

CHAPTER-I

1. INTRODUCTION

Infection within the subarachnoid space or throughout the leptomeninges is called meningitis (Forbes *et al.*, 2002). Meningitis is one of the quite serious, with fairly high death rate disease in developing countries like Nepal.

The meninges is the connective tissue coverings of the central nervous system and is the composite anatomical structure consisting of three different layers viz. duramater arachnoidmater and piamater from outside to inner (Wilson, 1995). When the inflammation occurs in the duramater the disease is termed as pachymeningitis and when the arachnoid and piamater is involved, disease is termed as leptomeningitis or meninges proper (Brain and Walton, 1969).

Based on the host's response to the invading microorganism, meningitis is divided into two major categories: purulent and aseptic meningitis (Forbes *et al.*, 2002). Meningitis can be caused by either a bacterial (usually purulent meningitis) or viral pathogen (usually aseptic meningitis), with the later being the less common form (JAMA, 1999). In immuno-compromised patients, it is caused by fungus. Among bacteria, *Neisseria meningitidis*, *Haemophilus influenzae* type b (Hib), and *Streptococcus pneumoniae* are common followed by Group B Sterptococci, *Staphylococcus* spp., *E. coli* and *Listeria monocytogenes* (Easmon, 1990). Viruses include herpes virus (HSV, VZV, CMV), enteroviruses and influenza A viruses (Koskiniemi *et al.*, 2001). Fungi include *Cryptococcus neoformans*, *Candida* spp., *Asppergillus* spp., *Histoplasma capsulatum*, *Coccidioides immitis* and *Blastomyces dermatitidis*. Tuberculous meningitis (TBM) occurs in 7-12% of the persons with tuberculous infections (Lang, 1998). The increase in prevalence of TBM during the past decade has been attributed in part to the increase of AIDS.

Risk factors for meningitis include immunodeficiency disorders, such as AIDS, recent respiratory tract infections, exposure to cigarette smoke and overcrowded living conditions (Weir, 2002). Populations most commonly affected by bacterial meningitis are infants and young children, elderly adults and large groups that share facilities, such as military recruits, foreign refugees and dormitory students (Parini, 2002). There is also recent evidence that recipients of cochlear implants are at an increased risk for contracting bacterial meningitis (Wooltorton, 2002). Additionally, researchers believe that illnesses or environmental conditions, such as dry air, may contribute to the thinning and damage of mucus membranes, and increase susceptibility to the disease. Evidence for this hypothesis is the greater prevalence rates of bacterial meningitis during the late winter and early spring months (Michael, 2002).

Acute bacterial meningitis remains a major cause of mortality and long-term neurological sequelae worldwide. Despite the availability of potent newer antibiotics, the mortality rate due to acute bacterial meningitis remains significantly high in developing countries, ranging from 16-32% (Mani *et al.*, 2007). The annual incidence was 1.7/100,000 to 7.2/ 100,000 inhabitants (mean, 3.8/100,000) (Sigurdardottir *et al.*, 1997). At least 1.2 million cases of meningitis are estimated to occur every year with 135000 deaths (Tikomirov *et al.*, 1997). In 1996, the largest outbreak ever reported occurred in the African meningitis belt; the total number of cases reported to the World Health Organization (probably a substantial underestimate) was 152,813, with 15,783 deaths (REMA, 1997). World wise two third of the cases of meningitis occur below the age of 15 years and most of bacterial meningitis (80%) occur in patient younger than 24 months. The most common causative organisms were *Neisseria meningitidis* (56%), *Streptococcus pneumoniae* (20%), *Listeria monocytogenes* (6%), and *Haemophilus influenzae* (5%). *Neisseria meningitidis* caused 93% of the infections in the 16 to 20 year old age group, but it caused only 25% of the infections in patients aged 45 years or older (Sigurdardottir *et al.*, 1997). More than 75% cases are caused by the three pathogens i.e. *Haemophilus influenzae* B (Hib), *Streptococcus pneumoniae* and *Neisseriae meningitidis*. (Ahmed *et al.*, 2004). Before the 1990s, *Haemophilus influenzae* type b (Hib) was the leading cause of bacterial meningitis, but new vaccines

being given to all children as part of their routine immunizations have reduced the occurrence of invasive disease due to *H. influenzae*. Today, *Streptococcus pneumoniae* and *Neisseria meningitidis* are the leading causes of bacterial meningitis (CDC, 2005).

Bacterial meningitis is a major cause of death and disability worldwide. Despite modern medical developments, approximately 20% of adult patients with bacterial meningitis died (Sigurdardottir *et al.*, 1997). It is estimated that 10-15% of all cases today are fatal and that 10-15% of survivors have long-term neurological damage, such as permanent hearing loss (CDC, 2003). A previous study found that over 25% of survivors have significant neuropsychological sequelae (Grimwood *et al.*, 1995).

Patients with meningitis may have nausea and vomiting, neurological abnormalities, and change in mental status (Baron, Peterson and Finegold, 1994). Severe headache is the most common symptom of meningitis (87%) followed by nuchal rigidity ("neck stiffness", 83%). The classic triad of diagnostic signs consists of nuchal rigidity (being unable to flex the neck forward), sudden high fever and altered mental status. All three features are present in only 44% of all cases of infectious meningitis. Other signs commonly associated with meningitis are photophobia (inability to tolerate bright light), phonophobia (inability to tolerate loud noises), irritability and delirium (in small children) and seizures (in 20-40% of cases). In infants (0-6 months), swelling of the fontanelle (soft spot) may be present (Van de *et al.*, 2004). The onset may be fulminating, acute or less commonly insidious. However, the signs and symptoms of meningeal irritation depend on age of patient and duration of illness. Other manifestations include lethargy, anorexia, irritability, photophobia, sometimes in infants, a temperature that is lower than normal.

During meningitis, the normal composition of cerebrospinal fluid (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 0-30 cells/ mm³ (in

infants) to 0-5 cells/mm³ (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. In former case, marked pleocytosis (mainly polymorphs) is observed whereas in latter case, moderate pleocytosis (mainly lymphocytes) is observed. 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). Thus, changes in the composition (increased protein) or in the appearance (cloudiness) of the CSF would suggest some neurological disease like meningitis.

Today meningitis has been one of the most important causes of morbidity and mortality in developing country like Nepal. Meningitis receives a high level of medical, public and media attention because of its rapid onset and high level of morbidity and mortality. Bacterial meningitis is a serious infection that particularly strikes infants, young children and old aged persons. If it is not cured on time it may lead to chronic complications like it may lead to deafness, learning disabilities, hydrocephalus (increase of CSF in brain), mental retardation, paralysis, seizures and vision loss (Brochert, 2000).

Bacterial meningitis is always a medical emergency that must be managed immediately which warrants early diagnosis and aggressive therapy to prevent mortality and long term morbidity. Most often therapy for bacterial meningitis has to be initiated before the etiology is known. The choice of initial antimicrobial therapy in community acquired acute bacterial meningitis is based on the most common pathogen prevalent in a particular geographical area and age group and its antibiotic sensitivity pattern. There is a need for a periodic review of bacterial meningitis worldwide, since the pathogens responsible for the infection vary with time, geography and patient age. Thus laboratory investigations of CSF samples from suspected meningitis patient is utmost important at the earliest possible moment. Therefore, the laboratory surveillance of isolates is crucial to identify targets for immunization, to formulate preventive strategies and to prescribe empirical treatment. Hence, the present study was carried out with aim to find out the

correlation of macroscopic, serological and microscopic findings with that of cultural findings of the CSF of suspected meningitis patients attending Patan Hospital.

CHAPTER-II

2. OBJECTIVES

2.1 GENERAL OBJECTIVE

To correlate macroscopic, serological and microscopic findings with cultural findings of CSF among the patients suspected of meningitis.

2.2 SPECIFIC OBJECTIVES

-) To isolate and identify bacteria and fungi from the cerebrospinal fluid sample taken from suspected meningitis patients attending Patan Hospital.
-) To correlate bacteriological findings with macroscopic and microscopic characteristic of cerebrospinal fluid samples.
-) To correlate the presence of leukocytes in cerebrospinal fluid with bacteriological findings.
-) To determine the protein and glucose level of cerebrospinal fluid samples and to correlate it with the cultural findings.
-) To detect *Haemophilus influenzae* type b in cerebrospinal fluid by Latex agglutination test.
-) To detect *Streptococcus pneumoniae* by Binax, pneumococcal antigen detection test.
-) To perform the antibiotic susceptibility testing of the bacterial isolates from cerebrospinal fluid.

CHAPTER-III

3. LITERATURE REVIEW

3.1 HISTORY OF MENINGITIS

Meningitis was first described in the 1020s in Avicenna's *The Canon of Medicine* (Skinner, 2001), and again more accurately by Avenzoar of al-Andalus in the 12th century. Symptoms of the disease were also noted in 1805 by the Swiss Gabinetto Vieusseux (a scientific-literary association) during an outbreak in Geneva, Switzerland. In 1887, Dr. Anton Weichselbaum (1845-1920) of Vienna became the first to isolate the specific germ, meningococcus (Martin-Araguz *et al.*, 2002).

3.2 MENINGITIS AS A DISEASE

Meningitis is an inflammation of the membranes surrounding the brain and spinal cord (meninges) which occurs either as primary or secondary to disease in some parts of the body (Easmon, 1990). People sometimes refer to it as spinal meningitis (CDC, 2005). It is usually caused by infection. The central nervous system, also called the CNS, consists of the brain and spinal cord which are protected within a bony compartment from outside and blood brain barrier (BBB) from blood circulation internally (Chaudhary, 1999). Three layers of fibrous tissue viz. duramater, arachnoid mater and pia mater from outside to inner (Wilson, 1995) cover the surfaces of the brain and spinal cord. These layers known as meninges cushion and protect the CNS.

Sometimes organisms, such as bacteria or viruses, can infect these layers. When this happens, the body takes steps to defend itself from infection. White blood cells and other infection-fighting substances pour into the cerebrospinal fluid, or CSF. This process results in a set of symptoms known as meningitis (Brochert, 2000). When the inflammation occurs in duramater, the disease is termed as pachymeningitis and when arachnoid and piamater are involved, it is called as leptomeningitis or meningitis proper (Brain and Walton, 1969).

3.3 ANATOMY AND PHYSIOLOGY

3.3.1 Anatomy of meninges

Because of the vital and essential role of the CNS in the body's regulatory processes, the brain and the spinal cord have two protective coverings: an outer covering consisting of bones and an inner covering of membranes called the **meninges**. The meninges is a collective term for three distinct layers surrounding the brain and the spinal column viz. the duramater, the arachnoid mater and the pia mater (Forbes *et al.*, 2002).

The outer duramater is the tough, fibrous membrane and serves as the internal periosteum of the skull to which it is closely applied. The middle layer of the meninges, between duramater and pia mater is the arachnoid mater which is a delicate connective tissue. The pia mater is the delicate innermost layer which directly covers the brain and dips down into the fissures between the raised ridges of the brain. It is also tightly attached to the spinal cord and roots. This membrane is lined with endothelial cells and most of the blood to the brain is supplied by the large number of blood vessels in the pia mater (Forbes *et al.*, 2002).

Between the duramater and periosteum of the vertebrae, there is an epidural space which is absent in case of the brain. It contains many blood vessels and some fat. Between the dura mater and arachnoid mater, there is a subdural space which does not contain any blood vessels. Between the arachnoid and pia mater is a network of trabecule and the subarachnoid space which contains cerebrospinal fluid (Carola *et al.*, 1992).

The extensions of subarachnoid space are called as subarachnoid cisterns. The cerebello medullary cistern lies between the inferior surface of the cerebellum and the posterior surface of medulla. The cistern points continuous with this lies anteriorly to the pons and continued forward into the cistern lying in front of the optic chiasma-the cistern chiasmatis (Brain and Walton, 1969).

3.3.2 Blood-brain barrier (BBB)

The structures of the brain capillaries are different from the capillaries of the rest of the body. The walls of the brain capillaries are formed by endothelial cells that are joined

by tight junctions also there is an investment, by the foot processes of the astrocytes, around the capillaries. The junctions actually merge the outer membranes of the adjoining cells. These relatively solid capillaries of the brain make up blood-brain barrier. The blood brain barrier helps to maintain the delicate homeostasis of the neurons in the brain by restricting the entrance of the potentially harmful substances from the blood, and by allowing essential nutrients to enter. Many substances, including many drugs, cannot cross the barrier. Oxygen, carbon dioxide, water, glucose, amino acids and electrolytes can cross the barrier easily (Carola *et al.*, 1992).

Large protein molecule and most of the antibiotics cannot enter at all. Among the antibiotics penicillin cannot cross where sulfonamides, tetracycline, chloramphenicol and many other lipid soluble drugs can. Inflammations can damage the tight junctions which results in the loss of the barrier function; in such cases penicillin can cross the barrier (Chaudhary, 1999).

3.3.3 The ventricles

Within the brain is the series of the connected cavities called ventricles. There are two lateral (of the cerebral hemispheres), one third (between the two thalami) and one-fourth (of pons and medulla oblongata) ventricles. Each lateral ventricle is connected to the third ventricle of the diencephalons through the small intra-ventricular foramen known as foramen of monro. The third ventricle is continuous with the fourth ventricle through a narrow channel called as the cerebral aqueduct of the mid brain (Carola *et al.*, 1992). Within the lateral ventricles and also in the third ventricle, choroid plexus, which produces the CSF, are present. The choroid plexuses are capillaries, which are derived from internal carotid and posterior arteries. These project in the cavities of the ventricle, which is covered by cubical epithelium of ependyma (Chaudhary, 1999).

3.3.4 Cerebrospinal fluid (CSF)

The cerebrospinal fluid (CSF) is the clear, colorless liquid that is essentially an ultrafiltrate of blood (the watery substance), that surrounds the brain and spinal cord

within subarachnoid space and within the ventricles and the central canal of the spinal cord (Carola *et al.*, 1992; Wilson, 1995).

Formation of CSF:-

Most of the cerebrospinal fluid is formed continuously at the choroids plexus of the lateral, third, and fourth ventricles by a combination of diffusion and active transport (Carola *et al.*, 1992). A very small part of CSF is probably also formed by brain parenchyma. The process of formation of CSF includes both filtration and secretion. It has also been suggested that a substantial amount of CSF (possibly 30%) may also be formed at sites other than the choroid plexus, particularly under pathological conditions. The ependymal lining of the ventricles and the endothelium of brain capillaries have been considered potential sites of extrachoroidal CSF production. As much as 12-20% of the total CSF volume may be extracellular fluid of capillary origin (Chaudhary, 1999).

Circulation of CSF:-

The CSF comes out of the lateral ventricle then enters the third ventricle through the foramen of monro. It then, passes through the aqueduct of sylvius to the fourth ventricle and escapes mainly through the foramen of Luschka and Magendie. It then occupies the subarachnoid space and is distributed all over the brain. A small part of it enters the central canal (Chaudhary, 1999).

Absorption of CSF:-

The CSF is secreted continuously at the rate of about 0.5ml per min, i.e. 720ml per day. The amount around the brain and the spinal cord remains fairly constant at about 120ml which means that absorption keeps pace with secretion. Thus, the pressure within the ventricles and other cavities containing CSF remains constant. However, conditions such as tumors, infections, and hemorrhages in the brain can disrupt the normal flow of CSF causing the pressure to change (Creager, 1983).

The endothelium of arachnoid villi shows pores. When the pressure of the CSF within the arachnoid villi is high, the pores open up and the fluid escapes into the venous blood, but the venous blood cannot enter the villi because (a) pressure within these sinuses are normally very low, (b) if there is any rise in venous pressure within these sinuses, the pores close down, (c) higher osmotic pressure of the venous blood than that of CSF also helps in the absorption of CSF into venous blood (Chaudhary, 1999).

Composition of CSF:-

CSF is generally similar to blood plasma in composition. It normally contains little protein and has a lower pH, and lower concentration of glucose, potassium, calcium, bicarbonate and amino acids than blood plasma. The sodium, chloride, and magnesium content, however, is greater in CSF than in blood plasma. Few cells are usually present in the CSF; 0-30 cells/ mm³ in infants and 0-5 cells/mm³ in adults is considered normal.

Table 1: Physical and chemical characteristics of CSF in normal condition

Physical characteristics of CSF in normal condition:-		Chemical composition of CSF in normal condition:-	
Appearance	Clear, colorless	Protein	15-45 mg/100ml
Volume	100-150ml (mean 120ml)	Glucose	50-80 mg/100ml
Specific gravity	1.003-1.008	Inorganic phosphorus	1.25-2 mg/100ml
Pressure: <i>New born</i>	30-80mm water	Uric acid	Trace
<i>Children</i>	50-100mm water		
<i>Adult</i>	70-200mm water (average 125)	Cholesterol	Trace
Cells	0-5 cells per mm ³	Calcium	5-6mg/100ml
		Sulfates	1mg/100ml
		Chlorine	125-750 mg/100ml

(Source- Brain and Walton, 1969)

Functions of CSF:-

CSF has several important functions (Wilson, 1995), such as

It supports and protects the brain and spinal cord.

It maintains a uniform pressure around the delicate structure.

It acts as a cushion and shock absorber between the brain and the cranial bones.

It keeps the brain and spinal cord moist and there may be interchange of substance between CSF and nerve cells such as nutrients and waste products.

3.4 METHODS OF CSF COLLECTION AND EXAMINATION:-

To obtain CSF for examination, it is necessary to puncture either the cerebral ventricles or the sub-arachnoid spaces, which may be reached most easily either in the cisterna magna or in the lumbar sac, where it extends beyond the lower end of the spinal cord (Brain and Walton, 1969).

Lumbar puncture: - Lumbar puncture is the simplest method of obtaining access to sub-arachnoid spaces. The spinal cord terminates at the lower border of the first lumbar vertebra in the adult and at a slightly lower level in the child. The arachnoid is continued downwards below the termination of the spinal cord as far as the second sacral vertebra. A needle can be introduced into the space without risk of injury to the spinal cord.

The CSF is collected by an experienced medical practitioner and introduction of infection should be prevented. Lumbar puncture may be performed with the patient either sitting or lying on one side. In either position the most important point is to secure the greatest possible degree of flexion of the lumbar spine. The puncture can be performed either between the third and fourth lumbar spine (L3-L4)(Adult) or fourth and fifth spine (L4-L5) (Children)(Brain and Walton, 1969).

In unusual circumstances, a tap can be done at higher levels. Even at these higher levels the probability of injuring the spinal cord is small. Nevertheless, this should be reserved for situations in which access to the usual sites has been obliterated (e.g., by extensive orthopedic fusion) and a specific need exists for the information obtained from the procedure. CSF also can be obtained from the cisterna magna by a tap below the external occipital protuberance (Waldman, 2005).

The skin is cleaned with alcohol and ether and painted with iodine: a local anesthetic may be applied so as to comfort patient (Brain and Walton, 1969). The needle is introduced midway between the spinous processes in the sagittal plane. A 20-gauge needle for adults, or a 22-gauge needle for children, is used typically (Waldman, 2005). At an average depth of about 4.5 cm there will be an increased resistance of the ligamentum flavum, and after penetrating a further 0.5 cm, it gets the access to the sub-arachnoid space. After the pressure has been measured, the fluid is collected in four sterile screw capped containers consecutively, about 1ml each (Forbes *et al.*, 2002). Only a few milliliters are needed for basic studies (e.g., protein, glucose). Specialized tests that require concentration of the CSF (e.g., cell count, specific antibody studies, fungi and mycobacteria) require more CSF (Waldman, 2005). Removal of the larger volume may lead to headache, and the rate of collection should be slow, about 4 to 5 drops a second (Collee *et al.*, 1996). When there is increased intracranial pressure, it may be unsafe to undertake lumbar puncture as removal of fluid may draw down the cerebellum into the foramen magnum and compress the medulla (Brain and Walton, 1969).

