CHAPTER-1

1.1 Introduction

Japanese encephalitis (JE) is an acute arboviral infection causing acute encephalitis syndrome (AES). Approximately 50,000 cases and about 15,000 deaths occur annually due to JE (Tsai, 1998). JE is wide spread in temperate and tropical Asian countries (South East Asia), the Indian subcontinent, China, Korea, Japan and parts of Oceania and is thus the most important causes of epidemic encephalitis worldwide (Solomon, 1998).

In the recent years, the number of JE patients has decreased dramatically from Japan, Korea and the People's Republic of China (PRC) with widespread use of JE vaccine and proper vector control systems. However, cases have been continuously increasing in Southeast Asia and Indian subcontinent with poor economic status and sanitation (Kabilan, 2004). In developed countries where children are protected by immunization, JE occurrence is increased in the elderly or old age, consistent with waning immunity with age (Vaughn and Hoke, 1992).

JE is a mosquito-borne acute viral infection of the central nervous system caused by Japanese encephalitis virus (JEV). Patients with JE typically present a few days of non-specific febrile illness followed by abrupt headache, vomiting and a reduced level of consciousness. It is primarily the disease of swine, equine and wading birds. The disease accidentally affects human population. Though there are many genera and species of mosquito vectors believed to be responsible for the disease transmission, *Culex* mosquito (*Cx. tritaeniorhyncus*) is the principal vector in Nepal. It often breeds in paddy ecosystem. Various vertebrate hosts (pigs, birds) are responsible for maintaining the natural virus cycle. Human is the incidental host. Clinical attack rate of the disease is low (1 in 300). JE virus infected persons do not have high titer

viremia and are therefore considered as "dead-end" hosts. They do not contribute to perpetuation of virus transmission (Brooks et al., 2004).

Approximately, 3 billion people and 60% of the world's population live in the JE endemic regions (Kabilan, 2004). The annual incidence of clinical infection in endemic areas ranges from 10 to 100 per 100,000 populations. More than 50% of the affected populations are children of less than 15 years of age. Case fatality rate range from 0.3% to 60%. There is nearly universal exposure to the virus by adulthood in JE endemic region. JE usually is severe, resulting in a fatal outcome in 25% of cases and residual neuropsychiatric sequelae in 30% of cases (Burke et al., 1988).

In Nepal, 24 districts of terai and inner terai regions are affected by JE and 12.5 million people are estimated to be at the risk of the disease. Since the first outbreak in 1978, seasonal outbreaks of JE have been reported annually. Now days, in terms of morbidity and mortality this disease is the major public health problem in Nepal. Annually 1000-3000 total cases and 200-400 deaths occur. Though, there is increase in the case incidence or improvement in the reporting status, case fatality rate (CFR) has been declined from previous years probably due to proper management and good nursing care provided at the hospitals and enhanced public health awareness etc (E D C D, 2005).

In Nepal, JE is principally a disease of rural agricultural areas, where vector mosquitoes live and grow in close association with the main vertebrate host. Some hyper endemic districts of Nepal represent the paddy field ecosystem with abundant *Culex* species and amplifying hosts like pigs and migratory birds indicating the potential epidemic in these districts. High humidity, summer temperature of 24°C-38°C and paddy field ecosystem of the terai region are the favorable conditions for breeding of *Culex* mosquitoes, the principal vector of JE in Nepal. Therefore, a high

prevalence of JE has been identified in the terai and inner terai regions where cross-border transmission is also possible around the border areas (EDCD, 2005). Sporadic cases have also been reported from other non-endemic districts.

Human become infected after the bite of an infective mosquito. Viral infection rates in the mosquitoes ranges from <1% to 3%. Because the paddy field-breeding *Culex* mosquitoes, which transmit JEV, are unavoidable, the majority of the population in rural Asia has been infected with the virus by early adulthood (Solomon et al., 2003).

JE cases in Nepal start to appear in the month of April-May reach its peak during late August to early September and start to decline from October (EDCD, 2005). There is always some periodic oscillation but when favouring climatic condition coincides with the natural period, fulminating outbreak of the vector borne diseases occurs (Mac Donald, 1957).

JE is generally diagnosed on the basis of clinical symptoms in the rural areas of Asia including Nepal. JE virus is rarely isolated from clinical specimens, probably because of short and low level of viraemia and rapid development of neutralizing antibodies. The laboratory diagnosis of JE, therefore, relies on serology.

For the laboratory diagnosis by serological methods, Haemagglutination test has long been employed, but this method has several practical limitations. Most importantly, it is time consuming, requires paired serum samples and cannot give early diagnosis. IgM capture enzyme linked immunosorbent assay (IgM-capture ELISA) has become the most reliable technique for the diagnosis of Japanese encephalitis. However, it requires relatively sophisticated equipment, and has been confined largely to few academic and referral centers in developing countries.

Laboratory diagnosis of JE in Nepal has been constrained by different factors. There is unavailability of JE diagnostic facility in many health centres. IgM-capture ELISA is expensive to be used in routine diagnosis of JE, as it requires relatively sophisticated equipment, which is impractical in the country like Nepal with poor socio-economic status. There is also lack of trained manpower in many health centres. As the diagnostic centers are only few early diagnoses of the patients is impossible which is crucial for the treatment of JE.

Recently, a simple particle agglutination assay (PA) has been developed for detecting anti-JEV IgM, which does not require specific laboratory facilities. PA can be performed at the room temperature in short period of time. It does not require ELISA reader, which is expensive to be used in diagnostic health centres. The sensitivity and specificity of particle agglutination assay is acceptably high and determined to be useful in rural areas of Asia including Nepal (Pandey et al., 2003). This study is focused primarily to compare the efficacy of particle agglutination assay with IgM-capture ELISA among the patients clinically diagnosed as Acute Encephalitis Syndrome (AES) and also to observe cross reactivity with other clinically infectious and tropical diseases.

CHAPTER II

2. OBJECTIVES

2.1 General objective

- To compare particle agglutination assay with IgM capture ELISA for the diagnosis of clinically suspected cases of JE.

2.2 Specific objective

- To observe age wise, sex wise and district wise status of JE among the patients included during study.
- To know the serological status of JE in patients of Bheri Zonal Hospital and Nepalgunj Medical College, Nepalgunj.
- To compare the sensitivity and specificity of particle agglutination assay with IgM capture ELISA.
- To know the cross reactivity pattern of particle agglutination assay with other clinically infectious and tropical diseases (dengue, malaria, typhoid, kala-azar and leptospirosis).

CHAPTER III

3. LITERATURE REVIEW

3.1 Introduction

Japanese encephalitis (JE) is the leading viral encephalitis in eastern and southern Asia. It is a mosquito borne arboviral infection caused by Japanese encephalitis virus (JEV). It is the most common cause of childhood viral encephalitis in the world. JE is an acute viral infection of the central nervous system. JE is wide spread in temperate and tropical Asian countries (South East Asia), the Indian subcontinent, China, Korea, Japan and parts of Oceania and is thus the most important causes of epidemic encephalitis worldwide. Annually a remarkable number of deaths due to JE are recorded worldwide. The high case fatality rate and frequent residual neuropsychiatric sequelae in the survivors make JE a significant public health problem (Solomon et al., 1998; Burke et al., 1988).

3.2 History:

The history of JE goes back to 1871 A.D. Even though cases and outbreaks of clinically resembling JE have been observed since 19^{th} century, the first outbreak of JE was occurred in Japan during 1924. Then it spread from East Asia to South East Asia (SEA) and then to South Asia. JE virus (JEV) was isolated from a clinical case in the first reported epidemic in Japan in 1924 (Miyake, 1964). Nakayama strain of JEV was first isolated from human cases in 1935 (Monath, 1985). It was called "summer encephalitis" till identified in 1936. A severe epidemic of JE has been recorded in Japan during 1964 with 6000 cases and case fatality rate (CFR) as high as 60%. Similarly, epidemics of JE were reported in many provinces of China in the early 1950s and with almost all provinces being affected by now (Yu Y, 1995). Merely three decades ago JE was endemic to only a few countries of East Asia like Japan, Korea and China, which later spread to other countries. Then it spread from

East Asia to South East Asia (SEA) and then to South Asia. This disease is spreading to America, Europe, Africa and part of Australia.

It is prevalent in Northern Thailand, as well as in Bihar, Uttar-Pradesh, Tamil Nadu and West Bengal states in India, in the terai areas of Nepal and in Sri Lanka. Cases have been reported in Bangladesh, Indonesia and Myanmar. The first indication of JE transmission in SEA region was from Sri Lanka where an outbreak was apparently reported in 1948 (Tsai, 1998). In India, epidemics of JE were first recognized around Vellore (Sehgal, 1989). In 1948 outbreak, 85% of donkey sera were JE antibody positive. In Nepal, clinical cases were reported even before 1975 and an epidemic of JE was first recognized in Rupandehi district of the Western Development Region (WDR) in 1978 (Joshi, 1986; Bista et al., 1999). The disease was then thought to be imported from Gorakhpur and surroundings areas of Uttar Pradesh of India, where JE epidemic had occurred (Umenai et al., 1985; Khatri et al., 1983, Joshi, 1983). Then subsequently epidemic occurred in Morang district of eastern Nepal gradually spreading into other district in successive years.

In recent years the epidemiological pattern and geographical distribution of Japanese encephalitis have changed in Asia. In Taiwan, Japan, South Korea and China clinical cases of JE have decreased dramatically. This has been possible through the integrated control effort comprising of human vaccination program, water management, and immunization of pigs, systematic piggery and community awareness program. On the other hand, the incidence of JE has increased in India, Nepal, Sri Lanka, Thailand, Bangladesh and Vietnam (Umenai et.al., 1985). Number of outbreaks occurred in India during 1948 to 1978. In 1955, first sero-survey was conducted in India. Haemagglutination inhibition test (HIT) was done on human and animal serum during 1978 to 1980 in Nepal (Bista and Shrestha, 2001). In Nepal, it is serologically diagnosed that pigs and ducks are main reservoir. During 1985-86

epidemics, JEV was isolated from human brain / CSF, sentinel pigs and pool of female mosquitoes.

3.3 Etiological Agent

A specific virus named Japanese encephalitis virus (JEV) is the causative organism of JE, which is under the genus Flavivirus and family Flaviviridae. JEV is one member of 70 viruses in the Flavivirus genus of the Flaviviridae family. It is antigenically related to St. Louis Encephalitis (SLE) virus, Rocio virus, Murray valley Encephalitis virus, West Nile virus and several other flaviviruses (Gubler et al., 1989). It agglutinates erythrocytes of chicken, pigeon and sheep, if the conditions of the tests are carefully controlled. This virus can be cultivated in either tissue cultures (C_6/36 cells) or chicken embryos.

Morphologically, the JEV virion is spherical, approximately 40-50 nm in diameter, with a lipid membrane enclosing isometric 30 nm diameter nucleocapsid core comprised of a capsid (C) protein and a single stranded messenger (positive) sense viral RNA of approximately 11kb. The RNA comprises a short 5' untranslated region (UTR), a longer 3' UTR and between them a single open reading frame (ORF) of approximately 10 kb (Chambers et al., 1990). This codes for a single polyprotein which is co- and posttranslational cleaved by viral and host proteases into three structural proteins (core-C, pre-membrane-PrM and envelope-E) and seven nonstructural (NS) proteins (NSI, NS2a, NS2B, NS3, NS4A, NS4B, NS5). The C protein is highly basic and combines with the RNA to form the nucleocapsid. The PrM is closely associated with the E protein, forming a heterodimer and is thought to act as a ' Chaperone' to it, impairing its function until after virion release. Immediately prior to virion release, the PrM protein is cleaved by a furin like protease to its mature M protein form. This allows the formation of E protein homodimers, which are thus 'activated' (Stadler et al., 1997). The E protein is the largest structural protein, consisting of nearly 500 amino acids with up to two glycosylation sites. It is the

mature target for the humoral immune response, and is thought to be important in viral entry into host cells (Solomon et al., 2003). Thus biological activities like mediation of viral bindings to susceptible cells, haemagglutination, possible participation in endosomal viral function and induction of host protective immune response are ascribed to "E" protein of the virus. Vaccination against JE utilizes mainly Structural protein Pre-membrane (prM) and envelope (E) protein as antigenic structure.

The virus can be inactivated at 56°C temperature in 30 minute. It can survive at 0°C upto 3 weeks, at -70°C upto several months, and very long time in 10% serum or milk (Shrestha, 2004).

The molecular phylogeny of JE viruses, based on the 240 base nucleotide sequence of viral PrM, divides JE isolates into four distinct genotypes, with a maximum divergence of 21% among the isolates. The largest genotype consists of viruses from Japan, Okinawa, China, Taiwan, Vietnam, Phillippines, Sri Lanka, India and Nepal. A second genotype consist isolates from northern Thailand and Cambodia, and a third, from southern Thailand, Malaysia, Sarawak, Australia and Indonesia. Five Indonesian isolates, two from Java, two from Bali and one from Flores, similar to each other and distinct from other Indonesian isolates, form the fourth genotype. Co-circulation of multiple genotypes was observed only in Thailand and Indonesia (Kumar, 1999).

Until now prevalence of three different strains of JEV have been reported in Nepal (Nepal 1/90, B-2524 and B-9548). A study on sero-epidemiology of JEV infection carried out by Kubo et al., in 1996, found out that the number of JE cases and deaths that occurred due to JE in Nepal for the last two decades correlated well with the findings in India. This was also proven by Kubo's antigenic study and is attributable to the free and frequent travel of the people of both countries through the open border.

3.4 Vectors

Thirty species of mosquitoes belonging to five genera of *Culex, Anopheles, Aedes, Humansonia and Amergeres* harbor JE viruses. They are mostly zoophilic. The mosquito borne mode of JE transmission was elucidated with the isolation of JE virus in 1983 and subsequently in other field studies that also established the role of aquatic birds and pigs in the viral enzootic cycle (Tsai and Yu, 1994). Entomological studies carried out during the outbreaks of 1981 to 1984, have shown Culicine mosquitoes namely *Cx tritaeniorhyncus, Cx gelidus, Cx vishnii, Cx pseudovishinii and Cx fuscocephala* as suspected vectors of transmitting JE virus both in animals and humans (Pradhan, 1981; Regmi et al., 1985; Khatri et al., 1983). As *Cx tritaeniorhyncus* is abundantly found in the rice fields ecosystem of the endemic areas during the transmission season and JE virus isolates have been obtained only from a pool of *Cx tritaeniorhyncus* female, this species is suspected to be the principal vector of JE in Nepal (Gubler et al., 1989; Darsie et al., 1989). Culicine mosquitoes breed in irrigated rice fields, shallow marshes, ponds, pools and ditches with fresh or polluted water with grass or aquatic vegetation in partial shade or full sun. Breeding preference of *Cx tritaeniorhyncus* and epidemics of JE associated with paddy fields ecosystem have been adequately substantiated by different studies (Tsai, 1997). Experts believe that rice fields are the probable predominant source of larval breeding in this country because they have demonstrated abundant presence of potential JE vectors in the rice fields.

Culex mosquitoes prefer to feed outdoor (exophagy) principally on vertebrate hosts other than human. They feed predominantly on cattle (85-88%); pigs (4-5%) and human (2-6%) (Reuben et al., 1997). Humans as mentioned earlier are the incidental hosts. The important factors governing spill over of the diseases to human are the related abundance of the vectors, the availability of amplifying hosts, the density and absolute number of mosquitoes, adequate human mosquitoes contact and longevity of vector (WHO, 1998). In endemic areas, up to 3% of the vector mosquitoes are

infected with JEV. Although the mosquitoes prefer to feed on large domestic animals and birds, if an infected mosquito bites a human, infection and subsequent illness can occur.

3.5 Vertebrate hosts as reservoir

It is well documented that swine and varieties of birds, both wild and domestic, are amplifying hosts of JE and serve as a source of infection for those mosquitoes that transmit JE to humans (Buescher et al., 1959; Carey et al., 1968; Dhanda et al., 1977). Pigs are important amplifying hosts of JE virus. Infected pigs generally do not manifest overt symptoms of illness. Virus of JE proliferates in pigs. The later remain viraemic for several days so that the biting mosquitoes become infected. Since pigs become infected as result of the bite of infected mosquitoes and can transmit the virus to many others, they are called amplifiers of virus transmission. In some places, up to 100% of pigs have antibodies to JE. They are generally symptomless during viraemia. Piglets (below 6 months age) show nonsuppurative encephalitis and nervous signs. Pregnant sow may abort or give stillbirth. Abnormal fetuses are found in the litter with the arrest of the growth at different critical periods. Hydrocephalus interna and defect of the brain is seen in the fetuses or stillbirth. There will be congestion and edema (dropsy) of scrotum of the affected boar. Perivascular infiltration of Lymphocytes at midbrain and proliferation of glia cells at the cerebrum are seen in abnormal newborn piglets. Inoculation test in suckling mice can be used for the isolation of JEV.

Bovines do not appear to serve as an amplifying host. The major vectors of JE feed on bovines and there is serological evidence of bovines being infected with the virus. However, viraemia is very difficult to be demonstrated. Also, bovines tend to live many years so the number of susceptible animals introduced every year is very few. Thus, bovines may act as mosquito attractants but are not considered as the natural hosts of the virus. Bovine and Sheep may not be natural hosts. Even with positive

antibody titer, virus is not found in circulating blood. They are symptomless and "dead end hosts".

