CHAPTER-I

1. INTRODUCTION

Meningitis is one of the quite serious, with fairly high death rate disease in developing countries like Nepal. Meningitis is a condition in which the meninges (coverings of the brain and spinal cord), which are protective membranes surrounding the brain and spinal cord, become infected and inflamed, causing a complex series of neurological consequences.

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. The duramater lines along the cranium and the piamater lies along the brain and the spinal cord. 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. When infection and inflammation occurs in brain proper, it is called as encephalitis. The infection may originate in the meninges (meningitis) or brain (encephalitis), then spread from one site to another (Wilson, 1995).

Meningitis can be caused by either a bacterial or viral pathogen, with the latter 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, *Staphlylococcus* spp, *E. coli* and *Listeria monocytogenes* (Easmon, 1990).Viruses include herpes virus (HSV, VZV, CMV), enteroviruses and influenza A viruses (Koskiniomi *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).

Bacterial meningitis is one of the most common serious infections of childhood. It is the major cause of morbidity and mortality in pediatric age group. Timely diagnosis and management with appropriate and the adequate antibiotic is of utmost importance to prevent mortality and long term morbidity. At least 1.2 million cases of meningitis are estimated to occur every year with 135000 deaths (Tikomirov *et al,* 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. So the major burden of this disease is being shared by the pediatricians. 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. 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 in children worldwide: >1,000,000 cases and 200,000 deaths are estimated to occur each year. (Naik and Seyoum, 2004). 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), and many of these difficulties are not evident until a child is school-aged.

Patients with meningitis may have fever, stiff neck, headache, nausea and vomiting, neurological abnormalities, and change in mental status (Baron, Peterson and Finegold, 1994). The onset may be fulminating, acute or less commonly insidious. However, the signs and symptoms depends of meningeal irritation depends 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 0-8 cells/ mm^3 (in infants) to 0-5 cells/mm^3 (in adults). But during meningitis, pleocytosis occurs. The number and types of leukocytes in the CSF is important in differentiating the pyogenic (bacterial) and aseptic (viral) meningitis. In formal case, marked pleocytosis (mainly polymorphs) is observed where as in later 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. Bacterial meningitis is a serious infection that particularly strikes infants and young children. Especially in case of children, it is more fatal. If it is not cured on time it may lead to chronic complications like it may lead to deafness, learning disabilities, hydrocephalous (increase of CSF in brain), mental retardation, paralysis, seizures and vision loss (Brochert, 2000).

Thus laboratory investigations of CSF samples from suspected meningitis children is utmost important at the earliest possible moment to guide the appropriate chemotherapy and hence prevention of life threatening outcome. This study was carried out with aims to find out the correlation of macroscopic and microscopic findings with that of cultural findings of the CSF of children attending Kanti Children's Hospital.

CHAPTER-II

2. OBJECTIVES

2.1 GENERAL OBJECTIVE

To correlate Microscopic findings with cultural findings of CSF among the children suspected of meningitis attending Kanti Children's Hospital.

2.2 SPECIFIC OBJECTIVES

- To correlate bacteriological findings with macroscopic characteristic of CSF samples of children.
- To correlate the presence of leukocytes in CSF with bacteriological findings.
- To determine the protein and glucose level of CSF samples and to correlate it with the cultural findings.
- To determine the efficiency of Gram staining from CSF samples directly in detection of meningitis.
- To detect Acid Fast Bacilli by ZN staining in suspected cases of Tuberculous Meningitis.
- To isolate and identify the organism isolated from the CSF sample.
- To perform the antibiotic susceptibility test of the isolates.

CHAPTER-III

3. LITERATURE REVIEW

3.1 MENINGITIS AS A DISEASE

Meningitis is an inflammation of the membranes that line the brain and spinal cord. 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 aracnoid and piamater are involved, it is called as leptomeningitis or meningitis proper (Brain and Walton, 1969).

3.2 ANATOMY AND PHYSIOLOGY

3.2.1 Anatomy of meninges

The brain and the spinal cord are surrounded by three protective membranes called meninges. These three membranes are viz. the duramater, the arachnoid mater and the pia mater.

The outer duramater is the tough, fibrous membrane and serves as the internal periostem 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.

Between the duramater and periostem 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 subrachnoid 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 chiamatis (Brain and Walton, 1969).

3.2.2 Blood-brain barrier (BBB)

The structures of the brain capillaries are different from the capillaries of the rest of the body. The walls of other capillaries contain penetrable gaps which allow most of the substances to pass through. The walls of the brain capillaries, however, are formed by endothelial cells that are joined by tight junctions. 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. That’s why many substances, including many drugs, cannot cross the barrier.

Through the blood-brain barrier, only lipid soluble molecules can pass because the plasma membrane of the endothelial cells is composed of primarily lipid molecules. Essential water soluble molecule like glucose, amino acids etc. are recognized by the carrier proteins and transported across the barrier (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 S, 1999).

3.2.3 Ventricles of the brain

Within the brain is the series of the connected cavities called ventricles. Each cranial ventricle is filled with cerebrospinal fluid and lined with the cuboidal epithelium called as ependyma. A network of blood vessels called choroids plexus is formed at several places where ependyma contacts the pia mater.

There are four ventricles numbered from top of the brain to downwards. They are left and right ventricles of the cerebral hemispheres, the third ventricle of diencephalon, and the fourth ventricle of the pons and medulla oblongata. 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).

3.2.4 Cerebrospinal fluid (CSF)

The cerebrospinal fluid (CSF) is 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 (Wilson, 1995). It is the clear, colorless liquid that is essentially an ultrafiltrate of blood (Carola *et al*, 1992).

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; 0-8 cells/ mm^3 in infants and 0-5 cells/mm^3 in adults is considered normal.

Physical characteristics of CSF in normal condition:-

Appearance	Clear, colorless
Volume	100-150ml (mean 120ml)
Specific gravity	1.003-1.008
Pressure: *New born*	30-80mm water
Children	50-100mm water
Adult	70-200mm water (average 125)
Cells	0-5 cells per mm^3 (mainly lymphocytes)

Chemical composition of CSF in normal condition:-

Protein	15-45 mg/100ml
Glucose	50-80 mg/100ml
Inorganic phosphorus	1.25-2 mg/100ml
Uric acid	Trace
Cholesterol	Trace
Calcium	5-6mg/100ml
Sulfates	1mg/100ml
Chlorine	125-750 mg/100ml

(Source- Brain and Walton, 1969)

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 (Chaudhary, 1999). 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.

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 adequate 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 The CSF is secreted continuously at the rate of about 0.5ml per min, i.e. 120ml per day. Just as CSF is continuously being formed, it is also reabsorbed into the blood at the same rate, so the volume of the CSF remains constant. 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).

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.

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) (Waldman, 2005).

CSF analysis also is an important diagnostic tool in subarachnoid hemorrhage and leptomeningeal carcinomatosis. Indeed, the extent to which CSF reflects the chemistry of surrounding tissue, and the significant differences in composition from plasma.

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).

Methods of obtaining CSF:-

To obtain CSF for examination, it is necessary to puncture either the cerebral ventricles or the sub-arachnoid spaces, which may reached most easily either in the cerbromedullary cistern 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 boarder 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 second sacral vertebra. The 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) or fourth and fifth spine (L4-L5) (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).

After making the patient ready for lumbar puncture, 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 two sterile screw capped containers consecutively, about 3 ml each. The needle is withdrawn (Brain and Walton, 1969). 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) require more CSF (Waldman, 2005). About 5 ml of

fluid is sufficient for the diagnostic purposes. 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.

3.3 POSSIBLE PATHOGENS

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*

Gram Negative Bacteria

- *Neisseria meningitidis*
- *Haemophilus influenzae* type b
- *Esherichia coli*
- *Klebsiella pneumoniae*
- Other enterobacteriaceae
- *Pseudomonas aeruginosa*
- *Proteus* spp
- *Salmonella* spp
- *Flavobacterium meningosepticum*
- *Bacteroids* spp

2. Spirochaetes:

Treponema pallidum

*Leptos*ppi*ra interrogans*

3. Fungus:

Cyrptococcus neoformans

Candida spp

Histoplasma capsulatum

Blastomyces dermatitidis

Coccodioides immitis

4. Virus:

Polio virus

Rabies virus

Herpes simplex virus

Varicella Zoster virus

Cytomegalo virus

Epstein Barr virus

ECHO virus

Coxackie virus

Equine encephalitis

Japanese B encephalitis

5. Parasites:

Trypanosoma spp

Nagleria fowleri

Acanthamoeba spp

Schistosoma spp

Echinococcus granulosus

Malarial parasites

Taenia spp

Toxoplasma gondii

(Source- Cheesebrough, 2000)

3.4 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:

Direct spread from an infected site: 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 (Baron *et al*, 1994). Organisms may also introduced by surgical procedures such as lumbar puncture (Brain and Walton, 1969).

Haematogenous spread: In this case, meningitis occurs by entry of organisms 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 (Baron, Peterson and Finegold, 1994). 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).

Anatomic defects in CNS structures: Anatomic defects as a result of surgery, trauma, or congenital abnormalities can allow microorganism easy and ready access to the CNS.

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.

3.5 PATHOGENESIS

Most of the 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 (Baron *et al*, 1994). For the organism to reach the CNS, host defense mechanism must be overcome.

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 IgA_1 proteases are major etiological agents (Landgraf, 1998). IgA_1 proteases released by pathogens desroy 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).

