *et al.* (2011) except for Chloramphenicol (Amikacin-87.5%, Cefotaxime-50%, Ceftazidime-75% sensitive). Similarly, Bhatt *et al.* (2014) in a hospital based study carried out in Nepal, showed Amikacin (90%) and Ciprofloxacin (59%) effective against *Staph. aureus*. Unlike the finding in this study regarding the sensitivity of Chloramphenicol (100%), Chugh *et al.* (2011) reported its sensitivity to only 29.17%. But Andargachew *et al.* (2005) showed 100% effectiveness of Chloramphenicol and Ciprofloxacin against *Staph. aureus* as similar to present study.

Ampicillin, Chloramphenicol, Ciprofloxacin, and Erythromycin were found to be 100% effective against *Strep. pneumoniae* in the present study which is comparable with the findings of Bhagawati, *et al.* (2014). They found *Strep. pneumoniae* to be 50%, 100% and 100% susceptible towards Chloramphenicol, Ciprofloxacin, and Erythromycin respectively. But the remarkable finding of this study is the level of Cotrimoxazole resistance and the result was in accordance with the findings of a study carried out in Bangladesh (Saha, *et al.*, 1999). Ansari and Pokhrel (2011) in their study in Patan Hospital, Nepal, also found Cefotaxime and Chloramphenicol effective against *Strep. pneumoniae* similar to this study. The increased resistance to Cotrimoxazole can possibly be correlated with the wide use this antibiotic in the communities because of its dose convenience, cost effectiveness, and easy availability over the counter.

CHAPTER VI
CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The isolation rate of pathogens causing meningitis is low (4.5%). This may be probably due to the fastidious nature of the organisms or prior exposure to antibiotics. *Staph. aureus* is the predominant bacterial isolate and *C. neoformans* is the only leading cause of fungal meningitis in this study. Analyzing the findings of the present study, it can be concluded that cytological examination, biochemical test and microbiological analysis is still helpful for early diagnosis of meningitis. If bacterial meningitis is suspected on admission or at any later stage of management of patients, initial antibiotics therapy with Chloramphenicol may be under taken as it is found to be most sensitive against all bacterial isolates identified in this study. However, empirical treatment with antibiotics is recommended only in life threatening cases.

6.2 Recommendations

1. Along with bacterial and fungal; viral and amoebic culture should also be carried out so that all etiological agents of meningitis can be isolated and identified.
2. For the rapid diagnosis and better management of patients test like antigen detection by Latex agglutination test is recommended.
3. Meningitis vaccine plan should be introduced to decrease mortality and morbidity caused by meningitis.
4. Regular prevalence and antibiotics susceptibility studies should be done to detect change in causative organism and to choose appropriate antibiotic.

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APPENDICES

APPENDIX I

CLINICAL AND MICROBIOLOGICAL PROFILE OF PATIENTS

A. Patient Information

Patient No	:	Date (dd/mm/yy)	: _ _/ _ _/ _ _
Name	:	Address	:
Age	:	Sex	: Male/Female
Patient	: OPD/ Emergency	Ward/Bed No.	:

B. Clinical Manifestation

Current Antibiotic Treatment: Yes/No

If yes, Antibiotic(s) taken: a)…….. b)……...

Duration of treatment : ……...

C. Microbiological Profile

Specimen: CSF *Collection Time:* *Time of Lab Receipt:*

i) Macroscopic Observation

☐ Clear ☐ Slightly turbid ☐ Cloudy ☐ Purulent ☐ Bloody

ii) Biochemical Analysis

Glucose (mg/dL): *Protein (mg/dL):*

iii) Cytological Investigation

Total Leukocytes Count (cells/mm^3):

Differential Leukocytes Count (%):
Lymphocytes: Neutrophils:

iv) Microscopy

Gram's Stain Result: ☐ Positive ☐ Negative ☐ Not Available

India Ink Preparation: ☐ Yes ☐ No

Culture Media:

☐ BA ☐ CA ☐ MA ☐ MSA ☐ NA ☐ SDA

Colony Characteristics:

Media	Shape	Size	Color	Texture	Opacity	Consistency	Others
BA							
CA							
MA							
MSA							
NA							
SDA							

Biochemical Tests:

☐ Coagulase test ☐ Catalase test ☐ Oxidase test

☐ Bile solubility test ☐ Indole test ☐ Methyl redtest

☐ Voges Proskauer test ☐ Citrate utilization test ☐ Motility test

☐ Oxidation & fermentation test ☐ Triple sugar iron test ☐ Urease test

☐ Gas production test

Microorganism Identified: ……

D. Antibiotic Susceptibility Test

Antibiotics Used	Zone of Inhibition (mm)	Interpretation
Amikacin		
Ampicillin		
Cefotaxime		
Ceftazidime		
Ceftriaxone		
Chloramphenicol		
Ciprofloxacin		
Cotrimoxazole		
Erythromycin		
Gentamycin		
Meropenem		
Ofloxacin		

Performed By: **Checked By:**

APPENDIX II

LIST OF EQUIPMENTS AND MATERIALS USED DURING THE STUDY

A. Equipment

Autoclave	Ravi (India)
Automated biochemical analyzer (Erba, XL-200)	Germany
Centrifuge	Remi (India)
Hot air oven	Advantec (Japan)
Incubator	Yamato (Japan)
Microscope	Olympus (Japan)
Refrigerator	Sanyo (Japan)
Water bath	NSW (India)
Weighing Machine	Chyo MP 300 (Japan)

B. Microbiological Media

Blood Agar Base	Chocolate Agar
Hugh Leifson Medium	Macconkey Agar
Mannitol Salt Agar	Mueller Hinton Agar
Mueller Hinton Broth	MR-VP Medium
Nutrient Agar	Simmons Citrate Agar
Sobouraud Dextrose Agar	Sulphur Indole Motility Agar
Triple Sugar Iron Agar	Urea Broth

C. Chemicals and Reagents

3% Hydrogen peroxide	Barritt's reagent
Crystal violet	Kovac's reagent
Gram's iodine	Barium chloride
Absolute (95%) alcohol	Sulphuric acid
Safranine	Turk's reagent
3 % sulphosalicylic acid	Normal saline
	India Ink

D. Antibiotic Discs

All the antibiotics discs used for the susceptibility tests were from Hi-Media Laboratories Pvt. Limited, Bombay, India. The antibiotics used were as follows:

Amikacin (30 µg)
Ampicillin (10 µg)
Cefotaxime (30 µg)
Ceftazidime (30 µg)
Ceftriaxone (30 µg)
Chloramphenicol (30 µg)
Ciprofloxacin (5 µg)
Cotrimoxazole (1.25/23.75 µg)
Erythromycin (15 µg)
Gentamicin (10 µg)
Meropenem (10 µg)
Ofloxacin (5 µg)

E. Identification Discs/Other Discs

Bacitracin, Optochin, 'X' factor 'V' factor and, 'XV' factor

F. Miscellaneous

Conical flasks
Cotton
Cover slips
Immersion oil
Distilled water
Droppers
Forceps
Glass slides
Inoculating loop
Inoculating wire
Lysol
Measuring cylinder
Petri dishes
Pipettes
Plastic containers
Spatula
Test tubes
Wooden applicator sticks

APPENDIX III

A. COMPOSITION AND PREPARATION OF CULTURE MEDIA

The culture media used were from Hi-Media Laboratories Pvt. Limited, Bombay, India. (All compositions are given in grams per liter and at 25^0C temperature)

1. Blood Agar Base (Hi Media Laboratories)
Blood agar base (infusion agar) + 5-10% sheep blood

Ingredients	**gm/liter**
Protease peptone	15.0
Liver extract	2.5
Yeast Extract	15.0
Sodium Chloride	5.0
Agar	15.0
Final pH (at 25^0C)	7.4±0.2

Direction: Blood agar base (infusion agar) + 5-10% sheep blood Direction: 42.50 grams of the blood agar base medium was suspended in 1000 ml distilled water, dissolved by boiling and sterilized by autoclaving at 1210C (15 lbs pressure) for 15 minutes. After cooling to 45-500C, 7% sterile defibrinated sheep blood was added aseptically, then mixed with gentle rotation and immediately poured in sterile petriplates.

2. Chocolate Agar (Hi Media Laboratories)
Preparation: It was prepared as described for blood agar except after adding blood; the medium was heated in a 70^0C water bath until it became brown in color. After allowing the medium to cool to about 45^0C, it was remixed and dispensed in sterile petri dishes as described for blood agar.

3. MacConkey Agar (Hi Media Laboratories)

Ingredients	**gm/liter**
Peptic digest of animal tissue	1.5
Casein enzymic hydrolysate	1.5
Pancreatic digest of gelatin	17.0
Lactose	10.0
Bile salts	1.50
Sodium chloride	5.0
Crystal violet	0.001
Neutral Red	0.03
Agar	15.0
Final pH (at 25^0C)	7.1±0.2

Direction: 51.5 grams of the medium was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121^0C (15 lbs pressure) for 15 minutes. The medium was poured into sterile petriplates.

4. Mueller Hinton Agar (Hi Media Laboratories)

Ingredients	gm/liter
Beef, Infusion form	300.0
Casein Hydrolysate	17.5
Starch	1.5
Agar	17.0
Final pH (at 25^0C)	7.4±0.2

Direction: 38 grams of the medium was suspended in 1000 ml distilled water and the medium was warmed to dissolve completely. It was sterilized by autoclaving at 121ºC (15 lbs pressure) for 15 minutes and poured into sterile petriplates.

5. Nutrient Agar (Hi Media Laboratories)

Ingredients	gm/litre
Peptic digest of animal tissue	5.0
Sodium Chloride	5.0
Beef Extract	1.5
Yeast Extract	1.5
Agar	15.0
Final pH (at 25^0C)	7.4±0.2

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

6. Mueller Hinton Broth (Hi Media Laboratories)

Ingredients	gm/liter
Beef, Infusion form	300.0
Casein Hydrolysate	17.5
Starch	1.5
Final pH (at 25^0C)	7.4±0.2

Direction: 21 grams of the medium was dissolved in 1000 ml of distilled water, boiled and dispensed into small containers. It was then sterilized by autoclaving at 121˚C for 15 minutes.

7. Nutrient Broth (Hi Media Laboratories)

Ingredients	gm/liter
Peptic digest of animal tissue	5.0
Sodium chloride	5.0
Beef extract	1.50
Yeast extract	1.50
Final pH (at 25^0C)	7.4±0.2

Direction: 13 grams of the medium was dissolved in 1000 ml of distilled water, boiled and dispensed into small containers. It was then sterilized by autoclaving at 121˚C for 15 minutes.

8. Sabouraud Dextrose Agar (Hi Media Laboratories)

Ingredients	gm/liter
Dextrose	40.000
Mycological, peptone	10.000
Agar	15.000
Final pH (at 25°C)	5.6±0.2

Direction: Suspend 65 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at15 lbs pressure (121°C) for 15 minutes.

9. Mannitol Salt Agar (Hi Media Laboratories)

Mannitol Salt Agar is used as a selective media for the isolation of pathogenic Staphylococci.

Ingredients	gm/litre
Proteose peptone	10.000
Beef extract	1.000
Sodium chloride	75.000
D-Mannitol	10.000
Phenol red	0.025
Agar	15.000
Final pH (at 25°C)	7.4±0.2

Directions: Suspend 111.02 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. If desired, add 5% v/v Egg Yolk Emulsion (FD045). Mix well and pour into sterile Petri plates.

B. COMPOSITION AND PREPARATION OF BIOCHEMICAL TEST MEDIA

1. MR-VP Medium (Hi-Media laboratories)

Ingredients	gm/litre
Peptone	5.0
Dextrose	5.0
Dipotassium Phosphate	5.0
Final pH (at 25^0C)	6.9±0.2

Direction: 15 gm powder was dissolved in 1000 ml of distilled water & mixed well. 3 ml of medium was distributed in each test tube and autoclaved at 121^0C for 15 minutes.

2. Sulphide Indole Motility (SIM) medium (Hi Media Laboratories)

Ingredients	gm/litre
Beef extract	3.0
Peptone	30.0
Peptonized iron	0.2
Sodium Thiosulphate	0.25
Agar	3.0
Final pH (at 25^0C)	7.3±0.2

Direction: 36 grams of the medium was suspended in 1000 ml of distilled water and dissolved completely. Then it was distributed in tubes to a depth of about 3 inches and sterilized by autoclaving at 121ºC for 15 minutes.