Bats have been shown to develolp viraemia. Horses are the only domestic animals so far known which show the sign of encephalitis due to JE Virus infection. Since they are not prevalent in large parts of Southeast Asia, they don't play a significant role in the transmission. Symptoms of paralysis of mouth lips, paddling of legs, unable to stand, circling and incoordination are seen. Signs of high fever, jaundice, rigid neck, petichiation of nasal mucosa, incoordination, paralysis, and convulsion are seen. Litters in the horse stable shows trace of circling movement of diseased horse. Lesions like marked congestion or hemorrhages under piamater of the cerebrum, dilation of the blood vessels under piamater and in parenchyma of the cerebrum and perivascular infiltration of the cells consisting of mononuclear cells at the white mater of the cerebrum are seen. Glia nodules are found at the white mater of the cerebrum. Neuronophagy are found at the Grey mater of the cerebrum.

Among birds, pond herons and cattle egrets may play an important part in the natural history of the JE virus. There is no convincing evidence that migratory birds can transfer the virus from one region to another. Pigeons and sparrows can develop viremia and infect mosquitoes. In Nepal infection in pigs and ducks has been proved through serological studies (Joshi, 1986) and Culicine mosquitoes have been found to be breeding and growing in close association with wading birds and ducks. In a sero-survey conducted in Nepal, 40% of pig, 35% of pond herons and cattle egrets and 6-7% of ducks have shown positive antibody titer for JE.

Table 1: Reservoir / animals Population in Nepal:

Region	Pig population	Duck population
National (Grand total)	912,350	411,410
In 24 endemic districts	358,850	331,646
Percentage in endemic districts	39%	81%

Source: Statistical information on Nepalese Agriculture, 2001/2002

3.6 Clinical Spectrum

3.6.1 Pathogenesis

Only about 1 in 25 to 1 in 1000 humans infected with JEV develops clinical features of infection. The factors determining which of all the humans infected develop disease are unknown, but could include viral factors such as route of entry, titre and neurovirulence of the inoculums, and host factors such as age, genetic make up, general health, and pre-existing immunity.

Human becomes infected after the bite of an infected mosquito vector. JEV multiplies locally and in regional nodes (Ahmed, 1999). A phase of transient viremia occurs before the invasion of central nervous system (CNS). Some data obtained from the experiment in mice and macaque monkeys suggest that the site of peripheral amplification is dermal tissue and then lymphocytes. In animal models, JEV strains differ in both their neuroinvasiveness (following peripheral inoculation) and neurovirulence (following intracranial inoculation). The means by which JEV crosses

the blood- brain barrier is unknown (Solomon et al., 2003). JEV is thought to invade brain via vascular endothelial cells by endocytosis (Liou and Hsu, 1998).

The gray matter is the principal target, but white matter is not totally exempt, the brain stem and the diencephalon are predominantly at risk. JE virus exerts lytic effect on neurons, sometimes on vascular endothelium; there are no inclusion bodies but perivascular cuffing with lymphocytes is invariable, involvement of meninges is usual and in severe cases, there is necrosis of brain. Iron concentration decreases. Hypoferraemia is associated with the accumulation of iron in spleen, accompanied by transient anemia (Mc Callum JD, 1991). Brain damage and severe/ permanent neurological sequelae including deficits in motor function and changes in behavior and intellectual function have been observed in more than half of the survivors.

CT and MRI scans reveal low density areas and abnormal signal intensities in thalamus, basal ganglia and putamen which correlate with clinical findings of tremor, rigidity and abnormal movements that are common in the acute phase of illness. Persistent ECG abnormalities are common in the children.

Experimental studies with a hamster model of related flavivirus (SLE virus) suggest that olfactory route as an important route of viral entry (Monath et al., 1983). However, immunohistochemical staining of human post mortem material has shown diffused infection throughout the brain, indicating a haematogenous route of entry (Desai et al., 1995; Johnson et al., 1985). Although experimental evidence suggests that replication within the endothelial cells may be an important means of crossing the blood-brain barrier in some flaviviruses, for JE virus passive transfer across the endothelial cells seems a more likely mechanism (Dropulie and Masters, 1990; Liou and Hsu, 1998).

Other factors which compromise the integrity of the blood-brain barrier have also been considered as risk factor for neuroinvasion (Solomon et al., 2003). It has also been suggested that head trauma (for example, due to road traffic accident) during the transient viremia could facilititate the viral entry into the CNS (Shiraki, 1970).

In the neurons, JEV replicates and matures in the neuronal secretory system, mainly the rough Endoplasmic Reticulum and Golgi apparatus, eventually destroying them (Hase et al., 1990).

3.6.2 Immunology

Both humoral (particularly against E and NS1 proteins) and cellular (including cytotoxic T lymphocytes) arms of immune system are involved in immunity to JEV (Johnson et al., 1985; Konishi et al., 1995). But the relative contribution of individual components has not been well understood (Tiroumourougane et al., 2002).

3.6.2.1 Humoral immunity

The humoral immune response in JE has been well characterized (Solomon et al., 2003). When disease is due to primary infection with JEV, a rapid and potent monotypic IgM response occurs in serum and cerebrospinal fluid (CSF), usually within 7 days of infection (Burke et al., 1985). By day 7 most patients have raised titres. The role of antibodies in protection is not yet clearly understood. Attempts to isolate virus during raised antibody titers, are usually negative. However, the failure to mount an IgM response is associated with positive virus isolation and a fatal outcome. Disappearance of neurological signs has been noted in the presence of IgM antibodies (Edleman et al., 1976). Antibodies to JEV probably protect the host by restricting viral replication during the viremic phase, before the virus crosses the blood- brain barrier (Hammon and Sather, 1973).

Evidence from other flaviviruses suggests that antibodies to JEV may also limit the damage during established encephalitis by neutralizing extracellular virus and

facilitating lysis of infected cells by antibody dependent cellular cytotoxicity (ADCC) (Carmenaga et al., 1974).

Within 30 days of infection the survivors exhibit IgG in the serum and CSF. Asymptomatic infection with JEV is also associated with raised IgM in serum, but not in CSF (Burke et al., 1985). An anamnestic antibody response with early rise in IgG and slow rise in IgM has been noted in patients with secondary infection (previously infected with antigenically related flaviviruses).

3.6.2.2 Cell mediated immunity

The importance of cell mediated immunity was recognized late. In animal models of JE, the cellular immune response seems to contribute to the prevention of disease during acute infection by restricting virus replication before the CNS is invaded (Solomon et al., 2003). JE virus induces human peripheral blood monocytes to secrete a chemotactic cytokine [human macrophage derived factor (hMDF)] that causes chemotaxis of neutrophils (Singh et al, 2000). JE virus may be degraded by neutrophils. Ability of neutrophils to degrade JE virus, via triggering of respiratory burst and generation of toxic radicals, has been investigated. JE or JE virus induced macrophage derived factor (MDF) induces increase in intracellular signals with generation of superoxide anion (O^{2-}) via activation of cytosolic NADPH and subsequent formation of hydrogen peroxide with maximum activity on day 7 post infection (Srivastava et al, 1999). From biochemical analysis of impact of Nitric oxide (NO) on JE virus replication in cell culture by Lin YL in 1997, NO was found to profoundly inhibit viral RNA synthesis, viral protein accumulation and virus release from infected cells. NO may play crucial role in innate immunity of host to restrict the initial stage of JE virus infection in CNS. Immunization with inactivated JE vaccine induces T-cells activation in vivo (Aihara et al., 2000). These studies reflect the protective role of cell-mediated immunity (CMI) in JE.

3.6.3. Clinical features

Most of the infection with JEV is often asymptomatic. The ratio of asymptomatic to symptomatic infection varies between 21:1 and 1000:1 (Halstead and Grosz, 1962; Thongcharoen, 1989). The mean is one symptomatic case for every 300 infections (Kalyanarooj, 1995). Incubation period is usually 5 to 14 days. Case fatality rates range from 0.3% to 60%. Both humoral and cellular immune responses attenuate the selective infection and destruction of neurons (Johnson, 1987).

Clinical features may range from a mild flu-like illness to a fatal meningoencephalomyelitis. Mild infections occur without apparent symptoms other than fever with headache. More severe infection is marked by quick onset, headache, high fever, neck stiffness, stupor, disorientation, coma, tremors, occasional convulsions (especially in infants) and spastic (but rarely flaccid) paralysis. Convulsions occur often in JE, and have been reported in up to 85% of children (Kumar et al., 1990) and 10% of adults (Dickerson et al., 1952; Poneprasert, 1989). In some patients, particularly older children and adults, abnormal behavior may be the only presenting clinical feature (Solomon et al., 1997).

The onset of illness can be abrupt, acute, sub-acute or gradual. The case progresses through four stages (CDC, 2007):

1. Prodromal illness stage (2-3 days)

2. Acute stage (3-4 days)

3. Subacute stage (7-10 days)

4. Convalescence stage (4-7 weeks)

1. Prodromal illness stage (2-3 days): Onset may be characterized by abrupt (sudden) headache, respiratory symptoms, anorexia (emotional disorder), nausea, abdominal

pain, vomiting and sensory changes, including psychotic episodes. A low grade fever or minor respiratory symptoms may be the only clinical expression of JE.

2. Acute stage (3-4 days): It is indicated by a high fever, convulsions, confusion, disorientation, delirium or somnolence (excited not able to think or speech clearly) & may progress to paralysis or coma. There may be oliguria, diarrhea and relative bradycardia. Fatal cases usually progress rapidly to coma and patient dies within 10 days.

3. Subacute stage (7-10 days): The severity of central nervous system lessens, but pneumonia, urinary tract infections, or bedsores and ulcer may be manageable problems and in some instances is life threatening.

4. Convalescence stage (Recovery period, 4-7 weeks): It is prolonged with weakness, lethargy, incoordinations, tremors and neuroses. Weight loss may be severe. Children, who survive, slowly regain the neurological function over several weeks. Sequelae are most common in patients who were younger than 10 year at the onset of disease. Mild cases may make a complete recovery (Bista and Banerjee, 2000). Only one third of the cases recover normally (so called abortive encephalitis). Spastic (rarely flaccid) paralysis may occur. Residual neurological impairment includes thick, slow speech, aphasia and paresis. Intellectual involvement may be found in 30% of cases, speech disturbance in 34% and motor deficits in 49% (Tiroumourougane et al., 2002).

Other sequelae in the patients recovering from JE may include:

- Behavior sequelae (aggressiveness 72%, depression 38%, attention deficits 55%).
- Intellectual sequelae (abnormal intelligence 44-72%, borderline intelligence 33%, mild mental retardation 11%, moderate mental retardation 11%).

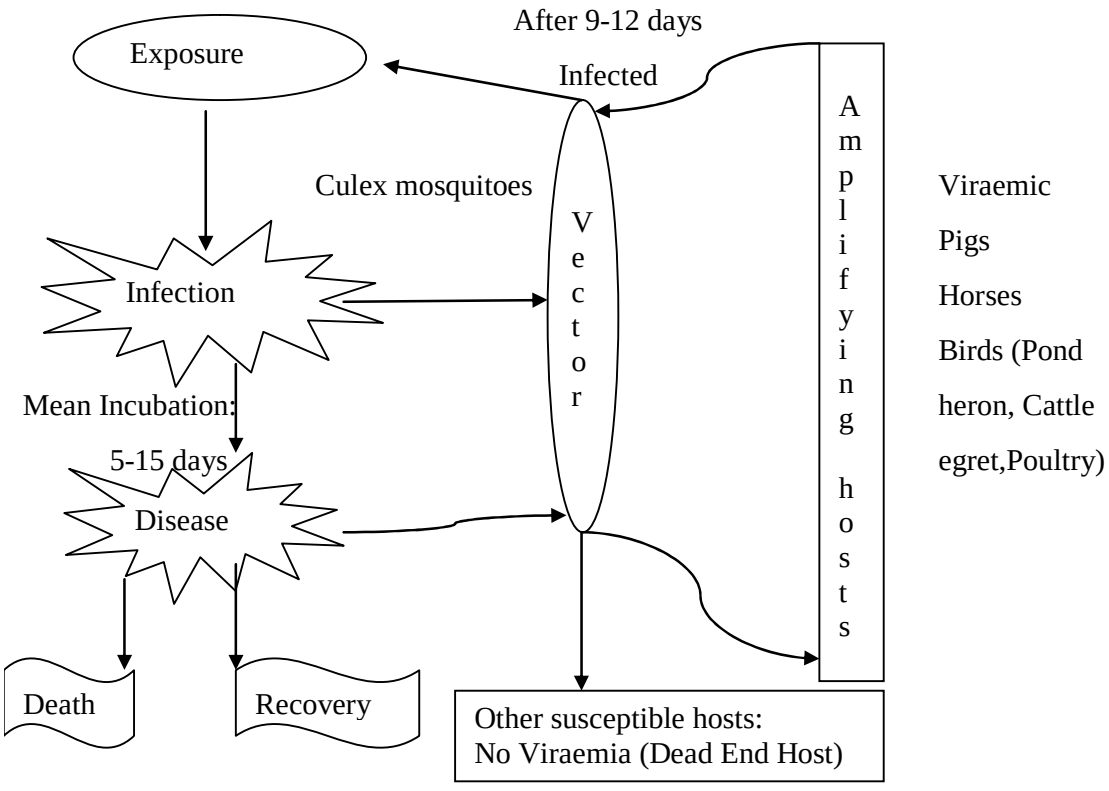
● Other neurological sequelae (epilepsy 16-20%, memory deficit 46%, cranial nerve paralysis 16%, blindness 2%).

JEV specific antibodies and JEV antigen can be detected in serum or CSF. Topographic distribution of tissue associated antigen in thalamus, hippocampus, substantia nigra and white matter of basal ganglia and medulla oblongata explains the evolution of post JE sequelae (Desain et al., 1995).

Recently, poliomyelitis like acute flaccid paralysis (AFP) has been identified in a subgroup of patients infected with JEV (Solomon et al., 1998). A small proportion of children may present with features of aseptic meningitis with no other clinical features of encephalopathy (Solomon et al., 1996). Seizure occurs in more than 75% of pediatric patients but is less frequently observed in adults (Tsai and Yu, 1994). Viral load may play a vital role in innate immunity of host to restrict the initial JEV infection in CNS. Limited data has also indicated that JE acquired during the first or second trimesters of pregnancy can cause intrauterine infection and miscarriage (Mathur et al., 1985). Infection during third trimester has not been associated with adverse effect in newborns.

3.7 Transmission cycle

Virus is maintained in nature through biological transmission between susceptible hosts by blood feeding arthopods (Mosquitoes, Psychodids, Grates, Ceratogonids and Ticks) (Bista and Shrestha, 2001). When the arthropod takes a blood meal, infectious virions are shed into its saliva and thence are injected into the victim's bloodstream. Mosquitoes transmit the virus to many species of birds and swine (Buescher and Scherer, 1959; Scherer et al., 1989). The risk for developing JE after a mosquito bite can be factored into a series of probabilities. Only bites of vector mosquitoes pose a risk and fewer than 3% of vector mosquitoes are likely to be infected (WHO, 2005).



(Source: Bista et al., 2001)

Fig 1: Basic Transmission Pathway of Japanese encephalitis

The important factors governing spill over of the diseases to human are the related abundance of the vectors, the availability of amplifying hosts, the density and absolute number of mosquitoes, adequate human mosquitoes contact and longevity of vector (WHO, 1998).

The maintenance and spread of JE virus appear to be mainly through a pig-mosquito-pig cycle (Gould et al., 1974; Johnson et al., 1974) and bird-mosquito-bird cycle (Joshi, 1998). Some studies suggest that virus may be transmitted transovarially in vector mosquitoes (Soman et al., 1985). Human is incidental and dead end host. JEV is not transmitted directly from person to person. In areas where JE vaccine coverage or natural immunity of residents is high so that endemic cases do not occur, JE virus may still be transmitted in the enzootic cycle and non-immune visitors to that area may still be at risk for disease. The epidemiology of the arboviral encephalitis must account for the maintenance and dissemination of the viruses in nature in the absence of humans (Brooks et al., 2004).

In tropical climates, where mosquito populations are present throughout the year, the cycle continues between mosquitoes and reservoir animals (Brooks et al., 2004). The mechanism of maintaining the virus over the winter in temperate areas has not been elucidated. Overwintering in the mosquitoes is a possibility either in infected hibernating mosquitoes or by transovarial passage (Rosen et al., 1980). Possible but unproved overwintering mechanisms include the following: (1) hibernating mosquitoes at the time of their emergence may re-infect birds; (2) the virus may remain latent in winter within birds, mammals, or arthropods; and (3) cold-blooded vertebrates (snakes, turtles, lizards, alligators, frogs) may act as winter reservoirs (Brooks et al., 2004).

3.8 Diagnosis

3.8.1 Clinical diagnosis:

Generally viral encephalitis shows similar clinical manifestation and therefore clinical diagnosis is made by the association of encephalitis and some symptoms and signs with possible viruses (Rao et al., 2000). In an area endemic to JE, when clinical features of a fever patient during transmission season resembles with JE infection, a case can be suspected as JE.

The most frequent alteration during viral infections is leucocytopenia with lymphocytopenia; but in JE, the leukocyte and neutrophil count is often raised. Differential counts reveal neutrophilia ranging between 51% and 90% whereas CSF examination shows a raised opening pressure, cell count of 10-980 x 10^6/litre, protein < 900 mg/liter and normal glucose level (Tiroumourougane et al., 2002).

In Nepal diagnosis of JE at National level is guided by the following case definitions (based on the recommendation of National Workshop on Prevention and Control of Vector Borne Diseases, 1997 and National recommended case definitions and surveillance standards, 1999):

"Any case having elevated temperature (over 38°C) altered consciousness or unconsciousness during epidemic in endemic areas will be considered as suspected Encephalitis and be referred for lumber puncture."

"If the suspected case has between 50 and 1000 cells (predominantly lymphocytes per cubic mm) in the CSF, it will be diagnosed as having Probable Viral Encephalitis."

"A case of probable viral encephalitis showing presence of Anti JE IgM in CSF or serum will be defined as a confirmed case of Japanese Encephalitis due to Japanese Encephalitis virus."

