CHAPTER-I

1 INTRODUCTION

Japanese encephalitis (JE) is a mosquito borne flavivirus infection of humans as well as swine, horse and other domestic animals. It is a leading cause of childhood encephalitis in Southeast Asia and annually a remarkable number of deaths due to same are recorded worldwide (CDC, 2008; WHO, 1998). JE virus produces human infection and disease throughout most of Asia and is the commonest cause of endemic encephalitis in that region (CDC, 2008).

JE is an acute infection of the central nervous system (CNS). Patients with JE typically present a few days of non specific febrile illness followed by an abrupt onset of headache, vomiting, and altered consciousness. The majority of human infections with JEV are asymptomatic and only about one in 250 infected people develop clinical symptoms (CDC, 2008). Seizures occur in over three fourths of pediatric patient but observed less frequently in adults (Solomon et al., 2002). JE usually is severe, resulting in a fatal outcome in 25% of cases and residual neuropsychiatric sequelae in 50% of cases (Vaughn and Hoke, 1992).

JEV is the most important cause of endemic encephalitis worldwide, with an estimated 35,000 to 50,000 cases and 10,000 to 15,000 deaths annually (Solomon, 2003). Most of the sporadic and epidemic cases of JE are reported from the Peoples Republic of China (PRC), Korea, Japan, Southeast Asia, the Indian subcontinent and parts of Oceania in the past (Solomon, 2003). Viral transmission occurs across a much broader area of the region than in a recognized by surveillance (CDC, 2008). Though, the numbers of cases have been decreasing in Japan, China and Korea but the cases have been continuously increasing in Southeast Asia and Indian subcontinent with poor socio economic condition and sanitation. Due to the high case fatality rate and frequent residual neuropsychiatric sequelae in the survivors of JE makes a significant public health problem in Southeast

Asia (CDC, 2008, and Solomon, 1997). Approximately, three billion people and 60% of the world population live in JE endemic regions (Kabilan, 2004).

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, 2001). Serological surveys have revealed that about 10 % of the people living in JE endemic areas are infected with the virus and most of them are infected before the age of 15 years (CDC, 1993; EDCD, 2001).

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, 2001). The plain region (terai and inner terai) of Nepal is always found to be endemic for JE. In Nepal, 24 districts of terai and inner terai regions are affected by JE and 12.8 million people are estimated to be at the risk of the disease (EDCD, 2006/2007). Moreover, the population of the high risk group (1-15 years) has reached 5.4 million, which reveals the scope of problem in Nepal. Since the first outbreak in 1978 (Joshi, 1983), seasonal outbreaks of JE have been reported annually. Annually 1000-3000 total cases and 200-400 deaths occur (EDCD, 2006/2007). Between 1978 and 2006, nationwide 32,481cases were identified and 5,902 deaths were reported with CFR of 18.17% (Joshi et al., 2005; EDCD 2005/2006). Although 24 districts are considered to be at constant risk of disease but sporadic cases of JE have been also reported 43 non endemic districts (Wierzba et al., 2008) and endemic cases have been reported from Kathmandu valley (Zimmerman et al 1997; Basnyat et al., 2001; Partridge et al., 2007). Thus, JE is a significant public health problem in Nepal.

For the laboratory diagnosis of JE several techniques have been employed such as virus isolation by cell culture, antigen detection, molecular methods viz. RT-PCR, real time PCR and serological methods for the detection of JEV antibody viz. haemagglutination inhibition (HI), complement fixation (CF), plaque reduction neutralization test (PRNT) and IgM/IgG ELISA. The HI test has long been employed for the diagnosis of JE, but this method has several practical limitations, which is not sensitive or specific and have a cross reactivity with other flaviviruses, time consuming, requires paired serum samples and cannot give early diagnosis. The PRNT is time consuming and difficult to perform which done only in reference laboratories and is not suitable for diagnosis of JE in context to our country. The IgM capture enzyme linked immunosorbent assay (IgM-capture ELISA) remains as the gold standard method for the diagnosis of the JE because it is sensitive, specific and does not have a cross reactivity with other flaviviruses and has become the most reliable technique for the diagnosis of JE in developing countries like Nepal..

This type of study is essential to be carried out in context to our country to know the sero-epidemiology of the disease. Appropriate diagnostic method will be a milestone for the stakeholders and policy makers for implementing the appropriate intervention and proper management, prevention and control of the disease.

CHAPTER-II

2. OBJECTVES

2.1 General objective

- To describe the epidemiological status of Japanese encephalitis in some selected hospitals of Nepal.

2.2 Specific objective

- To detect anti-JEV IgM in serum of patient by IgM capture ELISA.
- To observe socio-epidemiological status of JE among the patient included in the study.

CHAPTER-III

3. LITERATURE REVIEW

3.1 Japanese encephalitis

JE is a mosquito borne arboviral infection caused by Japanese Encephalitis Virus (JEV). JE virus a member of Flavivirus (*Flaviviridae*) is transmitted in an enzootic cycle between *Culex* mosquitoes and amplifying host vertebrate hosts, primarily pigs and wading birds (Tiroumourougane et al., 2002; Endy and Nisalak, 2002). Primarily *Culex tritaeniorhynchus* are the principal vectors. Humans accidentally acquire the infection by mosquito bites only when they encroach upon this enzootic cycle (Solomon, 2003) but JE virus infected persons do not have high titre viraemia and are therefore considered as dead end hosts (Solomon et al., 2000).

3.2 History

JEV infection and encephalitis was described as early as 1871 in Japan. The first major epidemic occurred in Japan in 1924 and involved 6000 cases. This was followed by a large outbreak in 1935 and then annual outbreaks from 1940 to 1952 (Endy, and Nisalak, 2002). Major epidemics were reported about every 10 years (Solomon et al., 2000). 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). Nakayama and Beijing-1 strains of JEV were first isolated from human case in 1934 and 1942. These strains were used in the development of mouse brain inactivated vaccine (Endy, and Nisalak, 2002; Solomon, 2003). The origins of the virus are uncertain, but phylogenetic comparisons with other flaviviruses suggest it evolved from an African ancestral virus, perhaps as recently as a few centuries ago (Solomon et al., 2003).

In the South East Asia Region (SEAR) JE 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 SEAR was from Sri Lanka where an outbreak was apparently reported in 1948 (Tsai, 1990). In India, first epidemics of JE were recognized around Vellore (Tsai, 1990). JE has also been expanding to the new areas including part of Australia (Hanna et al., 1996).

The history of JE in Nepal goes back to 1978 A.D. when the first case of JE was recognized. The epidemic of JE in Nepal was first recognized in Rupandehi district of the Western Development Region (WDR) in 1978 (Joshi, 1986 and Bista,. et.al., 1999). The disease was then thought to be imported from Gorakhpur and surroundings area of Uttar Pradesh of India where JE epidemic occurred (Joshi 1983 and Khatri et al., 1983). Then subsequently epidemic occurred in Morang district of eastern Nepal gradually spreading into other district in successive years (Bista et al., 1999).

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 is due to a combination of mass vaccination of children, spraying of pesticides, changing pig rearing practices, separation of housing from farming, better housing with air conditioning, less availability of mosquito breeding pools, and community awareness program. On the other hand, the incidence of JE has increased in India, Nepal, Sri Lanka, Thailand, Bangladesh and Vietnam (Solomon, et al., 2003; Bista et al., 1999).

There are two epidemiological patterns of JE which are recognized till now in the JE endemic areas. In Northern areas such as Northern Vietnam, Northern Thailand, Korea, Taiwan, Japan, China, Nepal, and Northern India huge epidemics occur during the summer months, whereas in Southern areas such as Southern Vietnam, Southern Thailand, Indonesia, Malaysia, Philippines, Sri Lanka, and southern India JE tend to be endemic, and cases occur sporadically throughout the year with a peak after the start of the of the rainy season (Vaughn and Hoke, 1992; Solomon, 2000). In endemic areas, the annual

incidence of disease ranges from 10-100 per 100,000 populations (Tiroumourougane et al., 2002).