3. Simmon's Citrate Agar (Hi Media Laboratories)

Ingredients	gm/litre
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)	6.8±0.2

Direction: 24.2 grams of the medium was dissolved in 1000ml of distilled water. 3ml 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.

4. Triple Sugar Iron Agar (TSI) (Hi Media Laboratories)

Ingredients	gm/litre
Peptone	10.0
Tryptone	10.0
Extract	3.0
Beef extract	3.0
Dextrose	1.0
Lactose	10.0
Sucrose	10.0
Ferrous sulphate	0.2
Sodium chloride	5.0
Sodium thiosulphate	0.3
Phenol red	0.024
Agar	12.0
Final pH (at 25^0C)	7.4±0.2

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

5. Urea Base Agar (Hi Media Laboratories)

Ingredients	gm/litre
Peptone	1.0
Dextrose	1.0
Sodium chloride	5.0
Dipotassium phosphate	1.2
Monosodium phosphate	0.8
Phenol red	0.012
Agar	15.0
Final pH (at 25^0C)	7.4±0.2

Direction: 24 grams of the medium was suspended in 950 ml of distilled water and sterilized by autoclaving at 121^0C for 121 minutes. After cooling to about 45^0C, 50 ml of 40% urea solution was added aseptically, mixed well and distributed 5 ml amount in sterile test tubes.

6. Hugh Leifson Medium ((Hi Media Laboratories))

Hugh Leifson Medium is used to distinguish between anaerobic and aerobic breakdown of carbohydrate (glucose).

Ingredients	**gms/litre**
Peptic digest of animal tissue	2.000
Sodium chloride	5.000
Dipotassium phosphate	0.300
Glucose	10.000
Bromothymol blue	0.050
Agar	2.000
Final pH (at 25°C)	6.8±0.2

Directions: Suspend 19.35 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Dispense into test tubes in duplicate for aerobic and anaerobic fermentation. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool the tubed medium in an upright position

C. COMPOSITIN AND PREPARATION OF DIFFERENT REAGENTS

1. For Gram's Stain

(a) Crystal Violet solution

Crystal Violet	20.0 g
Ammonium Oxalate	9.0 g
Ethanol or Methanol	95 ml
Distilled Water (D/W) to make 1 litre	

Direction: In a clean piece of paper, 20 gm of crystal violet was weighed and transferred to a clean brown bottle. Then, 95 ml of ethanol was added and mixed until the dye was completely dissolved. To the mixture, 9 gm of ammonium oxalate dissolved in 200 ml of D/W was added. Final volume was made 1 litre by adding D/W.

(b) Lugol's Iodine

Potassium Iodide	20.0 g
Iodine	10.0 g
Distilled Water	1000 ml

Direction: To 250 ml of D/W, 20 gm of potassium iodide was dissolved. Then 10 gm of iodine was mixed to it until it was dissolved completely. Final volume was made 1 litre by adding D/W.

(c) Acetone-Alcohol Decoloriser

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

Direction: To 25 ml D/W, 475 ml of absolute alcohol was added, mixed and transferred into a clean bottle. Then immediately, 500 ml acetone was added to the bottle and mixed well.

(d) Safranin (Counter Stain)

Safranin	10.0 g
Distilled Water	1000 ml

Direction: In a clean piece of paper, 10 gm of safranin was weighed and transferred to a clean bottle. Then 1 litre D/W was added to the bottle and mixed well until safranin dissolved completely.

2. Normal saline

Sodium Chloride	0.85 g
Distilled Water	100 ml

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

3. Biochemical Test Reagents

(a) Catalase Reagent (For Catalase test)

Hydrogen peroxide	3 ml
Distilled Water	97 ml

Direction: To 97 ml of D/W, 3 ml of hydrogen peroxide was added and mixed well.

(b) Oxidase Reagent (impregnated in a Whatman's No. 1 filter paper) (For Oxidase Test)

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

Direction: This reagent solution was made by dissolving 1 gm of TPD in 100 ml D/W. 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) Kovac's Indole Reagent (For Indole Test)

Isoamyl alcohol	30 ml
p-dimethyl aminobenzaldehyde	2.0 g
Conc. Hydrochloric acid	10 ml

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

(d) Methyl Red Solution (For Methyl Red Test)

Methyl red	0.05 g
Ethyl alcohol (absolute)	28 ml
Distilled Water	22 ml

Direction: To 28 ml ethanol, 0.05 gm of methyl red was dissolved and transferred to a clean brown bottle. Then 22 ml D/W was added to that bottle and mixed well.

(e) Barritt's Reagent (For Voges-Proskauer Test)

Solution A	
α-naphthol	5.0 g
Ethyl alcohol (absolute)	100 ml

Direction: To 25 ml ethanol, 5 g of α-naphthol was dissolved and transferred into a clean brown bottle. Then the final volume was made 100 ml by adding D/W.

Solution B	
Potassium hydroxide	40.0 g
Distilled Water	1000 ml

Direction: To 25 ml D/W, 40 gm of KOH was dissolved and transferred into a clean brown bottle. Then the final volume was made 100 ml by adding D/W.

(f) Urease Test

Ingredients	**gm/litre**
Yeast extract	0.100
Urea	20.000
Monopotassium phosphate	0.091
Disodium phosphate	0.095
Phenol red	0.010
Final pH (at 25°C)	6.8±0.2

Direction: Suspend 20.30 grams in 1000 ml distilled water. Mix well and sterilize by filtration. Do not autoclave or heat the medium. Dispense in sterile tubes as desired.

(g) Turk's Reagent

Glacial acetic acid	1.5ml
Distilled water	98.5ml

Direction: To 98.5 ml of distilled water, 1.5ml of Glacial acetic acid was added and mixed well. Then 2-3 drops of Crystal violet was added.

4. McFarland tube (No. 0.5)

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

APPENDIX IV

A. GRAM'S STAINING PROCEDURE

First devised by Hans Christian Gram during the late 19th century, the Gram-stain can 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 was out easily with the decolorizer 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 1 minute.
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 the 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 (safranine) for 1 minute 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. INDIA INK PREPARATION

1. Centrifuge CSF at 1500xg/3500 rpm for 10 minutes. Decant supernatant into separate sterile tube.
2. Place a drop of centrifuged CSF sediment on a clean glass slide.
3. Add a small loopful of India ink, mix and cover drop with coverslip.
4. Examine with reduced light under low and/or high power for budding yeasts with a well-defined capsule.