3.8.2 Laboratory diagnosis

The ideal assay for JE will have a high degree of sensitivity and specificity for diagnosing acute JE infection and encephalitis. Laboratory diagnosis of JE is based on four basic types of assays: serologic, virus isolation, molecular, and immunocytochemistry or demonstration of virus specific antigen.

3.8.2.1 Serology

Laboratory diagnosis of human arboviral encephalitis by serology has changed greatly over the last few years. In the past, identification of antibody relied on four tests namely haemagglutination inhibition (HI) test, complement fixation (CF) test, plaque reduction neutralization test (PRNT) and indirect fluorescent antibody (IFA) test. However, these tests are still in use in some laboratories. These assays are reliable when done and interpreted property (Beaty et al., 1989). Positive identification using these assays requires paired sera to demonstrate four fold rise in antibody titer between acute and convalescent serum samples (Bista and Banerjee, 2000). In recent decades, these conventional tests have been replaced by antibody capture enzyme linked immunosorbent assays (ELISAs), which are more sensitive and specific.

(a) Hemagglutination Inhibition (HI)

Hemagglutination Inhibition (HI) was the first to measure human antibody specific for the arthropod-borne viruses including JE as described by Clarke and Casals in 1958 (Clarke and Casals, 1958). The HI assay has remained largely unchanged since 1958 and is still a fundamental tool in arboviral and JE diagnostics. The precursor to

the HI assay is the hemagglutination assay (HA), which was developed by Sabin and colleagues when they observed that the arboviruses, in particular JE virus, were able to agglutinate certain types of erythrocytes. The finding that arbovirus-specific antibody was able to inhibit HA of erythrocytes created a relatively simple and inexpensive HI assay with which to measure JE-specific antibody (Clarke and Casals, 1958). In this assay, nonspecific inhibitors are removed from sera using acetone, ether or kaolin prior to performing the HI assay (Monath et al., 1970). Twofold serial dilutions of the test sera and standard positive and negative controls are placed into each well followed by the addition of 0.025ml of 50HA units of JE antigen. The HI titer is taken as the highest serum dilution that causes complete inhibition of agglutination. A fourfold increase in JE-specific HI titer between paired sera is indicative of a JE infection.

The value of the HI assay is that it can be performed with minimal laboratory equipment, reagents and expense. The major disadvantage is its failure to discriminate adequately between the closely related flaviviruses such as dengue and WN viruses. This produces results that are difficult to interpret in countries where these viruses co-circulate. Also, it is time consuming, requires paired serum samples and cannot give early diagnosis. Despite these limitations, the HI assay is a powerful technique that is still a standard assay for seroprevalence studies as well as in the diagnosis of acute primary and secondary dengue virus infections.

(b) IgM and IgG enzyme Immunoassay

The enzyme-linked immunosorbent assay (ELISA) is widely used in the diagnosis of many viral pathogens because of the relative ease of setting up this assay in a 96-well format and its high degree of reproducibility due to its ability to be automated using automatic plate washers and scanners. In 1980s, IgM and IgG ELISAs were developed which have become the accepted standard for diagnosis of JE (Solomon et al., 1998; Burke et al., 1982). The ability to detect IgM during acute JE virus infection

provides a highly specific antibody based assay without the problems of cross-reactivity that IgG antibody displays for other flaviviruses. After the first few days of illness, the presence of anti-JEV IgM in the CSF has a sensitivity and specificity of > 95% for CNS infection with the virus (before this, false negative may occur) (Burke et al., 1985). Nearly in all patients, after 7 days of onset of symptoms, specific anti-JEV IgM can be detected in CSF or in serum or in both. IgM antibody capture ELISA (MAC-ELISA) is the method of choice to demonstrate virus specific antibody in both blood/serum and CSF. Detection of JEV specific IgM is one of the most reliable indicators of JEV infection (Bundo and Igarashi, 1985). Moreover, presence of IgM in the CSF indicates local antibody formation associated with brain infection and is not seen in person with asymptomatic infection with JEV.

ELISA is a highly sensitive, specific, less time consuming and reproducible method for detection and quantification of many cytokines (Beech et al., 1997; Jung et al., 1998). The sensitivity of ELISA for JE was 89%, specificity 91% and accuracy 92% with reproducible results and was also able to detect a minimum concentration of 23 ng human macrophage derived factor (hMDF) per ml in test samples (Singh et al., 2000). Evaluation of a new commercially available IgM / IgG antibody capture ELISA for diagnosis of JE showed sensitivity of 88% with serum, 81% with CSF; specificity of 97% with sera from patients with primary and secondary dengue virus infections whereas specificity was 100% when samples from non flavivirus infections were tested (Cuzzubbo et al., 1999).

Avidin biotin system (ABC MAC-ELISA) (Chow et al., 1992), biotin labeled immunosorbent assay to sandwich ELISA (Chang et al., 1984), nitrocellulose membrane based IgM capture dot enzyme immunoassay (MAC DOT) (Solomon et al., 1998), and antibody capture radioimmunoassay (ACRIA) (Burke et al., 1982) are some of the newer modifications of MAC-ELISAs that have been used in antibody detection.

Yamamoto et al., (2000) described hydroxyapatite coated nylon beads (Ha-Ny beads) to be applicable for the development of a new JEV antibody-detection, kit which is simple, inexpensive and does not require specific laboratory facilities.

JEV specific IgM persists even till 116 to 350 days after acute illness (Edelman et al., 1976), which suggests that IgM antibody persistence is related to acute virulence rather than chronicity of JEV infection.

(c) Plaque Reduction Neutralization Titer Assay (PRNT)

In 1959, Henderson and Taylor developed a method to detect plaques of arboviruses using an agar overlay stained with neutral red (Henderson and Taylor 1959). This technique allowed an easy in-vitro assay to directly detect and measure the infectivity of a virus preparation or stock as plaque forming units. Its application to JE virus awaited the finding that JE virus produces cytopathic effects (plaques) in certain cell lines such as in cultured hamster kidney cells and in chick embryonic fibroblast monolayer cultures (Diercks and Hammon 1958). Yoshioka and colleagues developed a plaque reduction neutralization test (PRNT) for JE virus using cultured chick embryonic fibroblasts and demonstrated a good correlation to HI antibody titers. This technique was subsequently adapted to the green monkey kidney cell line, Vero cells, using a constant-virus decreasing serum technique and was found to be more sensitive than HI titers (Work, 1964).

Today a number of PRNT protocols are being used to measure JE neutralizing antibody using a variety of cell lines, including LLC-MK2, Vero and BHK21 cells. Most frequently cell lines are cultured to form healthy monolayers on 6-well culture plates. Reference stocks of JE virus are prepared at 100-50% tissue culture infectious doses (TCID50). Standard JE serotype-specific monkey sera are utilized in every PRNT assay as positive controls as well as negative controls that form the baseline

plaque count. The endpoint is the titer of antibody that will reduce a certain percentage of viral plaques such as a 50%, 70%, or 90% plaque reduction (PRNT50, PRNT70, and PRNT90, respectively) compared to control cultures. Higher PRNT values increase the specificity but decrease the sensitivity of the assay, therefore a PRNT50 is the most commonly used end point. The determination of the PRNT is by probit analysis using a log reciprocal dilution compared to the percent plaque reduction where the 50% end point (for PRNT50) is the dilution at which the line of best fit crosses the 50% probit line when plotted on probability two-cycle logarithmic paper or generated using a probit analysis computer program. A JE antibody titer as measured by the PRNT assay is interpreted in a number of ways. In a patient with suspected acute JE infection, a titer increasing by fourfold suggests an acute infection. A titer of 1:10 or more suggests past exposure or vaccination, and protective immunity.

(d). Western Blot

Western Blot is a technique that utilizes the characteristic of proteins to travel through a polyacrylamide gel, and to transfer onto nitrocellulose sheets when an electrical current is passed through the gel. Separation of proteins occurs as they travel through a gel at different speeds depending on their molecular weight and charge. The result, when applied to proteins derived from JE, is a spread of differing structural and nonstructural protein components across a gel, predominantly E, NS1, and pre-M. These proteins are then transferred onto nitrocellulose paper by an electrical current applied horizontally to the gel. Human sera can then be applied to the nitrocellulose strip and human antibody specific to JE protein will bind; an anti-human antibody conjugate system is then used to detect binding of the human antibody. The result is a band showing the presence of antibody to those proteins. This assay has limited diagnostic utility as JE and dengue virus proteins are highly cross-reactive and the western blot is unable to distinguish the two infections (Work, 1964).

3.8.2.2 Virus Isolation

The ability to isolate JE virus during an acute infection is extremely difficult probably because of the high levels of neutralizing antibody that exist at the time that clinical symptoms occur, frequent freezing / thawing of clinical materials, and the logistic difficulty in transport of specimens in the developing countries (Leake et al., 1996). Lack of skilled manpower and virus culture laboratories are also major hindrances to viral isolation in these countries. In one series of 101 patients with JE infection of the CNS, no virus was isolated when the geometric mean titer of neutralizing antibody exceeded 1:500 at the onset of clinical symptoms (Kimoto et al. 1968). In a similar series of patients in Thailand, among 49 cases of JE infection of the CNS, no plasma specimens or CSF yielded virus in the nonfatal cases. Only in 5 of the 15 fatal cases of JE was there virus isolation from the CSF and 7 of 15 had virus isolation from brain tissue (Leake et al. 1996). These studies illustrate the difficulties in isolating virus from nonfatal cases of JE. Viral isolation in fatal human cases of JE infection is best performed on tissue or autopsy specimens of brain tissue.

JE virus can be isolated by a variety of methods, the classic being intracranial inoculation of clinical specimens in suckling mice. Normally, 1 to 2 day old suckling mice (*Mus musculus*) are inoculated intra-cranially with 0.02 ml of a suspension of clinical material. Mice are observed twice a day for the first signs of encephalitis (failure to eat as evidenced by lack of milk in the stomach, color change, wasting, runting or tremors), which occur approximately 3-10 days after inoculation. Confirmation of JE virus is performed by staining the brain with either a polyclonal or monoclonal antibody specific for JE conjugated with a fluorescent tag.

A more direct method of JE virus isolation is by direct inoculation and isolation in a variety of mammalian and insect cell lines such as VERO and LLC-MK2 cells from monkey kidneys, BHK21 from hamsters, AP61 from *Ae. pseudoscutelaris,* C6/36 from *Ae. albopictus,* Sf9 from *Spodoptera,* and PS from swine (Igarashi et al., 1981).

Inoculation of patient sera into mosquitoes to amplify and propagate JE and other arboviruses was developed in the 1970s and took advantage of the natural vector of these arboviruses to amplify and propagate the virus (Rosen et al., 1980). A variety of mosquito vectors have been used including *Toxorhynchites splendens, Ae. albopictus,* or *Ae. aegypti*. *Toxorhynchites* mosquitoes have the advantage of being larger and easier to inoculate with human sera. In their laboratory, *T. splendans* is inoculated intrathoracically with 0.02 µl of human sera and after an incubation period of 10-14 days, the mosquitoes are sacrificed. JE is detected in the salivary glands by immunofluorescence antibody. If positive, the mosquito is ground up and filtered, and the filtrate is inoculated onto C_6/36 cell lines for virus expansion and typing.

3.8.2.3 Molecular: Polymerase Chain Reaction

The molecular fine mapping of important antigenic regions in JE over last few years has paved way for the future development in laboratory diagnosis (Tiroumourougane et al., 2002). Polymerase chain reaction (PCR) is a molecular technique based on the ability to amplify small amounts of RNA or DNA to detectable levels using molecular primers, a polymerase enzyme and a thermocycler. PCR has been used with success with other flaviviruses (Henchal et al., 1983; Lanciotti et al., 1992). The reverse transcriptase polymerase chain reaction (RT-PCR) amplification of viral RNA may help in specific and rapid detection of JEV in various samples (Meiyu et al., 1997; Paranjpe and Banerjee, 1998). However, its reliability as a routine diagnostic test has yet to be shown.

The limitations of PCR are similar to the limitations with the isolation of virus. Viremia and detectable viral RNA in the sera or CSF is found infrequently at the time of clinical illness. PCR may have a higher yield when performed using biopsy specimens although this has not been demonstrated in clinical studies.

3.8.2.4 Immunocytochemistry

Immunocytochemistry is the staining of tissue specimens for the presence of specific proteins as it applies to JE, the staining for JE antigen. JE antigen staining is a powerful technique to diagnose fatal cases of JE when serology or viral isolates are not available. A number of techniques have been used to detect arbovirus antigen in tissue specimens to include both direct and indirect fluorescent antibody staining, and enzyme conjugates using peroxidase and alkaline phosphatase conjugates. In laboratory, tissue specimens are fixed in Millonig's formalin for 2h, irradiated in a microwave oven, and then embedded in paraffin (Myint et al., 1999). JE antigen is detected by performing a modification of the immunoalkaline phosphatase method as described by Hall and colleagues (Hall et al., 1991). The staining method used for the tissue section involves deparaffinization in absolute alcohol and water, immersion in a 0.05% solution of protease VIII and blocking with normal horse serum and skim milk. The specimen is incubated overnight with a mouse polyclonal anti-dengue antibody followed by a secondary biotinylated horse anti-mouse IgG, streptavidin-alkaline phosphate then anti-streptavidin-B1 phosphate, hexazotized new fuchsin and levamisol as the chromogenic substrate. The tissue specimen is then counterstained with Mayer's hematoxylin. The value of immunohistochemistry is in diagnosing fatal cases of JE when sera or CSF are not available. In one series 62% of fatal cases of JE were diagnosed by immunohistochemical staining for JE antigen in brain tissue (Henchal et al., 1983).

3.8.2.5 Antigen detection

Various studies have proved the efficacy of JEV antigen detection in CSF using reverse passive haemagglutination (Ravi et al., 1989), immunofluorescence (Raghava and Badrinath, 1998) and Staphylococcal co-agglutination tests using polyclonal and monoclonal antibodies (Zhang et al., 1989) in rapid diagnosis of JE.

Twenty four CSF samples with anti-JEV IgM negative status were tested for the presence of JEV specific IgG by ELISA, which revealed that almost all samples

exhibited IgG1 suggesting its role in the clearance of virus from CNS (Thakare et al., 1991).

3.9 IgM Capture ELISA (MAC-ELISA)

The immunoglobulin M (IgM) antibody capture ELISA (MAC ELISA) for serum and cerebrospinal fluid (CSF) has become the accepted standard for diagnosis of JE. This assay is sensitive and specific; it is often positive for specimens collected on admission and distinguishes between JEV and the related dengue flaviviruses and other flaviviruses, which are serologically cross-reactive. Specific anti-JEV IgM can be detected in serum or CSF or both after 7 days of onset of symptoms.

Principle:

Assay plate was coated with anti-human IgM antibodies. Serum sample to be tested is added on the well. If the serum contains antibodies of the IgM class it will combine with anti-human IgM antibodies. The mixture complex of JE antigen and HRP-conjugated monoclonal antibody (MAb) is added to each well after removal of serum by washing. After incubation, the microwells are washed and a colourless substrate system, tetramethylbenzidine/hydrogen peroxide (TMB / $_{H2O2}$) is added. The substrate is hydrolysed by the enzyme and the chromogen changes to a blue colour. After stopping the reaction with acid, the TMB becomes yellow. Colour development is indicative of the presence of the respective anti- flavivirus IgM antibodies in the test sample.

3.10 Particle agglutination assay

Particle agglutination assay is an antibody detection kit, which utilizes hydroxyapatite beads as antigen carrier. It is a simple, reliable, inexpensive and preservable technique. It does not require any sophisticated equipment. It can be performed in diagnostic laboratories with insufficient resources.

Preparation of JEV antigen-coated Ha-Ny (Hyroxyapatite-coated nylon) beads

Hydroxyapatite and nylon beads were mixed to give hydroxyapatite-coated nylon beads. JEV antigen-coated Ha-Ny beads are prepared by mixing Ha-Ny beads with JEV antigen in Phosphate Buffer Saline (PBS). The remaining active areas on the surface of Ha-Ny beads were blocked with 0.4% (Wt. /V) blocking reagent. Antigen was fixed with 0.1% glutaraldehyde.

Principle:

The anti-human IgM antibody coated microplate captures human IgM antibodies in the serum samples. The surface of Ha-Ny beads is coated with JEV antigens. The beads can bind to anti-JEV specific IgM molecules, which are captured on the microplate, and adhere to the face of the wells. JEV antigen-coated Ha-Ny beads adhere to the face of the wells, when the tested sample is anti-JEV IgM positive. When the Ha-Ny beads form a button pattern at the bottom of the well, the sample is considered negative in Ha-Ny beads agglutination assays (Yamamoto et al., 2002).

PA assay can be divided into two types:

I. Qualitative assay and
II. Quantitative assay

Qualitative assay distinguishes whether the sample is positive or negative for the given disease i.e. JE whereas Quantitative assay gives antibody titer for the disease. Quantitative assay is particularly useful in case of cross reactivity with other flaviviral infection to distinguish the disease and observe rise in antibody titre in paired sample for confirmatory diagnosis.

3.11 Treatment

No specific treatment is indicated. Mainly supportive and symptomatic treatment is recommended. Meticulous nursing cares are required for unconscious patient.

3.12 Prevention and Control

3.12.1 Control of mosquito vector

Vector abundance which is directly associated with risk of human JEV infection is in turn associated with agricultural practices. Vector density and viral infection rate in vectors coincided with the distribution of cases in hyper endemic areas (Gajanana et al., 1996).