CSF Examination:-

In certain neurological disorders, alterations can occur in the cellular and chemical content of CSF as well as its pressure. In such cases, fluid has to be taken out for examination (Carola *et al.*, 1992). CSF analysis remains important in the diagnosis of infections (e.g., bacterial, mycobacterial, fungal, viral, and protozoan) and certain inflammatory diseases (e.g., multiple sclerosis, Guillain-Barre syndrome, vasculitis). CSF analysis also allows immunologic confirmation of certain infections (e.g., Lyme disease) and fractionation of CSF proteins (e.g., myelin basic protein) (Waldman, 2005).

3.5 POSSIBLE PATHOGENS OF MENINGITIS

Meningitis is caused by the variety of the organism viz. bacteria, viruses, fungi, spirochaetes and protozoans. The most commonest forms of bacterial meningitis is caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Haemophilus influenzae*

type b, *Staphylococcus aureus*, *Escherichia coli*, Group B Streptococci, *Listeria monocytogenes* (Easmon, 1990).

1. Bacteria:

Gram Positive Bacteria

Streptococcus pneumoniae
Staphylococcus aureus
Streptococcus agalactiae
Listeria monocytogenes
Bacillus anthracis
Enterococci

Mycobacteria

Mycobacterium tuberculosis

meningosepticum

2. Spirochaetes:

Treponema pallidum
Leptosppira interrogans

4. Virus:

Polio virus
Rabies virus
Herpes simplex virus
Varicella Zoster virus

Gram Negative Bacteria

Neisseria meningitidis
Haemophilus influenzae type b
Escherichia coli
Klebsiella pneumoniae
Other enterobacteriaceae
Pseudomonas aeruginosa
Citrobacter spp.

Proteus spp
Salmonella spp
Flavobacterium

Bacteroids spp

3. Fungus:

Cyrtococcus neoformans
Candida spp
Histoplasma capsulatum
Blastomyces dermatitidis
Coccidioides immitis

5. Parasites:

Trypanosoma spp
Nagleria fowleri
Acanthamoeba spp
Schistosoma spp

Cytomegalo virus	<i>Echinococcus granulosus</i>
Epstein Barr virus	Malarial parasites
ECHO virus	<i>Taenia</i> spp
Coxackie virus	<i>Toxoplasma gondii</i>
Equine encephalitis	
Japanese B encephalitis	

(Source- Cheesebrough, 2000)

3.6 PATHOGENESIS

Most cases of meningitis caused by bacteria or encephalitis caused by viruses share a similar pathogenesis (Baron *et al.*, 1994). The etiological agents that cause meningitis are commonly found in the mucous that lines the nose, throat and tonsils (Michael, 2002). Disease occurs only when this organism comes into contact with another strain acquired directly from an infected individual, or indirectly from an infection in another part of the body, as occurs with some postoperative patients (Weir, 2002).

An important host defense mechanism of the CNS is the blood-brain barrier; the choroids plexus, arachnoid membrane, and the cerebral micro vascular endothelium are the key structures. Because of the unique structural properties of the vascular endothelium, such as continuous intracellular junctions, this barrier minimizes the passage of infectious agents into CSF in addition to recognize the transport of plasma proteins, glucose, and electrolytes (Forbes *et al.*, 2002). For the organism to reach the CNS, host defense mechanism must be overcome.

Age of the host and other underlying host factors also contributes to whether an individual is predisposed to develop meningitis. Neonates have the highest prevalence of meningitis. This higher prevalence is probably due to the immature immune system of the newborn, the organism present in the colonized female vaginal tract, and the increased permeability of the blood brain barrier of newborns. Lack of demonstrable humoral antibody against Hib in children has been associated with an increased incidence of meningitis (Forbes *et al.*, 2002).

Once the pathogenic bacterium penetrates the mucus membrane layer, it can invade the blood-brain barrier, contaminate the bloodstream and spread throughout the meninges and tissue that surrounds the brain (Scheld *et al.*, 2002). Organisms can enter through loss of capillary integrity by disrupting tight junctions of the blood brain barrier or by the transport within circulating phagocytic cells or by crossing the endothelial cell lining within endothelial vacuoles. Bacterial products such as lipopolysaccharides from Gram negative bacteria and teichoic acid from Gram positive bacteria cause inflammatory responses that damage the meninges (Easmon, 1990). Among the virulence factor of different mucosal pathogens (including the major agents of bacterial meningitis *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Haemophilus influenzae*), the extracellular IgA₁ proteases are major etiological agents (Landgraf, 1998). IgA₁ proteases released by pathogens destroy the actions of the host's secretory IgA, thereby facilitating bacterial attachment to the epithelium (Plaunt, 1983). In addition, all of the most of the common etiological agents of the bacterial meningitis possess an antiphagocytic capsule that helps the organisms evade destruction by the host immune response. In patient with impaired host defenses, other etiological agents can gain a foothold and cause disease (Easmon, 1990).

Because the respiratory tract is the primary portal of entry for many etiologic agents of meningitis, factors that predispose adults to meningitis are often the same factors that increase the likelihood that the adult will develop pneumonia or other respiratory tract colonization or infection. Alcoholism, splenectomy, diabetes mellitus, prosthetic devices and immuno-suppression contribute to increased risk. Finally, patients with prosthetic devices, particularly CNS shunts, are also at increased risk of developing meningitis (Forbes *et al.*, 2002).

Once bacteria have penetrated the blood brain barrier, host defenses are incapable of inhibiting the growth of micro organisms. Fatty acids such as arachidonic acid and its derivative, the prostaglandins cause the reduction in reabsorption of CSF in the venous blood through arachnoid villi. Within the meninges the bacteria replicate in the subarachnoid space causing swelling and intracranial pressure (Michael, 2002). White

blood cells also begin to accumulate in the cerebrospinal fluid (CSF) and contribute to an inflammatory response (Phillips and Simor, 1998). This swelling of tissue, referred to as oedema, causes a temporary deficiency of blood flow to brain tissue. When tissue lacks a blood and oxygen supply, brain cells die and brain damage occurs. Because bacteria and white blood cells can obstruct the flow of CSF, proper drainage and circulation is hindered, which contributes to oedema and further constriction of blood flow (Michael, 2002).

Bacterial surface components complement, and inflammatory cytokines (eg, tumor necrosis factor, IL-1) draw neutrophils into the CSF space. The neutrophils release metabolites that damage cell membranes including those of the vascular endothelium. The result is vasculitis and thrombophlebitis, causing focal ischemia or infarction, and brain edema. Vasculitis also disrupts the blood-brain barrier, further increasing brain oedema. The purulent exudate in the CSF blocks CSF reabsorption by the arachnoid villi, causing hydrocephalus. Brain oedema and hydrocephalus increase intracranial pressure. Obstruction to spinal fluid outflow is probably responsible for the hydrocephalus seen in children with meningitis. Although with the use of modern medical facilities includes antibiotics, the mortality rate has decreased, yet the numbers of patients surviving with post meningitic hydrocephalus has been greatly increased (Dubey and Rao, 1997).

Systemic complications include hyponatremia due to the syndrome of inappropriate antidiuretic hormone (SIADH), disseminated intravascular coagulation (DIC), and septic shock. Occasionally, bilateral adrenal hemorrhagic infarction (Waterhouse-Friderichsen syndrome) result. Encephalomyelitis may occur occasionally as a complication of pyogenic meningitis either in acute phase or as a late manifestation in patient whom the infection has not been completely eradicated by the treatment (Brain and Walton, 1969).

Patients with prosthetic devices, particularly CNS shunts, are at the increased risk from less virulent species such as normal skin flora like aerobic and anaerobic diptheroids

and *Staphylococcus epidermidis*. Viruses, appears from viraemic phase, may also travel along the nerves (e.g. Herpes simplex virus, Adenovirus) to reach to brain. Infections adjacent to the brain such as sinusitis and brain abscess, may also lead to meningitis (Baron *et al.*, 1994).

3.6.1 Routes of infection

Inflammation of the meninges occurs either as a primary or secondary to disease in some other parts of the body (Easmon, 1990). Infection may originate in the meninges (meningitis) or in the brain (encephalitis) and then spread from one site to another (Wilson, 1995).

Organisms may gain access to CNS by several primary routes:

3.6.1.1 By the blood stream: It is followed by entry into the subarachnoid space through the choroids plexus or through other blood vessels of the brain. This is the most common way that CNS gets infected (Forbes *et al.*, 2002). In such cases, meningitis follows bacteraemia. It may be the only or the principal manifestation of this, as in so called primary pneumococcal meningitis, meningococcal meningitis, or the infection of the meninges may be secondary to focal infection elsewhere in the body, for example, pneumonia, empyema, osteomyelitis, erysipelas, typhoid fever, etc. in which case bacterium may or may not be associated with endocarditis due to infecting organism. Tuberculous meningitis may thus be the part of a general miliary dissemination of tuberculosis (Brain and Walton, 1969).

3.6.1.2 From an adjacent lesion: It may occur as a result of fracture in the skull, either in case of penetrating wounds of cranial vault or fractures of the base when organisms may spread to the meninges from the nasopharynx (Brain and Walton, 1969). The extension of an infection close to or contiguous with the CNS can occasionally occur; examples of such infections include otitis media (infection of middle ear), sinusitis and mastoiditis (Forbes *et al.*, 1994). Organisms may also introduced by surgical procedures such as lumbar puncture (Brain and Walton, 1969).

3.6.1.3 Iatrogenic infection: Anatomic defects as a result of surgery, trauma, or congenital abnormalities can allow microorganism easy and ready access to the CNS (Forbes *et al.*, 2002).

3.6.1.4 Travel along the nerves leading to the brain (direct intraneural): The least common route of CNS infection caused by organisms such as rabies virus which travels along peripheral sensory nerves, and herpes simplex virus (Forbes *et al.*, 2002).

3.7 CLINICAL MANIFESTATIONS

All forms of meningitis, whatever their cause, possess a number of symptoms in common. The onset may be fulminating, acute, or less commonly insidious. The general symptoms of an infection are usually conspicuous (Brain and Walton, 1969). Meningitis can be either acute, sub acute or chronic in onset or progression of disease (Baron, Peterson and Finegold, 1994).

Acute meningitis: Cases of acute meningitis is characterized by fever, stiff neck, headache, nausea and vomiting, neurological abnormalities, and change in mental status (Albright *et al.*, 1991). However signs and symptoms are largely dependent upon the age of the patient and duration of illness.

In children signs of meningitis may include: fever, lethargy, behavioral changes, arching of the back, refusal of food, vomiting, a bulging fontanelle ("soft spot" of the head), seizures (Parini, 2002) and, in about half of child cases, a deep red or purple rash (JAMA, 1999). These symptoms develop rapidly, over a period of a few hours, and can be followed by extreme drowsiness or lack of consciousness (JAMA, 1999). In adults, symptoms include fever, severe headache, stiffness of the neck, nausea or vomiting, sensitivity to light and changes in mental status (Parini, 2002).

Acute bacterial meningitis cannot be distinguished from viral or fungal meningitis on clinical ground alone. With acute bacterial meningitis, CSF usually contains large number of inflammatory cells (more than 1000 mm³), primarily polymorphonuclear neutrophils. The CSF shows decreased glucose level and increased protein level (Baron

et al., 1994). Acute bacterial meningitis is fulminant, often fatal pyogenic infection beginning in the meninges.

Table 2: CSF finding in different conditions

Condition	Glucose	Protein	Leucocytes or erythrocytes/mm³
Acute bacterial meningitis	Low	High	High, often > 300 (Mostly Neutrophils)
Acute viral meningitis	Normal	Normal or high	Mononuclear, < 300
Tuberculous meningitis	Low	High	Pleocytosis, mixed < 300
Fungal meningitis	Low	High	< 300
Malignant meningitis	Low	High	Usually mononuclear few to > 1000
Subarachnoid hemorrhage	Normal	Normal, or high	Erythrocytes

(Source: Provan *et al.*, 2005)

In children there are several potential disabilities which result from damage to the nervous system. These include sensorineural hearing loss, epilepsy, diffuse brain swelling, hydrocephalus, cerebral vein thrombosis, intra cerebral bleeding and cerebral palsy (Vasallo and Martland, 2004). Acute neurological complications may lead to adverse consequences. Those that contract the disease during the neonatal period and those infected by *S. pneumoniae* and gram negative bacilli are at greater risk of developing neurological, auditory, or intellectual impairments or functionally important behaviour or learning disorders which can manifest as poor school performance (Grimwood, 2001).

In adults central nervous system complications include brain infarction, brain swelling, hydrocephalus, intracerebral bleeding; systemic complications are dominated by septic shock, adult respiratory distress syndrome and disseminated intravascular coagulation (Pfister *et al.*, 1993). Those who have underlying predisposing conditions e.g. head injury may develop recurrent meningitis (Adriani *et al.*, 2007). Case-fatality ratio is highest for gram-negative etiology and lowest for meningitis caused by *H. influenzae*. Fatal outcome in patients over 60 years of age is more likely to be from systemic complications e.g. pneumonia, sepsis, cardio-respiratory failure; however in younger individuals it is usually associated with neurological complications (Adriani *et al.*, 2007). Age more than 60, low Glasgow coma scale at presentation and seizure within

24 hours increase the risk of death among community acquired meningitis (Durand *et al.*, 1993). Although the morbidity associated with meningitis today is still significant, the *Haemophilus influenzae* type b conjugate vaccine has played the major role in reducing post meningitis sequelae (Wenger, 1994).

Sub-acute and chronic meningitis: Sub-acute or chronic meningitis may have infectious or noninfectious causes and may be aseptic meningitis. Infectious causes include fungi (most commonly *Cryptococcus neoformans*), TB, Lyme disease, AIDS, actinomyces, and syphilis; noninfectious causes include sarcoidosis, vasculitis, Behçet's syndrome, and cancers such as lymphomas, leukemia, melanomas, certain carcinomas, and gliomas (particularly glioblastoma, ependymoma, and medulloblastoma). Other causes include chemical reactions to certain intrathecal injections. Immunosuppressant and the AIDS epidemic have increased the incidence of fungal meningitis (Merck, 2005).

Chronic meningitis often occurs in patients who are immuno-compromised, although this is not always a case. Patients experience an insidious onset of disease, with some or all of the following: fever, headache, stiff neck, nausea and vomiting, lethargy, confusion and mental deterioration. Symptoms may present for a month or longer before treatment is sought. The CSF usually manifests an abnormal number of cells (usually lymphocytic), elevated protein and some decrease in glucose level. The pathogenesis of chronic meningitis is similar to that of acute disease (Wilhelm and Ellner, 1986).

Tuberculosis meningitis normally develops more slowly and there may well be accompanying focal neurological signs including optochiasmatic tuberculoma (Akhadar *et al.*, 2001) as well as a history suggestive of tuberculosis (Easmon, 1990).

3.8 TYPES OF MENINGITIS

Depending upon the causative organism that cause inflammation of the meninges, meningitis may, broadly, be categorized as acute pyogenic meningitis, aseptic meningitis, tubercular meningitis, fungal meningitis and other types (Merritt, 1967).

3.8.1 Acute pyogenic (purulent) meningitis:

This is caused by infection of the subarachnoid space. Pia-arachnoid membrane becomes inflamed and exudate is added to the CSF, which is a good culture medium for many bacteria (Anderson, 1985). In pyogenic meningitis, the CSF is typically turbid due to the presence of leukocytes, e.g. from hundred to several thousands per cubic millimeter, most of which are polymorphonuclear cells (neutrophils) (Collee *et al.*, 1996). CSF pressure may be elevated.

Many bacteria can cause pyogenic meningitis, but most common are group B Streptococci (GBS) during the first 2 month of life and, thereafter, *Neisseria meningitidis* (meningococci) and *Streptococcus pneumoniae* (pneumococci) (Cheesebrough, 1984). These organisms usually pass to meninges from respiratory tract via blood stream (Baron *et al.*, 1994; Brain and Walton, 1969; Collee *et al.*, 1996; Merritt, 1967). These encapsulated bacteria are the major causes of respiratory and meningeal infection in children world wide (Vineusa and Lucas, 2001). They shows capsular antigen and evokes type 2 thymus independent antibody response but fail to generate conventional B-cell memory. Other bacteria, such as coliforms (particularly in neonates), pseudomonads, salmonellae, listeriae, and staphylococci are also cited (Baron, Peterson and Finegold, 1994; Brain and Walton, 1969; Cheesebrough, 1984; Collee *et al.*, 1996; Easmon, 1990; Kalpan, 1999; Kumari and Icchpujani, 2000; Merritt, 1967). Pyogenic meningitis is also associated with increased intra-cranial pressure, reduced glucose concentration and elevated protein concentration (Baron *et al.*, 1994; Cheesebrough, 1984; Collee *et al.*, 1996; Kumari and Icchpujani, 2000).

3.8.2 Aseptic meningitis:

Aseptic meningitis is the term used to describe those cases with the evidence of a meningeal reaction in the CSF in the absence of any infecting organisms (Merritt, 1967) that can be cultured in bacteriological cultures (Baron, Peterson and Finegold, 1994; Wilson 1995). Aseptic meningitis refers to non-bacterial causes of meningitis and includes infective etiologies such as viruses and fungi, neoplastic etiologies such as carcinomatous and lymphomatous meningitis, inflammatory causes such as sarcoidosis

(neurosarcoidosis)) and chemical causes such as meningitis secondary to the intrathecal introduction of contrast media.

Although the term "viral meningitis" is often used in any patient with a mild meningeal illness with appropriate CSF findings, certain patients will present with clinical and CSF features of viral meningitis, yet ultimately be diagnosed with one of the other conditions categorized as "aseptic meningitis". This may be prevented by performing polymerase chain reaction or serology on CSF or blood for common viral causes of meningitis (enterovirus, herpes simplex virus 2 and mumps in those not vaccinated for this) (Logan and MacMahon, 2008).

With the improved technology and availability of viral and other special techniques, many instances of the aseptic meningitis can now be assigned as a viral, leptospiral or other etiology. Viruses are the most common cause. Other causes may be infectious (eg, rickettsiae, spirochaetes, parasites) or noninfectious (eg, intracranial tumors and cysts, drugs, systemic disorders). Viruses that cause encephalitis typically also produce low-grade aseptic meningitis (Baron *et al.*, 1994).

Viral infections of the CNS are very rare. Viruses may cause meningitis, encephalitis or lesions of the neurons of the spinal cord and peripheral nerves. Most of the viruses are blood borne although a few travel along peripheral nerves, e.g., Rabies virus and possibly polio viruses. They enter the body via:-

-) Alimentary tract: - e.g. Poliomyelitis
-) Respiratory tract: - e.g. Shingles
-) Skin abrasions: - e.g. Rabies
-) Insect bites: - e.g. Encephalitis lethargia

The effects of virus infections vary according to the site and amount of tissue destroyed. Viruses are believed to damage nerve cells by taking over their metabolism-stimulating an immune reaction (Wilson, 1995).

The study of 3231 patients in Finland with acute CNS symptoms of suspected viral origin to elucidate the current etiological spectrum showed that Varicella zoster virus (VZV) was the major agent associated with encephalitis as well as meningitis and myelitis. VZV comprised 29% of all confirmed or probable etiological agents where as herpes simplex virus (HSV) and enteroviruses accounted 11% each and influenza 'A' virus accounted 7% (Koskiniemi *et al.*, 2001). The study of the expanding spectrum of herpes virus infection of the nervous system showed that acute meningitis was seen with VZV and HSV-2 and benign recurrent meningitis with HSV-2 (Kleinschmidt *et al.*, 2001). Infection due to echovirus 11 is most asymptomatic but can lead to serious outcomes such as meningitis and others during the first month of life (Rubinstein, Weisbrod and Garty, 2000). In the recent years, outbreaks of enterovirus infection caused by enterovirus-30 were increased in different countries. Major serious water borne meningitis outbreak was caused by this virus during the summer autumn period of 1997 in the city Gomel, Belarus (Amvrosyeva *et al.*, 2001).