C. 'X', 'V' and 'XV' Factor Discs Procedure

Used for the presumptive identification of Haemophilus species on the basis of their requirements for X or V factors or both.

Directions: Inoculate the surface of a Blood Agar (M073) plate or Brain Heart Infusion Agar (M211) plate with the test organisms by either streaking or surface spreading. Aseptically place the X (DD020), V (DD021) and X+V (DD022) factor discs on the plate, in the following positions:

Disc Position on the Agar plate
X factor disc 12 O' clock
V factor disc 4 O' clock
X+V factor disc 8 O' clock

Incubate the plates at 35 - 37°C for 24 - 48 hours. Observe for the growth in the neighbourhood of the discs.

APPENDIX V

BIOCHEMICAL TESTS FOR IDENTIFICATION OF BACTERIA

A. Catalase Test

This test is performed to demonstrate the presence of catalase, an enzyme that catalyses the release of oxygen from hydrogen peroxide. During aerobic respiration, in the presence of oxygen, microorganisms produces hydrogen peroxide, which is lethal to the cell itself. The enzyme catalase is present in most cytochrome containing aerobic and facultative anaerobic bacteria, the main exception being *Streptococcus* spp.

Procedure: 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

This test is performed for the detection of cytochrome oxidase in bacteria which catalyzes the transport of electrons between electron donors. In the presence of redox dye Tetramethyl-*p*-phenylene diamine dihydrochloride, the cytochrome oxidase oxidizes it into a deep purple colored end product Indophenol which is detected in the test. The test is used for screening species of *Neisseria, Alcaligenes, Aeromonas, Vibrio, Campylobacter* and *Pseudomonas* which give positive reactions and for excluding the Enterobacteriaceae, all species of which give negative reactions.

Procedure: A piece of filter paper was soaked with few drops of oxidase reagent (Whatman's No. 1 filter paper impregnated with 1% tetramethyl-p-phenylene diamine dihydrochloride). 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. Indole Production Test

This test detects the ability of the organism to produce an enzyme: 'tryptophanase' which oxidizes tryptophan to form indolic metabolites: indole, skatole (methyl indole) and indole acetic acid. The enzyme tryptophanase catalyses the deamination reaction attacking the tryptophan molecule in its side chain and leaving the aromatic ring intact in the form of indole.

Procedure: A smooth bacterial colony was stabbed on SIM (Sulphide Indole Motility) medium by a sterile stab wire and the inoculated media was incubated at 37°C for 24 hours. After 24 hours incubation, 2-3 drops of Kovac's reagent was added. Appearance of red color on the top of media indicates indole positive. Indole if present combines with the aldehyde present in the reagent to give a red color in the alcohol layer. The color reaction is based on the presence of the pyrrole structure present in indole.

D. Methyl Red Test

This test is performed to test the ability of an organism to produce and maintain stable acid end product from the fermentation of glucose to give a red color with the indicator methyl red and to overcome the buffering capacity of the system. Medium

used in the study was Clark and Lubs medium (MR/VP broth, pH 6.9). Methyl red is an indicator which is already acid and will denote changes in degree of acidity by color reactions over a pH range of 4.4- 6.0.

Procedure: A pure colony of the test organism was inoculated into 2 ml of MRVP medium and was incubated at 37°C 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 and negative with yellow color.

E. Voges-Proskauer (VP) Test

The principle of this test is to determine the ability of some organisms to produce a acetyl methyl carbinol, a neutral end product (acetoin) or its reduction product 2, 3-butanidiol during fermentation of carbohydrates. An organism of the Enterobacteriaceae group is usually either methyl red positive and Voges- proskauer-negative or methyl red negative and Voges-Proskauer positive. The Voges proskauer test for acetoin is used primarily to separate *E. coli* from *Klebsiella* and *Enterobacter* species.

Procedure: A pure colony of the test organism was inoculated into 2 ml of MRVP medium and was incubated at 37°C for 24 hours. After incubation, about 5 drops of Barritt'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.

F. Citrate Utilization Test

This test is performed to detect whether an organism utilizes citrate as a sole source of carbon for metabolism with resulting alkalinity. The medium used for citrate fermentation (Simmon's Citrate medium) also contains inorganic ammonium salts. Organisms capable of utilizing citrate as its sole carbon source also utilizes the ammonium salts present in the medium as its sole nitrogen source, the ammonium salts are broken down to ammonia with resulting alkalinity.

Procedure: A loopful of test organism was streaked on the slant area of Simmon's Citrate Agar medium and incubated at 37°C for 24 hours. 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 pH 7.6; a blue color develops due to alkalinity of the medium.

G. Motility Test

This test is done to determine if an organism was motile or non-motile. Bacteria are motile by means of flagella. Flagella occur primarily among the bacilli; however a few cocci forms are motile .Motile bacteria may contain a single flagella. The motility media used for motility test are semisolid, making motility interpretations macroscopic.

Procedure: Motility of organism was tested by hanging drop and cultural method. In cultural method, the test organism was stabbed in the SIM medium and incubated at 37ºC for 48 hours. Motile organisms migrate from the stabline and diffuse into the medium causing turbidity. Whereas non-motile bacteria show the growth along the stabline, and the surrounding media remains colorless and clear.

H. Triple Sugar Iron (TSI) Agar Test.

The TSI agar is used to determine the ability of an organism to utilize specific carbohydrate incorporated in the medium (glucose, sucrose and lactose in concentrations of 0.1%, 1.0% and 1.0% respectively), with or without the production

of gas (indicated by cracks in the media as well as an air gap at the bottom of the tube) along with determination of possible hydrogen sulfide production (detected by production of black color in the medium). A pH indicator (phenol red) included in the medium can detect acid production from fermentation of these carbohydrates and it gives yellow reaction at acidic pH, and red reaction to indicate an alkaline surrounding.

Procedure: The test organism was streaked and stabbed on the surface of TSI and incubated at 37°C 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. The results are interpreted as follows:

a. Yellow (Acid)/ Yellow (Acid), Gas, H_2S → Lactose/ Sucrose fermenter, H_2S producer.
b. Red (Alkaline) / Yellow (Acid), No Gas, No H_2S → Only Glucose, not lactose/ Sucrose fermenter, not aerogenic, No H_2S production.
c. Red (Alkaline) / No Change → Glucose, Lactose and Sucrose non-fermenter.
d. Yellow (Acid)/ No Change → Glucose- Oxidiser.
e. No Change / No Change →Non-fermenter.