3.3 Morphology and Structure

JEV is a member of the genus Flavivirus in the family *Flaviviridae*, which contains many closely related human pathogens, including yellow fever virus (YFV), dengue virus (DENV), West Nile virus (WNV), Murray Valley encephalitis virus (MVEV), St Louis encephalitis virus (SLEV), tick-borne encephalitis virus (TBEV), Omsk haemorrhagic fever virus(OHFV) and Kyasanur Forest disease virus (KFDV). The family *Flaviviridae* also contains the genus Hepacivirus, hepatitis C virus (HCV). Five distinct genotypes of JEV have been described but only four have been rigorously confirmed (Gould et al., 2008).

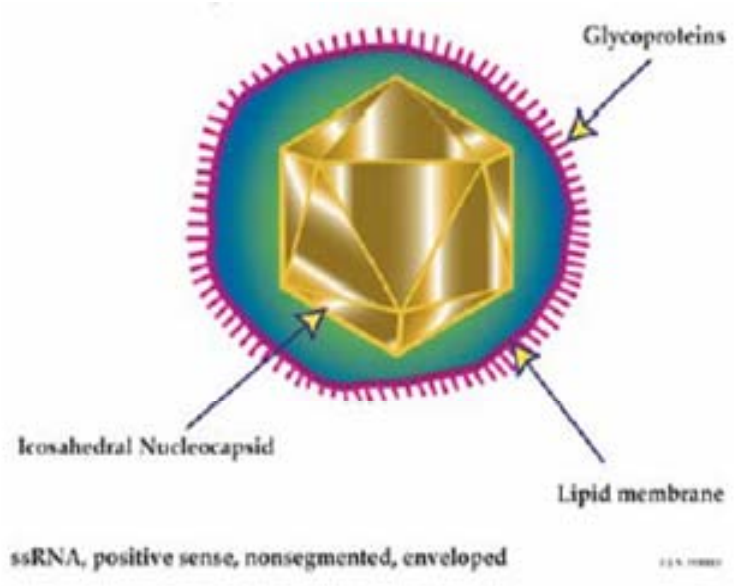


Fig. 1: Structure of Japanese encephalitis virus

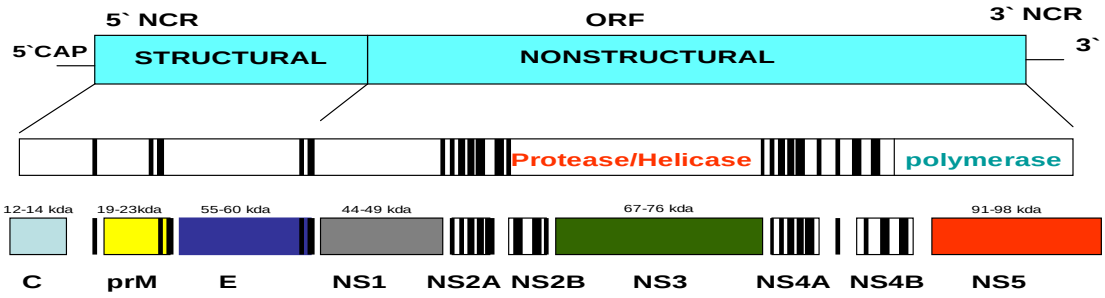


Fig. 2: Mature proteins generated by proteolytic processing cascade

(**Source**: Fields virology 2001)

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' nontranslated region (NTR), a longer 3' NTR 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. 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, 2003).

Based on limited nucleotide sequencing of C/PrM and E genes, four distinct genotypes of JEV have been identified and three genotypes have been fully sequenced (Solomon et al., 2003). Genotype I includes isolates from Northern Thailand, Cambodia and Korea, genotype II includes isolates from Southern Thailand, Malaysia, Indonesia and Northern Australia, genotype III include isolates from mostly temperate regions of Asia including Japan, China, Taiwan, Vietnam, Philippines, Sri Lanka, India and Nepal and genotypes IV includes isolates from Indonesia. A strain of JEV isolated in Singapore in 1952 from a patient who originated in Muar, Malaysia (Muar strain) may represent a fifth genotype (Solomon et al., 2003). Co-circulation of multiple genotypes was observed only in Thailand and Indonesia (Kumar, 1999). Until now prevalence of three different strains of JEV has been reported in Nepal (Bista et al., 1999).

3.4 Replication

JEV replicates in a variety of cultured cells of vertebrate and arthropod origin. JEV is thought to gain entry into the cells via adsorption of the viral spikes onto the plasma membrane at the adsorption site, and penetrating into the cytoplasm through the plasma membrane disruption. Virions seem to disintegrate at or near the penetration sites. RNA synthesis takes place in the perinuclear region with virion production at the rough endoplasmic reticulum, maturation with in the golgi and release from the cell by exocytosis. Ultrastructural studies reveal that viral particles can be seen in cisternae of the rough endoplasmic reticulum. Cell architecture is not heavily disrupted by infection (Vaughn and Hoke 1992).

3.5 Vectors

JE is a vector borne disease transmitted by mosquito, an arthropod. Thirty species of mosquito belonging to five genera of *Culex*, *Anopheles, Aedes, Mansonia*, and *Amergeres* harbor JEV (EDCD, 2001). They are mostly zoophilic or feed on animal blood. The mosquito borne mode of JE transmission was elucidated with the isolation of JEV in 1983 and subsequently in other field studies, which also establishes the role of aquatic birds and pigs in the viral enzootic cycle (Tsai et al., 1994). Entomological surveys carried out from

1981-1989 in Nepal indicated that the culicine mosquitoes namely *Cx. tritaeniorhynchus, Cx. gelidus, Cx. vishnui* and *Cx. fuscocephalus* as the suspected vectors of transmitting JEV to both animals and humans (Pradhan, 1981; Regmi et al., 1984; Khatri et al.,1983). Since *Cx. tritaeniorhynchus* is found abundant in the rice field ecosystem of the endemic areas during the transmission season, and because JEV isolates have been obtained from a pool of *Cx. tritaeniorhynchus* females, this species is suspected to be the principal vector of JE in Nepal (Bista et al., 1999; Endy, and Nisalak, 2002).

Culicine mosquitoes breed in irrigated rice fields, shallow marshes, ponds, and ditches with fresh or polluted water with grass or aquatic vegetation in partial shades or full sun. Breeding preference of *Cx. tritaeniorhynchus* and epidemics of JE associated with paddy fields ecosystem have been adequately substantiated by different studies (Tsai et al., 1994). 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 and Gajanana, 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. Mosquito become infective 14 days after the entry of JEV from the viraemic host (WHO, 1998).

3.6 Reservoir Hosts

The 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. The virus

does not cause any disease among its natural hosts and the transmission continues unnoticed through mosquitoes (Endy and Nisalak, 2002).

Pigs are important amplifying hosts of JE virus. Infected pigs generally do not manifest overt symptoms of illness (Bista et al., 1999). 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 (Endy and Nisalak, 2002).

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 viraemia and infect mosquitoes. In Nepal infection in pigs and ducks has been proved through serological studies and culicine mosquitoes have been found to be breeding and growing in close association with wading birds and ducks (Joshi, 1986).