Bacteria may also cause aseptic meningitis; such type of bacteria includes spirochaetes (in syphilis, Lyme disease, or leptospirosis) and rickettsiae (in typhus, Rocky Mountain spotted fever, or ehrlichiosis). CSF abnormalities may be transient or chronic. Bacterial infections such as mastoiditis, sinusitis, brain abscess, and infective endocarditis can result in aseptic meningitis because widespread inflammation produces vasculitis, which leads to CSF pleocytosis without bacteria in the CSF (Merck, 2005).

3.8.3 Tuberculous meningitis (TBM):-

Tuberculous meningitis is one of the commonest chronic infection of the CNS (Katti, 2001) caused by *Mycobacterium tuberculosis*. The involvement of the central nervous system is always secondary to a primary lesion elsewhere (Girgis *et al.*, 1998; Satya, 1995; Subanadze, 1999). Of all forms, TB of the nervous system is the most serious, constituting 4%–6% of the extrapulmonary cases (Barr and Menzies, 1994; Satya, 1995). Neuro-tuberculosis constitutes almost half of extra pulmonary tuberculosis (Bajpai *et al.*, 2000) and is one of the most serious forms (Lang, 1998). It is probably always secondary to a tuberculous focus in some other parts of the body (Easmon,

1990). The primary focus of infection is most commonly in the lungs but may be in the lymph glands, bones, sinuses, gastrointestinal tract, or any other organ in the body (Merritt, 1967). However, because it is often difficult to determine the site of primary infection, even at necropsy, the disease not frequently appears to be primary (Easmon, 1990).

Meningitis is the most common manifestation of neuro-tuberculosis. No part of the central nervous system (CNS) is spared; the disease can affect all tissue components of the brain and its coverings. Infection of the meninges by TB bacilli is usually caused by rupture of the subependymal tubercle into the subarachnoid space rather than by haematogenous seeding of the meninges. It can also be a complication of miliary TB. TB of the CNS can manifest itself as meningitis, encephalitis or encephalopathy, tuberculoma, vasculitis or arteritis, spinal arachnoiditis, radiculomyelitis and even behavioural changes or demyelination to a lesser extent (Al-Abbasi, 2002).

TBM is still a common and serious type of extra pulmonary tuberculosis in developing countries, associated with a high mortality rate and significant complications (Al-Abbasi, 2002). In developing countries, tuberculous meningitis constitutes 7-12% of active tuberculosis (Lang, 1998) but in developed countries, it is less frequent, however, it is increasing with HIV pandemic (Grag, 1999; Lang, 1998). HIV-infected patients with tuberculosis are at increased risk for meningitis, but infection with HIV does not appear to change the clinical manifestations or the outcome of tuberculous meningitis (Berenguer *et al.*, 1992). The incidence of TBM in the resident children (upto 14 yrs of age) in New South Wales was found to be 0.053 per 100,000 (Arestis *et al.*, 1999). It is fatal if it is not treated early and in surviving patient neurological sequelae are common (Lang, 1998) and hydrocephalous if is the late complication of the tuberculous meningitis (Bajpai *et al.*, 2000).

TBM differs from that caused by most of the other common bacteria in that the course is more prolonged (chronic), the mortality rate is much higher i.e. 52.1% (Karstaedt *et al.*, 1998) and the changes in CSF is less severe (Merritt, 1967). It is rare during the first

year but occur at any age (Brain and Walton, 1969). More cases occurred in children between one year and four years of age and in adults above forty-five. The study of 224 cases at El-Khateeb Hospital for Infectious Diseases in Baghdad showed that TBM comprised 5% of the total admissions of meningitis cases to the hospital and mainly affected the younger age groups (Al-Abbasi, 2002). Reactivation of tuberculosis, secondary to severe immuno-compression is likely to affect the age distribution of systemic complication of tuberculosis such as meningitis (Easmon, 1990). When a study was done to find out whether BCG vaccination prevent or postpone the occurrence of TBM or not, it was concluded that single dose of vaccination only postpones rather than prevents the occurrence of TBM (Mittal *et al.*, 1996). BCG vaccination offered significant protection against both the morbidity and mortality of TBM (Al-Abbasi, 2002).

In a study done by Tiwari (2002) found 6.67% tuberculous meningitis among total meningitis (15) cases when studied among 150 suspected cases.

3.8.4 Fungal Meningitis:-

The meninges and the CNS are occasionally invaded in the course of infection by a kind of pathogenic fungi and in recent years, there has been an increase in the incidence and severity of this type of infection (Merritt, 1967). The causative agents are *Cryptococcus neoformans*, *Candida* spp, *Histoplasma capsulatum*, *Coccidioides immitis* and *Blastomyces dermatitidis*. The later three are more common in the US (Easmon, 1990). Cryptococcosis is the most frequently recognized as a disease of CNS although the primary site of infection is the lungs (Evans, 2001). *Cryptococcus neoformans*, capsulated yeast, spreads from a focus in the lungs into the blood stream and hence in the meninges (Easmon, 1990), which can cause life threatening meningoencephalitis in patients with and without impaired immune function. Most symptoms of cryptococcal meningitis are attributable to cerebral edema and are usually nonspecific, including headache, blurred vision, confusion, depression, agitation, or other behavioral changes. Except for ocular or facial palsies, focal signs are rare until relatively late in the course. Cryptococcosis is usually an opportunistic infection in the patients with compromised

immunity as consequence of HIV infection, steroid administration, cancer therapy, sarcoidosis, diabetes or inherent immune defects (Evans, 2001). Infections in immunocompetent hosts are confined to lungs and are usually self limiting and asymptomatic, while in AIDS patient the yeast tends to disseminate throughout the body. Meninges is the best destination. The incidence of cryptococcal meningoencephalitis among patient with AIDS has to be 6-10% (Rappelli, 1998). Cryptococcal meningitis in patients with AIDS may produce minimal or no symptoms and normal CSF parameters except for the presence of many yeasts.

There are four types of the capsulated yeast, *Cryptococcus neoformans* (A, B, C and D) that represent two varieties of the organism, namely, *C. neoformans* var. *neoformans* (A and D) and *C. neoformans* var. *gatti* (B and D) (Evans, 2001). *C. neoformans* var. *neoformans* has been found predominantly in clinical samples while *C. neoformans* var. *gatti* infection has been also reported (Benerjee *et al.*, 2001). Capsule is the virulence factor of the cryptococci. The thickness of the capsule is different in different organs (lungs > brain > in vitro culture) and organ related genes are responsible for the capsule thickness (Rivera *et al.*, 1998).

The natural environment of *C. neoformans* is the excreta of wild and domestic birds throughout the world (5×10^7 cells/g of pigeon's droppings) and *C. neoformans* var. *gatti* is associated with the flowers of *Eukalyptus camaldulensis* (red-river gum tree) (Evans, 2001). *C. neoformans* var. *neoformans* was isolated in various environmental niches, including hospital environment in Vellore, India where cryptococci were isolated from 67% samples of pigeon's droppings collected from hospital environment (Abraham *et al.*, 1997).

3.8.5 Neurosyphilis:-

Treponema pallidum is one of the etiological agents responsible for chronic meningitis (Baron *et al.*, 1994). It is non infectious syphilis and occurs in about 10% of untreated syphilis (Cheesbrough, 1984). It has been supposed that certain strains of spirochaetes possess an affinity for the nervous system, while others do not. Spirochaetes may reach

to nervous system during the primary stage and before the development of the cutaneous exanthem. Slight changes in CSF and low grade head ache occurs in the primary stage and considerable severity exist about one year after infection during secondary stage.

3.9 TYPES OF MENINGITIS BY AGE GROUP

Which the etiological agents involved as the cause of any occurrence of bacterial meningitis depends upon several underlying host factors, but age of the patient is most important (Baron, Peterson, Finegold, 1994). On the basis of this, they may be “Neonatal meningitis”, “Meningitis in children” and “Meningitis in adults”.

3.9.1 Neonatal meningitis: - Neonatal meningitis is inflammation of the meninges due to bacterial invasion in the first 90 days of life. The age group with the highest prevalence of meningitis is that of the newborn with the concomitant increased mortality rate (as high as 20%). The high prevalence of the organism in this period of life is probably due to immature system, the organisms present in the colonized female genital tract, as well as the increased permeability of the blood-brain barrier in the newborns. Neonates are likely to be infected with, in order of incidence, Group B streptococci, *E.coli*, other gram negative bacilli, *Listeria monocytogenes*, and other organisms (Baron, Peterson and Finegold, 1994). Group B streptococcus is the most common pathogen associated with meningitis in newborns. *Listeria monocytogenes* is also recognized as a significant cause of meningitis in newborns and the elderly in the United States (Schuchat, 1997).

In the neonatal period common causative organisms are: *Escherichia coli*, *Streptococcus pneumoniae*, *Salmonella* group of organisms, *Pseudomonas aeruginosa*, *Streptococcus fecalis* and *Staphylococcus aureus* (Tiwari, 2003). *Citrobacter koseri* is the best known citrobacter that cause sepsis and meningitis leading to CNS abscess in neonates and young infants (Doran and Terence, 1999). Neonatal meningitis most frequently results from the bacteremia that occurs with neonatal sepsis; the higher the colony counts in the blood culture, the higher is the risk for meningitis. Meningitis may

also result from scalp lesions, particularly when developmental defects lead to communication between the skin surface and the subarachnoid space, which predisposes to thrombophlebitis of the diploic veins. Rarely, there is direct extension to the CNS from a contiguous otic focus (eg, otitis media) (Merck, 2005).

3.9.2 Meningitis in children: - Ninety-five percent of cases of meningitis occur in patients less than 5 years old (Baron, Peterson and Finegold, 1994). *Haemophilus influenzae* type b is the commonest in the children (Baron, Peterson and Finegold, 1994; Howard and Ison, 1996; Slack, 2001, Vandepitte *et al.*, 2004). Staphylococci and other unusual organisms are associated with congenital anatomical defects in child. TBM remains the most common forms of neurotuberculosis in children. *Flavobacterium meningosepticum* has been associated with waterborne outbreaks of meningitis in children. Lack of demonstrable humoral antibody against this bacterium in children has been associated with increased incidence of meningitis (Baron *et al.*, 1994).

3.9.3 Meningitis in adults: - Respiratory tract infection is the primary route of entry of many etiological agents of meningitis in adults. Alcoholism, splenectomy, diabetes mellitus, prosthetic devices, immunosuppression contribute to increased risk. Important agents are meningococci, pneumococci, *Listeria monocytogenes*, and less commonly *Staphylococcus aureus* and Gram negative Bacilli. The later organisms reach to meninges via. hematogenous seeding from various sources, including urinary tract infections (Lack of demonstrable humoral antibody against this bacterium in children has been associated with increased incidence of meningitis (Baron *et al.*, 1994). In North America and Europe, because of the vaccine related decline in *H. influenzae* disease, *S. pneumoniae* and *N. meningitidis* remain important pathogens in children and young adults (Schuchat, 1997).

3.10 BACTERIA IN CSF OF CONFIRMED CASES OF MENINGITIS BY AGE GROUP:

Neonates:

- Group B Streptococcus (esp early cases) - 75%
- Gram negatives (esp *E. coli*) - 20%
- Others include *Listeria monocytogenes*

Infants & Children:

- *Streptococcus pneumoniae* - 40%
- *Neisseria meningitidis* - 40%
- *Haemophilus influenzae* - now rare
- Group B Streptococci

Adults:

- *Strep. pneumoniae* - 40%
- *Neisseria meningitidis* - 40%
- *Haemophilus influenzae* - now rare

Elderly:

- *Strep. pneumoniae* (most common) - 50%
- Gram negatives (incl. *E. coli*) - 25%
- *Listeria* - < 10% (Source- Kalpan, 1999)

3.11 BACTERIAL MENINGITIS

Bacterial meningitis is an important serious illness worldwide. Bacterial infections account for about 60% of reported cases of meningitis. Prior to the introduction of antibiotics in the 1940s, case fatality rates for epidemic and endemic bacterial meningitis exceeded 70%. Since then, use of antibiotic has reduced case fatality rates for meningitis caused by most bacteria to 25% or less, but no further reduction has been documented in the past 20 years. Despite advances in vaccine development and

chemoprophylaxis, bacterial meningitis remains a major cause of death and long-term neurological disabilities, such as mental retardation, convulsions and hydrocephalus. These are best prevented by early diagnosis and appropriate treatment of the disease (Rao *et al.*, 1998). Bacterial meningitis is being reported predominantly in adults in USA because of the immunization practices adopted and also due to a relative increase in frequency of nosocomial meningitis (Schuchat, 1997).

Neisseria meningitidis, *Streptococcus pneumoniae* and *Haemophilus influenzae* type b represent the triad responsible for over 80% of all cases of bacterial meningitis. Other bacteria causing the disease include Gram-negative rods (especially *Escherichia coli*), streptococci (other than *S. pneumoniae*), *Listeria monocytogenes*, and staphylococci. Of special concern are hearing problems which often are bilateral and profound. Although epidemics of meningococcal disease attract much public attention, endemic bacterial meningitis causes substantial illness and death, as well as persistent neurological defects, particularly among infants and young children. Throughout the world, bacterial meningitis may be the most important cause of acquired deafness (WHO, 1998).

3.11.1 Epidemiology of meningococcal meningitis:-

Neisseria meningitidis was first identified as the causative agent of bacterial meningitis by Weichselbaum in 1887. However, clinical meningococcal disease was described by Vieusseux in 1805 during an outbreak in the vicinity of Geneva, Switzerland. During the 20th century, major outbreaks were noted during World War I and World War II. On the African continent epidemic meningitis has been known to occur for a long time. It was reported from the West coast of Africa by G. William in 1909 and has occurred from then onwards. In the Sudan, cerebrospinal meningitis has been present from time immemorial. However, the disease probably did not appear in the Northern savanna of Africa before the 1880s. Since World War II, epidemic meningitis caused by group A meningococcus has been infrequent in developed countries with a temperate climate, whereas meningococcal disease in its epidemic form has periodically continued to devastate sub-Saharan territories of Africa. At least 340,000 cases with 53,000 deaths

occurred in the 10-year period 1951-1960 in the countries of this region (estimated total population of 35 million) (WHO, 1998).

Meningococcal meningitis has repeatedly caused outbreaks worldwide. Meningococcal infections occur worldwide as endemic disease (Achtman, 1995; Caugant, 1998). Epidemiological studies by modern molecular methods have disclosed a complex picture of a few pathogenic meningococcal clones spreading worldwide. It appears that the occurrence of invasive meningococcal disease is not determined solely by the introduction of a new virulent bacterial strain but also by other factors that enhance transmission (Stephens, 1999).

Major meningococcal meningitis epidemics around the world (Source: WHO, 1998)

COUNTRY AREA	YEAR	NO. OF CASES	ATTACK RATE per 100,000 pop.	CFR	SERO-GROUP
EUROPE					
Finland	1973-74*	1,300	14.6*	4.3	A
Norway (North)	1975*-78	404	23.9*	13.7	B
Iceland	1976	86	37.7	10.3	B
Faroe Islands	1980-81*	74	95.0*	NA	B
SOUTH AND CENTRAL AMERICA					
Chile, Iquique	1966	46	31.2	NA	B
Brazil, Sao Paulo	1971-72	2,005	11	NA	C
Brazil, Sao Paulo	1974	30,555	370.0	NA	A
Cuba	1980-84*	NA	14.4*	14.9	B
ASIA					
Mongolia	1973-74	NA	141.0 -194.0*	11.8	A
Viet Nam, Ho Chi Minh	1977	1,015	>20.0	27.4	C
Nepal, Kathmandu	1983	875	103.0	10.9	A
AFRICA					
Nigeria, Zaria	1977	1,257	360.0	8.3	A
Rwanda, Ruhengeri,	1978	1,182	223.0	4.8	A
Kigombe	1978	248	729.0	NA	A
Burkina Faso,					
Diapaga	1979	539	517.0	10.2	C
Côte d'Ivoire					
Boundiali	1983	414	207.0	NA	A
Ferkessedougou	1985	251	217.0	8.5	A
Korhogo	1985	367	92.0	8.5	A
Chad, N'Djamena	1988	4,542	826.0	9.5	A
Sudan	1988	32,016	133.0	NA	A
Ethiopia	1989	41,139	83.0	3.9	A
Addis Ababa	1989	7,000	420.0	NA	A
Kenya, Nairobi	1989	3,800	250.0	9.4	A
Burundi, Ruyigi	1992	1,615	608.0	8.0	A
Burkina Faso	1996	42,129		10.0	A
	1997	22,305		11.3	A
Mali	1996	7,254		11.5	A
	1997	11,228		10.1	A
Niger	1996	26,738			A
	1996	16,145		9.9	A
Nigeria	1996	108,568		11.2	A

N. meningitidis, the causative agent of meningococcal meningitis, is divided into 13 serogroups defined by specific polysaccharides designated A, B, C, H, I, K, L, M, X, Y, Z, 29E and W135 (serogroup D is no longer recognized) and most infections are caused by organisms belonging to serogroups A, B, C, Y and W-135 (Frasch *et al.*, 1985). Of the five common serogroups (A, B, C, Y and W135) responsible for about 90% of infections caused by *N. meningitidis*, serogroups A, B and C account for most cases of meningococcal disease throughout the world, with serogroups A and C predominating throughout Asia and Africa and serogroups B and C responsible for the majority of cases in Europe and the Americas (Rosenstein *et al.*, 1999; WHO, 1995). In recent years, the number of cases involving serogroup Y has increased; from 1996 to 1998, one third of cases in United States were due to serogroup Y (Connolly and Noah, 1999). Meningococci exist in the nasopharynx of about 5% of people and spread by respiratory droplets and close contact. Purulent meningitis due to these bacteria is usually accompanied by septicaemia and has incubation period of 2-10 days (Easmon, 1990). Cerebrospinal meningitis occurs only in limited proportion of the population at the risk (Fulton and Slack, 2001). Up to 10% of the people carry strains of *N.meningitidis* but once epidemic become established, carriage rates of 80-90% were observed (Easmon, 1990).

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 *N. meningitidis* strains prevalent and differences in socioeconomic and environmental conditions. The proportion of cases caused by each sero group varies by age group; more than half of cases among infants aged <1 year are caused by serogroup B, for which no vaccine is available (Connolly and Noah, 1999; Fischer and Perkins, 1997).

The quadrivalent polysaccharide vaccine that provides protection against serogroups A, C, Y and W-135 and the bivalent polysaccharide vaccines (A and C) are available worldwide. Routine childhood vaccination with the meningococcal polysaccharide vaccine is not recommended because of its relative ineffectiveness in young children

aged < 2 years, who have the highest risk of sporadic disease and a relatively short duration of protection (Rosenstein *et al.*, 1999).

3.11.2 Epidemiology of *Haemophilus influenzae* meningitis:-

Haemophilus influenzae is the important cause of the serious systemic disease in children throughout the world (Slack, 2001). It forms the part of normal commensal flora of the throat and nasopharynx of between 25-75% of the healthy persons and acts opportunistically as a secondary invader in a variety of respiratory tract infection (Howard and Ison, 1996). Meningitis is the most common clinical outcome. Meningitis caused by *Haemophilus influenzae* is met with in the age group from 3 month to 2-3 years. *Haemophilus influenzae* meningitis is usually associated with or follicular pharyngitis, otitis media, CSF leak, immunodeficiency states (Tiwari, 2003).

A minority of strains of *Haemophilus influenzae* is capsulated; these comprise six serotypes (a, b, c, d, e, f) according to the different antigens present in its polysaccharide capsule (Howard and Ison, 1996). One of these, type b is more virulent for than other varieties of this species and is responsible for a number of invasive infections (Baron *et al.*, 1994; Easmon, 1990; Howard and Ison, 1996; Slack, 2001) with the mortality rate of 3-6.5% (Easmon, 1990).