I. Urea Hydrolysis Test

This test demonstrates the urease activity present in certain bacteria which decomposes urea, releasing ammonia and carbon dioxide. Ammonia thus produced changes the color of indicator (phenol red) incorporated in the medium.

Procedure: The test organism was inoculated in a medium containing urea and the indicator phenol red. The inoculated medium was incubated at 37°C overnight. Positive organism shows 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 the indicator to pink.

J. Coagulase Test

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

(i) Slide coagulase test

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

Procedure: For slide coagulase test, a drop of physiological saline was placed on three places of a slide, and then a colony of the test organism was emulsified in two of the drops to make thick suspensions. Later a drop of 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.

(ii) Tube coagulase test

This test is carried out to detect production of free coagulase. Plasma contains coagulase reacting factor (CRF) which activates free coagulase. The activated coagulase acts upon prothrombin thus converting it to thrombin. Thrombin converts

fibrinogen into fibrin which is detected as a firm gel (clot) in the tube test. Tube test is performed when negative or doubtful results are obtained in slide coagulase test.

Procedure: In the tube coagulase test, plasma was diluted 1:10 in physiological saline. Four small tubes were taken, one for test organism, one for positive control, one for negative control, and one to observe self-clotting of plasma. Then 0.5 ml of the diluted plasma was pipetted into each tube and 0.5 ml of test organism, 0.5 ml of positive control (*S. aureus* culture), and 0.5 ml negative control (*S. epidermidis* culture) was added to three tubes, to the fourth tube, 0.5 ml 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. The clotting is observed by gently tilting the tube for positive coagulase test.

K. Bile Solubility Test for Identification of *Streptococcus pneumoniae*

A loop of suspect strain from fresh growth on blood agar plate was taken and bacterial suspension was prepared in 0.5 ml of sterile saline. The suspension of bacterial cell was made cloudy similar to that of 0.5 or 1.0 Mc Farland standard. The suspension was divided into equal amount in two sterile tubes, 0.25mL of saline was added onto one tube and 0.25mL of 2% Sodium Deoxycholate to another tube (2% concentration of bile salt was made by adding 0.2gm of Sodium Deoxycholate to 10ml of saline). Tubes were shaken gently and incubated at 37°C for 2 hrs. The clearing of tube or loss in turbidity is positive result; reported as bile soluble and when turbidity remains same as that in saline control tube; reported as negative for bile solubility (bile resistant).

L. Optochin Susceptibility Test

The Optochin Susceptibility Test is performed with 6mm, 5µg Optochin disk and is used to differenciate between *S. pneumoniae* and viridians Streptococci; Optochin Susceptibe strains can be identified as *S. pneumoniae.* The suspect α-hemolytic colony was touched with sterile bacteriological loop and streaked on blood agar plate in straight line. Optochin disk 6mm diameter (containing 5µg ethylhydrocupriene) was placed aseptically on the streak of inoculums. Plates incubated in candle jar at 37°C for 18-24 hrs. The results were interpreted: A hemolytic strains with zone if inhibition of growth greater than 14mm in diameter are pneumococci, the α hemolytic strains with zones of an inhibition ranging between 9mm and 13mm should be tested for bile solubility for further characterization and identification.

M. Oxidation-Fermentation Test

This method (Hugh & Leifson, 1953) depends up on the use of a semi-solid tubed medim containing the carbohydrate (glucose) together with a pH indicator. If acid is produced only at the surface of the medium, where conditions are aerobic, the attack on the sugar is oxidative. If acid is formed throughout the tube, including the lower layers where conditions are anaerobic, the break down is fermentative.

Procedure: The test organism was stabbed into the bottom of two sets of tubes with Hugh & Leifson's Media, bromothymol blue being the pH indicator. The inoculated medium in one of the tubes was covered with a 1 cm deep layer sterile paraffin oil. The tubes were then incubated at 37^0C for 24 hours. After incubation, the tubes were examined for carbohydrates utilization as shown by yellow color of media that denotes the 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.

APPENDIX VI

MORPHOLOGICAL AND CULTURAL CHARACTERISTICS OF ISOLATES FROM CSF SPECIMEN

Bacteria	Morphological Characteristics	Cultural Characteristics
Strept. pneumoniae	Gram positive ovoid or lanceolate cocci arranged in pairs 1-3µm×0.4-0.7µm size, aerobic and anaerobic, nonsporing, motile, capsulated.	On BA: Raised, circular about 1mm in diameter, grow well when supplemented with CO_2. The colonies are alpha (α) haemolytic. On MA: No growth
H. influenzae	Gram negative coccobacilli of 0.3-0.5µm × 0.5-1.0µm size, nonsporing, nonmotile and capsulated.	On BA: No growth, but grows only when *Staphylococcus aureus* is streaked across the plate on which specimen containing *Haemophilusinfluenzae* has been inoculated On CA: Translucent colonies are obtained
N. meningitidis	Gram negative oval or spherical cocci 0.6-0.8µm in size, typically arranged in pairs with adjacent sides flttened, capsulated, nonsporing and non- motile rods.	On BA: Smooth, small (about 1mm in diameter) translucent, round, convex, typically lenticular colonies are obtained. On MA: No growth
E. coli	Gram negative rod of 1-3µm×0.4-0.7µm size, aerobic and anaerobic, nonsporing, motile, noncapsulated	On BA: Large 1-4 mm in diameter, grayish white, moist, smooth, convex and opaque. The colonies may appear mucoid and some strains are haemolytic. On MA: Bright pink colonies due to lactose fermentation, smooth, glossy and translucent.
Staphy. aureus	Gram positive, spherical cocci, 0.8-1 µm in diameter, non sporing, facultative anaerobe, non-motile, except for rare strains, non-capsulated. They are arranged in characteristics grape like clusters or in small groups, pairs, singles and short chain(less than five cocci in line).	On BA: Large, 2-4 mm diameter. Circular, smooth with glistening surface, entire edge, soft butyrous consistency and opaque. The pigmentation is golden yellow to cream coloured. Some strains are beta-haemolytic when grown aerobically. On MA: Small (pin head size), 0.1-0.5mm, pink or pink orange due to lactose fermentation. Some strains are non-lactose fermenting.
Klebsiella spp.	Gram negative, short and thick rods of 1-2µm × 0.8µm size, nonsporing, nonmotile and capsulated.	Large dome shaped moist and usually viscid or mucoid colonies when cultured on BA and MA. Most Klebsiella species are lactose fermenting.
Ps. aeruginosa	Gram negative slender rods with 1.5-3µm x 0.5µm size, actively motile by polar flagellum, non sporing, most of the strains produce slime.	On NA: Large, opaque, irregular colonies with distinctive, musty odor and metallic sheen. On BA: Large flat colonies showing haemodigestion. On MA: Pale, nonlactose fermenting, colorless translucent colonies.
C. neoformans	Budding oval yeast cell of variable size (2-10 µm) surrounded by thick capsule	On SDA: produces white-cream colored colony, cells are capsulated