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 hydrocephalous 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 hydrocephalous 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 CLINICAL MANIFESTATIONS

All forms of meningitis, whatever their cause, posses 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 fontanel ("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).

In older children, the onset of the symptoms of meningitis is usually sudden with high fever, irritability, headache, vomiting and general malaise. There is usually photophobia. The temperature rises up to 39°C-40°C. Respiration may be irregular, periodic or Chyne Stoke's type. Convulsions are common. There is generalized hypertonia and marked neck rigidity. In infants and neonates bacterial meningitis sometimes has a more insidious onset. The neonate's vacant stare, alternating drowsiness and irritability, persistent vomiting with fever, refusal to suck, poor tone, poor cry, circulatory collapse, fever or hypothermia, tremor or convulsions and neurological deficits should arouse a suspicion of bacterial meningitis (Tiwari, 2003).

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 mm^3), 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.

The sequaelae of acute meningitis include cerebral oedema, hydrocephalus, cerebral herniation, and focal neurological changes. Permanent deafness can occur in 10% children who recover from bacterial meningitis. Paralysis, seizures and vision loss may occur in children as its long term effect (Brochert, 2000). 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 detioration. 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 if acute disease (Wilhelm and Ellner, 1986).

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

3.7 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).

3.7.1 Acute pyogenic (purulent) meningitis:

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 neutrophils (pus cells) (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 meningral infection in the infancy world wide (Vineusa and C. de 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).

3.7.2 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 is inflammation of the meninges with CSF lymphocytic pleocytosis and no cause apparent after routine CSF stains and cultures. 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 or noninfectious. Causes may be infectious (eg, rickettsiae, spirochaetes, parasites) or noninfectious (eg, intracranial tumors and cysts, drugs, systemic disorders). Aseptic meningitis may also be due to tumor, cysts, chemicals, sarcoidosis or other non infectious causes (Baron *et al*, 1994).

Enteroviruses, including echovirus and coxsackievirus, cause most cases. Mumps virus is a common cause worldwide but has been minimized in the U.S. by vaccination.

Enteroviruses and the mumps virus enter via the respiratory or GI tract and spread via the bloodstream. Mollaret's meningitis is a syndrome of self-limited, recurrent aseptic meningitis characterized by large atypical monocytes (once thought to be endothelial cells) in the CSF; it presumably is caused by herpes simplex virus type 2 or other viruses. Viruses that cause encephalitis typically also produce low-grade aseptic meningitis.

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 entero viruses 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 bengin 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 (Rubistein, Weisbroad 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 outbreask 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.

Noninfectious causes of meningeal inflammation include neoplastic infiltration, leakage of the contents of an intracranial cyst, intrathecal drugs, lead poisoning, and radiopaque agents. Infrequently, inflammation results from certain systemically administered drugs, presumably as a hypersensitivity reaction, antimicrobials (especially sulfa drugs), and immune modulators (e.g., IV immune globulins, monoclonal antibodies, cyclosporines, vaccine) (Merck, 2005).

3.7.3 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 tuberculosos (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 necroscopy, 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).

3.7.4 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 in increase in the incidence 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 dessiminate 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 ($5x10^7$ 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).

3.7.5 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 mat 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.

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”.

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). 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).

Meningitis in children:- 95% 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). 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).

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, immunosupression 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).

Distribution of bacteria by age group: (relative percentages are approximate)

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)

3.8 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).

3.8.1 Meningococcal meningitis:-

Meningoccocal 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).

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 this 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 (Falton 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).

3.8.2 *Haemophilus influenzae* meningitis:-

Haemophilus influenae 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).

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

3.8.3 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 carriage rate is 5-70% (Finch, 2001) and they are mostly secondary pathogens but are primary pathogens in immuno-compromised 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.

3.8.4 Meningitis caused by other Gram Positive Organism:-

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 immunosupperessed 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).

3.8.5 Meningitis caused by other Gram Negative Organism:-

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 Kl capsular antigen and meningitis. Pregnancy is associated with an increased rate of colonization with Kl strains of *E.coli* and these strains are the ones involved in subsequent neonatal meningitis. The colonization of the gastrointestinal tract with *E.coli* Kl 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).

3.9 LABORATORY INVESTIGATIONS 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 Icchpujani, 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).

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 (5-10ml) may be required (Baron, Peterson and Finegold, 1994). Two sterile screw capped container are required to collect CSF. In the first container, 1ml of CSF is collected for culture and in another container 2-3ml is collected for cell count, microscopy and biochemistry. This is because the first sample may contain blood (due to traumatic lumber 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).

Examination of CSF:-

A. 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 crops 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 only 0-5 leucocyte/mm^3, mainly lymphocytes, though in neonates up to 30 cells/mm^3, mainly polymorph is considered as normal (Collee *et al,* 1996). There is increase in leukocyte count during different form of meningitis.

B. 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. Detection of lipopolysaccharide in CSF reveals the meningitis caused by Gram negative bacteria (Easmon, 1990).

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 quantitive technique using Benedict's reagent is also useful (Cheesbrough, 1984).

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-quantitive 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).

Cultures:-

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% Co_2 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 in air 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).

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.

A. 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.

B. 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.

C. 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.

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

E. 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.

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.

3.10 GLOBAL SCENARIO OF MENINGEAL INFECTION

Bacterial meningitis is an important cause of childhood 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 pediatricians. 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 in the childhood. 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 under five year old children (Wanyoike *et al,* 1995).

In a prospective surveillance of neonatal meningitis in England over a period of seven years Hristeva (1993) deduced that the estimated incidence of bacterial meningitis was 0.25 per 1000 live births, 75% of which was due to group B streptococci in early onset cases and gram negative organisms accounted for 86% of the late onset cases. The overall mortality was 26%.

A 5-year retrospective study of all children 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).

In a study of bacteria meningitis in children at Al-Fateh Children's Hospital, Benghazi during the period from April 1994 to May 1995, 77 children with a presumptive diagnosis of acute bacterial meningitis were investigated. 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).

A study done in India showed Gram positive isolates in adults and gram negative bacilli in paediatric age group 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 among the children between 1 month to 59 months of age at three major referral hospitals in Guatemalan city found that Hib was identified in 20.0% of children 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, for children with Hib, *S. pneumoniae*, and culture-negative and antigen-negative meningitis. 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 for children 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 children 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 *Haemophilius influenza* (23.80%), *Streptococcus pneumonia*e (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 children within the age group of 2 months to 12 years 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 children 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, Staphlylococcus 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).

3.11 NATIONAL SCENARIO OF MENINGEAL INFECTION

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 *et al* (2002) at TUTH, highest incidence of meningitis was found in neonates (0-1month). The rate of incidence of meningitis among children up to 14 yrs was found to be 8.70%.

A prospective study of 42 children (with range of ages from 1 month to 44 month) admitted to Kanti Children's Hospital, Katmandu from the June 1993 to February1994 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 *Streptococus 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 leukocytosis/mm3 and blood contaminated samples (4/77) within normal blood leukocyte count, 4000-11000 leukocytosis/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 leukocytosis/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 indicate pyogenic or tuberculous meningitis (TBM) (Tiwari *et al,* 2004).

CHAPTER – IV

4. MATERIALS AND METHODS

This study was conducted at Kanti Children's Hospital's Microbiology lab from June, 2006 to September, 2006. During this period CSF samples from 431 patients suspected of meningitis were processed in the microbiology laboratory. The samples were processed according to the standard protocol. Briefly, the appearance of the samples was noted, cell count was done, Gram staining was done, protein and glucose levels were estimated and samples were cultured in different types of the 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 Hospital by experienced medical officer by puncturing the lumbar region at L3-L4 level. For every patient, duplicate sample was collected, one for the cell count and other for culture. The sample was immediately transported to the laboratory at the room temperature. As soon as the samples were received in the laboratory, the samples were processed.

4.2.2 Sample processing

Each CSF sample was processed macroscopically, microscopically and microbiologically.

4.2.3 Macroscopic observations

Each CSF sample was observed for its physical appearance with naked eye. The color and the turbidity of the 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.

4.2.4 Cytological examinations

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

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

Where,

Depth Factor = 10

Area counted = 4

4.2.5 Estimation of sugar and protein

Sugar and protein of the CSF samples were determined by the kinetic method.

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 is prepared. Preparation and concentration of standard set is mentioned in Appendix II. Then 0.5 ml of CSF sample was taken in a tube to which 1.5 ml of 3% sulphosalicylic acid solution was added. The tube was shaken well to mix the solution in the tube and waited for 10 mins. Then the turbidity of the tube was compared and matched with the standard and the concentration of protein was expressed in mg/dl.

4.2.6 Microscopic examinations

The CSF samples were centrifuged at 3000x g revolutions per minute for 10 minutes. Those samples which were received in a volume less than 2 ml were not centrifuged. From the deposit of the centrifuged samples and from uncentrifuged samples (directly), Gram staining was done according to the standard protocol. Gram staining procedure is given in

Appendix III. Gram stained smear was observed for the presence of microorganisms for its cell morphology and number.

ZN staining was also performed if it is requested by physicians for detection of AFB. For that CSF sample was centrifuged for 30 min with 3000x g revolutions per minute. ZN staining was done according to standard protocol. AFB staining procedure is given in Appendix IV. Then the slide was observed under microscope for the presence of acid fast bacilli.