Haemophilus influenzae type b (Hib) used to be the cause of most bacterial meningitis in children from North America and Europe, a fact that changed with systematic vaccination with anti-*H. influenzae* type b vaccine (Latoree *et al.*, 2000). Information from a limited number of developing countries in Africa, Asia, and Latin America indicates that Hib is an important cause of childhood morbidity and mortality, responsible for 25%–65% of bacterial meningitis as well as 15%– 25% of severe pneumonia in some areas (Levine *et al.*, 1999; Peltola, 2000). Case fatality rates for meningitis in developing countries are often higher than reported in developed countries because of delays in diagnosis and suboptimal antimicrobial therapy (Mulholland and Adegbola, 1998; Peltola, 2000).

Until the availability of conjugate vaccines, *Haemophilus influenzae* type b (Hib) has been the most common cause of bacterial meningitis in childhood outside epidemic periods. In several countries where Hib vaccines have recently entered the routine infant immunization schedule, Hib meningitis has nearly been eliminated. The annual incidence of Hib meningitis is about 1-3 per 100,000 populations. However, the age-specific incidence rate among children under 5 years of age is much higher, ranging from 20-60 cases per 100,000 per year. Ninety percent of patients with Hib meningitis are under 5 years of age and the peak age group affected is 6-11 months. The case-fatality rate in Hib meningitis is approximately 5-20%. Sequelae occur in approximately 10-30%, the most common being hearing loss (WHO, 1998).

Haemophilus influenzae type b conjugate vaccines have decreased the incidence of meningitis caused by Hib. Routine Hib vaccination beginning at 2 months of age is recommended.

3.11.3 Epidemiology of Pneumococcal meningitis:-

Pneumococcal meningitis is caused by *Streptococcus pneumoniae* and is also the common cause of bacterial meningitis (Easmon, 1990). It exists as the normal flora of naso and oro pharyngeal region (Ross, 1996). The annual incidence of meningitis caused by *S. pneumoniae* is 1-2 per 100,000 populations in most developed countries. Developing countries have higher incidence rates, up to 20 per 100,000. In temperate climates, incidence is higher in the cold season than in the warm season. The highest incidence is found in children less than 2 years of age. Incidence decreases in the subsequent age groups to a low level in young adults but again increases in the elderly. The case-fatality rate in pneumococcal meningitis is several times higher than in meningococcal or Hib meningitis. More than half of pneumococcal meningitis patients have one or more complications (WHO,1998).The carriage rate is 5-70% (Finch, 2001) and they are mostly secondary pathogens but are primary pathogens in immunocompromised patients (Ross, 1996). It usually follows pneumonia, sinusitis, endocarditis or old and recent head trauma, CSF leak, sinusitis, splenectomy, sickle cell disease, bone marrow transplantation (Tiwari, 2003). Meningitis due to this disease

always coexist with bacteraemia (Finch, 2001) and affects all groups but mainly in children and old adults are affected. Pneumococcal meningitis is not the epidemic disease; however the mortality rate is in between 20-40% (Easmon, 1990). Incidence of pneumococcal meningitis is decreasing because of routine vaccination (Merck, 2005). The vaccine is available in two forms: the pneumococcal conjugate vaccine (PCV7) and the pneumococcal polysaccharide vaccine (PPV23). All infants should be vaccinated with PCV7. Infant vaccination provides the earliest protection and infants <23 months of age have the highest incidence of pneumococcal disease.

3.11.4 RARE ETIOLOGICAL AGENTS OF MENINGITIS

Meningitis caused by *Staphylococcus aureus* is relatively infrequent cause of meningitis (Merritt, 1967) which may develop as a result of spread from furuncles on the face or from staphylococcal infections elsewhere in the body (Baron *et al.*, 1994). Meningitis caused by staphylococci may occur in conjunction with or follow umbilical infection, sinusitis, pyoderma, brain abscess, endocarditis or severe staphylococcal septicaemia or in conjunction with CNS shunts (Tiwari, 2003). Patients with prosthetic devices, particularly central nervous system shunts, are at the increased risk of developing meningitis caused by virulent species, such as organisms of the normal skin flora like aerobic and anaerobic diptheroids and *Staphylococcus epidermidis* (Baron *et al.*, 1994).

Meningitis caused by *Streptococcus agalactiae* (Group B streptococci) is the leading cause of bacterial meningitis and sepsis in neonates and they rarely cause adult infection, but the incidence of adult meningitis due to this has been increased in recent years (Esen *et al.*, 2000). *Listeria monocytogenes* often cause meningitis in neonates, in the aged and in the immunosuppressed patient. In neonates the source of listeria is the genital tract or subclinical infection of the mother which is transferred to the baby during birth (Tiwari, 2003). Listerial meningitis can occur at all ages and is particularly common among patients immuno-compromised because of chronic renal failure, hepatic disorders, or corticosteroid or cytotoxic therapy after organ transplantation (Merck, 2005). Enterococci is also the cause of meningitis in neonates (Merck, 2005; Tiwari, 2003).

Meningitis caused by Gram-negative bacteria (most often due to *Escherichia coli*, *Klebsiella* spp, or *Enterobacter* spp) can occur in immunocompromised patients or after CNS surgery, CNS trauma, bacteremia, or hospital-acquired infections (Merck, 2005). Infants in their first month of life are particularly predisposed to bacterial meningitis with *E.coli*. In neonates there is a strong association between the presence of K1 capsular antigen and meningitis. Pregnancy is associated with an increased rate of colonization with K1 strains of *E.coli* and these strains are the ones involved in subsequent neonatal meningitis. The colonization of the gastrointestinal tract with *E.coli* K1 is the portal of entry into the blood stream and subsequent meningitis (Tiwari, 2003).

Meningitis is also caused by *Pseudomonas aeruginosa* and may be a result of extension from a contagious structure like the ear, mastoid or paranasal sinuses, and direct inoculation into the subarachnoid space or bacteraemic spread from different foci of infection like the urinary tract, lung or endocardium. *Pseudomonas* spp occasionally causes meningitis in immunocompromised or colonized patients. Conditions like neutropenia, severe burns, and recent surgery or immunodeficiency states predispose to the development of meningitis caused by pseudomonas (Tiwari, 2003).

The incidence of typhoid meningitis is very low. It occurs in severely malnourished children and in low birth weight babies below 3 month of life. Meningitis caused by salmonella is usually a complaint of typhoid fever in older children but in the newborn and in the very small typhoid meningitis may be primary and prognosis is poor with nearly 100% fatal outcome (Tiwari, 2003). Enterotoxigenic *Bacteroides fragilis* also causes bacterial meningitis (Aucher, 1996).

3.12 LABORATORY DIAGNOSIS OF MENINGITIS

There is an urgent need for laboratory diagnosis of suspected meningitis, for bacterial meningitis is life threatening and requires appropriate antibiotic therapy (Collee *et al.*, 1996). It is also difficult to distinguish various forms of meningitis (Easmon, 1990) without laboratory investigations. The principle specimen is CSF (Collee *et al.*, 1996).

CSF and blood should be collected from the suspected cases of meningitis (Kumari and Ichhapujani, 2000) because blood culture can enhance the chances of diagnosis for those organisms, which follow the hematogenous route to meninges. Blood should be collected at the same time as the CSF, if possible before antibiotics are given (Collee *et al.*, 1996). Nasopharyngeal swab may be the value in meningococcal or haemophilus infections both for establishing etiology and for screening close contact (Easmon, 1990). Examinations of CSF by macroscopically, microscopically, chemically and immunologically and culture methods is necessary to distinguish meningitis from non specific syndromes and to distinguish among bacterial, fungal and aseptic meningitis (Easmon, 1990).

3.12.1 Collection and transport of specimen:-

The CSF should be collected by experienced medical officer. The collection process should be aseptic to avoid infection. CSF is usually collected from the subarachnoid space in the lumbar region at L4-L5 level (Baron *et al.*, 1994). A ventricular puncture is sometimes performed to collect CSF from infants (Cheesbrough, 1984). Only 3-5 ml of the fluid should be collected at the rate of 4-5 drops per second (Collee *et al.*, 1996). For mycobacteria and fungi, a larger volume of CSF (5-10ml) may be required (Baron, Peterson and Finegold, 1994). Three to four sterile screw capped container are required to collect CSF. This is because the first sample may contain blood (due to traumatic lumbar puncture), which will affect the accuracy of the cell count and biochemical estimations (Cheesbrough, 1984).

CSF should be hand delivered immediately to the laboratory (Baron *et al.*, 1994) and cultured as soon as possible after receipt (Kumari and Ichhpujani, 2000) as delay may result in the death of delicate pathogens, such as Meningococci, Haemophilus, Pneumococci and disintegration of leukocytes. If delay for a few hours is unavoidable, the specimen is best kept in an incubator at 37°C but refrigeration should be avoided which tends to kill *Haemophilus influenzae*. In suspected cases of viral infection, a portion of CSF is sent to virology laboratory on ice or a cold pack in an insulated

container and for tissue culture (e.g. for mumps, HSV) or other agents (*Mycoplasma pneumoniae*) (Collee *et al.*, 1996).

3.12.2 Macroscopic and microscopic examination: - The CSF should be examined with naked eyes for the presence of turbidity and any sign of contamination with blood from the puncture wound (Collee *et al.*, 1996). Initial processing of CSF for bacterial, fungal or parasitic studies includes centrifugation of all specimens greater than 1ml in volume for at least 15 minutes at 1500xg rpm. The supernatant is removed (Baron *et al.*, 1994) and after thoroughly mixing the sediment heaped drops placed on the sterile slide (without spreading which lowers the chances of detection of low number of organisms). If CSF is highly turbid and proteinaceous, a part of film should be thin as there is chance of being washed away off the slide in the course of staining (though dried and fixed) (Collee *et al.*, 1996). A very careful search for bacteria should be made. The findings of bacterial forms resembling meningococci, streptococci, haemophilus or listeriae should once be reported to the physician. Based on the demographic and clinical data of the patient and Gram stain morphology, the etiology of majority of the cases of bacterial meningitis can be presumptively determined within the first 30 minutes after receiving the specimen (Baron *et al.*, 1994). AFB is difficult to detect in CSF. The chances can be increased by centrifugation for 30 min with higher angular velocity (3600xg rpm) and utilizing several drops. Examination of auramine stained smear by fluorescence microscopy is a more sensitive method of detecting AFB in CSF (Cheesbrough, 1984). If insufficient quantity of CSF is received, the specimen should be used directly for smear and culture (Baron *et al.*, 1994).

The leucocytes in the CSF are counted by microscopical observation of well mixed, uncentrifuged fluid in a counting chamber. The relative polymorphs and lymphocytes should be noted along with the number of erythrocytes in specimen contaminated with the blood. Normal CSF contains 0-5 leucocyte/mm³, lymphocytes, though in neonates up to 30 cells/mm³, mainly polymorph is considered as normal (Collee *et al.*, 1996). There is increase in leukocyte count during different form of meningitis.

3.12.3 Chemical examinations: - The routine biochemical testing of CSF to investigate meningitis should include glucose estimation, protein estimation and screening test for raised globulin (Cheesbrough, 1984). Lactate dehydrogenase (LDH) and C-reactive protein (CRP) levels are useful investigation to distinguish bacterial meningitis from aseptic meningitis. Detection of lipopolysaccharide in CSF reveals the meningitis caused by Gram negative bacteria (Easmon, 1990).

3.12.3.1 Glucose estimation: -If CSF is not preserved with fluoride; the glucose must be estimated within 20 minutes of the fluid being withdrawn otherwise a false low result will be obtained due to glycolysis. The enzymatic or O-toluidine colorimetric techniques can be used. A semi quantitative technique using Benedict's reagent is also useful (Cheesbrough, 1984).

3.12.3.2 Total protein estimation and globulin test: - Protein can be estimated from fluoride oxalated CSF or from an unpreserved specimen. Total protein is estimated by precipitating with trichloroacetic acid using either colorimetric technique or semi-quantitative visual method. Pandy's test is recommended for screening increase in globulin. The normal protein level in CSF is 15-40 mg%. Values up to 100 mg% are normal for new born infants. Only traces of globulin are found in normal CSF (insufficient to give positive Pandy's test). Increase in later protein with positive Pandy's test occur in all form of meningitis, in amoebic and trypanosomiasis, meningoencephalitis, cerebral malaria, brain tumors, cerebral injury, spinal cord compression, poliomyelitis and polyneuritis (Cheesbrough, 1984).

3.12.4 Culture:-

Although culture is not rapid, it is important to obtain viable organisms for the antimicrobial susceptibility testing and for epidemiological investigations as well as for detailed identification (Easmon, 1990).

After vortexing the sediment and preparing smears several drops of the sediment should be inoculated to each media- chocolate agar plate, 5% sheep blood agar plate, MacConkey agar and an enrichment broth (usually thioglycollate broth without

indicator) which are used as routine culture media. The plates are incubated at 37°C in 5-10% CO₂ in humid environment for 72 hrs except MacConkey agar plate which is incubated at 37°C aerobically for 24 hrs. The broth should be incubated aerobically at 37°C for at least 5 days. The broth cap must be loose to allow free exchange of air. If fungal meningitis is suspected, the specimen is inoculated onto two Sabouraud Dextrose agar (SDA) slant and incubated for at least 7 days, one at 30°C and other at 35°C. If tuberculous meningitis is suspected, CSF sediment is centrifuged at 3600xg for 30 min to concentrate bacteria. The supernatant is decanted and sediment is vortexed thoroughly and inoculated on Lowenstein Jensen or modified Ogawa slant and incubates at 37°C for at least 8 weeks. If amoebic meningoencephalitis is suspected, free living amoebae can be cultivated on artificial media if they are supplied with a living nutrient, such as *Klebsiella pneumoniae* or *E.coli*. CSF may be directly inoculated to tissue culture medium like monkey kidney Hep-2 continuous cell line and human fetal diploid fibroblast cell cultures which are incubated at 25°C in air for various amount of time, depending on the cell culture system. Diagnosis of viral encephalitis is often accomplished by isolation of virus from throat swab culture, feces or blood, so these specimens should be submitted in addition to CSF, for the identification of an etiological agent (Baron *et al.*, 1994).

3.12.5 CSF serology:-

The antigens of many important bacterial pathogens responsible for meningitis can be detected in the CSF by means of specific antibodies. The sensitivity and specificity of antigen depend both on quality of antisera and the technique used. Serological procedures are rapid and particularly useful when patient has already received chemotherapy.

3.12.5.1 Counter current immunoelectrophoresis (CIE):- The antigen-antibody complex is detected as a line of precipitation in agarose gel. Electrophoresis is performed at the isoelectric point of immunoglobulin (pH 8.2-8.6). The antigen carries a negative charge and migrates towards anode and the antibody, being neutral, is carried

towards the cathode in the counter current endosmotic flow. The antigen detection limit is 30-100ng/ml.

3.12.5.2 Latex agglutination test (LAT):- Commercial systems use principle of antibody coated particles that will bind to specific antigen, resulting in macroscopically visible agglutination. The soluble capsular polysaccharide produced by most common etiological agents of meningitis and Group B streptococcal polysaccharide is detected. The agglutination tests require no specific equipments and are more sensitive than CIE. However, *Neisseria meningitidis* group B and *E.coli* serotype K1 present particular problems because of the poor immunogenecity as the antigen concerned and consequent lack of good antisera.

3.12.5.3 Quellung reaction: - CSF is mixed with antisera against suspected organisms (Hib, meningococci, pneumococci) and a drop of saturated aqueous methylene blue dye. The presence of organisms is indicated by the swelled capsular polysaccharide under microscope. The test requires considerable experience and is frequently not reproducible between reference laboratories.

3.12.5.4 Syphilis serology:-The VDRL test is useful test for detecting antibody against *Treponema pallidum* in CSF.

3.12.5.5 ELISA: - It is the rapid test and is used to detect antigens of pathogens (such as Hib, meningococci, pneumococci, mycobacteria) in CSF by utilizing enzyme labeled specific antibodies.

3.13 Molecular technique:

PCR- This method is potentially sensitive enough to detect a single microbe in any patient sample and being rapid, a large number of samples can be processed in a day. This method is of particular importance to detect slow growing organisms such as *Mycobacterium tuberculosis* (at least 8 weeks) and *Cryptococcus neoformans* (>2 days) and is equally important for other organisms.

3.14 GLOBAL SCENARIO OF MENINGEAL INFECTION

Bacterial meningitis is an important cause of morbidity and mortality worldwide (Weber *et al.*, 2002). Timely diagnosis and management with appropriate and the adequate antibiotic is of outmost importance to prevent mortality and long term morbidity. World wise two third of the cases of meningitis occur below the age of 15 years. So the major burden of this disease is being shared by the pediatrics. In the pediatric age group, more than 75% of the cases occur below the age of 5 and of these, 50% of the cases occur below the age of 2 years (Ahmed *et al.*, 2004). For Asia as a whole, of the total of 51272 cases with defined etiology, 34% were caused by Hib, 23% by pneumococci, 14% by meningococci, 4 % by salmonellae and 25% by other bacteria (Peltola, 1999).

Acute Bacterial Meningitis (ABM) is one of the most severe infectious diseases. The global burden of the disease is high. Apart from epidemic, at least 1.2 million cases of meningitis are estimated to occur every year with 135000 deaths (Tikhomirov *et al.*, 1997). It is caused by a variety of microorganisms but, beyond the neonate period, the most important ones are *Streptococcus pneumoniae* and *Haemophilus influenzae* (Molyneux *et al.*, 1998; Lehmann *et al.*, 1999; Palmer *et al.*, 1999).

The incidence of bacterial meningitis is higher in developing countries than developed countries (Murray and Lopez, 1996) and is particularly high in children under one year of age. Case fatality rates (CFR) for bacterial meningitis range from 4.5% in developed countries to 15-50% in developing countries (Barraf *et al.*, 1993). A further 15-20% of survivors sustain neurologic sequelae (Qazi *et al.*, 1996). *H. influenzae*, *S. pneumoniae* and *N. meningitidis* are the common bacteria causing meningitis in fewer than five year old children (Wanyoike *et al.*, 1995; Vandepitte *et al.*, 2004).

A 5-year retrospective study with acute pyogenic meningitis admitted to a district hospital in north-western Ethiopia was carried out from 1990 to 1994. A total of 132 cases of pyogenic meningitis were identified. The causative bacteria were identified in 85 (64%) patients. The most common pathogen was *Haemophilus influenzae* (40%)

with a case fatality rate of 29.4%, followed by *Neisseria meningitidis* (36.4%), which had a case fatality rate of 16.1%, and *Streptococcus pneumoniae* (20%) with a case fatality rate of 35.3%. The overall case fatality rate was 28%. The mortality rate of children below the age of 1 year was 38.4% and 13.8% for those above 1 year (Etsegenet and Rahlenbeck, 1995).

The incidence of acute bacterial meningitis was 0.8%, with a case fatality rate of 13.0%. Children of 1 year of age were more affected (64.9%). The total male to female ratio was 1.2:1. Gram stain detected more cases (85.7%) than culture (66.2%). A total of 48 isolates were identified by culture and their antibiotic sensitivity was determined. *Haemophilus influenzae* (33.8%) was the predominant organism identified, followed by *Streptococcus pneumoniae* (26.0%), *Klebsiella* spp (6.5%) and *Neisseria meningitidis* (2.6%). Many of the bacterial isolates were found to be sensitive to gentamicin, cefotaxime and ceftriaxone and least sensitive to tetracycline and ampicillin (Rao *et al.*, 1998).

In studies that include all age groups, typically with a majority of the cases occurring among children, *H. influenzae*, *N. meningitidis*, and *S. pneumoniae* cause 70 to 87 percent of all cases (Carpenter, 1962).

In a study of 696 episodes of community-acquired acute bacterial meningitis. The most common pathogens were *Streptococcus pneumoniae* (5%) and *Neisseria meningitidis* (37%) (Van de Beek *et al.*, 2005).

A study done in India showed Gram positive isolates in adults and gram negative bacilli were the predominant organisms that cause meningitis. *Streptococcus pneumoniae* (21.42%), -haemolytic streptococci (14.29%), *Staphylococcus aureus* (7.14%) and Micrococci (4%) were isolated in the adult group while *Escherichia coli*, *Streptococcus pneumoniae*, nonfermenters (6.38% each), *Klebsiella* and *Pseudomonas* species (2.12% each), and *Staphylococcus aureus* (4.25%) were isolated in the pediatric age group (Tankhiwale *et al.*, 2001).