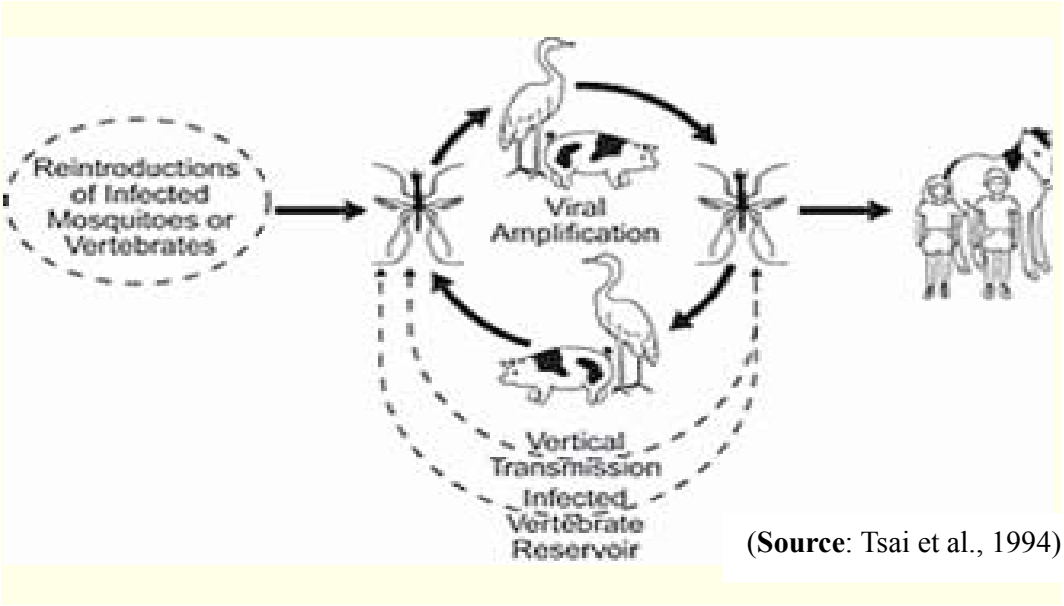
In a sero-survey conducted in Nepal, 40% of pig, 35% of pond herons and cattle egrets and 6-7% of ducks were found to be positive for anti-JE antibody (EDCD, 2001). A serological study for antibody to JE was conducted in Nepal from September 2003 to August 2004 by collecting 280 sera from pigs, ducks and horses covering 10 districts of which 43.92% were found to be positive for antibodies against JEV infection in Nepal. The sero-prevalence of JE in pigs, ducks, and horses was 48.11%, 26.79%, and 50% respectively (Pant, 2006). A regional survey was conducted in Nepal in 2002/2003 for the presence of anti-JEV antibody in domestic animals (pigs, limited no of ducks and horses) from 16 districts of Nepal showed that 55% were positive for the presence of anti-JEV antibody. There is also evidence for the presence of West Nile virus in Nepal (Pant et al., 2006).

Table 1: Potential Reservoir Population in Nepal, 2008

Particulars	Pig population	Duck population
Nationwide (75 districts)	10,13,359	3,90,748

Source: MOAC, 2008

3.6.1 Transmission Cycle



(**Source**: Tsai et al., 1994)

Fig. 3: Transmission cycle of Japanese encephalitis virus

The virus is maintained in nature in a transmission cycle which involves pigs, migrating birds, and ornithophilic mosquitoes, especially *Culex tritaeniorhynchus* play an important role in the amplification, dispersal and epidemiology of JEV (Gould et al., 2008; Vaughn and Hoke, 1992). Humans and horses are only incidental hosts for the virus. When the mosquito bites and takes the blood meal, JEV are shed into its saliva and eventually injected into the victim's blood stream. 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 (CDC, 1993). Man to man transmission has not been reported (Tiroumourougane et al., 2002).

The maintenance and spread of JE virus appear to be mainly through a pig-mosquito-pig cycle and bird-mosquito-bird cycle (Endy, and Nisalak, 2002; Joshi et al., 1998). Some studies suggest that virus may be transmitted transovarially in vector mosquitoes (Endy, and Nisalak, 2002). The epidemiology of the arboviral encephalitides must account for the maintenance and dissemination of the viruses in nature in the absence of humans (Brooks et al., 2004; Vaughn and Hoke, 1992).

In tropical climates, where mosquito populations are present throughout the year, the cycle continues between mosquitoes and reservoir animals (Brooks et al., 2004). There are four important mechanisms for the environmental maintenance of the JE virus in mosquitoes: overwintering of JE, transovarial infection, infection at ovipostion and sexual transmission. The mechanism of maintaining the virus over the winter in temperate areas has not been elucidated. Over wintering in the mosquitoes is a possibility either in infected hibernating mosquitoes or by transovarial passage (Endy, and Nisalak, 2002). 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 (Vaughn and Hoke, 1992).

3.7 Immunology

Interferon and interferon inducers are active against JE virus in mice and monkeys and endogenous interferon-α has been detected in the plasma and CSF of humans with JE (Solomon et al., 2000). Both humoral particularly against E and NS1 proteins and cellular including cytotoxic T lymphocytes arms of immune system are involved in immunity to JEV. But the relative contribution of individual components has not been well understood (Tiroumourougane et al., 2000).

3.7.1 Humoral immunity

The humoral immune response in JE has been well characterized. After primary infection with JEV, a rapid and potent monotypic IgM response occurs in serum and cerebrospinal

fluid (CSF), usually within 7 days of infection (Solomon et al., 2000). The role of antibodies in protection is not yet clearly understood. However, disappearance of neurological signs has been noted in the presence of IgM antibodies (Tiroumourougane et al., 2000). Antibodies to JEV probably protect the host by restricting viral replication during the viraemic phase, before the virus crosses the blood-brain barrier (Solomon et al., 2000).

3.7.2 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., 2000). Though cytotoxic T-lymphocyte response to flaviviral infection has been noted in men and mice, their role in JE is not very clear Immunization with inactivated JE vaccine induces T-cells activation in vivo. These studies reflect the protective role of cell mediated immunity (CMI) in JE (Tiroumourougane et al., 2000).

3.8 Pathogenesis of JEV

Only about one in 25 and 12 in 1000 humans infected with JEV develops clinical features of infection. These may range from a mild flu like illness to a fatal meningencephalomyelitis. The factors determining which of all the humans infected develops disease are unknown, but could include viral factors such as route of entry, titre and neurovirulence of the inoculum, host factors such as age, genetic makeup, general health and pre-existing immunity (Solomon et al., 2000).

After the bite of an infected mosquito, JEV enters dendritic cells under the skin. They transport it to peripheral lymph nodes where virus replication occurs. Following viraemia, usually lasting less than 1 week, most patients begin to recover. Alternatively, the virus enters the central nervous system probably through penetration of the vascular endothelium. Evidence of entry by way of the olfactory nerve has been observed in laboratory animals. Once in the brain, the virus infects neuronal cells and later, phagocytic

cells. CNS infection occurs within the hippocampus, thalamus, substantia nigra, and brain stem. The temporal lobes, the cerebellum and the upper spinal cord, particularly the anterior horn cells, may also be involved. These areas show inflammatory cell infiltration and oedema, with a predominance of activated T cells, macrophages and B cells. The inflammatory response is important in causing cerebral disease, although other mechanisms such as apoptosis and viral replication probably contribute to the damage (Mackenzie et al., 2005).

3.9 Clinical features

JE is an acute disease of the central nervous system and the incubation period of JE is five-15 days. Infection with JEV is often asymptomatic. The ratio of asymptomatic to symptomatic infection varies between 25:1 and 1000:1 (Tiroumourougane et al., 2002). The mean is one in 250 infections results in symptomatic illness (Kabilan et al., 2004). Both humoral and cellular immune responses attenuate the selective infection and destruction of neurons and grey matter is the principal target of JEV (Johnson, 1987).

Patients with JE typically present after a few days of non-specific febrile illness, which may include coryza, diarrhoea, and rigors (Solomon, 1997). This is followed by headache, vomiting and a reduced level of consciousness, often heralded by a convulsion. Convulsions occur often in JE, and have been reported in up to 85 % of children (Kumar et al., 1990) and 10 % of adults (Solomon et al., 2000). In some patients, particularly older children and adults, abnormal behaviors may be the only presenting clinical feature (Solomon et al., 1997).