For proper control of mosquito vector surveillance of the adult mosquito population has to be carried out throughout the year. Since nearly all vectors of JE virus utilize rice fields and other water logged sites as larval habitats, the reduction of habitat through water management and integrated farming should be practiced. Intermittent irrigation and periodic flushing of fields during rice cultivation should be followed. Various means of biological control have been successfully used against mosquitoes. The most successful biological interventions in rice fields have been the use of microbial control agents like *Bacillus thuringiensis var. israelensis*, *Bacillus sphaericus.* Growing of the water fern *Azolla microphyla* in rice field was also evaluated as a biological agent against mosquitoes breeding in rice fields (Rajendran and Reuben, 1991).

Due to wide disbursement of vectors with habit of outdoor feeding, insecticide spraying is not considered as a major strategy to control JE vectors. However, for immediate suppression of infective vectors, ultra low volume (ULV) or thermal fogging may be employed during the epidemic period. The principal vector of JE, *Cx tritaeniorhynchus*, has been found susceptible to deltamethrin and lambdacyhalothrin, but more information is required on the insecticide resistance in the JE vector and behaviour towards different insecticides (EHP, 2004). This mosquito is resistant to DDT in India. Although spraying of an appropriate insecticide in the resting places of mosquitoes is an easy method, environmental pollution due to insecticide and

development of resistance by mosquitoes against an array of insecticides have discouraged the use of insecticides in vector control programs.

3.12.2 Prevention of mosquito bite

The best preventive method against JE is protection against mosquito bites. Use of nets (normal and insecticide impregnated) and mosquito repellents (coils, creams and mats) by the population at risk and avoidance of outdoor sleeping in the tropics during evening hours, staying in screened houses, and wearing long sleeved shirts and long trousers (Tsai, 1992) reduce the risk of exposure to vectors. Pyrethroid impregnated curtains were found to be effective and proved its efficiency in the control of JE vectors (Theodore, 1990). Gurung et al., (2003) reported that mosquito-net nonusers are at 2.6 times greater risk of developing JE in comparison to net-users in Nepal.

3.12.3 Immunization of reservoirs

Animal husbandry practices should be improved. Building of piggeries away from human dwellings in the countries where pigs are reared near human settlement and making them mosquito proof would be desirable. Immunization of amplifier host of JE virus, like pigs, was suggested as a control measure for reducing the number of mosquitoes that become infected when they feed upon the swine. Immunization of pigs may reduce viral transmission by limiting or preventing viremia in pigs. SA 14-14-2 virus grown in cells is used to immunize pigs.

3.12.4 Public awareness programs

Realizing the limitation of control measures, public awareness should be created to emphasize and encourage personal protection by adopting various means of health education and strengthen the existing community participation. Awareness programs should focused on the hygiene and sanitation practices of the people to reduce the

mosquito breeding and also encourage the use of mosquito repellents and bed nets for personal protection. Use of broad casting media (radio, television etc.), news papers, hording boards and pamphlets can be implemented as the tools of public awareness programs.

3.12.5 Vaccination

JE is a vaccine preventable disease. There are two different schedules for vaccination. The first involves three injections over a period of 28 days and the second involves two injections given one to four weeks apart. Both provide protection for a year. Booster vaccination administered one year later extends protection to three years. People who have the vaccine should stay in areas where they have access to medical care for 10 days following a dose because vaccine occasionally causes a severe allergic reaction. However, vaccine does not give 100 percent protection.

Institutionalization of mass vaccination of those living in JE endemic outbreak prone areas was recommended as the most effective method of JE prevention in human population. The three JE vaccines (Theodore, 1990) in widespread production and in worldwide use for this purpose are: 1. Mouse brain-derived inactivated JE vaccine; 2. Cell culture-derived inactivated JE vaccine, and 3. Cell culture-derived live attenuated JE vaccine. Post vaccination neurological complications such as encephalitis and peripheral neuropathy have been reported in only 1 to 2.3 per million vaccines (WHO, 1998).

3.12.5.1 Mouse brain-derived inactivated JE vaccine:

The mouse brain-derived inactivated JE vaccine is produced in several Asian countries. The inactivated JE vaccines available for immunization purpose against JE are derived from infected mouse-brain, which was licensed in Japan in 1954. The commercially available mouse brain-derived JE vaccine is based either on the

Nakayama strain or on the Beijing-1 strain. Because of the higher antigenic yield in the mouse brain following inoculation of the Beijing strain 1, the Nakayama strain has been replaced in several mouse brain-derived JE vaccines. This vaccine is manufactured and commercially available in India, Japan, Korea, Taiwan, Thailand, Vietnam and United States of America (USA).

3.12.5.2. Cell culture-derived inactivated JE vaccine:

This vaccine is manufactured in China and based upon the Beijing P-3 strain of JE virus, which provides broad heterologous immunity in mice, and high antigenic yields when propagated in primary hamster kidney cells. In order to avoid brain antigens and allergic reactions associated with crude antigens and to ease production, tissue culture-derived vaccines were attempted in China. Although this enhanced inactivated vaccine was reported to be immunogenic, there were no reports on efficacy or persistence of immunity (Theodore, 1990).

3.12.5.3 Cell culture-derived live attenuated JE vaccine:

This Chinese vaccine is based on a stable neuro-attenuated strain (SA-14-14-2) of JE virus generated through a large number of passages in various cell culture systems. Efficacy trials in children of 1 to 10 years have yielded high protective rates. In non-endemic areas, a single dose of this vaccine induced an antibody response in 83% to 100% of children aged 6 to 7 years, and in older children immunized twice at intervals of 1 to 3 months, 94% to 100% showed a serological response. Side effects are reported to be minimal. Recent advances in molecular biology have emphasized to explore novel approaches for developing recombinant vaccines based on proteins, viruses and DNA (Kabilan, 2004).

In general vaccination is indicated in the following groups:

1. People living in endemic areas
2. Travelers spending 30 days or more in endemic area.

3. Travelers spending less than 30 days during epidemics or if extensive outdoor activity in rural areas is expected.
4. Lab workers with potential risk of exposure to JEV; since twenty- two cases of laboratory-acquired JE have been reported (WHO, 1998).

3.12.6 JE vaccination in Nepal:

Vaccination of high risks groups in endemic districts had an immense impact in decreasing case loads as well as death (EDCD, 2006). In 1983, 1152 subjects were immunized against JE using BIKEN killed lyophilized vaccine at British Military Hospital, Dharan.

In 1999, a live attenuated BHK vaccine trial was conducted in 3 districts (Bardiya, Banke and Kailali) with SA 14-14-2 single dose vaccine imported from South Korea. Out of 492,442 children between 1 and 15 years, only 224000 (45.5%) were vaccinated. Vaccine coverage was 83.5% in Bardiya followed by Banke (41.3%) and Kailali (22%) (EDCD, 2003).

During the year 2001 and 2002, two million doses of inactivated cell culture JE vaccines were donated by China. Although 4 doses were planned, only 3 doses were administered to the children of 6 months to 10 years age (EDCD, 2001). Single dose of SA 14-14-2 was proved to be highly effective in context of Nepal too (Bista et al., 2001; Ohrr et al., 2005).

Disease morbidity was decreased in the vaccinated districts and was lower as compared to the non-vaccinated districts. But there was again rise in morbidity due to discontinuation of the vaccination program. Because the vaccines were given to children, disease prevalence has been shifted to old age from under 15 years (Joshi et al., 2005).

3.13 Disease Distribution

3.13.1 Worldwide distribution

The history of the disease goes back to 1871. Epidemics of encephalitis were described in Japan from the 1870s onwards. A severe epidemic of JE has been recorded in Japan during 1924 with 6000 cases, 4000 deaths and case fatality rate as high as 60%. Major epidemics were reported about every 10 years (Miyake, 1964). During 1970s and 1980s, JE was endemic to only a few countries of East Asia like Japan, Korea and China. Then, it spread from East Asia to South East Asia (SEA) and then to South Asia. The virus was isolated in Japan in 1935, and has been recognized across Asia since then (Solomon et al., 2003).

JE is widespread in temperate & tropical Asian regions. Epidemic and sporadic cases of JE occur in many Asian countries including Cambodia, China, Indonesia, India, Japan, Malaysia, Myanmar, Nepal, Pakistan, Philippines, Korea, Sri Lanka, Thailand and Vietnam, and in the South Eastern Russian Federation (Tiroumourougane et al., 2002). It is spreading to America, Europe, Africa and part of Australia.

JE is a seasonal disease, with most cases occurring in temperate areas from June to September. Further south in subtropical areas, transmission begins as early as March and extends until October. Transmission may occur all year in some tropical areas (eg Indonesia). Although considered by many in the west to be a rare and exotic infection, JE is numerically one of the most important causes of viral encephalitis worldwide, with an estimated 50,000 cases and 15,000 deaths annually (Tsai, 1998; Solomon et al., 1998). Local incidence rates range from 1-10 cases per 100,000 persons but can reach more than 100 cases per 100,000 persons during outbreaks. The outcome of JE ends with the death of 25% of patients and residual severe neuropsychiatric sequelae in 30 to 60% of the survivors. Approximately, 60% of the world's population lives in

the JE endemic regions (Kabilan, 2004). Therefore, it has been considered as a global public health problem.

It is prevalent in Northern Thailand, as well as in Bihar, Uttar-Pradesh, Tamil Nadu and West Bengal states in India, in the Terai areas of Nepal and in Sri Lanka. Cases have been reported in Bangladesh, Indonesia and Myanmar (WHO, 1998). The first indication of JE transmission in SEA region was from Sri Lanka where an outbreak was apparently reported in 1948 (Tsai and Yu, 1994). In India, epidemics of JE were first recognized around Vellore (Sehgal, 1989). In 1948 outbreak, 85% of donkey sera were JE antibody positive.

In recent years the epidemiological pattern and geographical distribution of JE have changed in Asia. In Taiwan, Japan, South Korea and China clinical cases of JE have decreased dramatically. This has been possible through the integrated control effort comprising of human vaccination program, water management, and immunization of pigs, systematic piggery and community awareness program. On the other hand, the incidence of JE has increased in India, Nepal, Sri Lanka, Thailand, Bangladesh and Vietnam (Umenai et al., 1985). Number of outbreaks occurred in India during 1948 to 1978. Although the first epidemic of JE in India was recognized around Vellore in 1948 (Sehgal, 1989), the recognition of JE based on serological surveys, was first made in 1955 in Tamilnadu. Since then, many major outbreaks from different parts of the country have been reported, predominantly in rural areas. Subsequent surveys carried out by the National Institute of Virology (NIV), Pune indicated that about half of the population in South India had neutralizing antibodies to this virus (Park, 2002). Twenty-four states of India have reported JE including some states bordering to southern Nepal.

Gradual spread of disease to other non-Asian regions for example; Torres Strait of Australian mainland has been reported recently (Hanna et al., 1995). The first outbreak of JE (two clinical cases) in Australia was reported in 1995. These cases were identified on an island in the Torres Strait. No new case of JE was notified in Australia in 2002. An entomological investigation of an outbreak of JEV in the Torres Strait, Australia in 1998 recovered 43 isolates of JEV from adult mosquitoes (42 from *Cx. Sitiens* and one from *Ochlerotatus vigilax*) and also identified 2 confirmed human JE cases in that area and Cape York Perinsula in Northern Queensland (Johansen et al., 2001). Because of these outbreaks, mosquito borne arboviruses causing human diseases have been considered as important public health issues in Australia also.

In the US, JE mostly occurs among military personnel, expatriates, and, rarely in returning travelers. From 1978 to 1993, 12 cases of JE occurred in the US. The risk of symptomatic infection among travelers is estimated to be 1 per 150,000 person-months in an endemic area. Outbreaks are rare in the US territories of Guam and Saipan. There have been American and Australian military cases reported following the Korea and Vietnamese wars and postings to South East Asia (Burdon et al., 1994).

Two cases of JE in UK travelers have been documented. The first was a British woman who had been living and working in Hong Kong and was diagnosed with JE in 1982; she died as a result of cardiac and respiratory complication (Rose et al., 1983). Another case was a woman who had been to Thailand; she recovered completely after 4 months.

Since, most of the JE cases (35-50,000/year) are reported from the Asian countries especially South East Asian countries more research activities are also focused in these countries. In northern temperate region of Asia, JEV causes larger summer epidemics, whereas in southern tropical regions, it causes endemic disease year round

(Vaughn and Hoke, 1992). Cross sectional serological surveys have shown that in rural Asia, most of the populations are infected with JEV during childhood or early adulthood. About 10% of the susceptible population is infected each year; however, most infections are asymptomatic.

In Japan, annually, 6000 JE cases with up to 60% case fatality rate were reported during 1960s. In recent years Japan has decreased the JE cases to less than 100 patients per year after 1972. This may be due to development of inactivated JE vaccines and national immunization programs. An investigation conducted during 1967 to 1973 suggested that swine vaccination was not effective enough in Japan for the control of JE among humans because during this period, 10 human JE cases were reported. Out of 10 confirmed JE cases, 4 died in the Kurume region of Japan from 1984 to 1990 (Shoji et al., 1994). Three JE cases in the non-vaccinated US marines stationed on Okinawa were identified in 1991 (Saito et al., 1991). During the outbreaks of 1991 to 1993, 4 JE patients were reported in Kyushu island of Japan and all of them recovered completely (Shoji et al., 2002). Six patients unexpectedly presented with JE from early August to mid September 2002 in the Chungoku district of Japan which indicates that JE in Japan is still a threat to adults and elderly with decreased or absent immunity to JEV (Ayukawa et al., 2004).

The ratio of subclinical to clinical infection in vaccinated population was estimated to be 20,00,000:1, which was 2,000 to 80,000 times higher than the ratio previously reported for unvaccinated population (Konishi and Suzuki, 2002).

Korea demonstrated more than 1000 JE cases/year before 1969 but after the vaccination started in 1960 onwards the number of cases decreased dramatically. Vaccine coverage reached almost 100% in the 3-15 years age group in 1985. The

widespread use of Vaccine in children has been associated with a higher incidence of JE in those over 15 years (Vaughn and Hoke, 1992).

Although epidemics in northern Vietnam followed by 1965; currently, 1000-3000 cases/year are reported. In northern Vietnam, seasonal pattern of JE epidemic has been found as in other temperate areas whereas in southern Vietnam, sporadic cases have been reported throughout the year. During 1976 to 1991, Vietnam reported AES cases from all provinces with the highest number of cases (936) in 1980 and highest number of deaths (339) in 1977 (Ha et al., 1995). A serological study carried out in Gia Luong district of Vietnam after vaccination during 1993 to 1994 showed 71.66% JE positive cases out of 85 clinical encephalitis cases (Nga et al., 1995). None of the JE positive case was previously vaccinated which also supported the efficacy of vaccination in Vietnam. Virological and serological study conducted in Laos during 1993 to 1995 showed an increase in JE positivity with age. A case-control study on adult and paediatric AES patients admitted to Bach man Hospital, Vietnam, detected 67% positive cases out of 46 pediatric AES cases as compared to only 6% JE positive cases out of 33 adult AES patients (Lowry et al., 1998). A study conducted in 1998 in Vietnam detected 12 (55%) of the 22 children with acute flaccid paralysis (AFP) had evidence of acute JEV infection (Solomon et al., 1998).

Cases of JE were reported in Hongkong during the year 2000. In the history of China the first JE case was reported in 1935 with the first virus isolation in 1940. Epidemics of JE were reported in many provinces of China in the early 1950s and with almost all provinces being affected by now (Yu Y, 1995). There is currently 10,000-20,000 cases/year, although in the early 1970s it was over 80,000 cases per year (Vaughn and Hoke, 1992). Some studies conducted in China suggested early diagnosis, treatment and universal JE vaccination for all susceptible populations as the key reasons for decreasing incidence of sequelae and death due to acute JE. JE vaccination is

encouraged in China. Effectiveness of live attenuated JE vaccine (SA 14-14-2) for single dose was 80% and that for two doses was 97.5% (Hennesy et al., 1996).

In Thailand, 1,500-2,500 cases are reported annually. An antibody prevalence survey conducted in Thailand during 1989 studied 3,089-blood samples of children (aged 6 months to 14 years); out of which 27.45% of children possessed neutralizing antibody to JEV (Rajanasuphot et al., 1992). Strickman et al., (2000) studied distribution of dengue and JE among the school children of rural and suburban Thai villages where, out of the 1,477 children, 33/1000 had recent dengue and 7/1000 had recent JE infection. In a study on acute undifferentiated fever caused by infection with JEV, JE was reported in 22 (14%) individuals out of 156 adults; indicating JE as an underappreciated cause of acute undifferentiated fever in Asia (Watt and Jongsakul, 2003). This study was carried out in Chiangrai Regional Hospital, Thailand. Chokephaibulkit et al., (2001) conducted a perspective study of childhood encephalitis in Bangkok from 1996 to 1998. Among 26 (65%) children with identifiable viral agents, JE was reported in 6 children.

In Taiwan, the first clinical case of JE was recorded in 1931. The case incidence rate of JE during 1966 to 1977 showed a sharp decrease from 2.05/10,000 in 1967 to about 0.03/10,000 in 1997 reflecting the efficacy of JE vaccination started in 1968 onwards in Taiwan (Wu et al., 1999).

After JE was first reported in 1951 in Malaysia, only occasionally epidemics have been documented (in 1974, 1988 and 1992) with more than three fourth of the cases being children. The total number of cases is seemed to be too low, although the actual number of cases could probably be more (Haw, 1995). Out of 195 children with CNS symptoms admitted to pediatric ward of Penang hospital, Malaysia, 38.5% demonstrated the anti JEV IgM in their CSF (Cardosa et al., 1995).

Some countries in the South East Asia, including India, Nepal demonstrated a remarkable increase in the number of JE cases since 1970s. In 1948, Sri Lanka became the first country to report JE cases in South East Asia (SEA) region (Tsai, 1994). India is a JE endemic region that borders Nepal. Four districts of Uttar Pradesh (along Indo-Nepal border) have reported JE cases with frequent outbreaks (EHP, 2004). The pattern of JE epidemics reported in India correlates well with Nepal (Kubo et al., 1996).