A study conducted at three major referral hospitals in Guatemalan city found that Hib was identified in 20.0% with meningitis and *S. pneumoniae* in 12.9%. The average annual incidence of Hib meningitis was 13.8 cases per 100 000 children under 5 years of age, and 32.4% of meningitides caused by Hib and 58.7% of *S. pneumoniae* meningitides occurred prior to 6 months of age. Case fatality rates were 14.1%, 37.0%, and 18.0%, respectively. Over all, case fatality rate due to meningitis was found to be 23%. Prior antibiotic therapy was common and was associated with significant reductions in CSF–culture–positive results with other evidence of Hib or *S. pneumoniae* meningitis (Asturias *et al.*, 2003).

In a study conducted for 20 months period at Al-Thawrah hospital, Sana'a, Yemen 01/01/2001 to 23/8/2002, only 63 (81.81%) out of 77 suspected patients were diagnosed as acute bacterial meningitis (ABM). The most affected age group was 4 months and 3 years. Based on CSF examination; there were 23 with positive findings of CSF analysis (turbid, decrease glucose <40mg/dl, increase protein >80mg.dl and leucocytes neutrophils >5cells.cu.mm), 21 cases were confirmed with CSF culture, while the remaining 19 cases showed normal CSF pictures. The fatality rate was 14.28%. The predominant organism in CSF culture was *Klebsiella pneumoniae* (33.33%) followed by *Haemophilus influenza* (23.80%), *Streptococcus pneumoniae* (14.28%) then 2 cases each; *E.coli*, *Pseudomonas* (9.52%) while with one cases each of tubercles meningitis and *Staphylococcus aureus* (Sallam, 2004).

In a prospective surveillance study conducted in Iasi and Nonstanta districts of Romania, from March 2000 through March 2002, children <5 years of age hospitalized for bacterial meningitis were enrolled. A total of 56 cases of bacterial meningitis were identified, including 37 due to *Neisseria meningitidis* (22 per 100,000 per year), 13 due to *Haemophilus influenzae* type b (7.6 per 100,000 per year), and six due to *Streptococcus pneumoniae* (3.5 per 100,000 per year). Of the 31 meningococcal isolates that were serotyped, 12 were serogroup A, eight were serogroup B, and 11 were serogroup C. Among all cases of bacterial meningitis, 25 occurred in children <1 year

of age, including those due to meningococci ($n=14$), *H. influenzae* type b ($n=7$), pneumococci ($n=3$), and *Klebsiella pneumoniae* ($n=1$) (Luca *et al.*, 2004).

A study conducted in Pakistan among the suspected patients showed that three most common pathogens isolated were *Streptococcus pneumoniae* (22.5%), *Neisseriae meningitidis* (16.7%) and *Haemophilus influenzae* type b (9.2%) (Ahmed *et al.*, 2004).

A study conducted among 124 patients with meningitis to review the etiology, antimicrobial susceptibility and outcome of the disease in a Nigerian territory health facility showed 78.27% culture positive results and in the rest 21.8% diagnosis was based on gram staining of the CSF. *Streptococcus pneumoniae*, *Hemophilus influenzae* type b, *Neisseriae meningitidis*, *Staphylococcus aureus* and *Esherichia coli* were isolated in 33.9%, 33.9%, 5.6%, 2.4% and 2.4% samples respectively. All the isolates had 100% sensitivity to both ceftriazone and ciprofloxacin while sensitivities to penicillin and ampicillin were remarkably low. The mortality rate was 26.6 % while 17.6% of the survivors had various neurologic sequale (Ogunlesi *et al.*, 2005).

In a study conducted in Cuba, a nationwide observational study on children and adolescents from 1 to 18 years old was carried out from 1998 to 2003. The overall number of cases was 1023; the incidence ranged from 3.4 to 8.5 per 100 000 population, with the higher figures in children 1–5 years old (16.8 per 100000 population). *Streptococcus pneumoniae*, *Haemophilus influenzae* type b and *Neisseria meningitidis* serogroup B were the main identified agents. The average case-fatality rate was 10.5% and the most lethal agents were *Streptococcus pneumoniae* (27%) and *Haemophilus influenzae* type b (10.7%) (Perez and Dickinson, 2005).

3.15 SCENARIO OF MENINGEAL INFECTIONS IN NEPAL

A study done in the period of April 1974 to 1978, out of the 4.45% of the central nervous system disorder admitted in Kanti Children's Hospital 41.1% was meningitis out of which 33% comprised septic meningitis and 50% occurred in infants below one year of age, while 85% occurred below 5 years of age .The death rate was 24% (Shrestha, 1983).

A retrospective study conducted at Kanti children Hospital pointed out that 2.8% of the total children admitted at Kanti Childrens Hospital were patients with meningitis out of which 84.2% were with pyogenic meningitis, 15.7% with tubercular meningitis; 31.2% were below one year of age and 66.6% were males. The commonest modes of presentation were fever (91.6%) and vomiting (72.9%) (Sharma, 1983).

A study done to find out epidemiological aspects of Meningococcal Meningitis it was found that there was a high death rate (19.2%) of all admitted cases of meningitis in the different hospitals in Kathmandu valley (Pradhan, 1983). In other 2 year study at Kanti Children's Hospital, it was found that in the two year period, meningitis constituted 3.2% of the total admission at Kanti Childrens Hospital out of which 80.6% were due to pyogenic meningitis and 19.3% were tuberculous in origin. 23.5% were below the age of one year and 61.3% were males. The overall mortality rate was 7.9% (Sharma and Dixit, 1984).

A study done in the Pediatrics Ward of Bheri Zonal Hospital concluded that meningitis was the second most frequent disease of the central nervous system admitted in Bheri Zonal Hospital (Subedi, 1985).

In an epidemiological study, it was found that during the first six months of 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 100000 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).

In a study done by Tiwari (2002) at TUTH, highest incidence of meningitis was found. The rate of incidence of meningitis among patients was found to be 10.79%.

A prospective study in Katmandu from the June 1993 to February 1994 suspected to be suffering from meningitis and whose clinical features and microscopic and biochemical studies of the cerebrospinal fluid suggested bacterial meningitis showed Latex agglutination test (LAT) positive in 33 (80.5 %) out of the 41 cases. 18 (54.5%) of the LAT positive results revealed *Haemophilus influenzae*, 10 (30.3%) revealed *Neisseria meningitidis*, 4 (12.1%) revealed *Streptococcus pneumoniae*, and 1 (3.0%) revealed group B Streptococcus. LAT and positive result of Gram stain corresponded in 71.87% cases (Tiwari, 2003).

In a study done at Tribhuvan University Teaching Hospital (TUTH), a total of 77 Cerebrospinal fluid (CSF) samples from suspected meningitis cases were collected during September 2001 to March 2002, to observe leukocytes count in the fresh cerebrospinal fluid (CSF) samples and bacterial growth after cultivation. Growth was not revealed from the samples (64/77) containing <100 leukocytes/mm³ and blood contaminated samples (4/77) within normal blood leukocyte count, 4000-11000 leukocytes/mm³. Altogether five (5/77) isolates were recovered. Two third (66.7%) of the samples with the pleocytic range 100-200/mm³ were found to contain *Mycobacterium tuberculosis* (1/77) and *Aeromonas* spp (1/77). Similarly, 50.0% isolates from CSFs (2/4) containing 200-500 leukocytes/mm³ were found to be *Staphylococcus aureus* (1/77) and *Acinetobacter* spp (1/77). From a heavily blood contaminated CSF sample (more than the normal range of leukocyte count in blood, >11000 /mm³), *Klebsiella pneumoniae* was recovered. Concluding that the CSF containing 100-500 leukocytes/mm³ clearly indicates pyogenic or tuberculous meningitis (TBM) (Tiwari *et al.*, 2004).

In a study done by Tuladhar (2007) showed 4.87% meningitis cases among suspected cases (431).

CHAPTER – IV

4. MATERIALS AND METHODS

The present study was conducted at Patan Hospital from May, 2007 to March, 2008. During this period CSF samples from 466 patients suspected of meningitis were processed in the microbiology laboratory. The samples were processed according to the standard protocol. Briefly, the visible appearance of the CSF samples was noted, leucocyte count count was performed, Gram staining was performed, serological tests for *Haemophilus influenzae* type b and *Streptococcus pneumoniae* (latex agglutination, Binax) were also done, protein and glucose level was also determined in CSF. The samples were cultured on different culture media. After isolation antibiotic susceptibility pattern of different isolates were also observed.

4.1 MATERIALS

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

4.2 METHODS

4.2.1 Collection of samples

The CSF samples were collected in the Patan Hospital by experienced medical officer, puncturing the lumbar region at L3-L4 level. From every patient, four samples were collected, 1st for the cell count, 2nd for biochemical analysis, 3rd for culture and the 4th for cell count (repetition). The samples were immediately brought to the laboratory and processed immediately.

4.2.2 Macroscopic observation of CSF sample

Each CSF sample was observed for its physical appearance with naked eye. The color and the turbidity of the CSF samples were noted. The samples were categorized as clear, slightly turbid, turbid and highly turbid in terms of its turbidity and as watery, xanthochromic or traumatic (mixed with blood) in terms of its color. Based on the appearance, the interpretation was made on the basis of standard table.

4.2.3 Total leucocyte count

Each CSF sample was diluted in Turk solution in different proportions on the basis of the appearance and turbidity. A drop of CSF sample was taken from the fresh CSF sample with the help of sterile micropipette and transferred into a clean dried small test tube (5ml). Then a drop of Turk's Solution was added to the tube to obtain 1:2 dilutions if the CSF sample was clear. For the turbid CSF sample, further dilutions were made as follows: 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 1mm² area 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 micropipette was used to charge the counting chamber. The charged counting chamber was allowed to stand for about a minute and observed in the microscope under 10X objective. The leukocytes were counted on each corner square. Total number of leukocytes per mm³ was obtained by using following calculation:

$$\text{Total Leukocyte Count/mm}^3 = \frac{\text{Total cell X Dilution Factor X Depth Factor}}{\text{Area Counted}}$$

Where, Depth Factor = 10 Area counted = 4

4.2.4 Estimation of glucose and protein

Glucose and protein of the CSF samples were determined by the glucose oxidase peroxidase method and sulfosalicylic method respectively.

For the estimation of sugar, three test tubes were taken and then marked as T, S and B respectively. In all these three test tubes, 2000µl or 2ml of reagent was added. 20µl of CSF sample was added in the tube marked as T, 20µl of standard solution (100 mg %) was added in the tube marked as S and 20µl of distilled water was added in test tube marked as B. All the three tubes were then placed at water bath for ten minutes. After

10 min, the O.D of the test sample and standard were measured in colorimeter by using 520nm filter after setting the colorimeter at zero using blank. Then the sugar concentration of the sample was calculated by using the following formula:

$$\text{Sugar (mg \%)} = \frac{\text{O.D of the Test}}{\text{O.D of Standard}} \times \text{Concentration of Standard}$$

For estimation of protein, sulphosalicylic acid method was used which is based on the principle that proteins are precipitated by sulphosalicylic acid giving visual turbidity. For this, standard set of different concentration of protein was prepared (Appendix II). In a sterile test tube 0.05 ml of CSF sample was taken and 1.5 ml of 3% sulphosalicylic acid solution was added. The tube was shaken well and incubated for 10 minutes at room temperature. Then the turbidity of the tube was compared and matched with the standard and the concentration was expressed in mg/dl.

4.2.5 Latex agglutination test for *H. influenzae* type b

Specific antibodies are bound to the surface of latex beads. Latex particle aggregation becomes large enough to allow rapid visualization of positive agglutination in the presence of specific antigens. These specific soluble polysaccharide antigens in the CSF as a result of infection by *H. influenzae* type b (Directigen, 2005). The detailed protocol for this test is mentioned in appendix XIII.

4.2.6 Binax test for Pneumococcal antigen

The Binax *Streptococcus pneumoniae* test is an immunochromatic membrane assay used to detect pneumococcal soluble antigen in Human CSF. Rabbit anti-*S. pneumoniae* antibody, the sample line, is adsorbed onto nitrocellulose membrane. Control antibody is adsorbed onto the same membrane as a second stripe. Both rabbit anti- *S. pneumoniae* and anti-species antibodies are conjugated to visualizing particles that are dried onto an inert fibrous support. The resulting conjugate pad and striped membrane are combined to construct the test strip. This test strip and a well to hold the swab specimen are mounted on opposite sides of a hinged, book-shaped test device (Binax, 2005). The detailed protocol for this test is mentioned in appendix XIII.

4.2.7 Microscopic examination

The CSF samples were centrifuged at 3000x g revolutions per minute for 10 minutes. Gram staining of the deposit of the centrifuged was performed (Appendix III).

ZN staining was also performed in the case of samples collected for detection of AFB. CSF sample was centrifuged for 30 min with 3000x g revolutions per minute. ZN staining was done according to standard protocol (Appendix IV).

4.2.8 CSF culture

The CSF sample was inoculated on the chocolate agar plate, blood agar plate and MacConkey agar plate. Blood agar and chocolate agar plates were incubated anaerobically (5% CO₂) at 37°C for 24 hours and MacConkey agar plates were incubated at 37°C for 24 hours.

4.2.9 Identification of the isolate

The culture plates were examined after 24 hours 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 which include observation of colony morphologies, staining reactions, and biochemical properties (Cheesbrough, 1984 and Collee *et al.*, 1996).

Biochemical tests:-

Appropriate biochemical tests were performed for the confirmed identification of the bacterial isolates.

Gram-positive organisms were identified primarily on the basis of their response to gram's staining, catalase, oxidase and coagulase tests.

-) For the identification of *Streptococcus pneumoniae*, bile solubility test was done.

This test was done for the differentiation of pneumococci from viridans streptococci.

For this test, the isolate was first grown in 5 ml brain heart infusion broth for 18 hrs at 37°C.

While still warm, 0.5 ml of 10% sodium deoxycholate solution was added and reincubated at 37°C.

The result was then noted within 15 min where the initially turbid culture becomes clear and transparent due to the lysis of pneumococci.

-) The biochemical tests used for the identification of gram-negative bacterial isolates include Catalase test, Oxidase test, Indole test, Methyl red test, Voges Proskauer test, Citrate utilization test, Triple Sugar Iron (TSI) test, Urease test, Motility test and Gas production tests.
-) The *Haemophilus influenzae* were identified by 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 Muller-Hinton agar. Further, the colony was also confirmed by 'Satellitism' phenomenon on blood agar.

For this test, the pure isolate of *Haemophilus influenzae* was inoculated onto the blood agar plate.

A streak of *Staphylococcus aureus* was applied across the surface of an inoculated plate and incubated at 5% of CO₂ for 48 hrs.

After proper incubation, colonies of *Haemophilus influenzae* will be larger where they are within 1-3mm of the *Staphylococcus aureus*, which exerts 'V' factor. This phenomenon is known as 'Satellitism' (Cheesbrough, 1984; Collee *et al.*, 1996).

The composition and preparation of biochemical media and reagents used in the biochemical test are mentioned in the Appendix-II. The procedures for biochemical tests are mentioned in Appendix-V.

4.2.10 Antibiotic susceptibility pattern of bacterial isolates

The antimicrobial susceptibility testing of the isolates towards various antimicrobial discs was done by modified Kirby-Bauer disc diffusion method as recommended by Clinical and Laboratory Standards Institute (CLSI-M100, 2007) using Mueller Hinton agar (MHA).

The pure culture of the isolate was inoculated into Mueller Hinton broth and incubated for about 4 hours at 37°C and then using a sterile swab the organism was swabbed on Mueller Hinton agar plate. For the isolates like *S. pneumoniae*, Mueller Hinton agar containing 10% of sheep blood was used for antibiotic susceptibility testing. Antibiotic susceptibility pattern of *Haemophilus influenzae* was determined using chocolate agar. Commercially available antibiotic discs (Hi media) were used. Appropriate discs were placed on MHA containing 10% blood and chocolate agar plates and were incubated anaerobically (10% CO₂) for 24 hours.

After incubation the inhibition zone diameter was measured and the results were interpreted on the basis of the standard chart (Appendix-VIII and IX). All tests were performed with regular quality control. During the study, the sterility of each batch of the test medium was confirmed by incubating uninoculated plates and tubes overnight at 37°C. 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. Along with the test organism, *Pseudomonas aeruginosa* (ATCC27853) and *Staphylococcus aureus* (ATCC 25923) were used to check the quality of the medium and performance of the test.

4.2.11 Statistical analysis

Chi-square test was used to determine significant association of leucocyte count with that of culture result and association between culture results and genders (Appendix XI).

CHAPTER V

5. RESULTS

The present study was conducted among the patients suspected of meningitis attending Patan Hospital. A total of 464 CSF samples collected by the experienced physician from the patients were processed within the study period of 10 months (May 2007-March2008). The samples were processed in the Microbiology laboratory of Patan Hospital.

5.1 CULTURE POSITIVITY OF CSF

In all the samples cultured on Chocolate agar and blood agar, only 20 (4.31%) samples showed growth while remaining 95.69% were growth negative (Table 3).

Table3: Culture result

Total samples	Growth		No Growth	
	N	%	N	%
464	20	4.31	444	95.69

N= Number of CSF samples

5.2 CULTURE POSITIVITY WITH RESPECT TO AGE AND GENDER

Eight (3.23%) and 12 (5.52%) organisms were isolated from 247 male and 217 females respectively. The highest positive samples were (1.72%) from neonates (0-45 days) followed by age group 45days -1year (1.50%) (Table 4 and figures 1).

Table 4: Age and gender wise distribution of growth positive samples

Age	Male (N=247)		Female (N=217)		% among positives
	N	%	N	%	
0-45 days (Neonates) (N=299)	2	0.81	6	2.76	40
45D-1yr (N=65)	3	1.21	4	1.84	35
1Yr-5yrs (N=46)	0	0	1	0.46	5
5Yrs-40yrs (N=26)	0	0	1	0.46	5
>40yrs (N=28)	3	1.21	0	0	15
Total	8	3.23	12	5.52	100

N= Number of CSF samples

Male: female=1.14:1

5.3 CULTURE POSITIVITY WITH RESPECT TO APPEARANCE OF CSF

Of the total 20 positive culture results, 70% of the growth positive cases were from highly turbid samples, 25% from slightly turbid samples and remaining 5% were from traumatic samples (Table 5).

Table 5: Culture positivity with respect to appearance of CSF

Appearance of CSF	Growth		% among positives
	N	%	
Clear (N=334)	0	0	0
Xanthochromic (N=19)	0	0	0
Slightly turbid (N=23)	5	21.73	25
Highly turbid (N=44)	14	31.82	70
Traumatic (N=44)	1	2.27	5

N= Number of CSF samples

5.4 GRAM STAINING RESULT OF CSF

Out of 464 CSF samples received, presence of bacterial cells were observed in 26 (5.60 %) CSF samples. Out of 26 samples which showed organism during Gram stain, 14 (53.84%) were Gram positive and 14 (46.16%) were Gram negative organisms. Among Gram positive, diplococci (42.30%) were found to be predominant and among Gram negative, coccobacilli (34.61%) were found to be predominant (Table 6).

Table 6: Culture positivity with reference to Gram staining

Gram staining result	Growth		% among positives
	N	%	
Gram positive diplococci (N=11)	10	90.9	50
Gram positive cocci in cluster (N=2)	1	50	5
Gram negative coccobacilli (N=9)	6	66.67	30
Gram negative bacilli (N=2)	2	100	20
Gram negative diplococci (N=1)	0	0	0
Gram positive (Yeast) (N=1)	1	100	5
Total	20		100

N= Number of CSF samples

5.5 CORRELATION OF CULTURE POSITIVITY AND LEUCOCYTE COUNT OF CSF

Out of 464 CSF samples received in the laboratory, 248 samples had normal leucocyte count (0-5 cell/mm³). 163 CSF samples had cell count in the range of 5-100 cells/mm³. in all the CSF samples having leucocyte count between 5-100 cells/mm³, 4 (2.45%) isolates were obtained. Similarly 53 samples had >100 cells/mm³ and highest number of isolates i.e. 16 (30.18%) were obtained from these samples (Table 7).