4.2.7 Culture

The sample was inoculated onto the chocolate agar plate, blood agar plate and MacConkey agar plates in order. Blood agar and chocolate agar plates were incubated in candle jar (5-10% CO_2) at 37°C for overnight and MacConkey agar plates were incubated at 37°C in incubator for overnight.

4.2.8 Identification of the isolate

The culture plates were examined after overnight incubation and the organism showing the growth on the streaked line were identified with the use of standard microbiological techniques as described in the Bergey's manual which include observation of colony morphologies, staining reactions, and biochemical properties. Latex particle agglutination tests were also done for the confirmation of the bacterial isolates. Standard protocol provided by Cheesbrough, 1984 and Collee *et al,* 1996 was followed for identification of bacteria isolated from CSF specimens.

Biochemical tests:-

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

placed on the subcultured plate in order to differentiate pneumococcus from other gram positive cocci.

- 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 is especially done for the differentiation of pneumococci from viridans streptococci.
    - For this test, the isolate was first grown in 5 ml infusion broth for 18 hrs at $37^{o}C$.
    - While still warm, 0.5 ml of 10% sodium deoxycholatee solution was added and reincubated at $37^{o}C$.
    - 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 requirements of 'X' and 'V' factor was used to identify the colony of *Haemophilus influenzae.* This was done in disc test; the presence or absence of growth was observed around the paper disc impregnated with 'X' factor alone, 'V' factor alone or 'X+V' factor, placed on a nutrient agar. 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 CO_2 for 48 hrs.

- After proper incubation, colonies of *Haemophilus influenzae* will be larger where they are within 1-3mm of the *Staphylococcu*s aureus, which exerts 'V' factor. This phenomenon is known as 'Satellitism'.

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

Latex agglutination tests:-

This test was also performed for identification and serotyping the bacterial isolates like *Haemophilus influenzae*.

- For this test, the pure isolate of *Haemophilus influenzae* was emulsified with a drop of normal saline taken in a provided clean grease free glass slide.
- A drop of positive control reagent was also taken in other circle of same slide.
- A drop of Anti- Hib reagent was added to both circles and mixed thoroughly with the help of applicator.
- The slide was rotated for few seconds and agglutination was observed.

4.2.9 Antibiotic susceptibility pattern of the isolate

Standard Kirby-Bauer disc diffusion method was followed for the antibiotic susceptibility test. The pure culture of the isolate was inoculated into Mueller Hinton broth and incubated for about 4 hours at 37^0C and then using a sterile swab the organism was swabbed on Mueller Hinton agar surface plate. For the isolates like *S. pneumoniae* and *Niesseria meningitidis*, Mueller Hinton agar containing 10% of sheep blood is used for testing its antibiotic susceptibility and for *Haemophilus influenzae*, chocolate agar was used. Commercially available antibiotic discs manufactured by Hi media were used. The antibiotics discs were taken out from refrigerator and after bringing to room temperature, appropriate discs were placed over the agar plates containing the carpet culture of the

isolates with the help of sterile forceps. The plates were then incubated at 37^0C for overnight. Similarly, MHA containing 10% blood and chocolate agar plates were incubated anaerobically in CO_2 incubator for overnight.

Then after incubation the size of zone of inhibition were measured and the results were interpreted on the basis of the standard format.

4.2.10 Quality control for tests

All tests were performed with regular quality control.

During the study, the sterility of each batch of the test medium was confirmed by incubating uninoculated plates and tubes overnight at 37^oC. The incubated plates and tubes of the batch of the medium were not used if those plates and tubes showed the evidence of bacterial growth and other visual reactions after incubation.

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

4.2.11 Data analysis

Chi- square test was used to determine significant association of cell count with that of culture result. Test of present work are shown in Appendix XI.

CHAPTER- V

5. RESULTS

This study was conducted among the children suspected of meningitis attending Kanti Children's Hospital. A total of 431 CSF samples collected by the experienced physician from the children were processed within the study period of 4 months. The samples were processed in the Microbiology laboratory of Kanti Children's Hospital.

5.1 CLINICAL PATTERN OF THE RESULTS

Table 5.1 Age and gender wise distribution of the patients suspected of meningitis

Age Group	Male		Female		Total	
	No. of cases	Percentage	No. of cases	Percentage	No. of cases	Percentage
0-45 days (Neonates)	117	27.15	67	15.54	184	42.69
45 d-1 yr	41	9.51	35	8.12	76	17.63
1yr-5 yr	56	13.00	37	8.58	93	21.58
5 yr- 10yr	31	7.19	17	3.95	48	11.14
10yr-14yr	17	3.94	13	3.02	30	6.96
Total	**262**	**60.79**	**169**	**39.21**	**431**	**100**

As shown in the Table 5.1, out of 431 cases, 262 (60.79%) were male patients and 169 (39.21%) were female patients. The highest number of patients 184 (42.69%) were from the age group of 0-45days (neonates) followed by the age group 1yr-5yr which includes 93 (21.58%) patients. The least number of patients 30 (6.96%) were from the age group 10yr-14yr.

5.2 MICROBIAL PATTERN OF THE RESULTS

Table 5.2 Pattern of CSF culture results

Total no. of suspected cases	**Positive culture result**		**Negative culture result**	
	Total no. of positive cases	**Percentage**	**Total no. of negative cases**	**Percentage**
431	21	4.87	410	95.13

Table 5.3 Gender wise distribution of the no. of bacterial isolates from CSF samples

Gender	**Positive culture result**		**Negative culture result**	
	Total no. of positive cases	**Percentage**	**Total no. of negative cases**	**Percentage**
Male N=262	10	3.82	252	96.18
Female N=169	11	6.51	158	93.49
Total **N=431**	**21**		**410**	

Out of 431 CSF samples, only 21 (4.87%) showed positive growth during culture where as 410 (95.13%) of the samples showed no growth during the culture of CSF samples. Out of 21 positive culture cases, 10 (3.82%) CSF samples were from the male patients and 11 (6.51%) were from the female patients as shown in Table 5.2 and 5.3.

Table 5.4 Age and gender wise distribution of no. of bacterial isolates from CSF samples

Age group	Male		Female		Total	
	No. of positive cases	**Percentage**	**No. of positive cases**	**Percentage**	**No. of positive cases**	**Percentage**
0-45 days (Neonates)	3	14.28	4	19.05	7	33.33
45 d-1 yr	3	14.28	4	19.05	7	33.33
1yr-5 yr	1	4.76	0	0	1	4.76
5 yr- 10yr	3	14.28	1	4.76	4	19.05
10yr-14yr	0	0	2	9.53	2	9.53
Total	**10**	**47.61**	**11**	**52.33**	**21**	**100**

As shown in above table, the highest number of bacterial isolates was isolated from the age group 0-45 days i.e. from neonates and from the age group 45 days-1 yr (7 out of 21, 33.33%) followed by the age group 5yr-10yr (4 out of 21, 19.05%). The least number of bacterial isolates was isolated from the age group 1yr-5yr (1 out of 21, 4.76%). In all age groups, higher number of the isolates was isolated from female patients except in the age groups 5yr-10 yr and 1yr-5yr in which only 1 and no organism was isolated respectively.

Table 5.5 Pattern of bacterial isolates from the CSF positive cultures

S.N	Types of Organisms isolated	No. of isolates	Total percent (N=21)
1.	*Streptococcus pneumoniae*	2	9.53
2.	*Haemophilus influenzae* type b	2	9.53
3.	*Neisseria meningitidis*	1	4.76
4.	*Esherichia coli*	6	28.57
5.	*Staphylococcus aureus*	6	28.57
6.	*Klebsiella pneumoniae*	1	4.76
7.	Enterococci	1	4.76
8.	*Streptococcus viridans*	1	4.76
9.	*Pseudomonas aeruginosa*	1	4.76
	Total	**21**	**100**

As shown in Table 5.5, 9 different types of bacteria were isolated from the total 21 bacteria isolated from CSF samples. Among these bacteria, Gram negative organisms were the predominant among the study group (52.38%). *E.coli and Staphylococcus aureus* (28.57% each) were found to be the most predominant organism among the isolates which was followed by *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (9.53% each). Other isolates were *Neisseria meningitis* (4.76%), *Klebsiella pneumoniae* (4.76%), Enterococci (4.76%), *Streptococcus viridans* (4.76%) and *Pseudomonas aeruginosa* (4.76%).

Table 5.6 Gender wise distribution of bacterial isolates from CSF samples

S.N	Types of Organisms isolated	Male		Female		Total	Percent
		No.	**%**	**No.**	**%**		**(N=21)**
1.	*Streptococcus pneumoniae*	0	0	2	9.53	2	9.53
2.	*Haemophilus influenzae* type b	1	4.76	0	4.76	2	9.53
3.	*Neisseria meningitidis*	1	0	1	4.76	2	4.76
4.	*Esherichia coli*	4	19.05	2	9.53	6	28.57
5.	*Staphylococcus aureus*	2	9.53	4	19.05	6	28.57
6.	*Klebsiella pneumoniae*	1	4.76	0	0	1	4.76
7.	Enterococci	0	0	1	4.76	1	4.76
8.	*Streptococcus viridans*	1	0	0	0	1	4.76
9.	*Pseudomonas aeruginosa*	0	4.76	1	4.76	1	4.76
	Total	**10**	**47.61**	**11**	**52.69**	**21**	**100**

Out of 21 growth positive cases, 11(52.69%) were from female and 10 (47.31%) were from male patients. Among the different species of bacteria isolated, *E. coli* was the most predominant in male patients (19.05%) and *Staphylococcus aureus* in female (19.05%). Pneumococci were isolated from female patients only and meningococci from a male child. Distribution of other isolates is shown in above Table.