The onset of illness can be abrupt, acute, sub-acute or gradual. The course of disease can be conveniently divided into the following three stages:

a. A prodromal stage preceding CNS feature,
b. An encephalitis stage identified by CNS symptomatology, and
c. A late stage noticeable by recovery or persistence of signs of CNS injury Tiroumourougane et al., 2002).

The prodromal stage (2-3 days) is characterized by high grade fever with or without rigors, headache, general malaise, nausea and vomiting. Definitive clinical diagnosis is not possible in the prodromal stage. Encephalitis stage (3-5 days) manifests with altered sensorium, convulsions, neck stiffness, muscular rigidity, mask like facies, abnormal movements, dehydration, weight loss and thick and slow speech. Death usually occurs due to neurological illness in the first week (Tiroumourougane et al., 2000).

Children, who survive, slowly regain the neurological function over several weeks. Mild cases may make a complete recovery (Bista and Banerjee, 2000). Only one third of the cases recover normally known as abortive encephalitis. 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 (Desai et al., 1995). After chemotaxis, JEV may be degraded by neutrophils with the help of respiratory brust and toxic radical generation. JEV induces human peripheral blood monocytes to secrete a chemotactic cytokine, human macrophage derived factor that causes chemotaxis of neutrophils (Singh et al., 2000).

Poliomyelitis like acute flaccid paralysis (AFP) has been identified in a sub-group 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., 2000). Seizure occurs in more than 75 % of pediatric patients but is less frequently observed in adults. Viral load may play a vital role in innate immunity of host to restrict the initial JEV infection in CNS (Tsai et al., 1994).

3.10 Diagnosis

3.10.1 Clinical diagnosis:

Generally all viral encephalitides shows similar clinical manifestations and therefore clinical diagnosis is made by the association of encephalitis and some symptoms and signs with possible viruses (Rao et al., 2000). In JE, the leukocyte 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).

3.10.2 Laboratory diagnosis

Laboratory diagnosis of JE is based on virus isolation or demonstration of virus specific antigen or antibodies in CSF/blood. The laboratory diagnosis of a confirmed case of JE is based on one of the following (Tiroumourougane et al., 2002).

1. Fourfold or greater rise in serum antibody titre in paired sera, or
2. Isolation of virus from or demonstration of viral antigen or genomic sequences in tissue, blood, CSF, or other body fluid, or
3. Specific IgM antibody by enzyme immunoassay antibody captured in CSF or serum.

3.10.2.1 Culture

Isolation of JEV was conventionally carried out by intra-cerebral inoculation in suckling mouse brain. Various cell cultures that are being used more recently to isolate JEV include primary chick, duck embryo cells, and cell-lines of Vero, LLC/MK2, C6/36, and API cells. Virus can be isolated from the blood of patients in preneuroinvasive and neuroinvasive phases of illness, usually not later than six or seven days after onset of the symptoms

(Tiroumourougane et al., 2002). A sensitive mosquito inoculation technique has been described for the isolation of JEV (Gajanana et al., 1996). Identification of JEV in culture substrates was traditionally carried out by the complement fixation test and agar gel diffusion. The neutralization test, monoclonal based immunofluorescence technique, and enzyme immunoassay are presently being used (Tiroumourougane et al., 2002).

However, isolation of virus from a clinical specimen is generally considered a rare occurrence probably because of low viral titres, rapid production of neutralizing antibodies, and the logistic difficulty in transport of specimens in the developing countries and frequent freezing/thawing of clinical materials (Tiroumourougane et al., 2002). Lack of skilled manpower and virus culture laboratories are also major hindrances to viral isolation in these countries. For these reasons, serological diagnosis is more emphasized in the developing countries.

3.10.2.2 Antigen detection

Various studies have proved the efficacy of JEV antigen detection in CSF using reverse passive haemagglutination, Immunofluorescence, and staphylococcal co-agglutination tests using polyclonal and monoclonal antibodies in rapid diagnosis of JE (Tiroumourougane et al., 2002).

3.10.2.3 Antibody detection by serology

Laboratory diagnosis of human arboviral encephalitis by serology has been 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. Positive identification using these assays requires paired sera to demonstrate four fold rise in antibody titre between acute and convalescent serum samples (Tiroumourougane et al., 2002). In recent decades, these conventional tests have been replaced by antibody capture enzyme linked immunosorbent assays (ELISAs), which are more sensitive and specific.

3.10.2.3.1 Haemagglutination Inhibition (HI)

Haemagglutination 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 (Endy and Nisalak, 2002). The HI assay has remained largely unchanged since 1958 and is still a fundamental tool in arboviral and JE diagnostics. A fourfold increase in JE-specific HI titre 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 (Endy and Nisalak, 2002).

3.10.2.3.2 Plaque Reduction Neutralization Titre Assay (PRNT)

PRNT was developed by Henderson and Taylor in 1959. It is possible to confirm JE ELISA results using the sensitive PRNT method to differentiate JE antibody from other flaviviruses. The PRNT is a quantitative biological assay measuring neutralizing antibodies with the end-point determined by the neutralization of JE or other flavivirus plaques in cell monolayers, by the serum under test (Endy and Nisalak, 2002). This assay is considered more sensitive than ELISA for differentiating between different flaviviruses. However PRNT is time-consuming to perform, has a long incubation period and is labour intensive. It is recommended for use only in reference laboratories with experience in this assay and for samples which cannot be easily differentiated by ELISA methods (WHO, 2007).

3.10.2.3.3 Complement Fixation Test

The complement fixation test may be useful when both specimens are collected at a later stage and no rise in HI titres is shown since CF antibody develops later and thus the test

may be diagnostic in such cases. Since anti-JEV IgM may be present for 90 days following an illness, the CF test is rarely used (EWARS, 2005).

3.10.2.3.4 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 (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). 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 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, a Particle agglutination (PA) kit which is simple, inexpensive and does not require specific laboratory facilities.

Antibodies begin to appear soon after onset, but only about 70-75% of patients have IgM antibody in specimens collected up to four days after onset. However all patients will have antibody seven-10 days after onset (Burke et al., 1985; Han et al., 1988).

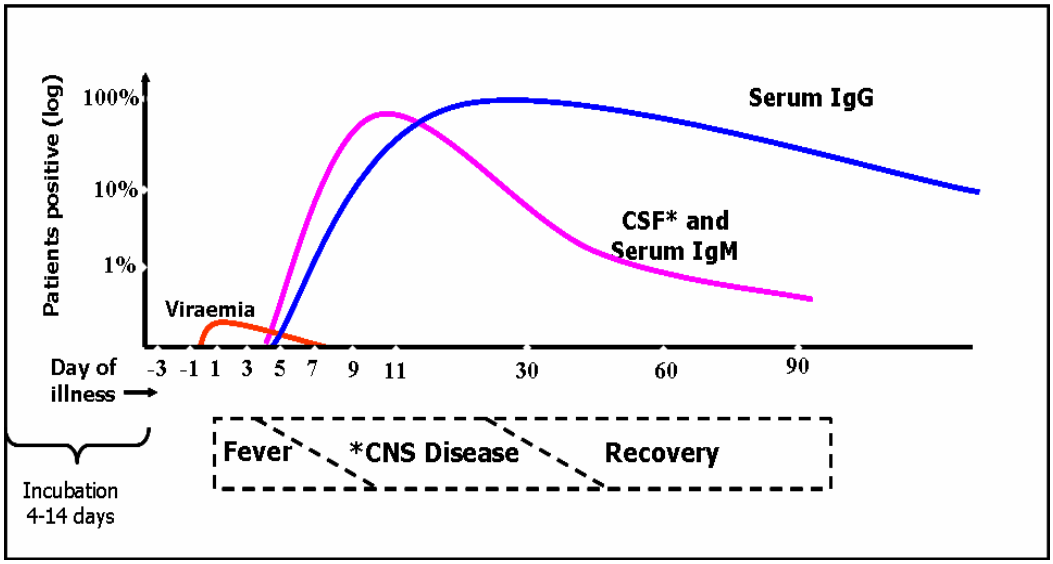


Fig. 4: Schematic antibody responses in JE infection (Solomon et al., 2003)

The level of anti-JEV antibody in serum is variable. The presence of anti-JEV IgM in day one is 68%, 100% in day seven, 96% in day 30 and 72% in day 180 (Burke et al., 1985). The anti-JEV IgM may 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 as the time pass by, IgM is then gradually replaced by

another antibody, anti-JEV IgG. The presence of anti-JEV IgG in day 1 is 47%, 89% in day 7, 100% in day 30, and 100% in day 180 (Burke et al., 1985).