APPENDIX VII

ZONE SIZE INTERPRETATIVE CHART

Antimicrobial Agents used	Symbol	Disc Content	Resistant (≤ mm)	Intermediate (=mm)	Susceptible (≥ mm)
Amikacin	AK	30µg	14	15-16	17
Amoxycillin/ Clavulanic acid	AC	20/10µg (30µg)	19	-	20
Ampicillin	A	10µg	18	19-21	22
Cefotaxime	CE	30µg	14	15-22	23
Ceftazidime	CAZ	30 µg	17	18-20	21
Ceftriaxone	CI	30 µg	13	14-20	21
Chloramphenicol	C	30µg	12	13-17	18
Ciprofloxacin	CIP	5 µg	15	16-20	21
Cloxacillin	CX	5 µg	12	12-13	14
Cotrimoxazole (Trimethoprim/ Sulphonamide)	COT	1.25/23.75 µg	10	11-15	16
Erythromycin	E	15 µg	13	14-22	23
Gentamicin	GEN	10 µg	12	13-14	15
Meropenem	MRP	10 µg	15	16-18	19
Ofloxacin	OF	5 µg	12	13-15	16
Penicillin When testing with *S. pneumoniae*	P	10 mcg	-	-	20

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

APPENDIX VIII

A. NORMAL VALUES OF CSF GLUCOSE

Age group	mmol/L
Newborn	1.8 - 6.6
45 days-14 years	3.3 - 4.4
Adults (15 years-50 years)	2.2 - 4.4

B. NORMAL VALUES OF CSF PROTEIN

Age group	mg/dL
≤ 1 week	15 - 170
≤ 45 days	15 - 150
46 days-14 years	15 - 40
14 years-60 years	15 - 60
>60 years	15 - 65

C. NORMAL CSF LABORATORY VALUES OF LEUKOCYTES

Age group	Leukocytes (per µL)	Predominant cell type (%)
Newborn	0-30	Lymphocytes (63-99%) Monocytes (3-37%) PMN (0-15%)
Adults	0-10	

(Marshall & Bangert, 1995; & Burtis *et al.*, 2006).

APPENDIX IX

GUIDELINES FOR CYTOLOGICAL AND BIOCHEMICAL ANALYSIS OF CSF

Clinical Setting	Appearance	Leukocytes per mm^3	Predominant cell type	Protein (mg/dL)	Sugar (mg/dL)
Normal	Clear and colorless	0-5	-	15-45	45-80
Purulent (pyogenic) meningitis	Cloudy or purulent, may contain clots	5-20000 (mean of 800)	Mostly Neutrophils	Elevated (>100)	Reduced
Viral (aseptic) meningitis	Clear or slightly cloudy	2-2000 (mean of 80)	Mostly lymphocytes	Normal or slightly elevated	Normal or slightly reduced
Tuberculous meningitis	Clear or slightly cloudy, fine clot may form	5-2000 (mean of 100)	Mostly lymphocytes	Elevated (>50)	Reduced
Cryptococcal meningitis	Clear or slightly cloudy	5-2000 (mean of 100)	Mostly lymphocytes	Elevated (>50)	Reduced
Leptospiral meningitis	Clear or cloudy	> 25	Mostly lymphocytes	Elevated (>50)	Normal
***L. monocytogenes* meningitis**	Slightly cloudy or purulent	>200	Mostly lymphocytes	Elevated (>50)	Reduced
Neurosyphilis	Usually clear, may contain fine clots	>20	Mostly lymphocytes	Normal or slightly elevated	Normal or slightly reduced

(Source- Cheesbrough, 1984; Baron *et al.*, 1994)