Table 5.7 Age wise distribution of types of bacterial isolates from CSF samples

Types of Organisms isolated	Age Group					Total
	0-45day	**45d-1yr**	**1yr-5yr**	**5yr-10yr**	**10yr-14yr**	
Streptococcus pneumoniae	0	2	0	0	0	2
Haemophilus influenzae type b	0	2	0	0	0	2
Neisseria meningitidis	0	0	0	1	0	1
Esherichia coli	4	0	1	1	0	6
Staphylococcus aureus	2	1	0	1	2	6
Klebsiella pneumoniae	0	1	0	0	0	1
Enterococci	0	1	0	0	0	1
Streptococcus viridans	0	0	0	1	0	1
Pseudomonas aeruginosa	1	0	0	0	0	1
Total	**7**	**7**	**1**	**4**	**2**	**21**

Distribution of isolates on the basis of age group as tabulated above in Table 5.7 showed that *Streptococus pneumoniae* and *Haemophilus influenzae* type b were isolated from the age group 45days to 1 year only. Similarly, *E. coli* and *Staphylococcus aureus* were found to be predominant among neonates (0-45 days).

5.3 CORRELATION OF MACROSCOPIC AND MICROSCOPIC FINDINGS WITH CULTURE RESULT

Table 5.8 Correlation of the no. of isolates with the appearance of CSF

S.N	Appearance of CSF	Total sample		Positive culture result	
		No. of samples	Percentage (%)	No. of isolates	Percentage of isolates (%)
1.	Clear	292	67.75	0	0
2.	Xanthochromic	46	10.67	0	0
3.	Slightly turbid	31	7.19	5	16.13
4.	Highly turbid	19	4.41	13	68.42
5.	Traumatic (Mixed with RBC)	43	9.98	3	6.98
	Total	**431**	**100**	**21**	**4.87**

Out of 431 CSF samples received, 292 (67.75%) were found to be clear and 46 (10.67%) were Xanthochromic in appearance and no organism was isolated from those samples. Only 19 (4.41%) CSF samples were found to be highly turbid and rate of isolation of bacteria from those CSF samples was highest (68.42%) followed by slightly turbid samples (68.42%) and traumatic samples (6.98%) respectively as shown in Table 5.8.

Table 5.9 Correlation of the no. of isolates with cell count of CSF

S.N	Cell count ($WBCs/mm^3$)	Total CSF samples		Positive culture result	
		No. of samples	Percentage (%)	No. of isolates	Percentage (%)
1.	0-5	292	67.75	0	0
2.	5-100	63	14.62	3	4.76
3.	>100	76	17.63	18	23.68
	Total	**431**	**100**	**21**	**28.44**

As shown in above table, out of 431 CSF samples received in the laboratory, 292 (67.75%) samples had normal cell count (0-5 $cell/mm^3$). 63 (14.62%) CSF samples had cell count in the range of 5-100 $cells/mm^3$ and 3 (4.76%) isolates were obtained from these samples. Similarly 76 (17.63%) samples had >100 $cells/mm^3$ and highest number of isolates i.e. 18 (23.68%) were obtained from these samples.

Table 5.10 Correlation of no. of bacterial isolates with the protein level of CSF samples

S.N	Protein level of CSF (mg/dl)	Total sample		Positive culture result	
		No. of samples	Percentage (%)	No. of isolates	Percentage (%)
1.	15-45 mg/dl (Normal level)	257	59.63	1	0.39
2.	<15 mg/dl	101	23.43	0	0
3.	45-100 mg/dl	58	13.46	13	22.41
4.	>100 mg/dl	15	3.48	7	46.67
	Total	**431**	**100**	**21**	**69.47**

As shown in Table 5.10, among 431 cases of suspected meningitis, 257 (59.63%) CSF samples were found to have normal protein level where as 101 (23.43%) of samples had protein level <15 mg/dl. Similarly, 15 (3.48%) CSF samples were found to have protein level >100 mg/dl and 58 (13.46%) samples had protein level in the range of 45-100 mg/dl. Out of all cases, highest number of isolates (46.67%) were obtained from those samples which had >100 mg/dl of protein level followed by the range of 45-100 mg/dl (22.41%). No organism was isolated from the CSF samples which had protein level less than 15 mg/dl.

Table 5.11 Correlation of no. of bacterial isolates with the Glucose level of CSF samples

S.N	Glucose level of CSF (mg/dl)	Total sample		Positive culture result	
		No. of samples	Percentage (%)	No. of isolates	Percentage (%)
1.	45-80 mg/dl (Normal level)	222	51.51	1	0.23
2.	>80 mg/dl	24	5.57	0	0
3.	<45 mg/dl	185	42.92	20	10.81
	Total	**431**	**100**	**21**	**11.04**

As shown in Table 5.11, out of 431 CSF samples, 222 (51.51%) of CSF samples had normal Glucose level and only 1 (0.23%) isolate was obtained from this group. Similarly, 185 (42.92%) of CSF samples had glucose level <than 45 mg/dl and highest number of isolates (20) were obtained from this group. Only 24 (5.57%) samples had >80 mg/dl of glucose level and no isolates were obtained from this group.

Table 5.12 Correlation of Gram staining with the no. of isolates of CSF samples

S.N	Gram staining result	Total samples		Culture positive	
		No. of samples	Percentage (%)	No. of samples	Percentage (%)
1.	Gram positive organism	20	4.64	10	2.32
2.	Gram negative organism	14	3.25	11	2.55
	Total (N=431)	**34**	**7.89**	**21**	**4.87**

Table 5.13 Gram staining result on the basis of morphology of the organism

S.N	Gram staining result	Total samples		Culture positive	
		No. of samples	Percentage (%)	No. of samples	Percentage (%)
1.	Gram positive diplococci	5	14.71	2	9.52
2.	Gram positive cocci in clusters	13	38.24	6	28.57
3.	Gram positive cocci in chain	2	5.88	2	9.52
4.	Gram negative coccobacilli	3	8.82	2	9.52
5.	Gram negative bacilli	9	26.47	8	38.10
6.	Gram negative diplococci	2	5.88	1	4.77
	Total	**34**	**100**	**21**	**100**

Table 5.12 and 5.13 showed that out of 431 CSF samples received, only 21 (4.87%) isolates were obtained where as when Gram Staining was performed, 34 (7.89 %) CSF samples showed organism. Out of 34 samples which showed organism during Gram stain, 20 (4.64%) were Gram positive and 14 (3.25%) were Gram negative organisms. Among Gram positive, cocci in clusters (38.24%) were found to be predominant and among Gram negatives, bacilli (26.47%) were found to be predominant.

5.4 ANTIBIOTIC SUSCEPTIBILITY PATTERN OF THE BACTERIAL ISOLATES OF CSF SAMPLES

Table 5.14 Antibiotic susceptibility pattern of individual Gram Negative organism isolated from CSF samples

S.N	Organisms isolated	Antibiotics used	Susceptibility pattern					
			Susceptible		Intermediate		Resistant	
			No.	%	No.	%	No.	%
1.	*Haemophilus influenzae* (type b) N=2	Ampicillin	0	0	0	0	2	100
		Amoxy-Clav	1	50	0	0	1	50
		Ceftriazone	1	50	0	0	1	50
		Chloramphenicol	2	100	0	0	0	0
		Ciprofloxacin	2	100	0	0	0	0
		Erythromycin	1	50	0	0	1	50
		Penicillin	1	50	0	0	1	50
2.	*Neisseria meningitidis* N=1	Ampicillin	0	0	0	0	1	100
		Ceftriazone	1	100	0	0	0	0
		Chloramphenicol	1	100	0	0	0	0
		Ciprofloxacin	1	100	0	0	0	0
		Cotrimoxazole	0	0	0	0	1	100
		Erythromycin	1	100	0	0	0	0
		Penicillin	1	100	0	0	0	0
3.	*E.coli* N=6	Amikacin	1	16.67	0	0	5	83.33
		Cefotaxime	2	33.33	0	0	4	66.67
		Ceftriazone	0	0	0	0	6	100
		Chloramphenicol	5	83.33	0	0	1	16.67
		Ciprofloxacin	2	33.33	2	33.33	2	33.33

		Cotrimoxazole	1	16.67	0	0	5	83.33
		Ofloxacin	4	66.67	1	16.67	1	16.67
4.	*Klebsiella pneumoniae* N=1	Amikacin	0	0	0	0	1	100
		Cefotaxime	1	100	0	0	0	0
		Ceftriazone	0	0	0	0	1	100
		Chloramphenicol	1	100	0	0	0	0
		Ciprofloxacin	0	0	0	0	1	100
		Cotrimoxazole	1	100	0	0	0	0
		Ofloxacin	1	100	0	0	0	0
5.	*Pseudomonas aeruginosa* N=1	Amikacin	1	100	0	0	0	0
		Cefotaxime	1	100	0	0	0	0
		Ceftriazone	0	0	0	0	1	100
		Chloramphenicol	0	0	0	0	1	100
		Ciprofloxacin	1	100	0	0	0	0
		Cotrimoxazole	1	100	0	0	0	0
		Ofloxacin	1	100	0	0	0	0

Among the gram negative isolates, *Haemophilus influenzae* type b was found to be 100% susceptible towards Chloramphenicol and Ciprofloxacin followed by Erythromycin, Penicillin and Amoxy-Clav (50%) and found to be 100% resistant towards Ampicillin.