3.10.2.3.5 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 virus and other flaviviruses, which are serologically cross-reactive. Specific anti-JEV IgM can be detected in serum or CSF or both after seven days of onset of symptoms.

Serum sample is added to 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. Concentrated JE antigens are diluted separately to the correct working volume with antigen diluents. An equal volume of the Horse Radish Peroxidase (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_2O_2) is added. The substrate is hydrolyzed 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-JEV IgM antibodies in the test sample (Panbio Diagnostics).

3.10.2.4 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 (Endy and Nisalak, 2002).

3.10.2.6 Molecular Techniques

3.10.2.6.1 Polymerase Chain Reaction (PCR)

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 (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). PCR assays are not recommended for routine diagnosis. Detection of virus genome is very specific for JE diagnosis; however, it is not sensitive. Virus is usually undetectable in a clinically ill JE case. Virus genome in CSF is usually only found in fatal cases. However PCR assays combined with sequencing can be useful for providing information about the molecular epidemiology and evolution of viruses. PCR testing is a function of the reference and specialized laboratories of the network (WHO, 2007). 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. Viraemia and detectable viral RNA in the sera or CSF are 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.

3.10.2.6.2 Real Time RT-PCR

The real time RT-PCR assay is a one step assay system using primer pairs and probes that are specific to JEV. The real time RT-PCR yields more rapid and sensitive test result than

conventional RT-PCR and it is less likely to produce false positive by contamination during preparing the sample. The use of a fluorogenic probe enables the rapid detection of the reaction products in real time which can reduce time consuming postPCR analysis such as gel electrophoresis. An advantage of this assay is the ability to determine viral load in a given sample, which is believed to be important in determining the severity of JE (Yang et al., 2004; Chao et al., 2007). A multiplex real-time reverse transcriptase polymerase chain reaction has been developed for the rapid detection and identification of eight medically important flaviviruses including JEV from laboratory reared, virus infected mosquito pools (Chao et al., 2007). TaqMan RT-PCR assay is one of the more rapid and sensitive real-time PCR based assay that has been extensively used in the detection and identification of the JEV RNA (Toriniwa and Komiya 2006).

3.10.2.6.3 Loop-Mediated Isothermal Amplification (LAMP)

Loop-Mediated Isothermal Amplification (LAMP) is a novel nucleic acid amplification method developed by Eiken Chemical Co., Ltd., Tokyo, Japan, and has the potential to replace PCR because of its simplicity, rapidity, specificity and cost effectiveness. One-step, real time quantitative reverse-transcriptase loop-mediated isothermal amplification assay (RT-LAMP) is used for the more rapid detection of JEV RNA (Toriniwa and Komiya 2006).

3.11 Nepal: National guidelines for JE diagnosis

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 will be considered as **"Suspected Meningitis/ 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 **"Probable Viral Encephalitis."**

- If a case of probable viral encephalitis showing presence of anti JEV-IgM in CSF or serum at the time of illness, the case will be considered as **"confirmed case of Japanese Encephalitis due to Japanese Encephalitis virus**."

3.11.1 Disease Surveillance

The JE cases were reported through Early Warning Reporting System (EWARS) is a hospital-based sentinel surveillance system which was established in 1997 first in 8 sentinel sites and expanded to 24 sites in 1998, 26 sites in 2002 and to 28 sites in 2003. The cases are reported through EWARS to the vector Borne Disease Research and Training center (VBDRTC) and EDCD which acts as focal point for EWARS (EWARS guidelines, 2005). The Department of Health Services of Nepal's Ministry of Health and Population in May 2004 instituted new JE surveillance guidelines in technical collaboration with WHO IPD which entailed enhanced case-based surveillance using a standardized definition for Acute Encephalitis Syndrome (AES), an increase in surveillance sites, and an increased access to laboratory facilities performing JE testing. In Nepal JE surveillance is based on experience implementing syndromic encephalitis surveillance and from integrating many JE surveillance activities with the infrastructure developed for Acute Flaccid Paralysis (AFP) surveillance. Sixty-four referral hospitals located throughout country were enrolled in laboratory-based JE surveillance. **"AES cases were defined as any patient presenting with acute onset of fever and a deterioration in mental status (e.g., confusion, disorientation, coma, or inability to talk) and/or new onset of seizures excluding simple febrile seizures" (Wierzba et al., 2008).**

13.12 Treatment

There is no specific therapy for JE. Treatment is only supportive.

3. 13 Prevention and control (WHO 2006)

Control programmes for JE have focused on three major areas:

3.13.1 Mosquito control

3.13.2 Amplifying host control and

3.13.3 Vaccination.

However, neither mosquito control nor amplifying host has been proven to be effective public health majors to control diseases.

13.3.1 Mosquito control

Mosquito control can include spraying, draining mosquito habitats and the use of bed nets. Such spraying is both resources intensive and expensive. While spraying is important in the control of many vector borne diseases, it is frequently ineffective in the control of JE and is not recommended. This is primarily because the principle vector (*Culex tritaeniorhynchus*) is an outdoor biter rester; residual spraying of insecticides and fogging operations have limited roles. To be effective these control majors must cover all mosquito habitats, which include rice paddy fields, puddles and drainage areas. This is difficult anytime, but especially difficult in rural rice growing areas where JE is most common. The time it takes a *Culex* mosquito to develop from an egg to an adult is 10-12 days. An average rice paddy field can produce 30,000 mosquitoes in one day which presents an incredible challenge. Indoor residual spraying has not been shown to be effective and fogging has only resulted in decreases of mosquito populations for one day with complete recovery in four days (WHO, 2006).

With increasing resistance to pesticides, it is now recognized that chemical control of JE mosquito populations for disease control is not effective. Similarly, non-chemical options, including alternative wet/dry irrigation and biological control measures, have shown temporary decreases in mosquito populations but none have been linked to a decrease in JE cases. Regardless of its effectiveness against JE, vector control is important for the control of many vector-borne diseases and should be maintained for the control of those diseases. Therefore, in addition the large area to be included in control programs, spraying must also be repeated very frequently every 10-12 days to control mosquito populations. That is why the use of insecticides have very limited role both time wise and cost wise in the control of JE.

Bed nets are not effective in the control of JE the reason is that the *Culex* mosquito bites in the twilight hours and most of the children are active in their house and surrounding during this time and so bed nets do not help (WHO). Use of nets both normal and insecticide impregnated and mosquito repellents eg. 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 (Tsai, 1990).

13.3.2 Amplifying host control

As the vector of JE is hard to control, additional efforts have been directed to the main amplifying host, the pig. Pig control has been attempted in three ways: segregation, slaughtering and vaccination. Pig must be segregated and contained at least 5km from humans (the flying radius of the mosquito vector) and is not practical in most rural settings. Slaughtering has a high economic impact and affects the livelihood of many families. Vaccination of pigs is costly, difficult and very time consuming. The window of opportunity for immunization is limited as pigs are often slaughtered at 6-8 months of age and vaccination too early has interference from maternal antibodies. Pig vaccination, therefore, has not been shown to significantly impact human cases of JE. In addition to the challenges of controlling pigs, multiple other animal hosts exist in the life cycle of JE virus. For example, birds have been implicated in several outbreaks in different settings. So, even with excellent control measures of pigs the risk of JE virus transmission is still present (WHO, 2006).