Table 7: Correlation of culture positivity and leucocyte count of CSF

Total samples	Leucocyte count/mm ³	Growth positive samples	
		N	%
464	<5 (N=248)	0	0
	5-100 (N=163)	4	2.45
	>100 (N=53)	16	30.18

N= No. of CSF samples

5.6 CORRELATION OF PROTEIN CONCENTRATION OF CSF WITH CULTURE POSITIVITY

Among 464 cases of suspected meningitis, 283 CSF samples were found to have normal protein level where as 12 of samples had protein level <15 mg/dl. Similarly, 75 CSF samples were found to have protein level >100 mg/dl and 94 samples had protein level in the range of 45-100 mg/dl. Most of the culture positive samples (17.33%) contained protein >100 mg/dl followed by 45-100 mg/dl (7.44%). No organism was isolated from the CSF samples which had normal protein level and protein level less than 15 mg/dl (Table 8).

Table 8: Correlation of protein concentration of CSF with culture positivity

Total samples	Protein level of CSF (mg/dl)	Growth positive samples	
		N	%
464	<15 (N=12)	0	0
	15-45 (Normal Level) (N=283)	0	0
	45-100 (N=94)	7	7.44
	>100 (N=75)	13	17.33

N= Number of CSF samples

5.7 CORRELATION OF GLUCOSE LEVEL WITH CULTURE POSITIVITY

Of the total 466 CSF samples, 351 of CSF samples had normal Glucose level and only 7 (1.99%) samples were growth positive. Similarly, 85 CSF samples had glucose level <40 mg/dl and highest number of samples 12 (13.79) were growth positive. Only 26 samples had >80 mg/dl of glucose level and only one sample was growth positive (Table 9).

Table 9: Correlation of glucose level with culture positivity

Total samples	Glucose level of CSF (mg/dl)	Growth positive samples	
		N	%
464	<40 (N=87)	12	13.79
	40-80 (Normal Level) (N=351)	7	1.99
	>80 (N=26)	1	3.84

N= Number of CSF samples

5.8 DETECTION OF *Haemophilus influenzae* type b IN CSF SAMPLES

Two hundred and sixteen samples (those having cell count >5) were tested for the presence of *Haemophilus influenzae* type b by latex agglutination. Only 9 samples showed agglutination and out of which only 6 (66.67%) were able to grow on culture media (Table 10).

Table 10: Detection of *Haemophilus influenzae* type b in CSF by Latex Agglutination test

Total sample	Latex Agglutination Test	Growth of <i>Haemophilus influenzae</i> type b on Chocolate agar
216	Positive	Positive
	9	6

5.9 DETECTION OF *Streptococcus pneumoniae* IN CSF BY BINAX TEST

Two hundred and sixteen samples (those having cell count >5) were tested for the presence of *Streptococcus pneumoniae* by Binax antigen detection test. Only 10 samples showed positive for the test and out of which 9 (90%) were able to grow on culture media (Table 11).

Table 11: Detection of *Streptococcus pneumoniae* in CSF by Binax test

Total sample	Binax Antigen Detection Test	Growth of <i>Streptococcus pneumoniae</i> on Blood agar
216	Positive	Positive
	10	9

5.10 MICROBIOLOGICAL PROFILE

In all the 464 samples of suspected meningitis patients, 20 were growth positive. Seven different genera were identified from culture positive samples. Among these, 19 were belonging to bacteria and remaining 1 was fungi. Out of 19 bacterial isolates, Gram positive organisms were predominant (52.38%). *Streptococcus pneumoniae* (40.90%) was found to be the most predominant organism among the isolates followed by *Haemophilus influenzae* type b (27.26%) (Table 12 and figure 2).

Table 12: Distribution of microorganisms in culture positive CSF samples

Organisms	N	% (N=20)
<i>Citrobacter freundii</i>	1	5
<i>Cryptococcus neoformans</i>	1	5
<i>Haemophilus influenzae b</i>	6	30
<i>Pseudomonas aeruginosa</i>	1	5
<i>Staphylococcus aureus</i>	1	5
<i>Streptococcus pneumoniae</i>	9	45
<i>Viridans streptococci</i>	1	5
Total	20	100

N= Number of CSF samples

5.11 MICROBIOLOGICAL PROFILE OF MENINGITIS PATIENTS (AGE WISE)

Most of the bacterial isolates were found in CSF samples taken from age group 0-45 days (40%) followed by age group 45days-1year (35%). *Haemophilus influenzae* and *Streptococcus pneumoniae* were isolated from the age group <5 years. *Cryptococcus neoformans* was isolated from age group >40 years (Table 13).

Table 13: Distribution of microorganisms on different age group of patients

Organisms (N=20) N= Number of cases	Age									
	0-45D		45D-		1-5Yrs		5-		>40Yrs	
	N	%	N	%	N	%	N	%	N	%
<i>Haemophilus influenzae b</i> (N=6)	2	10	4	20	0	0	0	0	0	0
<i>Streptococcus pneumoniae</i> (N=9)	3	15	3	15	1	5	0	0	2	10
<i>Citrobacter freundii</i> (N=1)	1	5	0	0	0	0	0	0	0	0
<i>Pseudomonas aeruginosa</i> (N=1)	1	5	0	0	0	0	0	0	0	0
<i>Viridans streptococci</i> (N=1)	1	5	0	0	0	0	0	0	0	0
<i>Cryptococcus neoformans</i> (N=1)	0	0	0	0	0	0	0	0	1	5
<i>Staphylococcus aureus</i> (N=1)	0	0	0	0	0	0	1	5	0	0
Total	8	40	7	35	1	5	1	5	3	15

5.12 ANTIBIOTIC SUSCEPTIBILITY PATTERN OF THE BACTERIAL ISOLATES OF CSF SAMPLES

Among the gram negative isolates, *Haemophilus influenzae* type b was 100% sensitive to Cefotaxime, Ciprofloxacin, Gentamicin and Ofloxacin followed by Chloramphenicol (83.34%) and Erythromycin (50%). Most of these isolates were resistant to Penicillin and Amoxicillin (84.34%) and Co-trimoxazole (100%). *Pseudomonas aeruginosa* was sensitive to Amikacin, Cefotaxime, Gentamicin and Tobramicin while they were resistant to Ciprofloxacin and Co-trimoxazole. *Citrobacter freundii* was sensitive to Chloramphenicol, Ciprofloxacin and Ofloxacin while it was resistant to Amikacin, Cefotaxime, Amoxicillin, Amoxyclav, Co-trimoxazole and Tobramicin.

Out of 9 isolates of *Streptococcus pneumoniae*, all were 100% sensitive to all the antibiotics used while some of these isolates were resistant to Co-trimoxazole (11.11%) and Erythromycin (88.89%). *Streptococcus viridans* was 100% sensitive to all the antibiotics used while it was resistant to Co-trimoxazole and Erythromycin. All the isolate (100%) *Staphylococcus aureus* were sensitive to Amikacin, Cefotaxime, Cloxacillin and Ofloxacin while it was resistant to Chloramphenicol and showed intermediate sensitivity to Ciprofloxacin and Ceftriaxone (Table 14).

Table 14: Antibiotic susceptibility pattern of bacterial isolates

Organisms	Antibiotics Used	Susceptibility Pattern					
		Sensitive		Intermediate		Resistant	
		N	%	N	%	N	%
<i>Haemophilus influenzae</i> (type b) N=6	Amoxicillin	0	0	1	16.66	5	83.34
	Cefotaxime	6	100	0	0	0	0
	Chloramphenicol	5	83.34	1	16.66	0	0
	Ciprofloxacin	6	100	0	0	0	0
	Co-trimoxazole	0	0	0	0	6	100
	Erythromycin	3	50	3	50	0	0
	Gentamicin	6	100	0	0	0	0
	Ofloxacin	6	100	0	0	0	0
	Penicillin	1	16.66	0	0	5	83.34

<i>Citrobacter freundii</i> N=1	Amikacin	0	0	0	0	1	100
	Amoxicillin	0	0	0	0	1	100
	Amoxyclav	0	0	0	0	1	100
	Cefotaxime	0	0	0	0	1	100
	Ceftazidime	0	0	0	0	1	100
	Chloramphenicol	1	100	0	0	0	0
	Ciprofloxacin	1	100	0	0	0	0
	Co-trimoxazole	0	0	0	0	1	100
	Ofloxacin	1	100	0	0	0	0
	Piperacillin +Tazobactam	1	100	0	0	0	0
	Tobramycin	0	0	0	0	1	100
<i>Pseudomonas aeruginosa</i> N=1	Amikacin	1	100	0	0	0	0
	Cefotaxime	1	100	0	0	0	0
	Chloramphenicol	0	0	1	100	0	0
	Ciprofloxacin	0	0	0	0	1	100
	Co-trimoxazole	0	0	0	0	1	100
	Gentamicin	1	100	0	0	0	0
	Ofloxacin	0	0	1	100	0	0
	Penicillin	0	0	0	0	1	
	Tobramycin	1	100	0	0	0	0
<i>Streptococcus pneumoniae</i> N=9	Amoxicillin	9	100	0	0	0	0
	Cefotaxime	9	100	0	0	0	0
	Chloramphenicol	9	100	0	0	0	0
	Co-trimoxazole	1	11.11	2	22.23	6	66.66
	Erythromycin	8	88.89	0	0	1	11.11
	Ofloxacin	9	100	0	0	0	0

	Penicillin	9	100	0	0	0	0
	Tetracycline	9	100	0	0	0	0
Viridans <i>streptococci</i> N=1	Amoxicillin	1	100	0	0	0	0
	Cefotaxime	1	100	0	0	0	0
	Chloramphenicol	1	100	0	0	0	0
	Co-trimoxazole	0	0	0	0	1	100
	Erythromycin	0	0	0	0	1	100
	Ofloxacin	1	100	0	0	0	0
	Penicillin	1	100	0	0	0	0
	Tetracycline	1	100	0	0	0	0
<i>Staphylococcus aureus</i> N=1	Amikacin	1	100	0	0	0	0
	Cefotaxime	1	100	0	0	0	0
	Ceftriazone	0	0	1	100	0	0
	Chloramphenicol	0	0	0	0	1	100
	Ciprofloxacin	0	0	1	100	0	0
	Cloxacillin	1	100	0	0	0	0
	Ofloxacin	1	100	0	0	0	0

N= Number of isolates tested

CHAPTER-VI

6. DISCUSSION AND CONCLUSION

6.1 DISCUSSION

The present study was conducted among the patients suspected of meningitis attending Patan Hospital, Patan, Nepal to correlate the macroscopy, serology and microscopy of CSF samples with that of culture. Four hundred and sixty-four CSF samples were collected from the patients suspected of meningitis and were subjected for the cell count, biochemical, serological and microbiological examination.

In the present study, altogether 464 CSF samples were processed and out of 464 samples only 20(4.31%) samples were culture positive. The result of the present study was similar with the study done by Seetha *et al.* (1999), Matee and Matre (2001) and Tuladhar (2007). However, culture positivity obtained in the present study was different from the study conducted by Weber *et al.* (2002); Asturias *et al.* (2003); Sallam (2004) and Ahmed *et al.* (2004). Tiwari (2002) in Nepal have reported 10.79% growth positivity. The lower growth positive result obtained in the present study may be due to the inclusion of every patient regardless of sign and symptoms of meningitis. Other reason could be the large number of patients being neonates and the patient may have started taking the antibiotics.

In the present study, highest numbers of suspected meningitis patients included in the study were from the age group 0-45 days (neonates) (64.43%) and the least number of patients were from the age group 5-40 yrs (5.60%).

The highest number of CSF samples which were culture positive was from the age group of 0-45 days (neonates) (40%). Tiwari (2002) also found the highest incidence of meningitis among the neonates (15.79%) in a study done at TUTH. This finding is also in agreement

with findings of Shrestha (1983) and Tuladhar (2007); they reported 50% and 33.33% of meningitis cases in the same age group respectively.

In the present study, maximum numbers of meningitis patients were children up to the age of 1 year. This result was found to be consistent with the findings of Rao *et al.* (1998) where 69.9% of the infected children were up to 1 year of age. A study done by Sallam in 2004 was also found to be similar with our finding where the most affected group was found to be those between 4 months to 3 years. Similarly in a study done by Asturias *et al.* in 2003, it was found that 54% percent of meningitis occurred prior to 6 months of age and 79.6% before 12 months of age. Tuladhar (2007) also reported the higher number of meningitis cases in children of age less than 3 years.

Among 20 bacterial isolates, 7 (35%) were Gram negative organisms where as 12 (60%) were Gram positive organisms. Thus Gram positive organisms were found to be predominant.

Altogether 7 different species of the isolates were isolated in our study. Among them, *Streptococcus pneumoniae* (45%) and *Haemophilus influenzae* type b (30%) were found to be most predominant which were followed by *Citrobacter freundii* (5%), *Cryptococcus neoformans* (5%) (Isolated from HIV positive patient of age >40 years), *Pseudomonas aeruginosa* (5%), *Staphylococcus aureus* (5%) and *Streptococcus viridans* (5%). Our findings were different to the statement “Gram negative enteric organisms appear to account for the majority of early onset (WHO, 1999)”. Similar results was obtained in a study done by Sallam (2004) where *Haemophilus influenzae* (23.80%) and *Streptococcus pneumoniae* (14.28%) were found to be predominant organism isolated in culture of CSF. Ahmed *et al.* (2004) and Ogunlesi *et al.* (2005) where *Streptococcus pneumoniae* and *Haemophilus influenzae* were the predominant organism.

No Group B streptococci (GBS) were isolated from neonates in present study because in developing country like ours, GBS appears to be much less frequent (WHO, 1999). The isolation of GBS is not universal (Nathoo *et al.*, 1991; Nel, 2000).

Haemophilus influenzae type b is uncommon after the age of 5 years (Vandepitte *et al.*, 2004). All the isolates (100%) of *Haemophilus influenzae* were from the age below 5 years. This result was in agreement with the findings made by Asturias *et al.* (2003) where, of the 71 children with Hib meningitis, 23 of them (32.4%) were younger than 6 months of age.

During the macroscopic examination of CSF, the CSF samples were categorized into five group viz. Clear, Xanthochromic, Slightly turbid, highly turbid and traumatic. Among 464 CSF samples received in the laboratory, 334 (71.98%) of the samples were clear and no organism was isolated from these samples.

Nineteen (4.09%) CSF samples were Xanthochromic in appearance and no organism was isolated from these samples too. Xanthochromia can be produced by spillover from a very high serum bilirubin level (i.e., >15 mg/dL) (Waldman, 2005) or due to old haemolysis (Kumari and Ichhapujani, 2000).

Only 44 (9.48%) CSF samples were found to be highly turbid and rate of isolation of bacteria from those CSF samples was (31.82%) followed by slightly turbid samples (21.73%). Ghimire *et al* (2003) reported high degree of positive correlation ($r= +0.7$) of turbidity with culture positivity.

In pyogenic meningitis, due to higher content of leukocytes (due to inflammation) become turbid. Hence, to initiate treatment of the patient suspected with meningitis, the physical examinations of fresh CSF samples can guide physicians to choose empirical therapy.

In all the samples, 248 (53.44%) of the samples contained leucocyte in normal range (0-5 cells/mm³) and no organism was isolated from these samples.

One hundred and sixty-three (35.12%) samples had the leucocyte count in the range of 5-100 cells/mm³ and many of these samples were negative for bacterial growth. Only four (2.45%) samples were culture positive. In a retrospective study done by Bema and Marvin (2005) among the patients who had low CSF cell count (0-30cells/mm³), acute bacterial meningitis was identified by culture in 0.3% of the study population. In the present study, 53 (11.41%) CSF samples had cell count of >100 cells/mm³ of which 30.18% of samples were growth positive. The specificity and sensitivity of leucocyte count were determined and were 55.85% and 100% respectively. Stephen *et al.* (2001) reported 88.64 and 69.61 as sensitivity and specificity of the leucocyte count method in bacterial meningitis.

Although CSF samples had cell count more than normal value, in most of the cases organisms were not isolated. This may be due to aseptic cases (viral meningitis) where lymphocytes are predominant or patients with meningism which can be correlated only with serological, chemical and clinical findings of the patient. In some cases, although there was higher count of neutrophils, organism was not isolated. This may be due to prior administration of antibiotics to the patients. Association of cell count with culture result was found to be statistically significant (P<0.05).

A differential cell count is important to categorize the meningeal reaction into pyogenic (bacterial and amoebic) lymphocytic (listerial, TBM, fungal, viral, leptospiral or neurosyphilitic) and eosinophilic (Helminthiasis) on the basis of predominance of respective leukocytes (Cheesbrough, 1984). Cellular reactions with differing pleocytosis are caused by different irritations within the space of CSF during the acute phase of meningitis with granulocytosis, lymphoid cells and plasmocytes (Olischer, 1998). Thus, a cytological examination of the fresh CSF samples is valueable for early diagnosis of pyogenic meningitis versus aseptic meningitis.

Among 464 cases of suspected meningitis, highest number of isolates (17.33%) were obtained from those samples which had >100 mg/dl of protein level followed by the range

of 45-100 mg/dl (7.44%). No organism was isolated from the CSF samples which had normal protein level and protein level less than 15 mg/dl. 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. Sensitivity and Specificity of determination of protein level were found to be 100% and 65.31% respectively. Similar result was mentioned by Stephen *et al.* (2001) in which the sensitivity and specificity were 79.22 and 89.04 respectively.

Similarly, most of the isolates (13.79%) were obtained from those CSF samples where the level of glucose was less than 40mg/dl. Seven (1.99%) isolates were obtained from those CSF samples which have normal glucose level and 3.84% isolates were obtained from those samples which had glucose level more than 80mg/dl. The isolation of bacteria from CSF with high glucose level was due to the patient was in last stage of life and during the last stage, the body functions may be receding, this results the less use of glucose which ultimately may accumulate in different body fluids. Sensitivity and Specificity of determination of glucose level were found to be 60% and 83.81% respectively. During the bacterial infection, due to the utilization of glucose for the multiplication of bacteria, the glucose level of CSF is reduced. Thus, by determining the glucose level of CSF, we can get the idea to distinguish meningitis from bacterial (where glucose level is reduced) with other forms (where glucose level is normal). Our finding is in agreement with the findings of Stephen *et al.* (2001) in which they found sensitivity and specificity to be 52.78 and 100.00 respectively.

In this study, Latex agglutination test detected more *Haemophilus influenzae* type b (4.17%) than culture which detected the organism in only (2.78%) of the cases. From this result the sensitivity and specificity determined were 100% and 98.57% respectively. Similarly, Binax test detected more *Streptococcus pneumoniae* (4.63%) than culture which

detected (4.17%). From this result the sensitivity and specificity determined were 100% and 99.52% respectively. In a study it was found that out of 6 samples tested for Hib only 2 gave positive latex agglutination test. Of the two positive only 1 gave culture positive (Stephen *et al.*, 2001). In a study done by Perkins *et al.* (1995) using Latex agglutination tests (LAT) for detection of capsular antigen of *Streptococcus pneumoniae*, *N. meningitidis* and *H. influenzae* (type b) in 185 CSF samples they found the test was positive in 101(54.6%) cases, accounting for 26.2% of the total 385 cases. A US-based study to determine the efficacy of antigen detection tests did not identify any false negative LAT, suggesting a high sensitivity of these tests (Perkins *et al.*, 1995).