Neisseria meningitidis was found to be 100% sucsceptible towards all antibiotics used except Ampicillin and Cotrimoxazole.

Chloramphenicol (83.33%) was found to be most effective against *E.coli* followed by Ofloxacin (66.67%) where as it was found to be 100% resistant towards Ceftriazone.

Similarly, *Klebseilla pneumoniae* showed 100% sensitivity towards Cefotaxime, Chloramphenicol, Cotrimoxazole and Ofloxacin and resistance towards Amikacin, Ceftriazone and Ciprofloxacin.

Pseudomonas aeruginosa was found to be susceptible towards Amikacin, Cefotaxime, Ciprofloxacin, Cotrimoxazole and Ofloxacin and resistance towards Chloramphenicol and Ceftriazone.

Table 5.15 Antibiotic susceptibility pattern of Gram Positive organism isolated from CSF samples

S.N	Organisms isolated	Antibiotics used	Susceptibility pattern					
			Susceptible		Intermediate		Resistant	
			No.	%	No.	%	No.	%
1.	*Streptococcus pneumoniae* N=2	Ampicillin	2	100	0	0	0	0
		Cefotaxime	2	100	0	0	0	0
		Chloramphenicol	2	100	0	0	0	0
		Ciprofloxacin	2	100	0	0	0	0
		Cotrimoxazole	0	0	0	0	2	100
		Erythromycin	2	100	0	0	0	0
		Penicillin	2	100	0	0	0	0
2.	*Staphylococcus aureus* N=6	Amikacin	6	100	0	0	0	0
		Cefotaxime	5	83.33	0	0	1	16.67
		Ceftriazone	5	83.33	0	0	1	16.67
		Chloramphenicol	5	83.33	0	0	1	16.67
		Ciprofloxacin	5	83.33	1	16.67	0	0
		Cloxacillin	5	83.33	0	0	1	16.67
		Ofloxacin	6	100	0	0	0	0

3.	Enterococci N=1	Ampicillin	0	0	0	0	1	100
		Cefotaxime	0	0	0	0	1	100
		Chloramphenicol	1	100	0	0	0	0
		Ciprofloxacin	1	100	0	0	0	0
		Erythromycin	1	100	0	0	0	0
		Penicillin	0	0	1	100	0	0
4.	*Streptococcus viridans* N=1	Ampicillin	1	100	0	0	0	0
		Cefotaxime	1	100	0	0	0	0
		Chloramphenicol	1	100	0	0	0	0
		Cotrimoxazole	0	0	0	0	1	100
		Erythromycin	1	100	0	0	0	0
		Penicillin	1	100	0	0	0	0

Out of 2 isolates of *Streptococcus pneumoniae,* both of them were found to be 100% sensitive towards all the antibiotics during susceptibility test except Cotrimoxazole which was found to be 100% resistant.

Amikacin and Ofloxacin showed 100% sensitive for *Staphylococcus auerus* and rest of the antibiotics used was found to be 83.33% sensitive.

Enterococci was found to be 100% sensitive towards Chloramphenicol, Ciprofloxacin and Erythromycin and 100% resistant towards Ampicillin and Cefotaxime.

Streptococcus viridans was found to be 100% sensitive towards all antibiotics used except Cotrimoxazole.

CHAPTER-VI

6. DISCUSSION AND CONCLUSION

6.1 DISCUSSION

This study was conducted among the children suspected of meningitis attending Kanti Childern's Hospital, Kathmandu, Nepal to correlate the macroscopy and microscopy of CSF samples with that of culture. Four hundred and thirty one CSF samples were collected from the children suspected of meningitis and were subjected for the cell count, biochemical and microbiological examination.

In this study, altogether 431 CSF samples were processed and out of 431 samples only 21(4.87%) samples showed culture positive. Our study was found to be similar with the study done by Seetha *et al* (1999) and Matee and Matre (2001) who gave 4.3% and 4.0% culture positive results respectively. Weber *et al* (2002) gave 16% of positive culture result where as Asturias *et al* (2003) reported 40.9% and Ahmed *et al* (2004) gave 49.2% of culture positive results which were found to be higher than this study. Sallam (2004) also gave 33.33% culture positive result in his study. The lower growth positive result in this study may be due to the time period of the study and short duration of the study and also due to the inclusion of septicaemic cases in neonates.

In this study, male child patients were 262 (60.79%) and female child patients were 169 (39.21%) with the sex ratio of male to female of 1.55:1. Thus the number of male patients was found to be higher than that of female patients. Rao *et al* (1998) and Tiwari *et al* (2002) gave the total male to female ratio of 1.2:1 and 1.28:1 respectively which coincides with our study. Higher number of male patients was found in the study done by Ahmed *et al* (2004) and Ogunlesi *et al* (2005) where the percentage of male patients were 57.5% and 71.0% respectively.

According to present study, highest number of patients was from the age group 0-45 days (neonates) which include 42.69% of total suspected cases and the least number of patients were from the age group 10-14 yrs which include only 6.96%.

The highest number of the isolates was obtained from the age group of 0-45 days (neonates) and 45 days-1yr with 33.33% each of positive culture result. Tiwari *et al* (2002) also gave the highest incidence of meningitis among the neonates (15.79%) in a study done at TUTH. This finding also correlates with the study done by Shrestha (1983) in Kanti Children's Hospital which showed 50% of the cases of meningitis occurred in the infants below 1 year of age.

In this study, maximum numbers of children infected were 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.

Among 21 bacterial isolates, 11 (52.38%) were Gram negative organisms where as 10 (47.62%) were Gram positive organisms. Thus Gram negative organisms were found to be predominant in this study group also which correlates with the study done by Gorman *et al* (1962) and Tankhiwale *et al* (2001). The predominance of Gram-negative organisms reported as etiological agents of bacterial meningitis was also seen in the study done by Rao *et al* (1998) where Gram-negative bacteria were isolated from 63.3%, while Gram-positive bacteria were found only in 36.7% of the cases.

Altogether 9 different species of the isolates were isolated in our study. Among them, *E.coli* (28.57%) and *Staphylococcus aureus* (28.57%) were found to be most predominant

one which is followed by *Streptococus pneumoniae* (9.53%) and *Haemophilus influenzae* type b (9.53%). 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 *Streptococus pneumoniae*(14.28%) were found to be second and third predominant organism isolated in culture of CSF with *Klebsiella pneumoniae* being the first (33.33%).

In this study, *E.coli* and *Staphylococcus aureus* were found to be predominant one which was found to be in contrast with the studies done by Etsegenet and Rahlenbeck (1995); Ahmed *et al* (2004) and_Ogunlesi *et al* (2005) where *Streptococus pneumoniae* and *Haemophilus influenzae* was the predominant organism. This may be because the highest number of patients included in this study was neonates and these organisms are unlikely to be isolated from them.

In a study conducted on neonatal meningitis by De Louvois *et al* (1991) and Holt *et al* (2001) in England and Wales, *E.coli* (26% and 18% respectively) was found to be second highest predominant organism isolated from neonates suspected of meningitis which supports our study. Also in a Canadian review of 101 cases of neonatal meningitis done by Newman *et al* (2001) determined 25% of the meningitis was caused by *E.coli*.

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

All the isolates (100%) of *Streptococcus pneumoniae* and *Haemophilus influenzae* were from the age group between 2 months and 1 year. 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, and 27 of the 46 children (58.7%) with *S. pneumoniae* meningitis were also less 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 431 CSF samples received in the laboratory, 292 (67.75%) of the samples were clear and no organism was isolated from these samples.

Forty six (10.67%) CSF samples were Xanthochromic in appearance and no organisms were isolated from these samples also. 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 19 (4.41%) CSF samples were found to be highly turbid and rate of isolation of bacteria from those CSF samples was highest (68.42%) followed by slightly turbid samples (68.42%) and traumatic samples (6.98%) respectively. Ghimire *et al* (2003) gave high degree of positive correlation (r= +0.7) of turbidity with bacterial findings.

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

When cell count of the CSF samples were done, 292 (67.75%) of the samples were found to have cell count in normal range (0-5 cells/mm^3) and no organism were isolated from these samples.

Sixty three (14.62%) samples had the cell count in the range of 5-100 cells/mm^3 with 3 (4.76%) isolates. In a retrospective study done by Bema and Marvin (2005) among the children who had low CSF cell count (0-30cells/mm^3), acute bacterial meningitis was identified by culture in 0.3% of the study population. In our study, 76 (17.63%) CSF samples had cell count of >100 cells/mm^3 and highest percentage (23.68%) of isolates were recovered from these CSF samples.

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 431 cases of suspected meningitis, highest number of isolates (46.67%) were obtained from those samples which had >100 mg/dl of protein level followed by the range of 45-100 mg/dl (22.41%). No organism was isolated from the CSF samples which had 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 95.24% and 87.07% respectively.

Similarly, most of the isolates (10.81%) were obtained from those CSF samples where the level of glucose was less than 45 mg/dl. Only one (0.23%) isolate was obtained from those CSF samples which have normal glucose level and no isolate was obtained from those samples which had glucose level more than 80mg/dl. Sensitivity and Specificity of determination of glucose level were found to be 95.24% and 59.76% 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).