In summary, control of JE through interventions among mosquitoes and pigs (amplifying host) is difficult due to

1. Limited knowledge of transmission dynamics of JE.
2. Outdoor habits of vectors.
3. Sporadic nature of occurrences.
4. Spread over relatively large areas.
5. Relative role of different zoonotic reservoir hosts.

6. Role of migratory birds.
7. Specific vectors for different geographical and ecological areas.

13.3.3 Vaccination

JE control through vaccination has been well established in many countries. The success of this intervention is best illustrated through the experience of Thailand. From 1973 until 1983, a vertical control programme for JE with vector control, case detection and outbreak response was used without much effect on disease burden. Since 1983 this programme has been integrated with the primary health care system as a horizontal control programme which also had little effect. However, when JE vaccine was introduced in a phased manner and as coverage increased, the incidence of JE fell dramatically. Recent work has shown that JE immunization is not only cost effective but also cost -saving. More cost effective and safe vaccines are now available and technical support through WHO and partners can help countries control JE throughout Asia. With the availability of safe, effective and affordable vaccines, JE control is now possible as an integrated part of the public health system. Vaccination now provides an effective and reliable public health intervention (WHO, 2006).

In the realistic background vaccination of the human is the only tool to control JE. Vaccination has been used to control JE in Japan, Korea, Taiwan, China, and Thailand. WHO has recommended the use of vaccine for JE control where the vaccine is affordable (WHO, 2006).

13.3.3.1 Immunization against JE:

JE is an immunization preventable disease (IPD). Vaccination of the population at risk is the method of choice for prevention of JE. The three JE vaccines (Tsai, 1990) in widespread production and in worldwide use for this purpose are: 1. inactivated mouse brain-derived JE vaccine; 2. inactivated primary hamster kidney (PHK) cell-derived JE Vaccine, and 3. Live attenuated JE vaccine (SA 14-14-2) and genetically derived vaccine is also being developed. Post vaccination neurological complications such as encephalitis

and peripheral neuropathy have been reported in only 1-2.3 per million vaccines (CDC, 1993; WHO, 1998).

13.3.3.1.1 Inactivated mouse brain-derived JE vaccines:

The mouse brain-derived inactivated JE vaccine is produced in several Asian countries. This is the only type of JE vaccine that is commercially available on the international market. The commercially available mouse brain-derived JE vaccine is based either on the Nakayama strain, which was isolated in Japan in 1935, or on the Beijing-1 strain. Currently, the mouse brain-derived vaccine is issued in China, India, Japan, South Korea, Sri Lanka, Thailand and Viet Nam (WHO 2006).

The mouse brain-derived JE vaccine is given subcutaneously in doses of 0.5 ml or 1 ml, the lower dose being for children aged 1-3 years. Due to likely interference of maternal antibodies, children are usually not vaccinated before the age of one year. The manufacturers of the internationally marketed vaccine recommend that primary childhood immunization involve 2 injections at an interval of 1-2 weeks. In several Asian trials, primary immunization has a disease preventing efficacy of > 95%; 91% efficacy was achieved in a placebo-controlled trial. There is no reduction in seroconversion rates when other childhood vaccines are given simultaneously. However, the primary vaccination schedules vary considerably among different Asian countries (WHO 2006).

13.3.3.1.2 Inactivated primary hamster kidney (PHK) cell cultured vaccines:

Formalin-inactivated cell cultured JE vaccine is prepared from the Beijing P-3strain of JE virus in PHK cells. This vaccine was manufactured exclusively in the people's republic of China and was China's principal JE vaccine since 1968 (Tsai, 1990). Primary immunization of infants with this formalin-inactivated vaccine results in about 85% protection. The vaccine is inexpensive, and 90 million doses are distributed for internal use in China every year (WHO 2006). However, it is now being substituted by the cell culture-derived live attenuated SA14-14-2 vaccine because of its cost and limited production scale (Oya et al., 2007).

13.3.3.1.3 Cell culture-derived live attenuated vaccines:

An attenuated strain of JEV was developed in China through serial passages of JEV on primary hamster kidney cells. The live attenuated vaccine was prepared from JEV; SA14-14-2 strain has been used as an effective and safe vaccine since 1989 in china (Oya et al., 2007). It has been reported that more than 100 million children received SA14-14-2 vaccine with no serious adverse affects. It is not conformed yet whether it grows in JEV vector mosquito, *Cx. tritaeniorhynchus* (Oya et al., 2007). The vaccine has a 96-98 % efficacy rate and Chinese studies have shown protective efficacy up to 12 years after a two shot regimen in China. The safety of vaccine has been studied multiple times in greater than 600,000 children 1-15 years of age with fever occurring in 1/500 and no associated encephalitis cases (WHO 2006). A 0.5ml dose is administered subcutaneously in children at one year of age and again at 2 years. In some areas, a booster dose is given at 6 years (Tsai, 1990). The SA14-14-2 vaccine is produced only in China and used in a limited number of countries including China. A study done in Nepal demonstrated a 99% efficacy with a single dose given prior to an outbreak (Bista et al., 2001).

In general vaccination is indicated in the following groups:

a. People living in endemic areas
b. Travelers spending 30 days or more in an endemic area.
c. Travelers spending less than 30 days during epidemics or if extensive outdoor activity in rural areas is expected.
d. Lab workers with potential risk of exposure to JEV; since twenty-two cases of laboratory-acquired JE have been reported (Tiroumourougane et al., 2000).

3.14 JE vaccination in Nepal

Mass vaccination in JE endemic areas is effective in controlling the disease in humans. The JE vaccination in Nepal was started in 1983, 1152 subjects were immunized against JE using three doses of 1ml (0.5ml for children <3 years) BIKEN killed lyophilized vaccine at British Military Hospital, Dharan (Henderson, 1984).

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 four hundred ninety thousands children (492,442) between 1 and 15 years only two hundred twenty four thousands (224000) i.e. 45.5% children were vaccinated. Vaccine coverage was 83.5 % in Bardiya followed by Banke (41.3 %) and Kailali (22 %); (EDCD, 2001). 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 group in Rupandehi, Dang, Banke, Bardiya, Kailali, and Kanchanpur (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, and Tandan et al., 2007). No significant adverse events in children or adults after immunization were reported from Nepal (Wierzba et al., 2008).

In 2006, JE mass vaccination programme using live attenuated SA-14-14-2 vaccine was conducted in six endemic districts namely Kanchanpur, Kailali, Bardiya, Banke, Dang, and Rupandehi respectively. That campaign was jointly organized by the EDCD and the Child Health Division (CHD) with technical support of WHO Immunization Preventable Disease (IPD) programme. Out of the targeted population of Twenty four million eighty two thousands six hundred seventy eight only twenty one million seventy eight thousands four hundred twenty two (87.74%) populations were vaccinated in these districts (EHA Newsletter, 2006; EDCD, 2006/2007). However, the efficacy of this vaccination programme is to be assessed.

Based on studies conducted in Nepal, the Government of Nepal has approved the use of the SA 14-14-2, a live-attenuated JE vaccine (Wierzba et al., 2008). All three studies conducted in Nepal in past suggest that this vaccine may be efficacious and safe (Wierzba et al., 2008). After the administration of 224,000 vaccine doses in the year 1999, a case-control study conducted in the Western terai suggested 99% protection for children when vaccine was administered only days to weeks before the seasonal increase in JE cases

(Bista et al., 2001). A second study found 98.5% protection 12 to 15 months after immunization (Ohrr et al., 2005) and a third study conducted 5 years after the immunization program found 96.2% protection (Tandan et al., 2007).