3.13.2 JE situation in Nepal

In Nepal, clinical cases of JE were reported in Nepal before 1975 and an epidemic of JE was recognized for the first time in Rupandehi district of Western Development Region (WDR) in 1978 (Joshi, 1986; Bista and Shrestha, 2005). The disease was then thought to be imported from Gorakhpur and surroundings areas of Uttar Pradesh of India, where a JE epidemic occurred in previous years (Umenai et. al., 1985). Subsequently, JE epidemics occurred in Morang district of Eastern Nepal from adjoining Bihar state of India and thus, the disease is gradually spread into other districts in the successive years.

JE has been recognized as a significant public health problem because of its severity and the increasing incidence rates. Though this disease is endemic in 24 districts (20 Terai and 4 inner Terai districts) starting from Jhapa in the east to Kanchanpur in the far west; however, sporadic cases from other districts have also been reported in recent years (EDCD, 2005). The plain areas (<1000m) were seen to be endemic, while the hills (1000-3000) and mountains (>3000m) seen to be affected sporadically in Nepal (Kubo et al., 1996). The mosquito, *Cx. tritaeniorhynclus* is considered as the principal vector of JE in Nepal. Three different strains of JEV isolated from Nepal are: Nep-1/90, B-2524 and B-9548.

Zimmerman et al., in 1997 first reported proven outbreak of JE occurring in the Kathmandu Valley of Nepal. Since then JE has been reported annually from Kathmandu Valley. However, the most patients are from out of the valley.

Conditions in the terai region are most favorable for the breeding of *Culex* mosquitoes. Although the disease has been reported throughout the year from endemic areas, epidemics occur during the monsoon season- starting in the April/ May period, peaking during August and September, declining in October, and leveling off in November (Joshi et al., 2005; EDCD, 2005).

Between 1978 and 2005, nationwide 31,029 cases were identified and 5,828 deaths were reported. Over that time the average CFR was 20 percent (EDCD, 2006; Joshi et al., 2005). However, because JE diagnostic laboratories have only recently become available, there is no data from some endemic areas. In this case most reported cases of JE are merely “suspected”.

Since 1978, several epidemics have been occurred and each successive epidemic has been found to be larger than the previous one. The highest CFR (46.3%) was observed in the year 1982 and the lowest (8.5%) in 2004. The largest epidemic in the history of Nepal was reported in 1997 with 2,953 cases and 407 deaths (CFR=13.8%) whereas the known smallest epidemic was reported in 1981 with 54 cases and 16 deaths. The highest number of casualty was reported in 1999 with 424 deaths out of 2942 cases and the least number of casualties was reported again in the year 1981 with only 16 deaths. A comprehensive result of 1978 to 2005 showed that more than 50% morbidity and 60% mortality have occurred in the age group below 15years (Bista and Shrestha, 2005).

Table 2: JE case and deaths in Nepal: 1978 to 2005

Total	No. of cases	Deaths	CFR %
1978	422	119	28.2
1979	182	49	26.9
1980	622	231	37.1
1981	54	16	29.6
1982	843	390	46.3
1983	242	36	14.9
1984	142	45	31.7
1985	629	183	29.1
1986	1615	415	25.7
1987	502	140	27.9
1988	1403	380	27.1
1989	868	227	26.2
1990	365	102	27.9
1991	650	145	22.3
1992	702	127	18.1
1993	446	108	24.2
1994	1836	383	20.9
1995	1246	257	20.6
1996	1450	263	18.1
1997	**2953**	407	13.8
1998	1161	149	12.8
1999	**2924**	434	14.8
2000	1729	169	9.8
2001	1908	277	14.5
2002	842	168	20.0
2003	931	161	17.3
2004	1538	131	8.5
2005	2824	316	11.2
Total	31029	5828	18.8

Source: EDCD, 2006; Joshi et al., 2005

The annual case incidence (CI) rate has been found to be increasing (leaving some exceptions) in Nepal over last few decades. CI rate (per 1,00,000) ranged from 0.2 in 1981 to 25 in 1997 (Joshi et al., 2005). Data analysis report of 1993 to 1997 showed the increasing pattern of CI rate in each successive year except in the year 1995 (Bista et al., 1999). During this period, the CI rate reached its peak (25.3) in 1997 with high CI in Mid-western and Far-western regions. The cumulative result of this 5 years period indicated below 15 years children as the most vulnerable group with 4,802 cases (61% of the total cases within this period) and male sex (57%) dominated the female (43%) cases (Bista et al., 1999).

Between 1993 and 1997 the districts affected with JE ranged from Jhapa district in the eastern Terai region to Kanchanpur in the Far West. Almost 85% of cases came from eight districts namely Kailali, Banke, Bardiya, Rupandehi, Parsa, Morang, Sunsari and Jhapa. Banke reported 30 percent of the total, Kailali 20%, Morang 10%, Bardiya 7%, and almost 6.5% from Rupandehi. Sunsari, Jhapa and Chitawan each reported less than 5%. The remainder reported 1 to 3 percent each and Udayapur, Rautahat, Bara and Makwanpur districts reported less than 1 percent of total cases. The case incidence (CI) between 1993 and 1997 ranged from almost 0.2 in Saptari (1995) to 343 in Banke (1997). The CI was always above 100 for Banke except in 1993 when it was 24 (Joshi et al., 2005).

During 1998, Kailali and Banke districts combined reported 485 cases and 45 deaths, which represent 42 percent and 30 percent of total cases and deaths in Nepal. In 1999, again Banke and Kailali reported the most cases—1042 and 1041 respectively. The CFR in these districts was 8 and 11 percent respectively. Banke and Kailali continued to report highest number of cases in the year 2000, 2001, 2002, 2003 and 2004. According to EDCD, in the year 2005, among 58 AES cases reporting districts, the highest number was reported from Kailali (435, 14.7%) followed by Dang, Bardiya, Kathmandu, Banke, Kanchanpur, Kapilvastu, Nawalparasi and Sunsari.

From 1998 through 2004, there was an increase in CI from 1998 (13) to 1999 (31.8) but 1999 onwards, the CI rate followed the clear-cut decreasing pattern except in the years 2001 and 2004 (Joshi et al., 2005). Also during this period, Mid-western and Far-western regions represented the most affected regions.

There are three designated referral laboratories, namely National Public Health Laboratory (Teku), Vector Borne Diseases Research and Training Center (Hetauda) and B.P. Koirala Institute of Medical Sciences (Dharan), for confirmatory diagnosis of JE. For external quality assurance of these laboratories, Armed Force Research Institute of Medical Sciences, Department of Virology, Bangkok, Thailand has been participated, especially for JE.

Of the 204 samples tested in 1999, 137 (67%) were found to be positive for JE whereas 47 samples would not be confirmed due to unavailability of the second samples (Bista and Banerjee, 2000). In the same year, some samples which were positive for JEV but showed low conversion titres, were tested against other flaviviruses at AFRIMS, Thailand and some samples showed extremely high titres against West Nile Virus (WNV) (Bista and Banerjee, 2000). In 1998 and 2000, 70% and 62% of cases were confirmed as JE positive (Joshi et al., 2005). In 2001, 43% (374/880) cases were found to be JE positive whereas the year 2002 detected 32.7% (290/888) JE positive cases but in 2003, quite high percentage 89.9%) of JE cases were confirmed among 277 tested cases (Bista and Shrestha, 2005). In 2005, out of the 1862 laboratories examined samples only 35.3% percent were positive for JE.

3.13.3 Risk for travelers

The disease is not common among travelers. Risk varies substantially by season (e.g. risk is highest in the rainy season), geographic location, duration of travel, outbreak presence and activities of the travelers (CDC, 1993).

Although JE is a substantial public health problem in Asian countries, transmission to short-term travelers to JE endemic countries has rarely been reported (CDC, 1993; Geraghty and McCarthy, 2004). Monthly incidence of JE in travelers is less than one per one million among short term and urban travelers but 0.25 to 1 per 5000 among rural travelers to endemic regions (Halstead and Grosz, 1962). Since 1981, only 5 cases of JE among Americans traveling or working in Asia are known to have occurred.

Vaccination is recommended in person spending more than three to four weeks in a region where the disease is prevalent during the transmission season, especially if travel will include rural areas. Under specific circumstances, vaccine should be considered for persons spending less then 1 month in endemic areas (e.g. travelers to areas experiencing epidemic transmission and persons' extensive outdoor activities in rural areas).

CHAPTER IV

4. MATERIALS AND METHODS

4.1 Materials

A complete list of equipments, reagents and chemicals required for particle agglutination assay and IgM capture ELISA is given in Annex I. The reagents for particle agglutination assay was supplied by Pentax Corporation, Japan and that for IgM capture ELISA was used from by Panbio diagnostics, Brisbane, Australia.

4.2 Methods

4.2.1 Study site

Serum samples of suspects of Acute Encephalitis Syndrome (AES), viral fever and malaria were collected from Bheri Zonal Hospital and Nepalgunj Medical College, Nepalgunj in the Year 2062/2063 B.S. Serum samples of suspects of dengue, typhoid, Kalaazar and leptospirosis were collected from Sukraraj Tropical and Infectious Disease Hospital, Teku and Everest International Clinic and Research Centre, Kalanki, Kathmandu during summer season. Samples were transported to the Everest International Clinic and Research Center, Kalanki, Kathmandu for diagnosis.

4.2.2 Study Period

The study was conducted from August 2006 to September 2007.

4.2.3 Sample size

Serum samples collected during the year 2062/2063 from suspects of AES and Viral fever and laboratory confirmed cases of malaria, dengue, kala-azar, typhoid and leptospirosis in Bheri Zonal Hospital, Nepalgunj Medical College, Nepalgunj, Sukraraj Tropical and Infectious disease Hospital, Teku and Everest International

Clinic and Research Centre, Kalanki, Kathmandu were included in this study. A total of 279 serum samples were collected during the study period.

4.2.4 Specimen collection, storage and transport

Five ml of venous blood was collected from adult by vein puncture from suspected AES cases during acute phase of illness and was put in a labeled, clean and dry test tube. In case of children, 3ml of blood was collected. The blood was allowed to clot for 15 minutes at room temperature. Then at 4°C the clot was allowed to retract. The test tube containing blood specimen was centrifuged and serum was transferred to a tightly stoppered sterile container. The container was sealed with parafilm and labeled properly. Serum samples were stored at 2-8°C until transported to the Everest International Clinic and Research Centre, Kalanki, Kathmandu. Samples were transported to the Clinic in icebox and stored at -20°C until tested.

4.3 IgM-Capture ELISA

IgM-capture ELISA is the standard test for the confirmation of suspected cases of AES during acute phase of illness. During the testing procedure, the protocol provided by the Panbio diagnostics was strictly followed to achieve high level of accuracy.

Procedure:

(1.) All reagents were equilibrated to room temperature (20-25°C) before commencing assay.

Serum dilution

(1). Positive control, Negative control, Calibrator and Patient serum samples were diluted. For this, 10 µl of each serum sample was diluted to 1000 µl of Serum diluent. It was mixed well.

Preparation of antigen

(1.) JE antigen was diluted in the ratio of 1:250 using 10 µl antigen as minimum to antigen diluent.

(2.) The required volume of diluted JE antigen was removed and mixed with an equal volume of MAb tracer in a clean glass vial. Antigen-MAb tracer solution was gently mixed and left at room temperature (20-25°C) until required.

Assay Plate

(3) The required numbers of microwells were removed from the foil sachet and were inserted into strip holder. Five microwells were required for Negative Control (N), Positive Control (P) and Calibrator (Cal) in triplicate.

(4.) Within 10 minutes after mixing the MAb tracer and diluted antigen, 100 µl diluted patient sample, controls and calibrator were pipetted into their respective microwells of the assay plate.

(5.) The plate was covered and incubated for 1 hour at 37°C.

(6.) After incubation, wells were washed six (6) times with diluted wash buffer.

(7.) The antigen-MAb tracer solution was mixed before transfer. 100 µl of JE antigen-MAb complexes was pipetted into the wells.

(8.) The plate was covered and incubated for 1 hour at 37°C.

(9.) The wells were washed six (6) times with diluted wash buffer.

(10.) 100 µl of TMB was pipetted into each well.

(11.) Timing from the first addition, the plate was incubated at room temperature (20-25°C). A blue colour was developed.

(12.) 100 µl of stop solution was pipetted into all wells in the same sequence and timing as the TMB addition. It was mixed well. The blue colour was changed to yellow.

(13.) The absorbance of each well was read within 30 minutes at a wavelength of 450 nm with a reference filter of 630 nm.

4.3.1 CALCULATIONS:

The average absorbance of the triplicates of the calibrator was calculated and multiplied by calibration factor. This is the cut-off value.

Cut-off value= average absorbance of calibrator x calibration factor

An index value was calculated by dividing the sample absorbance by the cut-off value.

$$\text{Index value} = \frac{\text{Sample absorbance}}{\text{Cut-offvalue}}$$

Panbio Units was calculated by multiplying the index value by 10.

Panbio units= Index value x 10

4.4 PARTICLE AGGLUTINATION ASSAY (PA)

During the testing procedure, the protocol provided by the Pentax Corporation was strictly followed to achieve high level of accuracy. PA assay can be divided into two types:

III. Qualitative assay and
IV. Quantitative assay

Procedure:

Reconstitution of JEV antigen coated Ha-Ny beads

(1.) 2 ml of beads diluent was transferred into one vial of freeze-dry JEV antigen-coated Ha-Ny beads and mixed by vortexing on maximum setting 3-4 times for 2-3 seconds each.
(2.) Beads were settled down at room temperature at least for 30 minutes.

Assay Procedure:

I. Qualitative assay

(1.) The reagents were brought to room temperature before use.

(2.) Samples were diluted 1:100 with serum dilution buffer.

(3.) Strips were washed three times with wash buffer and they were tapped onto Paper towel to remove liquid from them.

(4.) 50 µl of diluted samples were added on wells immediately after Step 4 to prevent the surface of the wells from drying. The wells may lose the ability to capture antibodies if dried completely.

(5.) The wells were incubated for 30 minutes at room temperature.

(6.) Strips were washed three times with wash buffer and tapped on Paper towel to remove any remaining liquid.

(7.) The JEV antigen-coated beads slurry was gently mixed.

(8.) 100 µl of the beads slurry was added to the wells and it was allowed to settle for one hour at room temperature.

(9.) Beads agglutination patterns was observed.

II. Quantitative assay

(1.) The reagents were brought to room temperature before use.

(2.) The samples were two-fold serially diluted from 1:100 to 1:12,800 with serum dilution buffer in microtubes. In the control lane, Positive control was two-fold serially diluted from 1:100 to 1:6400. Negative control is also diluted to 1:100.

(3.) Strips were washed three times with wash buffer and they were tapped onto Paper towel to remove liquid from them.

(4.) 50 µl of diluted samples were added on wells immediately after Step 4 to prevent the surface of the wells from drying. The wells may lose the ability to capture antibodies if dried completely.

(5.) The wells were incubated for 30 minutes at room temperature.

(6.) Strips were washed three times with wash buffer and tapped on Paper towel to remove any remaining liquid.

(7.) The JEV antigen-coated beads slurry was gently mixed.

(8.) 100 µl of the beads slurry was added to the wells and it was allowed to settle for one hour at room temperature.

(9.) Beads agglutination patterns was observed.

4.5 Statistical Analysis

Sensitivity, specificity, positive predictive value and negative predictive value of PA were calculated in comparison with IgM-capture ELISA. Chi-square tests were done using WIN-PEPI software to find out whether the findings were statistically significant.

CHAPTER-V

5. RESULTS

A total of 279 serum samples were collected in this study in the year 2062/ 2063. Out of 279 samples, 255 samples were clinical suspects of acute encephalitis syndrome (AES), 8 were suspects of viral fever, 5 were laboratory confirmed cases of malaria, 7 were laboratory confirmed cases of dengue, 2 were laboratory confirmed cases of typhoid and one each cases of kala-azar and leptospirosis. Clinical suspects of AES and Viral fever were used to compare particle agglutination assay (PA) and IgM capture ELISA for the diagnosis of Japanese Encephalitis (JE). Laboratory confirmed cases of malaria, dengue, typhoid, kala-azar and leptospirosis were used to observe cross reactivity of PA.

The laboratory result is divided into 2 parts. First, for the comparison of PA with IgM capture ELISA and second for the observation of cross reactivity of PA with different infectious diseases prevalent in same areas.

5.1 Comparison of PA with IgM Capture ELISA for diagnosis of JE

A total of 263 samples of clinical suspects of AES and viral fever were used to compare PA and IgM Capture ELISA. Out of 263 samples, 255 samples were clinical suspects of AES and 8 were clinical suspects of viral fever.

5.1.1 Results from IgM capture ELISA

Table 3. Anti-JE IgM-positive rate among suspected patients of AES and viral fever by IgM capture ELISA

Clinical diagnosis	Total	Positive	Positive %	% Of total positive cases	P-value
AES	255	107	41.6	98.2	0.15
Viral fever	8	2	25	1.8	
Total	263	109	41.4	100	

As shown in Table 3, Out of total 255 samples from the suspected cases of AES, 107 (41.6%) samples showed positive result for anti-JE IgM and 148 samples showed negative result for anti-JE IgM by IgM capture ELISA. Out of 8 suspects of viral fever, 2 (25%) showed positive result for anti-JE IgM and 6 showed negative result. Overall positivity for anti-JE IgM was 41.4%. Statistically, there is no significant difference between AES and viral fever for the occurrence of JE ($P > 0.05$).