In this study, Gram stain detected organism in more cases (7.89%) than culture which detect the organism in only 4.87% 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 *et al* (2002) also gave high degree of positive correlation (r= +0.8) of direct Gram staining and growth of organism.

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.

The incidence of TBM infection in children (up to 14 years) is very low i.e. 0.053% (Arestis *et al,* 1999). In our study, out of 431 suspected cases, only 30 (6.96) were suspected of TBM and when AFB staining of those samples was performed, no acid fast bacilli were seen during microscopy. Culture of specimen is more sensitive than microscopy but due to limited laboratory facility, culture could not be done.

Among the isolates, most of the organisms were found to be susceptible to Chloramphenicol and Cefotaxime. The isolates of *Haemophilus influenzae* and *Neisseria meningitidis* showed low susceptibility towards Ampicillin and Penicillin which was found to be similar with the findings made by Ogunlesi *et al* (2005). *Neisseria meningitidis* was found to be 100% sucsceptible towards all antibiotics used except Ampicillin and Cotrimoxazole.

Ceftraizone was found to be resistant towards most of the organism isolated. Our findings were to be in contrast with the findings made by Sallam (2004) where Ceftriazone was found to be susceptible and Chloramphenicol was found to be resistant towards majority of isolates.

Among 6 isolates of *E.*coli, Chloramphenicol (83.33%) was found to be most effective followed by Ofloxacin (66.67%) where as Ceftriazone was found to be 100% resistant towards *E.coli. Klebseilla pneumoniae* showed 100% sensitivity towards Cefotaxime, Chloramphenicol, Cotrimoxazole and Ofloxacin and resistance towards Amikacin, Ceftriazone and Ciprofloxacin. *Pseudomonas aeruginosa* was found to be sensitive towards Amikacin, Cefotaxime, Ciprofloxacin, Cotrimoxazole and Ofloxacin and resistance towards Chloramphenicol and Ceftriazone.

Out of 2 isolates of *Streptococcus pneumoniae,* both of them were found to be 100% sensitive towards all the antibiotics during susceptibility test except Cotrimoxazole which was found to be 100% resistant. Amikacin and Ofloxacin showed 100% sensitive for

Staphylococcus auerus and rest of the antibiotics used was found to be 83.33% sensitive. Enterococci was found to be 100% sensitive against Chloramphenicol, Ciprofloxacin and Erythromycin and 100% resistant against Ampicillin and Cefotaxime. *Streptococcus viridans* was found to be 100% sensitive against all antibiotics used except Cotrimoxazole.

In a study done by Dutta and Bhatnagar (2001) on a rational antibiotics therapy in bacterial meningitis, they stated that the neonatal 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 microscopic and cultural examination of CSF samples collected from children attending Kanti Childern’s Hospital was done. The study revealed that there is significant association between the microscopic findings like cell count and Gram stain with that of cultural findings. Gram staining seemed to be more sensitive and rapid than culture in case of diagnosis of bacterial meningitis.

CHAPTER-VII

7. SUMMARY AND RECOMMENDATIONS

7.1 SUMMARY

1. Altogether 431 CSF samples were processed and out of 431 samples only 21(4.87%) samples showed culture positive.
2. The highest number of the isolates was obtained from the age group of 0-45 days (neonates) and 45 days-1yr with 33.33% each of positive culture result.
3. Among 21 bacterial isolates, 11 (52.38%) were Gram negative organisms where as 10 (47.62%) were Gram positive organisms. Thus Gram negative organisms were found to be predominant in our study group.
4. Altogether 9 different species of the isolates were isolated in our study. Among them, *E.coli* (28.57%) and *Staphylococcus aureus* (28.57%) were found to be most predominant one which is followed by *Streptococus pneumoniae* (9.53%) and *Haemophilus influenzae* type b (9.53%). Only one isolate each of *Neisseria meningitidis, Klebseilla pneumoniae,* Enterococci, *Streptococcus viridans* and *Pseudomonas aeruginosa* were obtained from culture.
5. Rate of isolation of bacteria from turbid CSF samples was highest (68.42%) followed by slightly turbid samples (68.42%) and traumatic samples (6.98%) respectively.
6. In our study, 76 (17.63%) CSF samples had cell count of >100 cells/mm^3 and highest percentage (23.68%) of isolates were recovered from these CSF samples. 63 (14.62%) samples had the cell count in the range of 5-100 cells/mm^3 with 3 (4.76%) isolates. Association of cell count with culture result was found to be statistically significant ($P<0.05$).
7. Out of all cases, highest number of isolates (46.67%) were obtained from those samples which had more than100 mg/dl of protein level followed by the range of 45-100 mg/dl (22.41%). 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 95.24% and 83.02% respectively.
8. Highest number of isolates i.e. 20 (10.81%) were obtained from CSF samples that had glucose level less than 45 mg/dl. Sensitivity and Specificity of determination of glucose level were found to be 95.24% and 59.76% respectively.

9. Gram stain of CSF samples detected more organism than culture and Sensitivity and Specificity of Gram stain was found to be 100% and 96.82% respectively.
10. No acid fast bacilli were seen when ZN staining of CSF samples was done in suspected cases of tuberculous meningitis.
11. Antibiotic susceptibility pattern of the isolates showed that Chloramphenicol was susceptible to most of the isolates. Ceftriazone was found to be resistant among most of the Gram negative isolates.

7.2 RECOMMENDATIONS

1. It is recommended to conduct this kind of study in other parts of the country also so that actual incidence of meningitis among the children in whole country can be calculated.
2. As Gram stain detected more case than culture, Gram staining should be performed in all CSF samples that are received in the laboratory for culture as it provide rapid diagnosis of bacterial meningitis.
3. Along with bacterial culture, fungal, viral (cell culture) and amoebic culture should also be carried out so that all etiological agents that cause meningitis can be isolated and identified.
4. Extensive studies on geographical and seasonal variations of meningitis should be carried out nationwide.
5. The incidence and case fatality rates of bacterial meningitis is high in developing country like Nepal than developed country and is particularly high in children under one year of age. There is also great variability in the quality of laboratory diagnosis and there is a lack of good microbiological data from developing countries as compared to developed countries. So, there is a need for development of simple tests to diagnose bacterial meningitis, particularly in developing countries like ours.
6. Ideally, diagnosis of bacterial meningitis is established by isolation of organism by culture but true incidence in outbreaks may not be obtained if the laboratory diagnosis is based only on isolation of organism by standard smear and culture technique, as previous antibiotic therapy alter the Gram stain and culture results. Thus, recently developed techniques like detection of antigen in CSF by latex agglutination test should also be employed as it helps to record true incidence of infection.

7. 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 morbitidy caused by bacterial meningitis.