3.15 Disease Distribution

3.15.1 Worldwide Distribution and Research Activities

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).

In the past 50 years, the geographical area affected by JEV has expanded. The timing of the first reported cases or new epidemics in each area gives an impression of the relentless spread of JE. The disease has occurred on the western pacific islands with outbreaks in Guam in 1947 and Saipan in 1990. JE is endemic in Indonesia, and 1000-2500 cases of encephalitis are reported annually. In the far eastern Russian states, JE first occurred in 1938. The first cases of JE were reported in Burma and in Bangladesh in 1970s. The Sporadic cases of JE cases were occurred in the Philippines, New Guinea, and Pakistan (Solomon et al., 2000).

In Taiwan, the first clinical case of JE was recorded in 1931. The case incidence rate of JE during 1966-1997 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). In Malaysia, the disease is endemic and the virus was first isolated in 1960s and about 100 cases are reported annually. The first outbreak of JE with three clinical cases in Australia was reported in 1995 in Torres Strait and then the JE cases were reported for the first time in north of Cairns on the Australian mainland in 1998 (Solomon et al., 2000).

JEV causes larger summer epidemics in northern temperate region of Asia whereas it causes endemic disease through out the year in southern tropical regions, (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 (CDC, 1993); however, most infections are asymptomatic.

Annually, 6000 JE cases with upto 60 % case fatality rate were reported during 1960s in Japan. 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, national immunization programs, combination of mass vaccination of children, spraying of pesticides, changing pig rearing practices, separation of housing from farming, better housing with air conditioning, and less availability of mosquito breeding pools (Solomon et al., 2000). Three JE cases in the non vaccinated US marines stationed on Okinawa were identified in 1991 (Saito et al., 1999). 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).

Large epidemics of JE were reported in northern Vietnam in 1965 and currently 1000-3000 cases/years is reported throughout the country. 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 (Solomon et al., 2000). During 1976-1991, 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 1993-1994 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 1993-1995 showed an increase in JE positivity with age.

In the history of China the first JE case was reported in 1935 with the first virus isolation in 1940. 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; Solomon et al., 2000). 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 % (Vaughn and Hoke, 1992).

The large numbers of epidemics of JE were reported in Chiang Mai in Northern Thailand in 1969. Since then 1500-2500 cases are reported annually (Solomon et al., 2000). An antibody prevalence survey conducted in Thailand during 1989 studied 3089 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).

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 et al., 1994). In 1985 first epidemics of JE occurred in Srilanka with 410 cases and 75 deaths (Solomon et al., 2000).

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, 2003). The pattern of JE epidemics reported in India correlates well with Nepal (Kubo et al., 1996). Although the first epidemic of JE in India was recognized around Vellore in 1948 (Tsai, 1990), the recognition of JE based on serological surveys, was first made in 1955 in Tamilnadu. Since then, large outbreaks with 2000-7000 cases a year have been reported from different parts of the country especially from eastern and northeastern states predominantly in rural areas (Solomon et al., 2000).

Twenty four states of India have reported JE including some states bordering to southern Nepal. In India, children are mostly affected with the morbidity rate of 0.3 to 1.5/100,000 populations and case fatality rate of 10 % to 60 % (Reuben and Gajanana, 1997). National data of 1996 to 2000 shows an average of over 2500 JE cases and about 550 deaths per year in India (Park, 2002). A prospective serological community based study of subclinical flavivirus infection in children during 1989-1991, showed an overall incidence of 15 JE cases per 10,000 children for the age group of 5-9 years (Gajanana et al., 1996). Gajanana et al. (1996) conducted a study during the transmission seasons of 1993-1995 and found 62.4 % JE cases out of 85 acute encephalitis or other related CNS disorders.

Rao et al. (2000) studied the epidemic of JE in Andhra Pradesh during October to November 1999, and recorded 873 cases and 178 deaths. An epidemic of viral encephalitis was reported from July through November 2005 in Gorakhpur. More than 5737 persons were affected in 7 districts of the eastern Uttar Pradesh, and 1344 persons died. The etiologic agent was confirmed to be JEV by analyzing 326 acute phase serum for virus-specific antibodies and by viral RNA and by virus isolation (Parida et al., 2005). An epidemic of encephalitis was reported in August–September 2001 from 16 districts of Uttar Pradesh which reported 443 encephalitis cases and 96 deaths (Sapkal et al., 2007).

3.15.2 JE among travelers

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 (Tiroumourougane et al., 2000).

Although the overall risk for infection among travelers is very low, 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). Travelers living for prolonged periods in rural areas where JE is endemic or epidemic are at greatest risk. Travelers with extensive unprotected outdoor, evening and night-time exposure in

rural areas might be at high risk even if their trip is brief (Tiroumourougane et al., 2000). In general, vaccine should be offered to persons spending one month or more in JE endemics areas during the transmission season, especially if travel will include rural areas. Under specific circumstances, vaccine should be considered for persons spending less than one month in JE endemic areas (CDC, 1993).

3.15.3 JE situation in Nepal and research activities

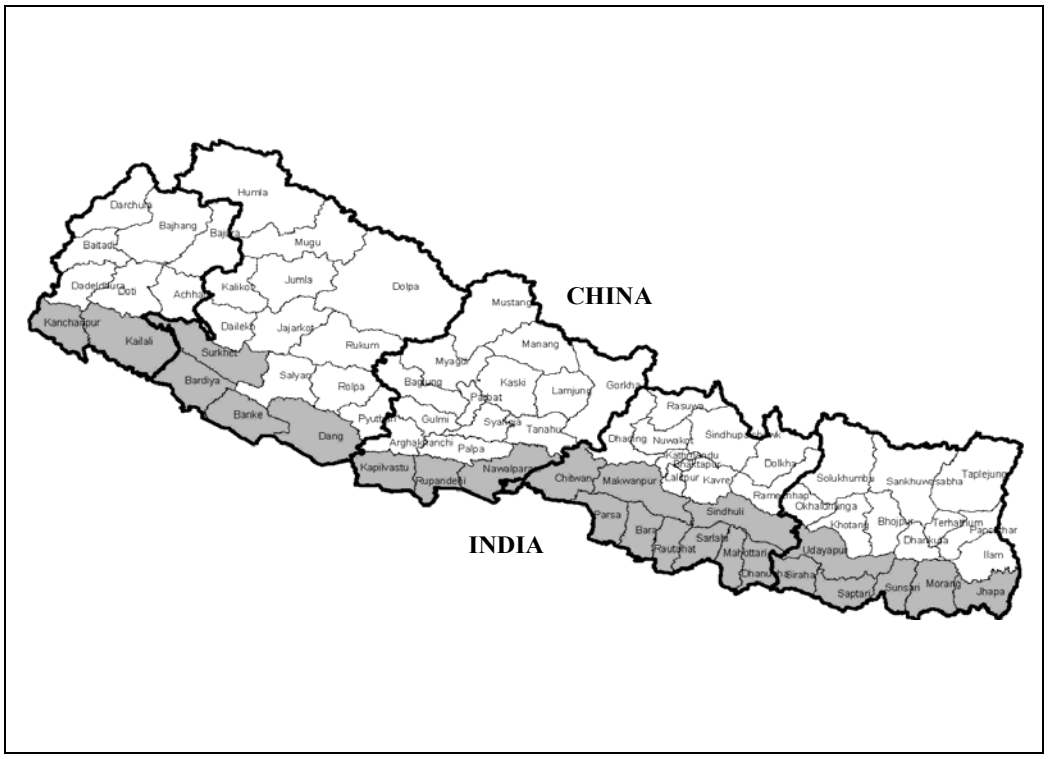


Fig. 5: Japanese encephalitis endemic districts (n = 24) of Nepal (EDCD, 2005)

In Nepal the JE is known as "the visitation of the goddess of the forest" (Henderson, 1983). In Nepal, JE has been recognized as a significant public health problem because of its severity and the increasing incidence rates. Clinical cases of JE were reported in Nepal even before 1975; however, the first epidemic was identified in Rupandehi district of Western Development Region (WDR) from adjoining Uttar Pradesh state of India during 1978 (Joshi, 1986; Bista and Shrestha, 2005). 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. The mosquito, *Cx. tritaeniorhynchus* 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.