Table 4 Sexwise positivity of anti-JEV IgM among suspected AES and viral fever cases by IgM Capture ELISA

Sex	No. Of cases	Positive	Positive %	% Of total positive cases	P value
Male	135(51.3%)	46	34.1	42.2	0.023
Female	128(48.7%)	63	49.2	57.8	
Total	263	109	41.4	100	

As shown in Table 4, among the 135 male cases of AES and viral fever tested, 46 (34.1%) showed positive result for anti-JEV IgM by IgM capture ELISA, which accounts for 42.2% of total positive cases. Similarly, out of 128 female cases of AES and viral fever tested, 63 (49.2%) showed positive result for anti-JE IgM by IgM capture ELISA, which accounts for 57.8% of total positive cases. Sero-positivity was seen higher in female however number of male cases was observed high (51.3%). Statistically, significant difference between the male and female was found for the occurrence of the disease ($P < 0.05$).

Table 5: Districtwise distribution of positive cases in suspected AES and viral fever cases by IgM capture ELISA

District	No. Of cases	Positive	Positive %	% Of total positive cases
Banke	108	44	40.7	40.35
Bardiya	69	33	47.8	30.3
Dang	52	16	30.8	14.7
Kailali	17	7	41.2	6.4
Surkhet	11	6	54.5	5.5
Rukum	2	0	0	0
Salyan	2	2	100	1.8
Kalikot	1	0	0	0
Mugu	1	1	100	0.95
Total	263	109	41.4	100

As shown in table 5, samples from 9 districts were collected in Bheri Zonal Hospital and Nepalgunj Medical College, Nepalgunj. Banke showed maximum number of samples (108) followed by Bardia (68), Dang (51), Kailali (16), Surkhet (11), Rukum (2), Salyan (2) and 1 each from Kalikot and Mugu. Sero-positivity for anti-JE IgM was observed high (100%) in samples from Salyan and Mugu followed by Surkhet (54.5%), Bardiya (47.8%), Kailali (41.2%), Banke (40.7%) and Dang (30.8%) by IgM capture ELISA. No positivity for anti-JE IgM was observed in samples from Rukum and Kalikot. However, high number of positive cases was observed in Bankey (44) followed by Bardiya (33), Dang (16), Kailali (7), Surkhet (6), Salyan (2) and Mugu (1).

Table 6: Agewise distribution of positive cases among suspected AES and viral fever cases by IgM capture ELISA

Age	No. Of cases	Positive	Positive %	% Of total positive cases	P-value
Below 10	79	32	40.5	29.4	0.24
10-20	73	30	41.1	27.5	
20-30	38	21	55.3	19.3	
30-40	22	6	27.3	5.5	
40-50	22	7	31.8	6.4	
50-60	12	4	33.3	3.7	
60-70	11	7	63.6	6.4	
Above 70	6	2	33.3	1.8	
Total	263	109	41.4	100	

As shown in Table 6, age wise distribution of cases of AES and viral fever shows maximum number of cases in the age group below 10 years (79) followed by age group 10-20 years (73), 20-30 (38), 30-40 (22), 40-50 (22), 50-60 (12), 60-70 (11) and above 70 (6). Sero-positivity for anti-JE IgM was seen highest in age group 60-70 (63.6%) followed by age group 20-30 (55.3%), 10-20 (41.1%), below 10 (40.5%), 50-60 (33.3%), above 70 (33.3%), 40-50 (31.8%) and 30-40 (27.3%) by IgM capture ELISA. Highest number of positive cases was seen in age group below 10 (32) followed by age group 10-20 (30), 20-30 (21), 40-50 (7), 60-70 (7), 30-40 (6), 50-60 (4) and above 70 (2). Statistically, there was not significant difference between age and the occurrence of disease ($P > 0.05$).

5.1.2 Results from Particle agglutination assay (PA)

Table 7: **Anti-JE IgM-positive rate among suspected AES and viral fever cases by PA**

Clinical diagnosis	Total	Positive	Positive %	% Of total positive cases	P-value
AES	255	136	53.3	98.6	0.18
Viral fever	8	2	25	1.4	
Total	263	138	52.5	100	

As shown in Table 7, out of total 255 samples from the suspected cases of AES, 136 (53.3%) samples showed positive result for anti-JE IgM and 119 samples showed negative result by PA. Out of 8 suspects of viral fever 2(25%) showed positive result for anti-JE IgM and 6 showed negative result. Overall positivity for anti-JE IgM was 52.5%. Statistically, there is no significant difference between AES and viral fever for the occurrence of JE ($P > 0.05$).

Table 8: Sexwise Positivity of anti-JEV IgM among suspected AES and viral fever cases by PA

Sex	No. Of cases	Positive	Positive %	% Of total positive cases	P-value
Male	135(51.3%)	63	46.3	45.6	0.012
Female	128(48.7%)	75	58.6	54.4	
Total	263	138	52.5	100	

As shown in Table 8, among the 135 male cases of AES and viral fever tested, 63(46.3%) showed positive result for anti-JE IgM by PA, which accounts for 45.6% of total positive cases. Similarly, out of 128 female cases of AES and viral fever, 75(58.6%) showed positive result for anti-JE IgM by PA, which accounts for 54.4% of total positive cases. Positivity for anti-JE IgM was seen higher in female however

number of male cases was observed high (51.3%). Statistically, significant difference between the male and female was found for the occurrence of the disease ($P < 0.05$).

Table 9: Districtwise distribution of Positive cases among suspected AES and viral fever cases by PA

District	No. Of cases	Positive	Positive %	% Of total positive cases
Banke	108	59	54.6	42.7
Bardiya	69	37	53.6	26.8
Dang	52	22	42.3	16
Kailali	17	8	47.1	5.8
Surkhet	11	9	81.8	6.5
Rukum	2	0	0	0
Salyan	2	2	100	1.5
Kalikot	1	0	0	0
Mugu	1	1	100	0.7
Total	263	138	52.5	100

As shown in Table 9, districtwise sero-positivity was observed high (100%) in samples from Salyan and Mugu followed by Surkhet (81.8%), Banke (54.6%) Bardiya (53.6%), Kailali (47.1%), and Dang (42.3%) by PA. No positivity was observed in samples from Rukum and Kalikot. However, high number of positive cases was observed in Bankey (59) followed by Bardiya (37), Dang (22), Kailali (8), Surkhet (9), Salyan (2) and Mugu (1).

Table 10: Agewise distribution of positive cases among suspected AES and viral fever cases by PA

Age	No. Of cases	Positive	Positive %	% Of total positive cases	P-value
Below 10	79	43	54.4	31.2	0.26
10-20	73	38	52.1	27.5	
20-30	38	25	65.8	18.1	
30-40	22	10	45.4	7.2	
40-50	22	7	31.8	5.1	
50-60	12	5	41.7	3.6	
60-70	11	7	63.6	5.1	
Above 70	6	3	50	2.2	
Total	263	138	52.5	100	

As shown in Table 10, age wise positivity was seen highest in age group 20-30(65.8%) followed by age group 60-70(63.6%), below 10(54.4%), 10-20(52.1%), 30-40(45.4%), 50-60(41.7%), above 70(33.3%), and 40-50(31.8%) by PA. Highest number of positive cases was seen in age group below 10(43) followed by age group 10-20(38), 20-30(25), 30-40(10), 40-50(7), 60-70(7), 50-60(5) and above 70(3). Statistically, there was not significant difference between age and the occurrence of disease (P> 0.05).

5.1.3 Comparison of PA with IgM capture ELISA

Table 11: Comparision of PA with IgM capture ELISA among suspected AES and viral fever cases

Particle agglutination assay	IgM Combo ELISA		Total
	Positive	Negative	
Positive	106	32	138
Negative	3	122	125
Total	109	154	263

As shown in Table 11, out of total 263 serum samples of AES and Viral fever, 138 (52.5%) were positive for anti-JE IgM by PA and 106 (77%) of the 138 PA IgM-positive samples were also IgM positive for anti-JE IgM by IgM-capture ELISA. Thirty-two (23%) of the 138 PA IgM-positive samples were IgM negative by IgM-capture ELISA. Three samples which were IgM negative for anti-JE IgM by PA were positive by IgM capture ELISA. One hundred twenty-two serum samples were negative for anti-JE IgM by both IgM-capture ELISA and PA. Thus, PA assay had a sensitivity of 97% and specificity of 79% in comparision with IgM-capture ELISA. A positive predictive value of 0.77 and negative predictive value of 0.98 was observed with PA in comparision with IgM-capture ELISA.

5.2 Observation of cross reactivity of PA with different diseases.

Table 12 Quantitative assay for the observation of cross reactivity of dengue samples with JE

Sample No.	Anti-JE IgM titre	Anti-dengue IgM titre
1.	1:400	1:1600
2.	Negative (< 1:100)	1:3200
3.	Negative (<1:100)	1:6400
4.	1:400	1:3200
5.	1:800	1:3200
6.	Negative (<1:100)	1:1600
7.	Negative (<1:100)	1:6400

Cross reactivity of PA was observed with clinically infectious and tropical diseases (malaria, dengue, typhoid, kala-azar and leptospirosis). A total of 16 laboratory confirmed serum samples were taken to observe cross reactivity of PA. Among which

5 were the malaria cases, 7 were dengue cases, 2 were typhoid cases and 1 each cases of kala-azar and leptospirosis (See Appendix III for method of test done to confirm the sample). Cross reactivity of PA was seen with dengue during qualitative assay. However with quantitative assay all the dengue samples showed four fold more antibody titre for dengue than JE, which shows the samples to be confirmed for dengue. PA was found to be cross reactive with malaria in one sample out of five samples which was also found to be cross reactive with IgM capture ELISA indicating the chances of mixed infection of JE with malaria. No cross reactivity of both PA and IgM capture ELISA was seen with typhoid, kala-azar and leptospirosis.

CHAPTER VI

6. DISCUSSION AND CONCLUSION

6.1 Discussion

Japanese encephalitis (JE) is the most important form of viral encephalitis worldwide, causing approximately 50,000 cases and 15,000 deaths annually. The annual incidence of clinical infection in endemic areas ranges from 10 to 100 per 100000 populations (Solomon, 1998).

Approximately, 3 billion people and 60% of the world's population live in the JE endemic regions (Kabilan, 2004). In recent years the epidemiological pattern and geographical distribution of JE have changed in Asia. In Taiwan, Japan, South Korea and China clinical cases of JE have decreased dramatically. On the other hand, the incidence of JE has increased in India, Nepal, Sri Lanka, Thailand and Bangladesh and Vietnam (Umenai et al., 1985). Due to its fatal outcome in approximately 25% of cases and residual neuropsychiatic sequelae in 30% of cases (Burke et. al. 1998) JE has been a serious health problem in Asia and in Nepal too.

In Nepal, since the first outbreak in 1978, seasonal outbreaks of JE have been reported annually. Now a day, in terms of morbidity and mortality this disease is the major public health problem in Nepal.

JE is generally diagnosed on the basis of clinical symptoms in the rural areas of Asia including Nepal. IgM capture ELISA has been the most reliable technique for the confirmatory laboratory diagnosis of JE. But, this technique is expensive and relatively sophisticated. Laboratory diagnosis of JE is difficult to perform in Nepal due to insufficient resources and diagnostic facilities in many health centres.

In this study, Particle Agglutination assay (PA) which is simple, reliable and inexpensive method suitable for rural areas of Asia is compared with IgM capture ELISA for the diagnosis of JE and cross reactivity of it was observed with other infectious and tropical diseases.

A total of 279 samples were collected in this study in the year 2062 /2063. Out of 279 samples, 255 samples were clinical suspects of acute encephalitis syndrome (AES), 8 were suspects of viral fever and 5 were laboratory confirmed cases of malaria, 7 were laboratory confirmed cases of dengue, 2 were laboratory confirmed cases of typhoid and one each cases of kala-azar and leptospirosis. Clinical suspects of AES and viral fever were used to compare PA and IgM capture ELISA for the diagnosis of JE. Laboratory confirmed cases of malaria, dengue, typhoid, kala-azar and leptospirosis were used to observe cross reactivity of PA.

A total of 263 samples of clinical suspects of AES and viral fever were used to compare PA and IgM Capture ELISA. Out of total 255 samples from the suspected cases of AES, 107 (41.6%) samples showed positive result for anti-JE IgM and 148 samples showed negative result by IgM-capture ELISA whereas 136 (53.3%) samples showed positive result for anti-JE IgM and 119 samples showed negative result by PA. Out of 8 suspects of viral fever 2 (25%) showed positive result for anti-JE IgM and 6 showed negative result by both IgM-capture ELISA and PA. Overall positivity for anti-JE IgM was 41.4% with IgM capture ELISA and 52.5% with PA. This shows that cases diagnosed as viral fever may be the mild febrile manifestation of Japanese encephalitis. Statistically, there is no significant difference between AES and viral fever for the occurrence of JE ($P > 0.05$).

Overall positivity was seen higher which is in accordance with similar type of previous reports from Nepal. A study carried out in Bheri Zonal Hospital by

Bajracharya et al. (2001) demonstrated a positivity rate for anti-JE IgM of 47.2%. In 1999, 67% of the tested samples out of the 204 were JE positive (Bista and Banerjee, 2000) whereas in 1998 and 2000, 70% and 62% of cases were confirmed as JE positive (Joshi et al., 2005). In 2001, 43% (374/880) cases were found to be JE positive and in 2003, quite high percentage (89.9%) of JE cases were confirmed among 277 tested cases (Bista and Shrestha, 2005).

However, positivity was quite high in comparison with national figure. In the year 2005, out of 1862 laboratory examined samples only 657(35.3%) were positive for JE. The positivity difference between the previous reports and present finding could be due to small sample size and localized nature of study in hyper-endemic region in contrary to wide coverage of surveillance samples. The negative cases could be due to over diagnosis or other causes of acute encephalitis syndrome.

Among the 135 (51.3%) male cases of AES and viral fever tested, 46 (34.1%) showed positive result for anti-JE IgM by IgM Capture ELISA and 63 (46.3%) showed positive result by PA. Similarly, out of 128 (48.7%) female cases of AES and viral fever, 63 (49.2%) showed positive result for anti-JE IgM by IgM Capture ELISA and 75 (58.6%) showed positive result by PA. Positivity for anti-JE IgM was seen higher in female however number of male cases was observed high (51.3%). The predominance of male cases was usually expected because male works more in the outdoor environment than the female and thus has higher chances of being bitten by mosquitoes. This was in accordance with previous reports. However, positivity rate for anti-JE IgM was observed higher in female by both PA and IgM-capture ELISA. Statistically, significant difference between the male and female was found for the occurrence of the disease ($P < 0.05$). There is no explanation for higher positivity in female. This may be due to poor immunological status among women due to social discrimination and nutritional status in socioeconomically poor community.

Between 1993 and 1997, of the 7,931 people affected with JE, 4,495 were males and 3,358 females. This breaks down to 57% male and 43% female. The male-female ratio was approximately 1.3 to 1. In the year 2000, of the 1,729 people affected with JE, 932(54%) were males and 797(46%) were females (Joshi et al., 2005).

District wise distribution of cases showed maximum number of cases from Banke (108) followed by Bardiya (68), Dang (51), Kailali (16), Surkhet (11), Rukum (2), Salyan (2) and 1 each from Kalikot and Mugu. Sero-positivity for anti-JE IgM was observed high (100%) in samples from Salyan and Mugu followed by Surkhet (54.5%), Bardiya (47.8%), Kailali (41.2%), Banke (40.7%) and Dang (30.8%) with IgM-capture ELISA. Similarly with PA districtwise positivity for anti-JE IgM was observed high (100%) in samples from Salyan and Mugu followed by Surkhet (81.8%), Banke (54.6%), Bardiya (53.6%), Kailali (47.1%) and Dang (42.3%). However, high number of positive cases was observed in Bankey followed by Bardiya, Dang, Kailali, Surkhet, Salyan, Mugu and India by both PA and IgM-capture ELISA. Higher positivity isn samples from Salyan and Mugu is due to fewer number of cases. Bankey, Bardiya, Dang and Kailali showed more than 90% of positive cases. These four districts were hyperendemic districts for JE. No positivity for anti-JE IgM was observed in samples from Rukum and Kalikot by both IgM-capture ELISA and PA. Sporadic cases were observed from Rukum, Salyan, Kalikot and Mugu. Surkhet is the endemic district for JE, which showed 11 cases. The results were in correlation with previous reports. Sporadic cases in Rukum, Salyan, Kalikot and Mugu may be due to expansion of diseases from endemic districts to these regions or due to frequent travel of the patients to endemic regions. Environmental conditions of paddy field ecosystem in the Terai region are most favourable for the breeding of *Culex* mosquitoes indicating its higher incidence in that region.

According to EDCD, in the year 2005, among 58 AES cases reporting districts, the highest number was reported from Kailali (435, 14.7%) followed by Dang, Bardiya, Kathmandu, Banke, Kanchanpur, Kapilvastu, Nawalparasi and Sunsari.

Between 1993 and 1997 case incidence was always above 100 for Banke except in 1993 when it was 24. Case incidence rate was highest in the Mid-western development region, which was 27.8 followed by Far western development regions which was 16. The number of cases in the same year was 836 in MWDR and 228 in FWDR, which were 54.4% and 14.8% respectively (Joshi et al., 2005).

Serological surveys conducted in Mid-western regions of Nepal have found 62 percent significant antibody titres to JE virus (Bajracharya et al., 2001).