In this study, Gram stain detected organism in more cases (5.60%) than culture which detected the organism in only 4.31% of the cases. This result was in agreement with the study done by Rao *et al.* in 1998, where Gram stain detected more cases (85.7%) than culture (66.2%). Similar result was also obtained in a study done by Ahmed *et al* (2004) where Gram staining was positive in 68 (56.7%) cases and CSF cultures were positive in 59 (49.2%) cases only. Tiwari (2002) also gave high degree of positive correlation ($r=+0.8$) of direct Gram staining and growth of organism. A simple Gram stained smear can offer immediate clues to aid a diagnosis of pyogenic meningitis. Some studies have reported a CSF Gram stain sensitivity of 60-90% and a high specificity of >97%, stressing its importance in the rapid and accurate diagnosis of the causative bacteria (Chinchankar *et al.*, 2002; Van de Beek *et al.*, 2004).

During Gram staining, Gram stain detected more number of Gram positive diplococci, Gram negative coccobacilli and Gram negative diplococci than culture. The lower rate of the culture result of these organisms may be due to the fastidious and delicate nature of these organisms.

When sensitivity of Gram stain was determined, the test was found to be highly sensitive (100%). Rapid diagnosis of bacterial meningitis is essential to avoid a poor outcome

(Goetghebuer *et al.*, 2000). Hence, Gram staining of fresh CSF sample provides valuable information about the organisms that can guide the physician to choose proper antibiotics before receiving culture result. Our result is in consistent with the findings of Stephen *et al.* (2001) in which they found the sensitivity and specificity to be 47.73 and 100.00. In a study, Gram stain provided an evidence of the causative bacteria in 253 (65.7%) patients. Of these 138 CSF samples yielded growth on culture, while 115 were culture negative. Fifty-five of the culture negative cases were positive by antigen detection tests. Thus in a total of 60 culture negative cases (23-antigen negative and 37-antigen detection not done) the etiological pathogen was recognized only by Gram stain (Chandramukhi *et al.*, 1989).

Among the isolates, most of the Gram negative organisms were found to be susceptible to Chloramphenicol and Cefotaxime. The isolates of *Haemophilus influenzae* showed low susceptibility towards Ampicillin and Penicillin which was found to be similar with the findings made by Ogunlesi *et al.* (2005) and Tuladhar (2007). In a study it was found that two of the four isolates of *H. influenzae* that could be tested were sensitive to ampicillin, cefuroxime and chloramphenicol (Mani *et al.*, 2007).

Amoxicillin was found to be resistant towards most of the Gram negative organism isolated. Similar results were obtained by Mani *et al.* (2007) in which they found that other gram negative bacilli (except *Haemophilus influenzae* and *Neisseria meningitides*) showed a high percentage of resistance to several common drugs tested; ampicillin (79%), amikacin (26.3%), gentamicin (21%), ciprofloxacin (47.4%), ofloxacin (58%), cefotaxime (52.6%), ceftazidime (63.2%) and ceftriaxone (52.6%).

Out of 9 isolates of *Streptococcus pneumoniae*, all was found to be 100% sensitive towards all the antibiotics during susceptibility testing except Co-trimoxazole (11.11%) and Erythromycin (88.89%). In a study it was found that all the 116 isolates of *S. pneumoniae* were sensitive to penicillin and vancomycin where the resistance pattern to other drugs

tested was ofloxacin (23.2%), cotrimoxazole (22.5%), cefotaxime (7.8%), ceftriaxone (6.8%) and tetracycline (2.5%) (Mani *et al.*, 2007).

Streptococcus viridans was found to be 100% sensitive towards all the antibiotics except Co-trimoxazole and Erythromycin.

Amikacin, Cefotaxime, Cloxacillin and Ofloxacin showed 100% sensitive for *Staphylococcus aureus* and rest of the antibiotics used was found to be either resistant or intermediate.

In a study done by Dutta and Bhatnagar (2001) on a rational antibiotics therapy in bacterial meningitis, they stated that the meningitis is best treated with a combination of ampicillin and a third generation cephalosporin for the wide range of Gram positive and Gram negative bacilli and third generation cephalosporin with or without ampicillin for pneumococci, haemophilli and meningococci. They further supported that the therapy should be modified, if necessary, on availability of culture susceptibility pattern.

Lu, Chang and Chang in 2000 stated besides the evolution of newer pathogens there has been increasing incidence for nosocomially acquired bacterial meningitis, with the emergence of resistant strains presenting a therapeutic challenge in recent years.

6.2 CONCLUSION

Hence, comparative evaluation of macroscopic, microscopic, serologic and cultural examination of CSF samples collected from suspected meningitis patients attending Patan Hospital was performed. The present study revealed that there is significant association between the microscopic findings like cell count and Gram stain, serological findings like Latex agglutination (for *Haemophilus influenzae* type b) and Binax test (for *Streptococcus pneumoniae*) with that of cultural findings. Gram staining and serological tests were more sensitive and rapid than culture method for the laboratory diagnosis of meningitis.

CHAPTER-VII

7. SUMMARY AND RECOMMENDATIONS

7.1 SUMMARY

1. Altogether 464 CSF samples were processed and out of 464 samples only 20 (4.31%) samples showed culture positive.
2. The highest numbers of CSF samples were growth positive from the age group of 0-45 days (neonates) and 45 days-1yr with 40% and 35% respectively.
3. Among 20 isolates, 8 (40%) were Gram negative organisms where as 12 (60%) were Gram positive organisms. Thus Gram positive organisms were predominant.
4. In all the CSF isolates identified in the present study, *Streptococcus pneumoniae* (45%) and *Haemophilus influenzae* type b (30%) were predominant followed by *Citrobacter freundii* (5%). *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus viridans* and *Cryptococcus neoformans* were detected in 5% samples.
5. Rate of isolation of bacteria from highly turbid CSF samples was highest (31.82%) which was followed by slightly turbid samples (21.73%).
6. In the present study, 53 (11.42%) CSF samples had leucocyte count of >100 cells/mm³ and highest percentage (30.18%) of isolates were isolated from these CSF samples. One hundred and sixty-three (35.12%) samples had the cell count in the range of 5-100 cells/mm³ with 4 (2.45%) isolates. Association of cell count with culture result was found to be statistically significant ($P<0.05$).
7. In all the samples, highest number of isolates (17.33%) were obtained from those samples which had more than 100 mg/dl of protein level followed by the range of 45-100 mg/dl (7.44%). No organism was isolated from the CSF samples which had protein level less than 15 mg/dl. Sensitivity and Specificity of determination of protein level were found to be 100% and 65.31% respectively.
8. Highest number of isolates i.e. 12 (13.79%) were obtained from CSF samples that had glucose level less than 40 mg/dl. Sensitivity and Specificity of determination of glucose level were found to be 60% and 83.81% respectively.

9. Latex agglutination antigen detection test for (Hib) (N=216) detected more Hib (9) than culture (6) and Sensitivity and Specificity of Latex agglutination antigen detection test for (Hib) was found to be 100% and 98.57% respectively.
10. Binax (antigen detection test for Pneumococci) (N=216) detected more Pneumococci (10) than culture (9) and Sensitivity and Specificity of Binax test was found to be 100% and 99.52% respectively.
11. Gram stain of CSF samples detected more organisms (5.60%) than culture (4.31%) and Sensitivity and Specificity of Gram stain was found to be 100% and 98.64% respectively.
12. No acid fast bacilli were seen when ZN staining of CSF samples was done in suspected cases of tuberculous meningitis.
13. Antibiotic susceptibility pattern of the isolates showed that Cefotaxime and Chloramphenicol were susceptible to most of the isolates. Amoxycillin and Penicillin were found to be resistant among most of the Gram negative isolates.
14. Co-trimoxazole was found to be resistant among the Gram positive isolates.

7.2 RECOMMENDATIONS

1. It is recommended that this kind of study should be conducted in other parts of the country so that actual incidence of meningitis among the suspected patients in whole country could be known.
2. Rapid antigen detection tests should be performed in all CSF samples received in the laboratory for culture to diagnose and manage timely with appropriate and adequate antibiotic to prevent mortality and long term morbidity.
3. As Gram stain detected more cases than culture, Gram staining should be performed in all CSF samples as a rapid diagnostic method of meningitis.
4. Along with bacterial culture, fungal, viral (cell culture) and amoebic culture should be done so that all etiological agents that cause meningitis can be isolated and identified.
5. Extensive studies on geographical and seasonal variations of meningitis should be carried out nationwide.
6. Vaccination programmes against three major pathogens i.e. *Streptococcus pneumoniae*, *N. meningitidis* and *H. influenzae* type b should be implemented

among the children nationwide to decrease mortality and morbidity caused by bacterial meningitis.

Chapter - VIII

8. REFERENCES

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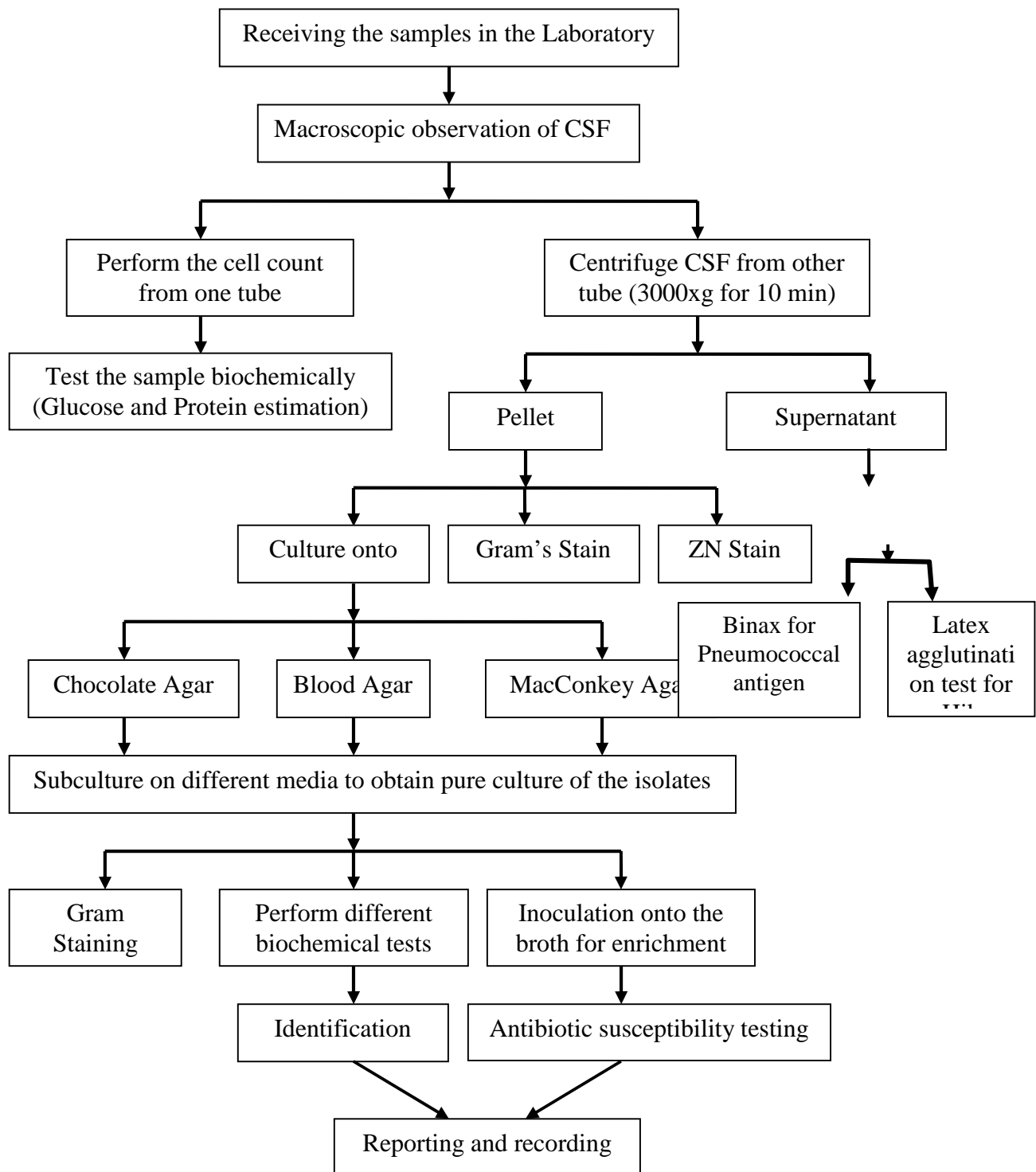
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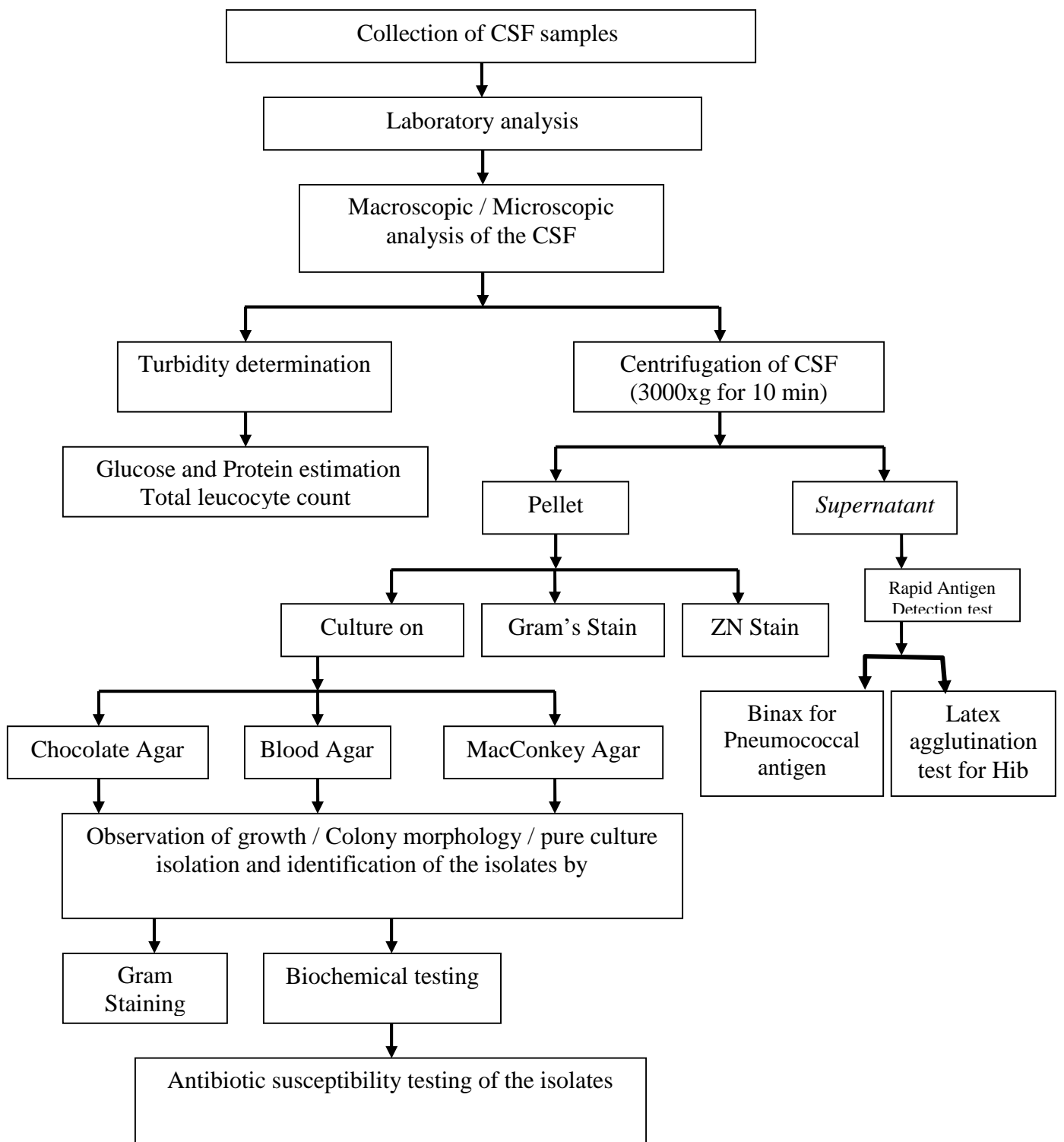
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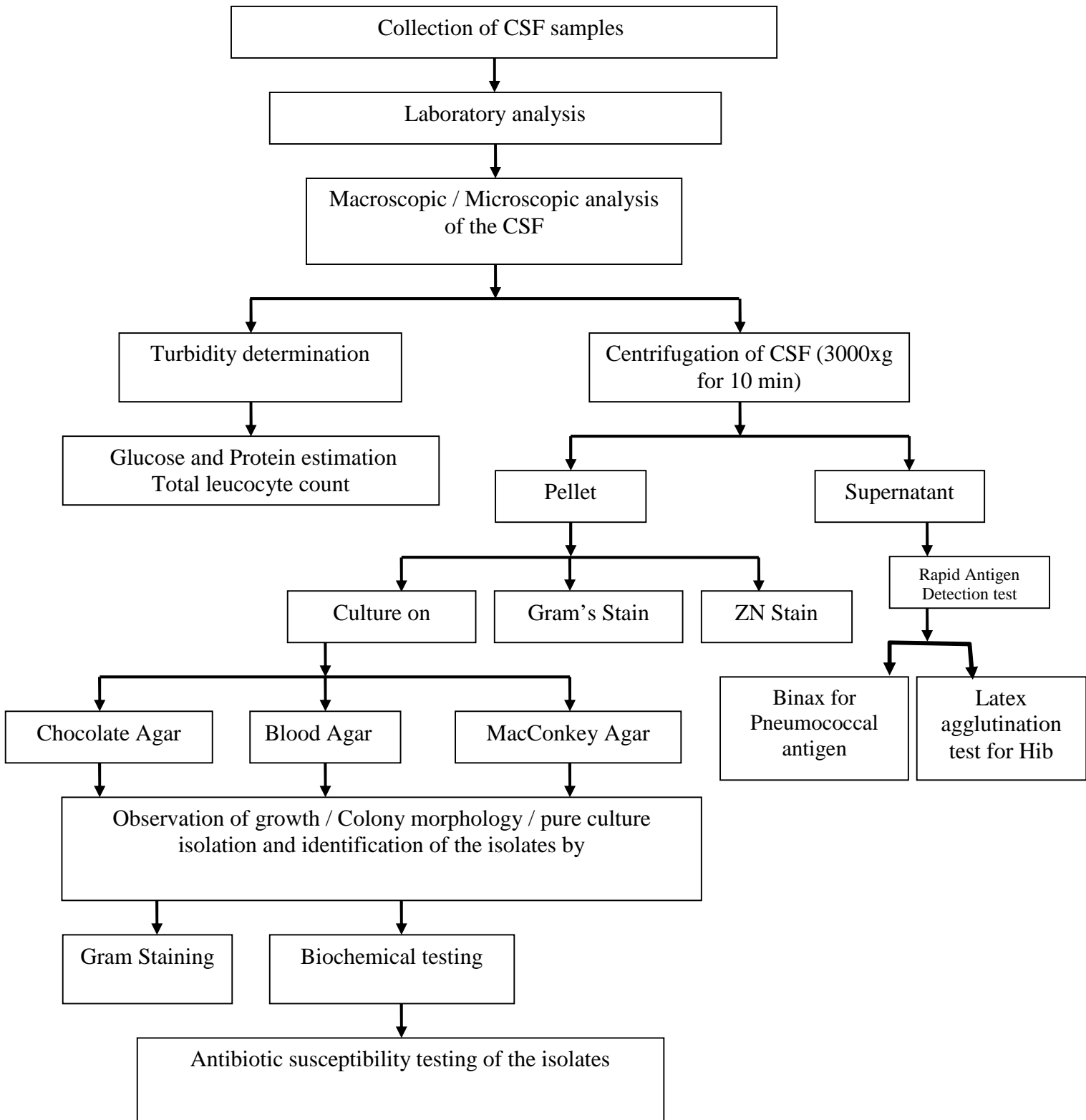
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APPENDIX-I

LIST OF EQUIPMENTS AND MATERIALS USED DURING THE STUDY

A. EQUIPMENTS

Hot air oven	Perkin Chemical Industries (India)
Incubator	Scientific Products (USA)
	Sienco Instruments (USA)
Autoclave	SA350 Sturdy Industrial Company
Refrigerator	Samsung Company Inc. (Korea)
	Electrolus (Sweden)
Ultra low Temp. freezer	New Brunswick Scientific (England)
Microscope	Olympus (Japan)
Centrifuge	Remi (India)
Weighing Machine	Cent-O-Gram (USA)
Chemical Analysers	Trace 25 (Germany)
	Erba-Chem 5 plus, Transasia (India)

B. MICROBIOLOGICAL MEDIA

Blood agar base	Mueller Hinton broth
MacConkey agar	Simmons Citrate agar
MR-VP medium	Sulphur Indole Motility agar
Mueller Hinton agar	Triple Sugar Iron agar
Nutrient 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

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

Ampicillin (10mcg)	Cloxacillin (1mcg)
Amikacin (30mcg)	Cotrimoxazole (1.25/23.75mcg)
Cefotaxime (30mcg)	Erythromycin (15mcg)
Ceftriazone(30mcg)	Ofloxacin (5mcg)
Chloramphenicol (30mcg)	Penicillin (10 mcg)
Ciprofloxacin (5mcg)	Piperacillin/Tazobactam (100/10=110mcg)
Tetracycline (30mcg)	Tobramycin (10mcg)
Amoxyclav (20/10=30mcg)	Gentamicin (10mcg)

E. IDENTIFICATION DISCS/OTHER DISCS

Bacitracin, Optochin/ 'X' factor, 'V' factor

F. RAPID ANTIGEN DETECTION KITS

Binax for Pneumococcal Antigen	Binax Inc. (USA)
Latex agglutination test for Hib	Directigen (USA)

G. MISCELLANEOUS

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

APPENDIX-II

A. COMPOSITION AND PREPARATION OF DIFFERENT CULTURE MEDIA

The culture media used were from two companies:

- a. Hi-Media Laboratories Pvt. Limited, Bombay, India.
- b. Oxoid Unipath Ltd. Basingstoke, Hampshire, England

(All compositions are given in grams per liter and at 25⁰C temperature)

1. Blood agar base (Oxoid, England)

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

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

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. 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 ⁰ C)	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 121⁰C (15 lbs pressure) for 15 minutes. The medium was poured into sterile petriplates.