APPENDIX X

BAR DIAGRAMS TO DEMONSTRATE KEY RESULTS

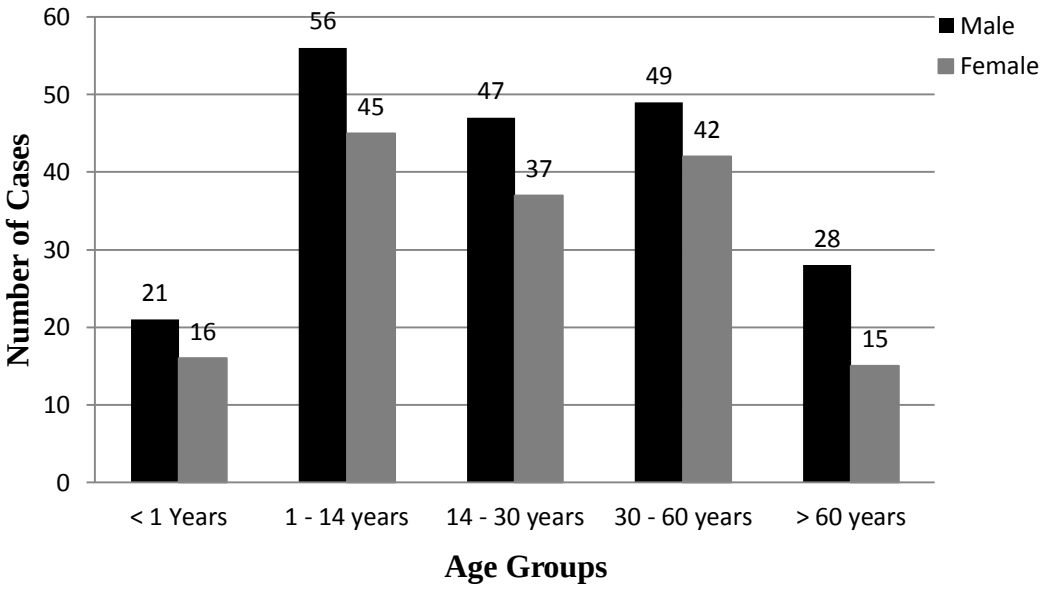

Figure: Age and Sexwise Distribution of Suspected Cases of Meningitis

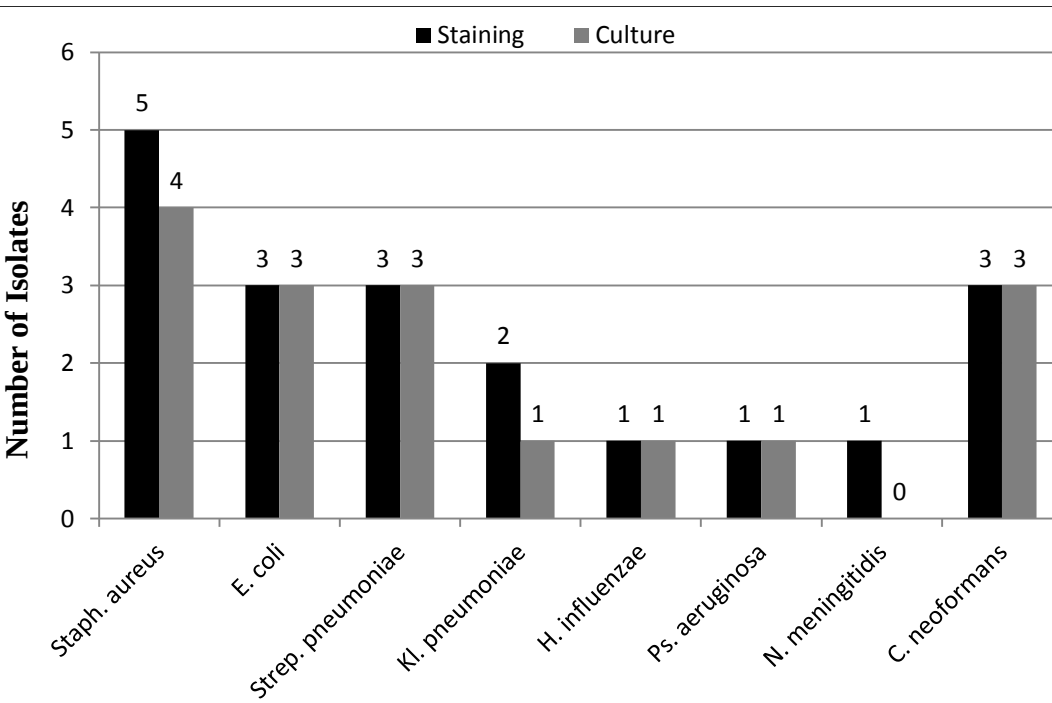

Figure: Isolates Identified on CSF Staining and Culture

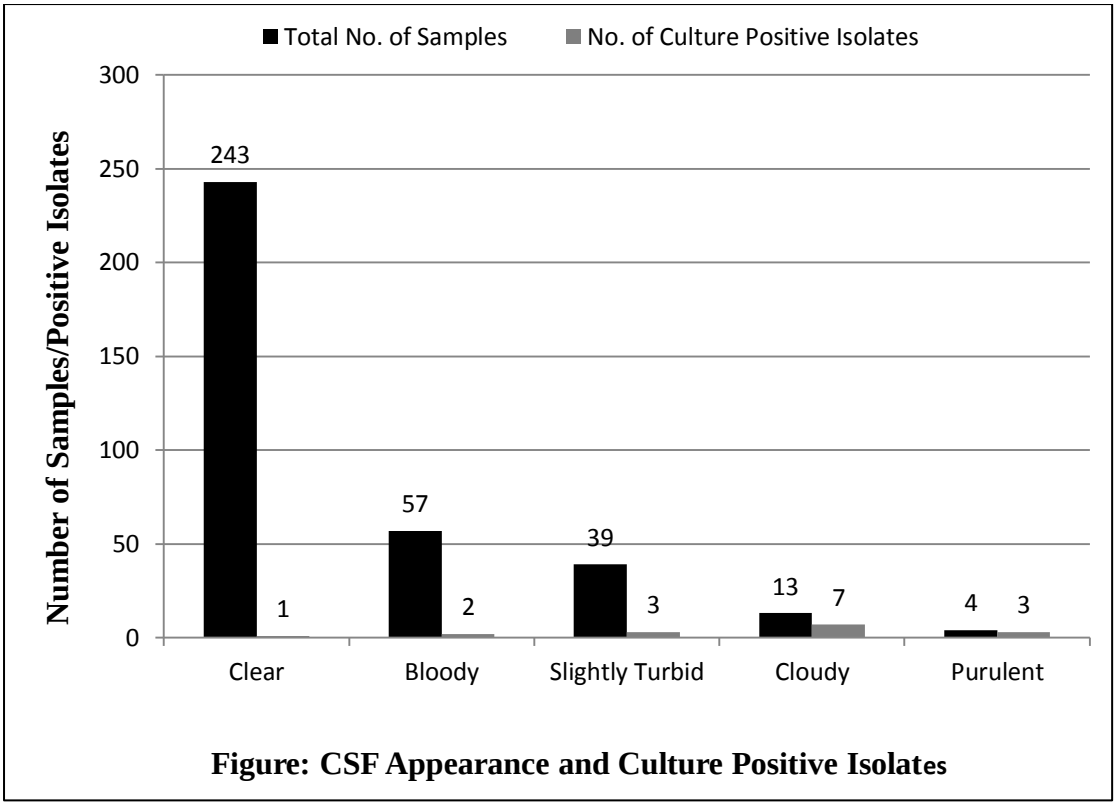

Figure: CSF Appearance and Culture Positive Isolates