Age wise distribution of AES cases show maximum number of cases in the age group below 10 years (79) followed by age group 10-20 years (73), 20-30(38), 30-40(22), 40-50(22), 50-60(12), 60-70(11) and above 70(6). Positivity for anti-JE IgM was seen highest in age group 60-70(63.6%) followed by age group 20-30(55.3%), 10-20(41.1%), below 10(40.5%), 50-60(33.3%), above 70(33.3%), 40-50(31.8%) and 30-40(27.3%) with IgM-capture ELISA. Similarly with PA age wise positivity for anti-JE IgM was seen highest in age group 20-30(65.8%) followed by age group 60-70(63.6%), below 10(54.4%), 10-20(52.1%), 30-40(45.4%), 50-60(41.7%), above 70(33.3%), and 40-50(31.8%). Highest number of positive cases was seen in age group below 10(43) followed by age group 10-20(38), 20-30(25), 30-40(10), 40-50(7), 60-70(7), 50-60(5) and above 70(3) with IgM-capture ELISA. Similarly with PA highest number of positive cases was seen in age group below 10(32) followed by age group 10-20(30), 20-30(21), 40-50(7), 60-70(7), 30-40(6), 50-60(4) and above 70(2). Approximately 58% of total cases were in the age group below 20. This may be due to poor immunological status and previous non-exposure of children to JE. This is in accordance with previous national reports. However, statistically, there was not significant difference between age and the occurrence of disease ($P > 0.05$). Due to vaccination among children the disease may have shifted to other elderly age group. In developed countries where children are protected by immunization, JE occurrence is increased in the elderly or old age, consistent with waning immunity with age (Vaughn and Hoke, 1992).

Analysis by patient age in disease- endemic areas revealed that 60 percent of patients were below 15 years (Joshi et al., 2005). During the period of 1993-1997, age wise distribution of cases showed 42% of total cases in the age group below 10 years. During the year 2001, age wise distribution of JE cases showed 51% of total cases in the age group below 15 years (Joshi et al., 2005). Five to fifteen years children are the most active and they always roam outside home to play around water lodged areas and rice fields. Because of high temperatures of terai region during the mosquito-breeding season, children play outside the home in the evening time unknowingly putting themselves at the risk of mosquito bite.

Out of total 263 serum samples of acute encephalitis syndrome (AES) and Viral fever, 138 (52.5%) were positive for anti-JE IgM by particle agglutination assay and 106 (77%) of the 138 PA IgM-positive samples were also IgM positive for anti-JE IgM by IgM-capture ELISA. Thirty-two (23%) of the 138 PA IgM-positive samples were IgM negative for anti-JE IgM by IgM-capture ELISA. Three samples that were IgM negative for anti-JE IgM by PA was positive by IgM capture ELISA. One hundred twenty-two serum samples were negative for anti-JE IgM by both IgM-capture ELISA and PA. Thus, PA assay had a sensitivity of 97% and specificity of 79% in comparision with IgM-capture ELISA. A positive predictive value of 0.77 and negative predictive value of 0.98 was observed for PA in comparision with IgM-capture ELISA. This shows that there is a high level of compatibility between the PA assay and IgM-capture ELISA. PA could be an alternative for the diagnosis of JE in rural areas of Nepal.

Cross reactivity of PA was seen with Dengue during qualitative assay. However with quantitative assay for both JE and Dengue all the dengue samples showed four fold greater titre for dengue confirming the diagnosis as dengue. One sample out of five samples of Malaria showed positive result with PA, which was also positive by IgM, capture ELISA. This may be due to mixed infection of JE with Malaria. It was

observed during previous studies and according to the manufacturer that IgM-capture ELISA was generally not cross-reactive with Malaria. No cross reactivity was seen with Typhoid, Kala-azar and Leptospirosis by both PA and IgM capture ELISA.

Previous studies on PA by Pandey et al., (2003) in Nepal showed a sensitivity of 99%, a specificity of 88%, a positive predictive value of 0.83 and a negative predictive value of 0.99 for PA in comparision with IgM-capture ELISA which is quite similar to this study.

However there are several practical limitations in this study. Particularly for the observation of cross reactivity, the number of samples was very few (16). For the confirmatory diagnosis of JE paired sera during acute and convalescent phase is desirable to observe the rise in anti-JE IgM titre that could not be collected during this study. This study is primarily focused in hyper-endemic district, so the result may be quite variable with National figure.

6.2 Conclusion:

The result from PA is highly compatible with IgM capture ELISA. Both sensitivity and specificity of PA was acceptable in comparision with IgM-capture ELISA. With quantitative assay performed for both dengue and Japanese encephalitis it was observed that it could distinguish JE and dengue as well. Sexwise distribution showed highest number of cases in male. Below 20 age group showed more than 50% of cases indicating child are more susceptible to the disease. The higher number of cases was observed in hyperendemic terai regions favourable for growth of mosquitoes. Sporadic cases were observed in some hilly regions indicating the expansion of the disease to that region.

CHAPTER VII

7. SUMMARY AND RECOMMENDATIONS

7.1 Summary

1. A total of 279 serum samples were collected in this study in the year 2062/ 2063. Out of 279 samples, 255 samples were clinical suspects of AES, 8 were suspects of viral fever, 5 were laboratory confirmed cases of malaria, 7 were laboratory confirmed cases of dengue, 2 were laboratory confirmed cases of typhoid and one each cases of kala-azar and leptospirosis.

2. Out of total 255 samples from the suspected cases of AES, 107 (41.6%) samples showed positive result for anti-JE IgM by IgM-capture ELISA whereas 136 (53.3%) samples showed positive result by PA. Out of 8 suspects of viral fever 2(25%) showed positive result for anti-JE IgM by both IgM-capture ELISA and PA. Overall positivity for anti-JE IgM was 41.4% with IgM capture ELISA and 52.5% with PA.

3. Among the 263 cases of AES and viral fever, 51.3% were male and48.7% were female. Majority of the cases (about 58%) were of below 20 years.

4. Highest number of positive cases was observed in Bankey (59) followed by Bardiya (37), Dang (22), Kailali (8), Surkhet (9), Salyan (2) and Mugu (1).

5. Out of total 263 serum samples of AES and Viral fever, 138 (52.5%) were positive for anti-JE IgM by PA and 106 (77%) of the 138 PA IgM-positive samples were also IgM positive for anti-JE IgM by IgM-capture ELISA. Thirtytwotwo (23%) of the 138 PA IgM-positive samples were IgM negative for anti-JE IgM by IgM-capture ELISA. Three samples which were IgM negative for anti-JE IgM by PA were positive by IgM

capture ELISA and 122 serum samples were negative for anti-JE IgM by both IgM-capture ELISA and PA.

6. It was found during this study that PA assay had a sensitivity of 97% and specificity of 79% in comparision with IgM-capture ELISA. A positive predictive value of 0.77 and negative predictive value of 0.98 was observed for PA in comparision with IgM-capture ELISA.

7. Cross reactivity of PA was seen with Dengue during qualitative assay. However with quantitative assay for both JE and Dengue all the dengue samples showed four fold greater titre for dengue confirming the diagnosis as dengue.

8. One sample out of five samples of malaria showed positive result with PA for JE, which was also positive by IgM, capture ELISA. No cross reactivity was seen with typhoid, kala-azar and leptospirosis by both PA and IgM capture ELISA.

7.2 Recommendations

1. Both sensitivity and specificity of PA was found to be acceptable in comparision with IgM-capture ELISA. Thus, on the basis of our findings it can be recommended that PA can be used for the diagnosis of JE in rural areas of Nepal, which does not require specific diagnostic facilities. However, the result should be well correlated with clinical findings.

2. For the observation of cross reactivity, the number of serum samples tested was very few, so it is recommended that cross reactivity studies should be performed with large sample size.

3. For the confirmatory diagnosis of JE, it is essential to test paired serum samples during acute and convalescent phase of the disease, which could not be collected during this study. So, it is strongly recommended that in future paired serum samples should be tested for further verification of the test.

4. In this study, during the testing of laboratory confirmed cases of malaria, both PA and IgM-capture were found to be cross reactive to malaria in one of the five samples. It was observed during previous studies and according to the manufacturer that IgM-capture ELISA was generally not cross-reactive with malaria. The result in our study may be due to mixed infection of JE with malaria. So, the chances of mixed infection of JE with malaria in these regions should be studied in future.

5. Vaccination and other preventive measure such, as use of bed net, mosquito repellant should be focused in hyperendemic regions to contol the disease.

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APPENDIX I

Materials

Equipments, chemicals and reagents available at Everest International Clinic and Researh Centre, Kathmandu and Central Department of Microbiology, Tribhuvan University were used during this study. The reagents for particle agglutination assay was supplied by Pentax Corporation, Japan and that for IgM capture ELISA was used from Panbio diagnostics, Brisbane, Australia.

A. Equipments & supplies

Adjustable micropipettors

Aluminium foils (Hindalco, India)

Beakers (100 ml, 500 ml and 1000 ml)

Centrifuge

Cotton

Disposable gloves

Disposable pipettes (5-1000µl)

Disposable syringes (3 ml and 5 ml) (Lifeline, Everest Med Pvt. Ltd., Nepal)

ELISA plate reader (ELISKAN)

Eppendorf tubes

Freezer (-20°C)

Incubator

Liquid household bleach for inactivating clinical specimens

Measuring cylinders (50 ml, 100 ml, 500 ml and 1000 ml)

Paper towels

Parafilm

Reagent bottles (500 ml)

Refrigerator (2-8°C)

Serum vials (2 ml)

Sink or bucket

Stirring rod

Test tube racks (3 x 10 holes)

Test tubes

Timer

Tip boxes

Volumetric flasks (500 ml and 1000 ml)

Vortex mixer

Washing bottles

B. Reagents and chemicals

For IgM capture ELISA (Supplied by Panbio diagnostics, Brisbane, Australia)

Antigen diluent (Phosphate Buffer containing preservatives)

Anti-human IgM Coated Microwells- (Assay plate)

Dengue 1-4 Antigens (Recombinant)

Dengue Calibrator Serum

Distilled or deionized water

Flavivirus Negative Control Serum

Flavivirus Positive Control Serum

HRP (Horseradish peroxidase) Conjugated Monoclonal Antibody Tracer

Japanese encephalitis Antigen (Recombinant)

JE Calibrator Serum

Serum diluent (Tris buffered saline with preservatives)

Stop Solution (1M Phosphoric acid)

Tetramethylbenzidine (TMB) (mixture of 3,3',5,5'-tetramethylbenzidine and hydrogen peroxide in a citric acid citrate buffer)

Wash buffer (concentrate of phosphate buffered saline with Tween 20)

For Particle agglutination assay (PA)

Beads diluents

Distilled or deionized water

Freeze-dry JEV antigen-coated Ha-Ny beads

Human IgM-capture microplate

Negative control (Anti-JEV IgM negative human serum)

Positive control (Anti-JEV IgM positive human serum)

Serum diluent

10x Wash buffer

ANNEX II

Protocol of the Test

A. IgM CAPTURE ELISA (Panbio diagnostics, Brisbane, Australia)

JAPANESE ENCEPHALITIS – DENGUE IgM COMBO ELISA TEST

(E-JED01C)

Intended use

The Panbio Japanese Encephalitis – Dengue IgM Combo ELISA is for the qualitative presumptive detection of IgM antibodies to Japanese encephalitis and dengue virus in serum as an aid in the clinical laboratory diagnosis of Japanese encephalitis and dengue virus infection in patients with clinical symptoms consistent with encephalitis or dengue fever. This assay is a serological aid to diagnosis of Japanese encephalitis or dengue infection and positive results should be confirmed by PRNT or current CDC guidelines.

Assay performance characteristics have not been established for automated instruments.

Principle

Each serum sample is added to 2 wells of the Assay plate. Serum antibodies of the IgM class, when present, combine with anti-human IgM antibodies attached to the polystyrene surface of the microwell test strips (Assay plate). Concentrated Japanese encephalitis and dengue 1-4 antigens are diluted separately to the correct working volume with antigen diluent. The antigens are produced using an insect cell expression system and immunopurified utilizing a specific monoclonal antibody. An equal volume of the HRP-conjugated monoclonal antibody (MAb) is added to each diluted antigen, which allows the formation of antigen- MAb complexes. Residual serum is removed from the Assay plate by washing, and complexed antigen-MAb is added to the assay plate. After incubation, the microwells are washed and a colourless substrate system, tetramethylbenzidine/hydrogen peroxide (TMB/ H_20_2) is added.

The substrate is hydrolysed by the enzyme and the chromogen changes to a blue colour. After stopping the reaction with acid, the TMB becomes yellow. Colour development is indicative of the presence of the respective anti-flavivirus IgM antibodies in the test sample.

Materials provided

Materials provided for IgM capture ELISA by Panbio diagnostics is listed in Appendix I.

Precautions for in vitro diagnostic use

(i) This test should be performed on serum only. The use of whole blood, plasma or other specimen matrix has not been established.

(ii) Icteric or lipaemic sera, or sera exhibiting haemolysis or microbial growth should not be used.

(iii) Do not heat inactivate sera.

(iv) All reagents must be equilibrated to room temperature (20-25°C) before commencing the assay. The assay will be affected by temperature changes. Do not remove microwells from closed bag until they have reached room temperature (20-25°C).

(v) Dispense reagents directly from bottles using clean pipette tips. Transferring reagents may result in contamination.

(vi) Unused microwells should be resealed immediately and stored in the presence of desiccant. Failure to do this may cause erroneous results.

(vii) Substrate System:

(a) As TMB is susceptible to contamination from metal ions, do not allow the substrate system to come into contact with metal surfaces.

(b) Avoid prolonged exposure to direct light.

(c) Some detergents may interfere with the performance of the TMB.

(d) The TMB may have a faint blue colour. This will not affect the activity of the substrate or the results of the assay.

(viii) **WARNING:** Some kit components contain sodium azide, which may react with lead or copper plumbing to form highly explosive metal azide compounds. When disposing of these reagents through plumbing fixtures, flush with a large volume of water to prevent azide build-up in drains.

(ix) Sodium azide inhibits conjugate activity. Clean pipette tips must be used for the conjugate addition so that sodium azide is not carried over from other reagents.

Specimen collection and preparation

Blood obtained by venipuncture should be allowed to clot at room temperature (20-25°C) and then centrifuged according to the National Committee for Clinical Laboratory Standards (NCCLS) (Approved Standard- Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture, H3-A4, 1998).

The serum should be separated as soon as possible and refrigerated (2-8°C) or stored frozen (-20°C or colder) if not tested within two days. Self-defrosting freezers are not recommended for storage. The use of icteric sera or sera exhibiting haemolysis, lipaemic or microbial growth is not recommended. The NCCLS provides recommendations for storing blood specimens (Approved Standard – Procedures for the Handling and Processing of Blood Specimens, H18-A2, 1999).

Test Procedure

Note: Ensure all reagents are equilibrated to room temperature (20-25°C) before commencing assay. Performing the assay outside the time and temperature ranges

provided may produce invalid results. Assays not falling within the established time and temperature ranges must be repeated.

Serum Predilution

(i) Remove the required number of microwells from the foil sachet and insert into strip holder. Five microwells are required for Negative Control (N), Positive Control (P) and Calibrator (CAL) in triplicate. Ensure the remaining unused microwells are sealed tightly in the foil sachet.

(ii) Using suitable test tubes or a microtitre plate, dilute the Positive Control, Negative Control, Calibrator, and patient samples:

(a) To 10 µl serum add1000 µl of Serum Diluent. Mix well.

Alternatively,

(b) To 10 µl serum add 90 µl of Serum Diluent. Take 25 µl of the diluted serum and add 225 µl Serum Diluent. Mix well.

ELISA procedure

(a) Antigen

(i) Determine the required number of wells for your assay. Dilute both antigens 1/250 using the antigen diluents. It is recommended, as a minimum, to dilute 10 µl of antigen into 2.5 ml of Antigen Diluent. A volume of 0.5 ml of diluted antigen is required per strip (8 wells). Ensure the remaining unused concentrated antigen remains at 2-8°C.

(ii) Remove the required volume of diluted JE antigen and mix with an equal volume of MAb tracer in a clean glass or plastic vial. Repeat the process with the diluted Dengue antigen in a separate vial, ensuring each vial is well labeled. Gently mix the two antigen-MAb tracer solutions and leave at room temperature (20-25°C) until required. Discard any unused diluted antigen.

(b) Assay plate

(iii) Within 10 minutes after mixing the MAb tracer and diluted antigen, pipette 100 µl diluted patient sample and controls into their respective microwells of the assay plate.

(iv) Cover the plate and incubate for 1 hour at 37°C ±1°C.

(v) Wash six (6) times with diluted Wash buffer

(vi) Mix the antigen-MAb tracer solution before transfer. Pipette 100 µl of antigen-MAb complexes into the appropriate wells.

(vii) Cover plate and incubate for 1 hour at 37°C ±1°C.

(viii) Wash six (6) times with diluted Wash Buffer.

(ix) Pipette 100 µl TMB into each well.

(x) Incubate for 10 minutes at room temperature (20-25°C), timing from the first addition. A blue colour will develop.

(xi) Pipette 100 µl of Stop solution into all wells in the same sequence and timing as the TMB addition. Mix well. The blue colour will change to yellow.

(xii) Within 30 minutes read the absorbance of each well at a wavelength of 450nm with a reference filter of 600-650nm.

Note: If a dual wavelength spectrophotometer is available, set the reference filter between 600-650 nm. Reading the microwells at 450 nm without a reference filter may result in higher absorbance values due to background.

Washing Procedure

Efficient washing to remove uncomplexed sample or components is a critical requirement of the ELISA procedure.

(a) Automated Plate Washer

(1) Completely aspirate all wells.

(2) Fill all wells to rim (350 µl) during wash cycle.

(3) On completion of 6 washes, invert plate and tap firmly on absorbent paper towel to ensure all wash buffer is removed.

(4) Automated plate washers must be well maintained to ensure efficient washing. The manufacturer's cleaning instructions should be followed at all times.

(b) Manual washing

(1) Discard contents of plate in appropriate waste container.

(2) Fill wells with Wash Buffer using a suitable squeeze bottle. Avoid bubbling of wash buffer as this may reduce wash efficiency. Discard wash buffer from wells immediately.