3. 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 ⁰ C)	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 121°C (15 lbs pressure) for 15 minutes and poured into sterile petriplates.

4. 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 ⁰ C)	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 121⁰C (15 lbs pressure) for 15 minutes.

5. 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 ⁰ C)	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.

6. 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 ⁰ C)	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.

B. COMPOSITION AND PREPARATION OF DIFFERENT BIOCHEMICAL TESTS 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 ⁰ C)	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 121⁰C 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 ⁰ C)	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 121°C 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 ⁰ C)	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 121⁰C 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
Yeast 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 ⁰ C)	7.4±0.2

Direction: 65 grams of the medium was dissolved in 1000ml of distilled water and sterilized by autoclaving at 15 lbs (121⁰C) 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 ⁰ C)	7.4±0.2

Direction: 24 grams of the medium was suspended in 950 ml of distilled water and sterilized by autoclaving at 121⁰C for 121 minutes. After cooling to about 45⁰C, 50 ml of 40% urea solution was added aseptically, mixed well and distributed 5 ml amount in sterile test tubes.

C. COMPOSITIN AND PREPARATION OF DIFFERENT STAINING AND TESTS 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. For Ziehl Neelsen stain

(a) Carbol fuschin

Solution A

Basic fuschin	10 gm
Ethyl alcohol (absolute)	100ml

Solution B

Phenol crystals	50 gm
Distilled water	1000ml

Direction: In 100ml of absolute ethanol, 10 gm of basic fuschin was added and mixed well to dissolve. 50 gm of phenol was weighed and dissolved in small amount of water taken in a beaker and then water was added to make volume of 1 liter. This solution was added to previous solution of stain in bottle.. Final preparation was filtered and stored in a clean bottle.

(b) Decoloriser (3% acid alcohol)

Ethyl alcohol (absolute)	97ml
Hcl	3ml

Direction: 97 ml of methylated alcohol was taken in a flask. The flask was placed in 2-3 inches of water in a sink. Then, 3 ml of Hydrochloric acid was added and mixed well.

(c) Methylene blue (counter stain)

Methylene blue	1 gm
Distilled water	1000ml

Direction: 1 gm of methylene blue was weighed and transferred into clean bottle. Then 1000ml of distilled water was added and mixed well to dissolve methylene blue completely.

3. 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.

4. 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 <i>p</i> -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
<i>p</i> -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

-naphthol	5.0 g
Ethyl alcohol (absolute)	100 ml

Direction: To 25 ml ethanol, 5 g of -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) 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.

5. McFarland tube (No. 0.5)

0.5 ml of 0.048 M BaCl₂ (1.17% w/v BaCl₂.H₂O) was added to 99.5 ml of 0.18 M H₂SO₄ (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.

6. Preparation of Standard Set

100 mg% of protein solution in normal saline from protein standard or human serum protein was prepared in the following way.

Suppose serum protein is 7 g/dl

$$S1 = 7.0 \text{ g \% or } 7000 \text{ mg \%}$$

$$S2 = 100 \text{ mg \%}$$

$$V1 = ?$$

$$V2 = 100 \text{ ml.}$$

We know, $S1 \times V1 = S2 \times V2$

$$V1 = \frac{100 \times 100}{7000}$$

$$= 1.43 \text{ ml}$$

Therefore 1.43 ml of 7 g % human serum and up to 100 ml. normal saline gives 100 mg % protein solution.

Tube No.	1	2	3	4	5	6	7	8	9	10
100 mg % protein solution (ml)	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Normal Saline (ml)	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	00
3 % sulphosalicylic acid (ml)	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Final concentration of protein standard (mg/dl)	10	20	30	40	50	60	70	80	90	100

APPENDIX-III

GRAM-STAINING PROCEDURE

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 covered 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 covered 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 covered with counter-stain (safranin) 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.

APPENDIX-IV

ZIEHL-NEELSON (ZN) STAINING PROCEDURE

The following steps are involved in ZN stain:

1. A thick smear of the centrifuged deposit was made on a clean grease free slide and was air dried.
2. The smear was heat fixed and placed on the staining rack.
3. Carbol fuschin was flooded on the slide and heated from underneath the slide for approximately 5 minutes.
4. The slide was allowed to cool to room temperature and washed with water.
5. The slide was decolorized with 3% acid alcohol properly and washed with water.
6. Then methylene blue was flooded on the slide for few minutes and washed with water.
7. The slide was allowed to dry in air and observed under the microscope under oil immersion at 100X.

APPENDIX-V

METHODOLOGY OF BIOCHEMICAL TESTS USED 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 produce 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% H₂O₂ 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-butanediol 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 37°C 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, H₂S Lactose/ Sucrose fermenter, H₂S producer.
- b. Red (Alkaline) / Yellow (Acid), No Gas, No H₂S Only Glucose, not lactose/ Sucrose fermenter, not aerogenic, No H₂S 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.

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.

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 37⁰C 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.

APPENDIX-VI
DETERMINATIVE CHARACTERISTICS OF BACTERIAL AND
FUNGAL ISOLATES

Bacteria	Morphological Characteristics	Cultural Characteristics
<i>Streptococcus pneumoniae</i>	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 () haemolytic. On MA: No growth
<i>Haemophilus influenzae</i> type b	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 <i>Staphylococcus aureus</i> is streaked across the plate on which specimen containing <i>Haemophilus influenzae</i> has been inoculated On CA: Translucent colonies are obtained
<i>Citrobacter freundii</i>	Gram negative, 1-3µm×0.4-0.7µm size, non sporing, non capsulated, motile rods.	Smooth, convex 2-4 mm colonies, sometimes rougher mucoid forms occur On BA: Non-haemolytic On MA: Non-lactose fermenting colonies Or slow lactose fermenting colonies
<i>Staphylococcus aureus</i>	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	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

	chain(less than five cocci in line).	fermenting.
<i>Streptococcus viridans</i>	Gram positive cocci, non sporing and non-motile.	On BA: Alpha () haemolytic colonies are obtained.
<i>Pseudomonas aeruginosa</i>	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.

CHARACTERISTICS OF YEAST LIKE FUNGI

<i>Cryptococcus neoformans</i>	Gram positive spherical budding cell, 5-15 µm in diameter having a prominent polysaccharide capsule	On BA: Creamy white mucoid
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APPENDIX-VII

Table: Distinguishing reactions of the commoner and pathogenic Enterobacteriaceae

Species	Test/ substrate											
	lac	mot	gas	ind	VP	cit	PDA	ure	lys	H ₂ S	inos	ONPG
<i>E. coli</i>	+	+	+	+	-	-	-	-	+	-	-	+
<i>Shigella</i> groups A, B, C	-	-	-	±	-	-	-	-	-	-	-	-
<i>Sh. Sonnei</i>	-	-	-	-	-	-	-	-	-	-	-	+
<i>Salmonella</i> (most serotypes)	-	+	+	-	-	+	-	-	+	+	±	-
<i>S. Typhi</i>	-	+	-	-	-	-	-	-	+	+	-	-
<i>S. paratyphi A</i>	-	+	+	-	-	-	-	-	-	-	-	-
<i>C. freundii</i>	±	+	+	-	-	+	-	±	-	±	-	+
<i>C. koseri</i>	±	+	+	+	-	+	-	±	-	-	-	+
<i>K. pneumoniae</i>	+	-	++	-	+	+	-	+	+	-	+	+
<i>K. oxytoca</i>	+	-	++	+	+	+	-	+	+	-	+	+
<i>E. aerogenes</i>	+	+	++	-	+	+	-	-	+	-	+	+
<i>E. cloacae</i>	+	+	+	-	+	+	-	±	-	-	-	+
<i>Hafnia alvei</i>	-	+	+	-	+	-	-	-	+	-	-	+
<i>Serratia marcescens</i> ^b	-	+	±	-	+	+	-	-	+	-	±	+
<i>P. mirabilis</i>	-	+	+	-	±	±	+	++	-	+	-	-
<i>P. vulgaris</i>	-	+	+	+	-	-	+	++	-	+	-	-
<i>M. morgani</i>	-	+	+	+	-	-	+	++	-	±	-	-
<i>Providencia rettgeri</i>	-	+	-	+	-	+	+	++	-	-	+	-
<i>P. stuartii</i>	-	+	-	+	-	+	+	±	-	-	+	-
<i>P. alcalifaciens</i>	-	+	+	+	-	+	+	-	-	-	-	-
<i>Yersinia enterocolitica</i> ^c	-	-	-	±	-	-	-	±	-	-	±	+
<i>Y. pestis</i>	-	-	-	-	-	-	-	-	-	-	-	±
<i>Y. pseudotuberculosis</i>	-	-	-	-	-	-	-	+	-	-	-	±

^a lac, inos, fermentation of lactose, inositol; mot, motility; gas, gas from glucose; ind, indole production; VP, Voges-Proskauer; cit, Citrate utilization (Simmons'); PDA, phenylalanine deaminase; ure, urease; lys, lysine decarboxylase; H₂S, H₂S produced in TSI agar; ONPG, metabolism of *o*-nitrophenyl- β -D-galactopyranoside.

^b Some strains of *Serratia marcescens* may produce a red pigment

^c *Yersinia* are motile at 22°C. {Key: +, 85% of strains positive; -, 85% of strains negative; 16-84% of strains are positive after 24-48 hour at 36°C}
(Source: Collee *et al.* 1996)

APPENDIX-VIII

ZONE SIZE INTERPRETATIVE CHART

Antimicrobial Agents used	Symbol	Disc Content	Resistant (mm or less)	Intermediate (mm)	Susceptible (mm or more)
Amikacin	Ak	30µg	14	15-16	17
Amoxicillin/Clavulanic acid	Ac	20/10µg (30µg)	19	-	20
Ampicillin	A	10µg	18	19-21	22
Cefotaxime	Ce	30µg	14	15-22	23
Ceftriaxone	Ci	30 µg	13	14-20	21
Chloramphenicol	C	30µg	12	13-17	18
Ciprofloxacin	Cf	5 µg	15	16-20	21
Cloxacillin	Cx	5 µg	12	12-13	14
Cotrimoxazole (Trimethoprim/ Sulphonamide)	Co	1.25/23.75µg	10	11-15	16
Erythromycin	E	15 µg	13	14-22	23
Ofloxacin	Of	5 µg	12	13-15	16
Penicillin When testing with <i>S.pneumoniae</i>	P	10 mcg	-	-	20
Tobramycin	Tb	10mcg	12	13-14	15
Gentamicin	G	10mcg	12	13-14	15
Piperacillin/Tazobactam	Pt	100/10mcg	17	18-20	21
Amoxyclav	Ac	20/10mcg	13	14-17	18

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

APPENDIX-IX

ZONE SIZE INTERPRETATIVE CHART

For *Streptococcus pneumoniae*:

Antimicrobial Agents used	Symbol	Disc Content	Resistant (mm or less)	Intermediate (mm)	Susceptible (mm or more)
Ampicillin	A	10µg	13	14-16	17
Chloramphenicol	C	30µg	12	13-17	18
Ciprofloxacin	Cf	5 µg	15	16-20	21
Cotrimoxazole (Trimethoprim/ Sulphonamide)	Co	1.25/23.75µg	10	11-15	16
Penicillin	P	10µg	19	20-27	28

For *Haemophilus influenzae*:

Antimicrobial Agents used	Symbol	Disc Content	Resistant (mm or less)	Intermediate (mm)	Susceptible (mm or more)
Ampicillin	A	10µg	13	14-16	17
Amoxicillin/Clavulanic acid	Ac	20/10µg (30µg)	14	15-16	17
Chloramphenicol	C	30µg	12	13-17	18
Ceftriaxone	Ci	30 µg	-	-	26
Ciprofloxacin	Cf	5 µg	15	16-20	21
Cotrimoxazole (Trimethoprim/ Sulphonamide)	Co	1.25/23.75µg	10	11-15	16
Penicillin	P	10µg	19	20-27	28

APPENDIX-X

CALCULATION OF SENSITIVITY, SPECIFICITY, POSITIVE AND NEGATIVE PREDICTIVE VALUE AND EFFICIENCY

Tests	True positive (a)	False positive (b)	False negative (c)	True negative (d)
Protein level	20	154	0	290
Sugar level	12	75	8	369
Gram Stain	20	6	0	438
Cell count	20	196	0	248
Latex agglutination test for Hib(N=216)	6	3	0	207
Binax for S. pneumoniae(N=216)	9	1	0	206

Calculation of sensitivity

Sensitivity can be calculated as

Sensitivity of Protein level

Sensitivity of Sugar level

Sensitivity of Gram Stain

Sensitivity of cell count

Sensitivity of latex agglutination

test for Hib

Sensitivity of Binax test for Pneumococci

$$\text{Sensitivity} = a / (a+c) \times 100\%$$

$$= 20 / (20+0) \times 100\% = 100\%$$

$$= 12 / (12+8) \times 100\% = 60\%$$

$$= 20 / (20+0) \times 100\% = 100\%$$

$$= 20 / (20+0) \times 100\% = 100\%$$

$$= 6 / (6+0) \times 100\% = 100\%$$

$$= 9 / (9+0) \times 100\% = 100\%$$

Calculation of Specificity

Specificity can be calculated as

Specificity of Protein level

Specificity of Sugar level

Specificity of Gram Stain

Specificity of cell count

Specificity of latex agglutination

test for Hib

Specificity of Binax test for Pneumococci

$$\text{Specificity} = d / (b+d) \times 100\%$$

$$= 290 / (154+290) \times 100\% = 65.31\%$$

$$= 75 / (75+369) \times 100\% = 83.81\%$$

$$= 438 / (6+438) \times 100\% = 98.64\%$$

$$= 248 / (196+248) \times 100\% = 55.85\%$$

$$= 207 / (3+207) \times 100\% = 98.57\%$$

$$= 206 / (1+206) \times 100\% = 99.52\%$$

Calculation of Positive Predictive Value (PPV)

PPV can be calculated as

PPV of Protein level

PPV of Sugar level

PPV of Gram Stain

PPV of cell count

$$\text{PPV} = a / (a+b) \times 100\%$$

$$= 20 / (20+154) \times 100\% = 11.49\%$$

$$= 12 / (12+75) \times 100\% = 13.79\%$$

$$= 20 / (20+6) \times 100\% = 76.92\%$$

$$= 20 / (20+196) \times 100\% = 9.25\%$$

PPV of latex agglutination test for Hib	$=6 / (6+3) \times 100\%$	$=66.67\%$
PPV of Binax test for Pneumococci	$=9 / (9+1) \times 100\%$	$=90\%$

Calculation of Negative Predictive Value (NPV)

NPV can be calculated as	$NPV=d/(c+d) \times 100\%$	
NPV of Protein level	$=290 / (0+290) \times 100\%$	$=100\%$
NPV of Sugar level	$=369 / (8+369) \times 100\%$	$=97.87\%$
NPV of Gram Stain	$=438 / (0+438) \times 100\%$	$=100\%$
NPV of cell count	$=248 / (0+248) \times 100\%$	$=100\%$
NPV of latex agglutination test for Hib	$=207 / (0+207) \times 100\%$	$=100\%$
NPV of Binax test for Pneumococci	$=206 / (0+206) \times 100\%$	$=100\%$

Calculation of Efficiency

Efficiency can be calculated as	$Efficiency= a+d/ (a+b+c+d) \times 100\%$
Efficiency of Protein level =66.81%	$=20+290 / (20+154+0+290) \times 100\%$
Efficiency of Sugar level =82.11%	$=12+369 / (12+75+8+369) \times 100\%$
Efficiency of Gram Stain =98.70%	$=20+438 / (20+6+0+438) \times 100\%$
Efficiency of cell count =57.75%	$=20+248 / (20+196+0+248) \times 100\%$
Efficiency of latex agglutination test for Hib =98.61%	$=6+207 / (6+3+0+207) \times 100\%$
Efficiency of Binax test for Pneumococci =99.54%	$=9+206 / (9+1+0+206) \times 100\%$

APPENDIX-XI

DATA ANALYSIS (CHI-SQUARE TEST)

1. Association of cell count with culture result

Cell count	Culture positive	Culture negative	Total
0-5	0	248	248
5-100	4	159	163
>100	16	37	53
Total	20	444	464

Test statistic is χ^2

Ho: There is no significant association of cell count and culture result.

H1: There is significant association of cell count and culture result.

From $\chi^2 = \sum \frac{(O-E)^2}{E}$ we find $\chi^2 = 89.202$

Thus $\chi^2_{cal} (89.202) > \chi^2_{tab}$ at $\alpha = 0.05$ and d.f. = 2 i.e. 5.99

Hence, Ho is rejected i.e. the higher proportion of positive culture results seen when there is high cell count is statistically significant.

2. Association of Culture results between genders

		Culture result		Total
		+ve	-ve	
Gender	Male	8	239	247
	Female	12	205	217
Total		20	444	464

Test statistic is χ^2

Ho: There is no significant association of gender and culture result.

H1: There is significant association of gender and culture result.

From $\chi^2 = \sum \frac{(O-E)^2}{E}$ we find $\chi^2 = 0.975$

Thus $\chi^2_{cal} (0.975) < \chi^2_{tab}$ at $\alpha = 0.01$ and d.f. = 1 i.e. 3.84

Hence, Ho is accepted i.e. there is no significant difference in frequency of isolation of organisms from CSF samples in males and females ($p < 0.01$).

APPENDIX-XII

Table: Guidelines for the interpretation of results following hematological and chemical analysis of CSF from children and adults

Clinical Setting	Appearance	Leukocytes per mm ³	Predominant cell type	Protein (mg/dL)	Sugar (mg/dL)
Normal	Clear and colorless	0-5	-	15-45	40-80
Purulent	Cloudy or	5-20000	Mostly	Elevated	Reduced

(pyogenic)meningitis	purulent, may contain clots	(mean of 800)	Neutrophils	(>100)	
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
<i>L. monocytogenes</i> 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)