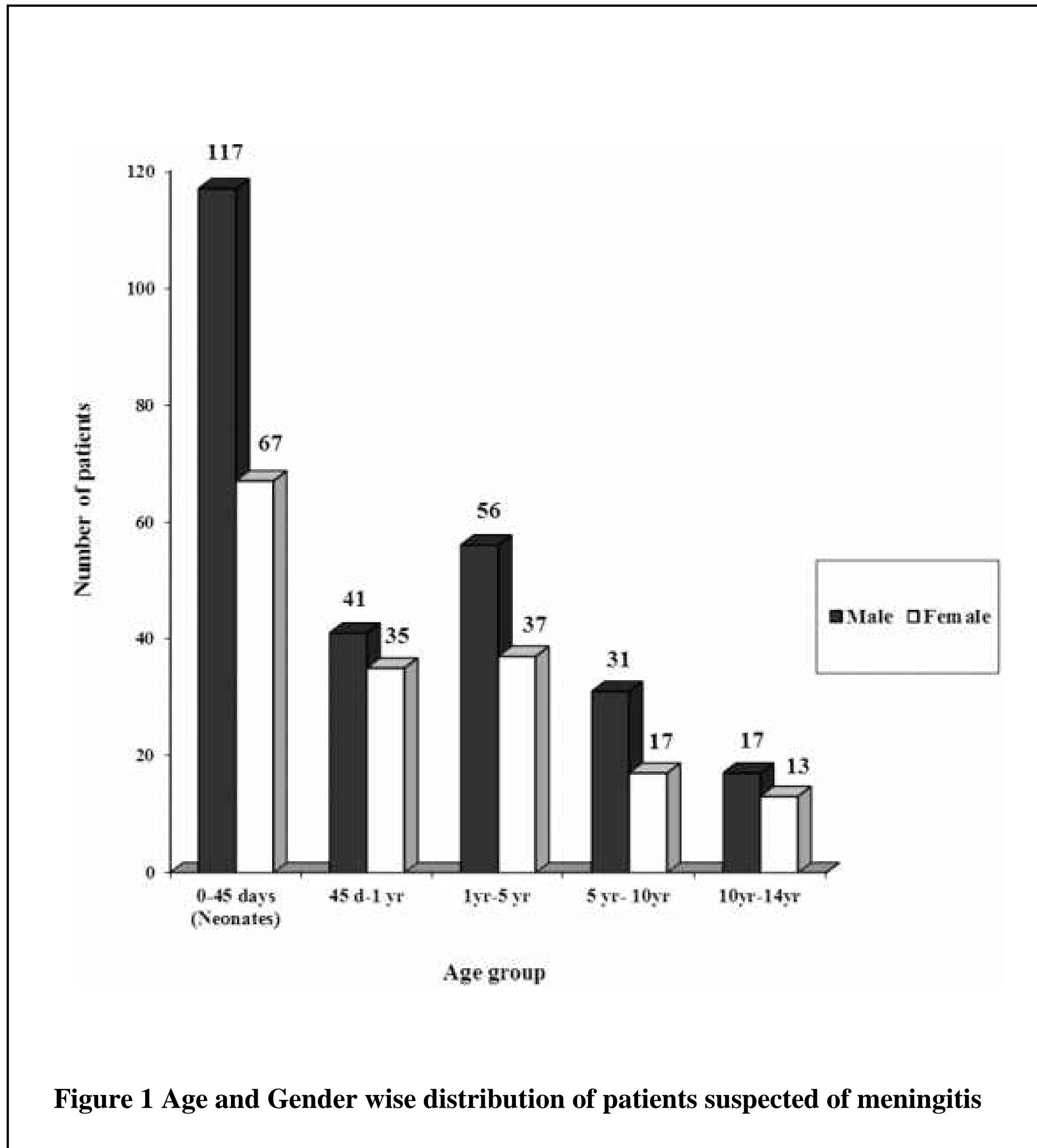

Figure 1 Age and Gender wise distribution of patients suspected of meningitis

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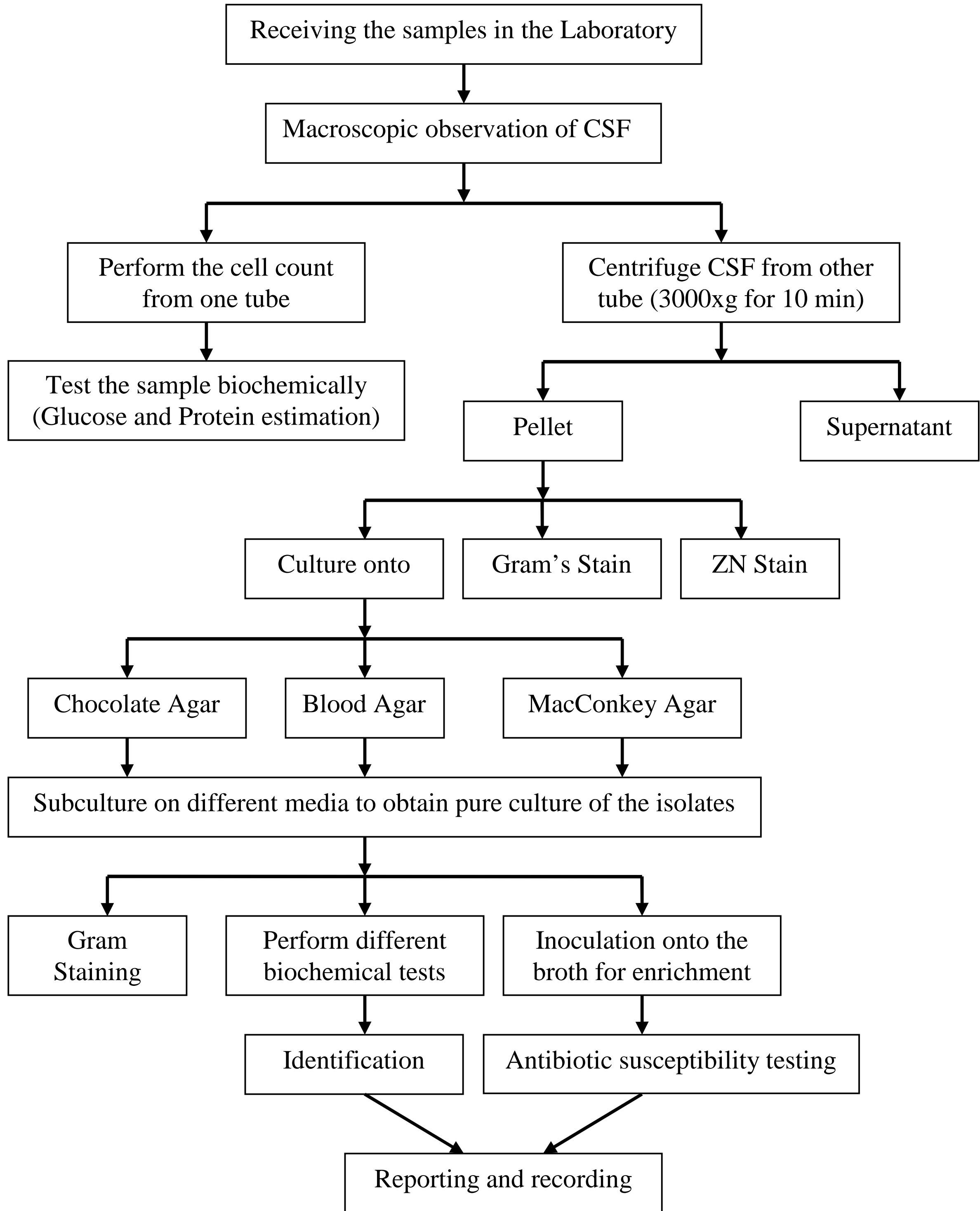
Receiving the samples in the Laboratory
Macroscopic observation of CSF
Perform the cell count from one tube
Centrifuge CSF from other tube (3000xg for 10 min)
Test the sample biochemically (Glucose and Protein estimation)
Pellet
Supernatant
Culture onto
Gram's Stain
ZN Stain
Chocolate Agar
Blood Agar
MacConkey Agar
Subculture on different media to obtain pure culture of the isolates
Gram Staining
Perform different biochemical tests
Inoculation onto the broth for enrichment
Identification
Antibiotic susceptibility testing
Reporting and recording

APPENDIX-I

LIST OF EQUIPMENTS AND MATERIALS USED DURING THE STUDY

A. EQUIPMENTS

Hot air oven	Advantec (Japan)
Incubator	Yamato (Japan)
Autoclave	Ravi (India)
Refrigerator	Sanyo (Japan)
Microscope	Olympus (Japan)
Centrifuge	Remi (India)
Weighing Machine	Chyo MP (Japan)

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)	

E. IDENTIFICATION DISCS/OTHER DISCS

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

F. 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 25^0C 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 25^0C)	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 121^0C (15 lbs pressure) for 15 minutes. After cooling to 45-50^0C, 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 25^0C)	7.1±0.2

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

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 25^0C)	7.4±0.2

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

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 25^0C)	7.4±0.2

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

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 25^0C)	7.4±0.2

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

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 25^0C)	7.4±0.2

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

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 25^0C)	6.9±0.2

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

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

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

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

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

Ingredients	gm/litre
Magnesium sulfate	0.2
Mono-ammonium phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Agar	15.0
Bromothymol Blue	0.08
Final pH (at 25^0C)	6.8±0.2

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

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

Ingredients	**gm/litre**
Peptone	10.0
Tryptone	10.0
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 25^0C)	7.4±0.2

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

5. Urea Base Agar (Hi Media Laboratories)

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

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

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.

3. 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	1gm
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 *p*-phenylene diamine dihydrochloride (TPD)	1 gm
Distilled Water	100 ml

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

(c) Kovac's Indole Reagent (For Indole Test)

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

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

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

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

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

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

Solution A

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

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 g % or 7000 mg %

S2 = 100 mg %

V1 =?

V2 = 100 ml.

We know, S1 X V1 = S2 X V2

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

= 1.43 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

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

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

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 produces hydrogen peroxide, which is lethal to the cell itself. The enzyme catalase is present in most cytochrome containing aerobic and facultative anaerobic bacteria, the main exception being *Streptococcus* spp.

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

B. Oxidase test

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

Procedure: A piece of filter paper was soaked with few drops of oxidase reagent (Whatman's No. 1 filter paper impregnated with 1% tetramethyl-p-phenylene diamine dihydrochloride). Then the colony of the test organism was smeared on the filter paper. The positive test is indicated by the appearance of blue-purple color within 10 seconds.

C. Indole Production test

This test detects the ability of the organism to produce an enzyme: 'tryptophanase' which oxidizes tryptophan to form indolic metabolites: indole, skatole (methyl indole) and indole acetic acid. The enzyme tryptophanase catalyses the deamination reaction attacking the

tryptophan molecule in its side chain and leaving the aromatic ring intact in the form of indole.

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

D. Methyl Red test

This test is performed to test the ability of an organism to produce and maintain stable acid end product from the fermentation of glucose to give a red color with the indicator methyl red and to overcome the buffering capacity of the system. Medium used in the study was Clark and Lubs medium (MR/VP broth, pH 6.9). Methyl red is an indicator which is already acid and will denote changes in degree of acidity by color reactions over a pH range of 4.4-6.0.

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

E. Voges-Proskauer (VP) test

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

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

F. Citrate Utilization test

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

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

G. Motility test

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

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

H. Triple Sugar Iron (TSI) Agar Test.

The TSI agar is used to determine the ability of an organism to utilize specific carbohydrate incorporated in the medium (glucose, sucrose and lactose in concentrations of 0.1%, 1.0% and 1.0% respectively), with or without the production of gas (indicated by cracks in the media as well as an air gap at the bottom of the tube) along with determination of possible hydrogen sulfide production (detected by production of black color in the medium). A pH indicator (phenol red) included in the medium can detect acid production from fermentation

of these carbohydrates and it gives yellow reaction at acidic pH, and red reaction to indicate an alkaline surrounding.