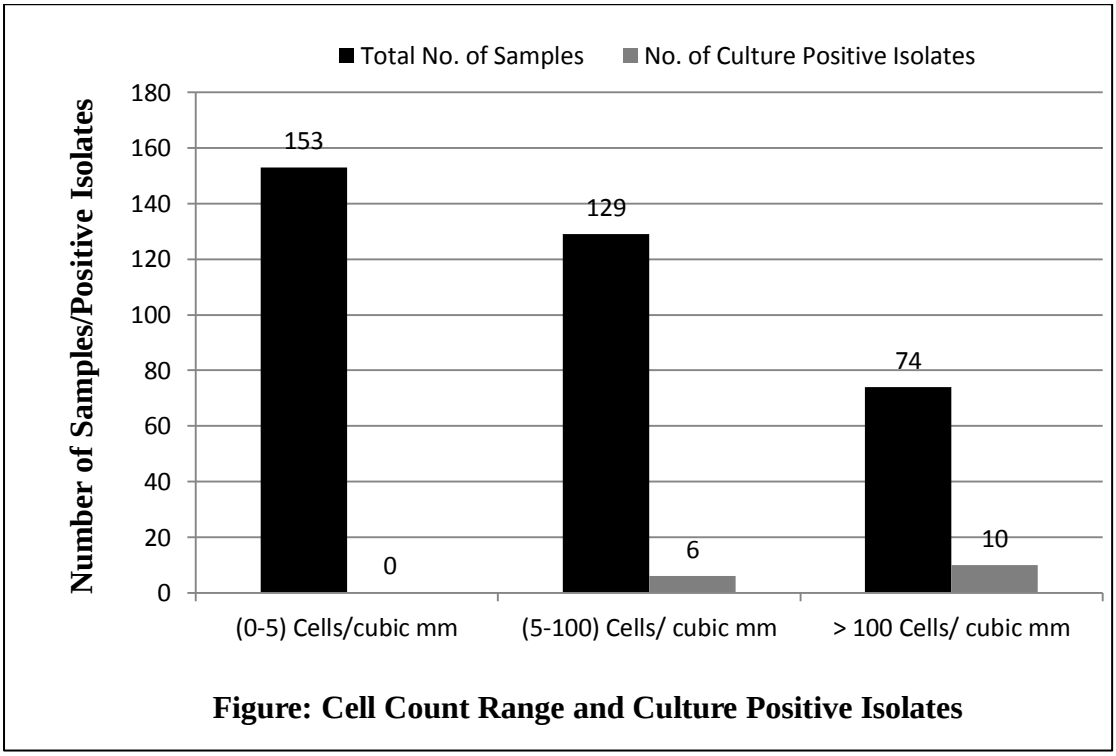

Figure: Cell Count Range and Culture Positive Isolates

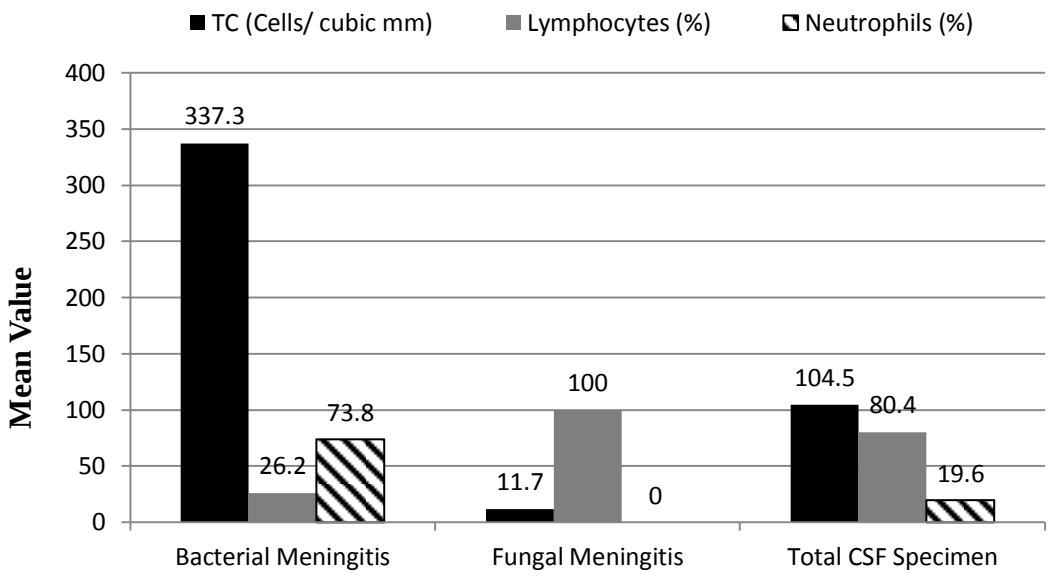

Figure: Cytological Paramaters of Different Types of Meningitis

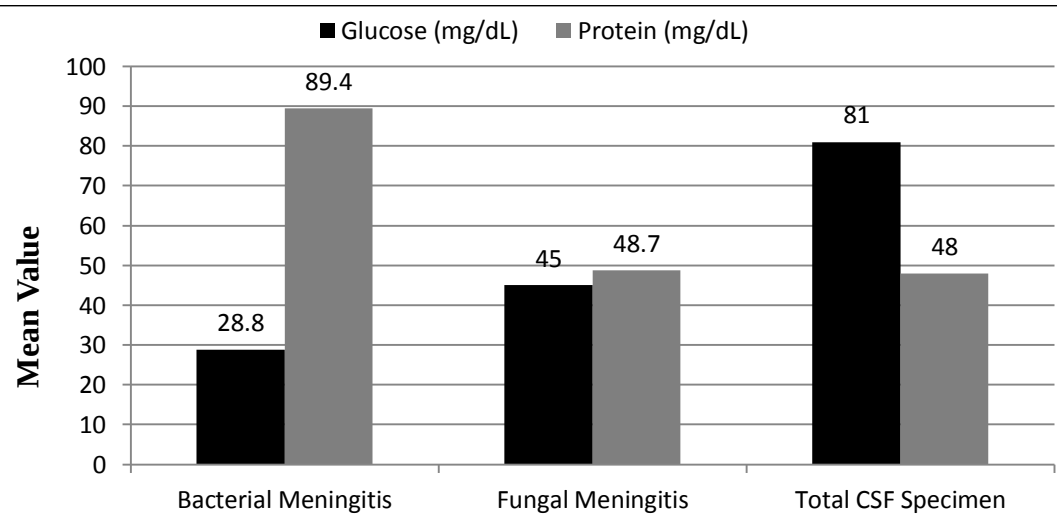

Figure: Biochemical Parameters of Different Types of Meningitis