(3) Refill wells with wash buffer and discard immediately.

(4) Repeat step 3 another four times. This will make a total of six washes with wash buffer.

(5) After the final wash, discard contents of wells and tap the plate on absorbent paper towel to ensure all wash buffer is removed.

Quality Control

Each kit contains two Calibrators, Positive and Negative Control sera. Acceptable values for these sera are found on the accompanying specification sheet. The Negative and Positive Controls are intended to monitor for substantial reagent failure. The

Positive Control will not ensure precision at the assay cut-off. The test is invalid and should be repeated if the absorbance readings of either the Controls or the Calibrator do not meet the specifications. If the test is invalid, patient results cannot be reported.

Quality Control (QC) requirements must be performed in conformance with local, state, and/or federal regulations or accreditation requirements and your laboratory's standard QC procedures.

It is recommended that the user refer to NCCLS C24-A and 42 CFR 493.1202(c) for guidance on appropriate QC practices.

Calculations

IMPORTANT NOTE: The calibration factor is batch specific and different for JE and Dengue. This is detailed in the specification sheet. Obtain the calibration factor values before commencing calculations.

Ratios are to be calculated separately for the JE and dengue antigen wells using the appropriate calibrator as follows:

(1) Calculate the average absorbance of the triplicates of the Calibrator and multiply by the calibration factor. This is the Cut-off value.

(2) An index value can be calculated by dividing the sample absorbance by the Cut-off value (calculated in step 1 above).

Alternatively;

(3) Panbio Units can be calculated by multiplying the index value (calculated in step 2 above) by 10.

$$\text{Index Value} = \frac{\text{Sample absorbance}}{\text{Cut-off value}}$$

Example: Sample A absorbance= 0.949

Sample B absorbance= 0.070

Mean absorbance of Calibrator= 0.802

Calibration Factor= 0.62

Cut-off value=0.802 x 0.62= 0.497

Sample A (0.949/0.497) =1.91 Index value

Sample B (0.070/0.497) =0.14 Index value

Panbio units= Index value x 10

Sample A 1.91 x 10= 19.1 Panbio units

Sample B 0.14 x 10= 1.4 Panbio units

Interpretation of results

Diagnosis of Dengue Infection is characterized by the presence of a significant and/ or rising level of IgM 3-5 days after the onset of infection.

The cut-off has been determined using endemic populations from South-East Asia / South America and a population from Queensland, Australia, of 208 characterized negative (208/409), positive (91/409) and disease control samples (110/409). The cut-off was determined by two-graph receiver operating characteristic analysis (TG-ROC). A cut-off ratio of 1.0 was selected based on the optimal F value for sensitivity and specificity.

Diagnosis of JE infection:

The cut-off has been determined using endemic populations from South-east Asia and non-endemic populations from Australia and the USA, consisting of 317 characterized negative (317/376) and positive samples (59/376). The cut-off was determined by two-graph receiver operating characteristic analysis (TG-ROC). A cut-off ratio of 1.0 was selected based on the optimal F value for sensitivity and specificity.

Japanese encephalitis infection is characterized by the presence of a significant and/ or rising level of IgM 3-5 days after the onset of infection. As serological cross-reactivity across the flavivirus group is common, the JE result on its own does not distinguish between JE and dengue infection. When examined in conjunction with the dengue IgM result, the 2 results can assist in more accurate diagnosis, since clinical trials with the Panbio Dengue IgM Capture ELISA alone have observed limited cross-reactivity with JE. Therefore samples those are positive in the JE antigen wells and negative in the dengue antigen wells are indicative of JE infection. Samples those are either positive or negative in the JE antigen wells and positive in the dengue antigen wells are indicative of recent dengue infection.

JE-Dengue IgM Combo Capture ELISA

JE Panbio units	JE IgM result	Dengue Panbio units	Dengue IgM result	Interpretation
<9	Negative	<9	Negative	No detectable IgM antibody. The result does not rule out JE or dengue infection. An additional sample should be tested in 7-14 days if early infection is suspected. Further testing should be performed to rule out acute infection.
<9	Negative	9-11	Equivocal	Samples should be re-tested

<9	Negative	>11	Positive	Presence of detectable IgM antibody, presumptive infection with dengue virus.*
9-11	Equivocal	<9	Negative	Samples should be re-tested
9-11	Equivocal	9-11	Equivocal	Samples should be re-tested
9-11	Equivocal	>11	Positive	Presence of detectable IgM antibody, presumptive infection with dengue virus.*
>11	Positive	<9	Negative	Presence of detectable IgM antibody, presumptive infection with JE virus.*
>11	Positive	9-11	Equivocal	Samples should be re-tested
>11	Positive	>11	Positive	Presence of detectable IgM antibody, presumptive infection with dengue virus.*

*Results should be confirmed by PRNT or current CDC guidelines.

The following is a recommended method for reporting the results obtained: "The following results were obtained with the Panbio Japanese encephalitis – Dengue IgM Combo ELISA. Values obtained with different methods may not be used interchangeably. The magnitude of the measured result, above the cut-off, is not indicative of the total amount of antibody present." The result should be reported as positive, negative or equivocal for either dengue or JE, and not as a numeric value. The reported results should contain an appropriate interpretation.

Test Limitations

Note: If specimen remains equivocal following repeat testing then the specimen should be tested by an alternate method or another patient specimen obtained and tested.

1. The clinical diagnosis must be interpreted with clinical signs and symptoms of the patient. The results from this kit are not by themselves diagnostic and should be considered in association with other clinical data and patient symptoms.

2. Population sero-epidemiology may vary over time in different geographical regions. Consequently, the cut-off may require adjustment based on local studies.

3. Screening of the general population should not be performed. The positive predictive value depends on the likelihood of the virus being present. Testing should only be performed on patients with clinical symptoms consistent with encephalitis or dengue fever.

4. Heterophilic antibodies are a well- recognized cause of interference in immunoassays. These antibodies to animal IgG may cross-react with reagent antibodies and generate a false positive signal. This must be excluded before confirmation of diagnosis.

5. The performance characteristics have not been established for visual result determination.

6. This assay employs insect-expressed proteins. The cross-reactivity or interference of human anti-insect antibodies is unknown with the assay's results.

7. All sera demonstrating a positive result by the Panbio JE-Dengue IgM Combo ELISA should be referred to a reference laboratory for confirmation of positivity and epidemiological recording.

8. Serological cross-reactivity across the flavivirus group is common. Other flavivirus diseases must be excluded before confirmation of diagnosis. In-house studies with sera from infected Thai and Vietnamese patients have shown that the use of the Panbio JE-Dengue IgM Combo ELISA can be used to distinguish between JE and dengue infections.

9. Secondary dengue infections that are characterized by a sharp increase in anti-dengue IgG level with no apparent IgM increase will not be detected by the assay and require additional testing.

B. PARTICLE AGGLUTINATION ASSAY (Pentax Corporation, Tokyo, Japan)

Intended Use

PENTAX Hapylase JE-M PA kit is to be used for the in vitro qualitative and quantitative determination of anti-JE virus IgM antibody in human serum and plasma.

Assay Principle

This kit is a particle agglutination test kit for Japanese encephalitis virus (JEV) specific IgM in human serum samples. The test kit essentially consists of two parts, anti-human IgM antibody-coated microplate and JEV antigen-coated Ha-Ny (hydroxyapatite-coated nylon) beads. The microplate captures human IgM antibodies in the serum samples. The surface of Ha-Ny beads is coated with JEV antigens. The beads can bind to anti-JEV specific IgM molecules, which are captured on the microplate, and adhere to the face of the wells.

The positive and negative agglutination reactions are represented as the schemes in Fig. 1. JEV antigen-coated Ha-Ny beads adhere to the face of the wells, when the tested sample is anti-JEV IgM positive (Fig. 1a). When the Ha-Ny beads form a button pattern at the bottom of the well (Fig. 1b), the sample is considered negative in Ha-Ny beads agglutination assays.

Materials provided

Materials provided for Particle agglutination assay (PA) by Pentax Corporation is listed in Appendix I.

Disposal Note

This kit contains materials such as sodium azide and human serum which may necessitate special disposal procedures. Before disposing of leftover reagents, check local regulations regarding disposal of medical waste to ensure compliance with local laws. Take care in discarding the reagents containing sodium azide.

Reconstitution of JEV antigen coated Ha-Ny beads

Transfer 2 ml of Beads diluents into one vial of freeze-dry JEV antigen-coated Ha-Ny beads. Two ml of the reconstituted beads could be applied for sample of two strips. Mix by vortexing on maximum setting 3-4 times for 2-3 seconds each. Settle beads down at room temperature at least for 30 minutes.

Once reconstituted, store at 4°C

Use reconstituted beads within 4 weeks.

Precautions and Recommendations

- Do not freeze any kit components- store at 4°C
- Use only the wells provided with the kit.
- Do not mix reagents from different kits.
- The serum calibrator and negative control are HIV 1 and 2-negative, Hepatitis B and C-negative, but like all serum samples, should be treated as infectious agents. Do not ingest, expose to open wounds or breathe aerosols. Wear protective gloves and dispose of biological samples properly.
- Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.
- Do not permit contact of reagents with skin, metal or oxidizing agents.

Assay Procedure

[I] Qualitative assay

1. Bring the reagents to room temperature before use.

2. Dilute samples 1:100 with serum dilution buffer.

3. Make a cut on the Human IgM (green collared plate) capture microplate by a cutter for the number of strips needed and remove the seal film from the strips. In addition to the number of samples, 2 wells will be required for Positive and Negative controls.

4. Wash strips three times with wash buffer and tap onto paper towel to remove liquid from them.

5. Add 50 µl of diluted samples on wells. Samples should be added immediately after Step 4 to prevent the surface of the wells from drying. The wells may lose the ability to capture antibodies if dried completely.

6. Incubate the wells for 30 minutes at room temperature.

7. Wash the strips three times with wash buffer and tap on Paper towel to remove any remaining liquid as previously.

8. Gently mix the JEV antigen-coated beads slurry.

9. Add 100 µl of the beads slurry to the wells and let them settle for one hour at room temperature.

Note: It may be false negative if the beads suspension is added too much. Keep the amount of regulations.

10. Observe beads agglutination patterns.

[II] Quantitative assay

1. Bring the reagents to room temperature before use.

2. Two-fold serially dilute the samples from 1:100 to 1:12,800 with serum dilution buffer in microtubes. In the control lane, Positive control is also two-fold serially diluted from 1:100.

3. Make a cut on the Human IgM (green collared plate) capture microplate by a cutter for the number of strips needed and remove the seal film from the strips. In addition to the number of all the samples, one strip will be required for Positive control and Negative control.

4. Wash strips three times with wash buffer and tap onto Paper towel to remove liquid from them.

5. Add 50 µl of diluted samples on wells. Samples should be added immediately after Step 4 to prevent the surface of the wells from drying. The wells may lose the ability to capture antibodies if dried incompletely.

6. Incubate the microplate for 30 minutes at room temperature.

7. Wash the microplate three times with distilled water and tap on Paper towel to remove any remaining liquid.

8. Gently mix the JEV antigen-coated beads slurry.

9. Add 100 µl of the beads slurry to the microplate and let them settle for one hour at room temperature.

Note: It may be false negative if the beads suspension is added too much. Keep the amount of regulations.

10. Observe beads agglutination patterns.

Interpretation of Results

1. Qualitative assay

If the button is observed in the well within one hour of addition of beads slurry then the sample is considered negative and if button is not observed in the well within one hour of addition of beads slurry then the sample is considered positive.

2. Quantitative assay

- Highest dilution showing complete agglutination is taken as the anti-JEV IgM titer of samples.
- Cut off value: Positive ≥ 1:100
  Negative < 1:100

Quality control

The titers of positive and negative controls in the quantitative assay are as follows:

Positive control	x400 ~ x 1600 (Variable, according to kit lot)
Negative control	< x100

If the PA titer of the positive control is lower or that of the negative control is higher than above, the test is not valid and must be repeated. In the qualitative assay, the positive and negative controls always show positive and negative, respectively.

Test performance

PENTAX Hapalyse JE-M PA kit is highly specific to anti-JEV virus IgM antibody. Sensitivity and specificity of this kit are 85.1% and 97.8%, respectively. Cross reactivity of the kit is observed for Dengue serum.

Additional Information

- A weak positive reaction often makes determination of titers difficult.
- The JEV antigen was inactivated completely by treatment with beta-propiolactone.
- The cross-reactions among flaviviruses are common. Therefore, results of the assay should be interpreted carefully.

Storage

Store in the refrigerator at 2-8°C for storage.

Expiry

The expiry date is shown on the box.

Warnings and precautions

- All blood samples should be treated as potentially infectious.
- Wear disposable gloves and eye protection throughout the assay.
- Avoid microbial contamination of reagents after opening the reagent bottle.
- Controls in this kit were inactivated for 30 minutes at 56°C.
- Before discarding the instruments used for the examination, immerse in 1% sodium hypochlorite solution (available chlorine concentration 10,000 ppm) for more than 1 hour, or autoclave for 20 minutes at 121°C.
- Make sure to keep this kit at 2-8°C.
- Do not use a kit after its expiry date.

APPENDIX III

Test method for confirmatory diagnosis of malaria, dengue, typhoid, kalaazar and leptospirosis.

Diseases	Test	Place
Malaria	Blood smear	Bheri Zonal Hospital
Dengue	IgM capture ELISA	Everest International Clinic and Research Center
Typhoid	Widal & Blood culture	National Public Health Laboratory
Kalaazar	K-39 dipstick diagnosis	National Public Health Laboratory
Leptospirosis	Rapid test	National Public Health Laboratory

APPENDIX IV

Questionnaire for the clinical and laboratory information on patients

Name:

Age / sex:

Address:

Religion:

Education:

Occupation:

Hospital Bed No:

Date of admission:

Date of onset of symptoms:

Date of serum collection:

Date of discharge:

Clinical Presentation:

Sample No:

Remarks:

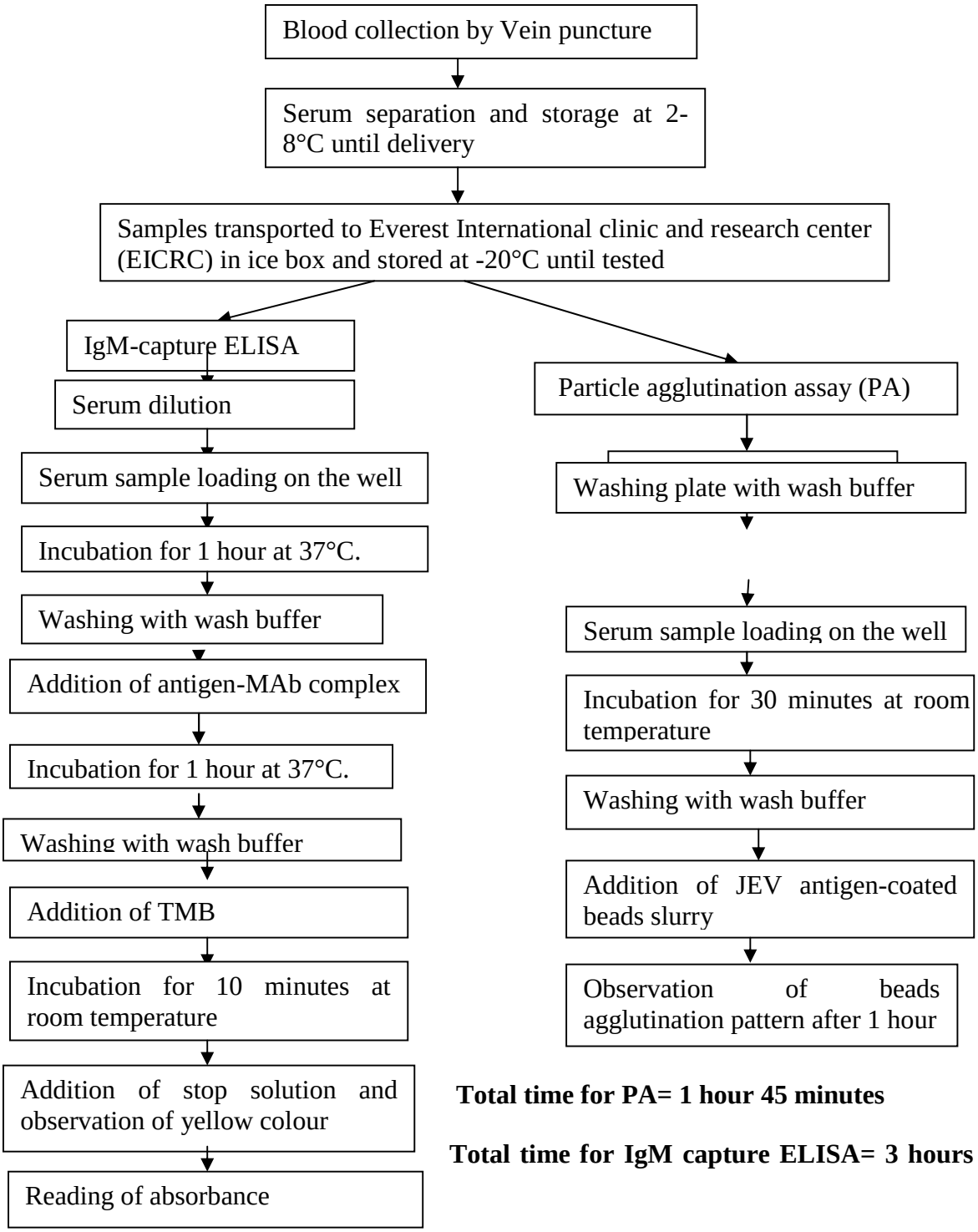

Figure 3: Flow Chart of Methodology