Procedure: The test organism was streaked and stabbed on the surface of TSI and incubated at 37°C for 24 hours .Acid production limited only to the butt region of the tube is indicative of glucose utilization, while acid production in slant and butt indicates sucrose or lactose fermentation. The results are interpreted as follows:

a. Yellow (Acid)/ Yellow (Acid), Gas, H_2S Lactose/ Sucrose fermenter, H_2S producer.

b. Red (Alkaline) / Yellow (Acid), No Gas, No H_2S Only Glucose, not lactose/ Sucrose fermenter, not aerogenic, No H_2S production.

c. Red (Alkaline) / No Change Glucose, Lactose and Sucrose non-fermenter.

d. Yellow (Acid)/ No Change Glucose- Oxidiser.

e. No Change / No Change Non-fermenter.

I. Urea Hydrolysis test:

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

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

J. Coagulase test

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

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 37^0C on a water bath for 6 hours and observed for gel formation in every 30 minutes. The clotting is observed by gently tilting the tube for positive coagulase test.

APPENDIX-VI

MORPHOLOGICAL AND CULTURAL CHARACTERISTICS OF BACTERIA ISOLATED FROM CSF SAMPLE

Bacteria	**Morphological Characteristics**	**Cultural Characteristics**
Streptococcus pneumoniae	Gram positive ovoid or lanceolate cocci arranged in pairs 1-3µm×0.4-0.7µm size, aerobic and anaerobic, nonsporing, motile, capsulated.	On BA: Raised, circular about 1mm in diameter, grow well when supplemented with CO_2. The colonies are alpha ( ) haemolytic. On MA: No growth
Haemophilus influenzae 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 *Staphylococcus aureus* is streaked across the plate on which specimen containing *Haemophilus influenzae* has been inoculated On CA: Translucent colonies are obtained
Neisseria meningitidis	Gram negative oval or spherical cocci 0.6-0.8µm in size, typically arranged in pairs with adjacent sides flttened, capsulated, nonsporing and non motile rods.	On BA: Smooth, small (about 1mm in diameter) translucent, round, convex, typically lenticular colonies are obtained. On MA: No growth
Escherichia coli	Gram negative rod of 1-3µm×0.4-0.7µm size, aerobic and anaerobic, nonsporing, motile, noncapsulated	On BA: Large 1-4 mm in diameter, grayish white, moist, smooth, convex and opaque. The colonies may appear mucoid and some strains are haemolytic. On MA: Bright pink colonies due to lactose fermentation, smooth, glossy and translucent.

Staphylococcus aureus	Gram positive, spherical cocci, 0.8-1 µm in diameter, non sporing, facultative anaerobe, non-motile, except for rare strains, non capsulated. They are arranged in characteristics grape like clusters or in small groups, pairs, singles and short chain(less than five cocci in line).	On BA: Large, 2-4 mm diameter. Circular, smooth with glistening surface, entire edge, soft butyrous consistency and opaque. The pigmentation is golden yellow to cream coloured.Some strains are beta-haemolytic when grown aerobically. On MA: Small (pin head size), 0.1-0.5mm, pink or pink orange due to lactose fermentation. Some strains are non-lactose fermenting.
Klebsiella spp.	Gram negative, short and thick rods of 1-2µm × 0.8µm size, nonsporing, nonmotile and capsulated.	Large dome shaped moist and usually viscid or mucoid colonies when cultured on BA and MA. Most Klebsiella species are lactose fermenting.
Enterocooci	Gram positive oval cocci in pairs or short chains.	On BA: Non-haemolytic colonies with 1-2 mm in diameter are obtained.
Streptococcus viridans	Gram positive cocci, non sporing and non-motile.	On BA: Alhpa ( ) haemolytic colonies are obtained.
Pseudomonas aeruginosa	Gram negative slender rods with 1.5-3µm x 0.5µm size, actively motile by polar flagellum, non sporing, most of the strains produce slime.	On NA: Large, opaque, irregular colonies with distinctive, musty odor and metallic sheen. On BA: Large flat colonies showing haemodigestion. On MA: Pale, nonlactose fermenting, colorless translucent colonies.

APPENDIX-VII

Table: Distinguishing reactions of the commoner and pathogenic Enterobacteriaceae

Species	Test/ substrate											
	lac	mot	gas	ind	VP	cit	PDA	ure	lys	H_2S	inos	ONPG
E. coli	+	+	+	+	-	-	-	-	+	-	-	+
Shigella groups A, B, C	-	-	-	±	-	-	-	-	-	-	-	-
Sh. sonnei	-	-	-	-	-	-	-	-	-	-	-	+
Salmonella (most serotypes)	-	+	+	-	-	+	-	-	+	+	±	-
S. typhi	-	+	-	-	-	-	-	-	+	+	-	-
S. paratyphi A	-	+	+	-	-	-	-	-	-	-	-	-
C. freundii	±	+	+	-	-	+	-	±	-	±	-	+
C. koseri	±	+	+	+	-	+	-	±	-	-	-	+
K. pneumoniae	+	-	++	-	+	+	-	+	+	-	+	+
K. oxytoca	+	-	++	+	+	+	-	+	+	-	+	+
E. aerogenes	+	+	++	-	+	+	-	-	+	-	+	+
E. cloacae	+	+	+	-	+	+	-	±	-	-	-	+
Hafnia alvei	-	+	+	-	+	-	-	-	+	-	-	+
Serratia marcescens[b]	-	+	±	-	+	+	-	-	+	-	±	+
P.mirabilis	-	+	+	-	±	±	+	++	-	+	-	-
P. vulgaris	-	+	+	+	-	-	+	++	-	+	-	-
M. morganii	-	+	+	+	-	-	+	++	-	±	-	-
Providencia rettgeri	-	+	-	+	-	+	+	++	-	-	+	-
P. stuartii	-	+	-	+	-	+	+	±	-	-	+	-
P. alcalifaciens	-	+	+	+	-	+	+	-	-	-	-	-
Yersinia enterocolitica[c]	-	-	-	±	-	-	-	±	-	-	±	+
Y. pestis	-	-	-	-	-	-	-	-	-	-	-	±
Y. pseudotuberculosis	-	-	-	-	-	-	-	+	-	-	-	±

[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; H_2S, H_2S produced in TSI agar; ONPG, metabolism of *o*-nitrophenyl- -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
Amoxycillin/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 *S.pneumoniae*	P	10 mcg	-	-	20

(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
Amoxycillin/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	53	1	357
Sugar level	20	165	1	245
Gram Stain	21	13	0	397

Calculation of sensitivity
Sensitivity can be calculated as:
Sensitivity = a/ (a+c) ×100%
Sensitivity of Protein level = 20/ (20+1) ×100%=95.24%
Sensitivity of Sugar level = 20/ (20+1) ×100%=95.24%
Sensitivity of Gram Stain= 21/ (21+0) ×100%=100%
Calculation of Specificity
Specificity can be calculated as
Specificity=d/ (b+d) ×100%
Specificity of Protein level=357/ (53+357) ×100%=87.07%
Specificity of Sugar level=245/ (165+245) ×100=59.76%
Specificity of Gram Stain= 397/ (13+397) ×100%=96.82%
Calculation of Positive Predictive Value (PPV)
PVP can be calculated as
PVP=a/ (a+b) ×100%
PVP of Protein level =20/ (20+53) ×100%=27.40%
PVP of Sugar level=20/ (20+165) ×100%=10.81%
PVP of Gram Stain=21/ (21+13) ×100%=61.76%
Calculation of Negative Predictive Value (NPV)
PVN can be calculated as
PVN=d/(c+d) ×100%

PVN of Protein level =357/ (1+357) ×100=99.72%
PVN of Sugar level=245/ (1+245) ×100=99.59%
PVN of Gram Stain =397/ (0+397) ×100=100%

Calculation of Efficiency

Efficiency can be calculated as
Efficiency= a+d/ (a+b+c+d) ×100%
Efficiency of Protein level=20+357/ (20+53+1+357) ×100%=87.47%
Efficiency of Sugar level=20+245/ (20+165+1+245) ×100%=61.48%
Efficiency of Gram Stain=21+397/ (19+16+2+394) ×100%=96.98%

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	292	**292**
5-100	2	61	**63**
>100	19	57	**76**
Total	**21**	**410**	**431**

Test statistic is ^{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 $^2 =$ (0-E) ^{2}/E we find ^{2}=85.2

Thus ^{2}cal (85.2) > ^{2} tab at ℑ=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.

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	45-80
Purulent (pyogenic)meningitis	Cloudy or purulent, may contain clots	5-20000 (mean of 800)	Mostly Neutrophils	Elevated (>100)	Reduced
Viral (aseptic) meningitis	Clear or slightly cloudy	2-2000 (mean of 80)	Mostly lymphocytes	Normal or slightly elevated	Normal or slightly reduced
Tuberculous meningitis	Clear or slightly cloudy, fine clot may form	5-2000 (mean of 100)	Mostly lymphocytes	Elevated (>50)	Reduced
Cryptococcal meningitis	Clear or slightly cloudy	5-2000 (mean of 100)	Mostly lymphocytes	Elevated (>50)	Reduced
Leptospiral meningitis	Clear or cloudy	> 25	Mostly lymphocytes	Elevated (>50)	Normal
***L. monocytogenes* meningitis**	Slightly cloudy or purulent	>200	Mostly lymphocytes	Elevated (>50)	Reduced
Neurosyphilis	Usually clear, may contain fine clots	>20	Mostly lymphocytes	Normal or slightly elevated	Normal or slightly reduced

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