In Nepal, JE 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, 2001). The plain areas were seen to be endemic, while the hills seen to be affected sporadically in Nepal (Kubo et al, 1996).

Environmental conditions of paddy field ecosystem in the terai region are the most favorable for the breeding of *Culex* mosquitoes. There is considerable seasonal variation in the number of JE cases each year, though the disease has been recorded throughout the year from endemic areas. Epidemic/outbreak generally starts in the month of April/May, reaches its peak during August and September, declines in October and levels off in November (Joshi et al., 2005; EDCD, 2001).

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 (6.2 %) in 2006. The largest epidemic in the history of Nepal was reported in 1997 with 2953 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. Comprehensive result of 1978 to 2003 showed that more than 50 % morbidity and 60 % mortality have occurred in the age group below 15 years (Bista and Shrestha, 2005).

The major epidemics in Nepal were identified in the years; 1986, 1988, 1994, 1995, 1996, 1997, 1999, 2000, 2001, 2004, and 2005 with 1615, 1403, 1836, 1246, 1450, 2953, 2924, 1729, 1888, 1538, and 2824 cases respectively (Joshi et al., 2005 and EDCD, 2004/2005).

An epidemiological, study of viral encephalitis in Nepal conducted by Khatri et al., during late 1980 and early 1981 showed that a total of 1225 cases and 399 deaths were reported in different hospitals of terai districts due to suspected JE during the year 1978, 1979, and 1980. The total number of cases was 422 in 1978, 182 in 1979, and 622 in 1980 respectively (Khatri et al., 1982). Similarly a sero-epidemiological study for JE carried out during late 1980 and early 1981 showed that 447 cases were children < 14 years among 1225 cases out of which 133 died during the same period due to JE. Out of the 64 serum sample tested for JE by HI test in same year showed 14(21.88%) were positive for HI antibody titre (Joshi, 1983).

Similarly, a serological study conducted in Kathmandu by Rai et al., in 1987, showed that 4(2.6%) out of 154 cases tested were positive for antibodies against JE by HI and NT tests (Rai et al., 1987). An epidemiological study of JE carried out in all epidemic districts of Nepal in 1989 the seasonal variation for the occurrence of JE was observed. Cases were increased in June/July and reached peak in September/October. All ages and both sex groups were affected from JE, children below 14 years constituted the largest number of cases. The male were affected about 1.5 times more than females in all age groups. The overall case fatality rate was 26.6% (Parajuli, et al., 1992).A serological study was conducted by Kubo et al., in 1993 antibodies against JE were examined in 356 serum samples from individuals visiting Tribhuvan University Teaching Hospital (TUTH) using neutralization test showed 13.2% seropositivity (Kubo et al., 1993). Similarly, a study conducted by Kubo et al., in TUTH 15.4% (98/638) seropositivity was observed (Kubo et al., 1996).

Out of 15 patients of meningo-encephalitis 8 patients died in 1995, the mortality rate was 53%. Anti-JEV IgM in the CSF was found in 2 cases. All cases were the dwellers of Kathmandu valley (Zimmerman et al., 1997). 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).

Of the 7 CSF samples tested in 1996, 3 (42.8%) were found to be positive for anti-JEV IgM. Similarly, of the 14 patients tested for anti-JEV IgM in 1998, 9 (64.2%) were found to be positive for anti-JEV IgM (Basnyat et al., 2001). Of the 53 serum samples tested for anti-JEV IGM by IgM capture ELISA in 1998, 41(77.3%) were found to be positive for anti-JEV IgM (Akiba et al., 2001). Serum and CSF samples were colleted from 94 childrens admitted in BPKISH Dharan in 2000-2001. Of 94 samples 58(61.7%) were found to positive for anti-JEV IgM. Of 94, 50(86.2%) patients were found to be positive for anti-JEV IgM in both serum and CSF. 4 (7%) patients were positive for anti-JEV IgM in serum and 4 (7%) patients were positive for anti-JEV IgM in CSF (Rayamajhi et al., 2007). Of the 466 samples tested in 2001, 220(62.6%) were found to be positive for anti-JEV IgM and 220 (47.2%) were found to be positive for anti-JEV IgG respectively (Bajracharya et al., 2001). In the same year, out of the 279 samples (244 serum and 35 CSF) tested, 80 (28.7%) were found to be positive for anti-JEV IgM (Sherchand et al., 2001).

In 2000, Pandey et al. (2003) collected 193 serum samples from Western Nepal that were tested by Particle agglutination assay (PA) for anti-JEV IgM and compared with IgM capture ELISA. Of the 193 samples, 86 (44.5%) were IgM positive by the PA assay, and 71(82.5%) of 86 were also positive by capture ELISA (sensitivity, 99%; specificity, 88%; positive predictive value, 0.82; negative predictive value, 0.99).

In 2005, a total of 2952 AES cases were reported from Nepal. Of the 2239 serum and CSF sample tested for anti-JEV IgM by IgM capture ELISA, 723(32.3%) were found to be

positive for anti-JEV IgM. Among positive cases, 420(58.1%) were male and 303(41.9%) were female. Age group 5-15 years showed both highest no of cases (41.2%) and seropositivity (36%); (Dumre, 2006).

In 2006, 1481 AES cases were reported from Nepal through the national surveillance network. Of the 1291 clinical cases with serum or CSF samples, 262 (23%) were confirmed as JE at NHPL or at BPKISH. In the Kathmandu valley, 350 AES cases were detected, of which 318 (91%) had at least one serum or CSF samples and 48 (15%) were confirmed as JE. Among the 48 JE cases in the Kathmandu valley, 40 cases were verified to be the residents of the valley (Partridge et al., 2007). Wierzba et al. 2008 also reported the endemic cases of JE from Kathmandu (Wierzba et al., 2008). Of 263, serum samples 138(52.5%) samples were found to be positive for anti-JEV IgM by PA among which 106(77% of PA) were also positive for anti-JEV IgM by IgM capture ELISA. PA showed 97% sensitivity, 79% specificity, positive predictive value of 0.77 and negative predictive value of 0.98 (Khanal, 2008).

CHAPTER-IV

4. MATERIALS AND METHODS

4.1 Materials

A complete list of equipments, reagents, chemicals and other supplies required for IgM capture ELISA is given in Appendix I. The reagents for IgM capture ELISA was supplied by Panbio Diagnostics, Brisbane, Australia.

4.2 Methods

4.2.1 Study Design

The study was designed as a descriptive cross-sectional study.

4.2.2 Study period

The study was conducted from July 2007 to August 2008.

4.2.2 Study site

Serum Samples of suspects of AES and viral fever were collected from Bheri Zonal Hospital (BZH) Nepalgunj, Lumbini Zonal Hospital (LZH) Rupendehi and Tribhuvan University Teaching Hospital (TUTH) Kathmandu in the Year 2007 and 2008 A.D. Serum samples were transported to the Everest International Clinic and Research Center (EICRC), Kalanki, Kathmandu for further study. A part of laboratory work was done in Central Department of Microbiology (CDM), T.U. Kirtipur.

4.2.3 Sample size

The serum samples collected during the year 2007 and 2008 from suspects of AES cases in BZH, LZH and TUTH were included in the study. A total of 267 serum samples were collected during the study period.

4.2.4 Specimen collection, storage and transport

Five ml of venous blood was collected by vein puncture from suspected AES and viral fever cases during acute phase of illness and was kept in a labeled, clean and dry test tube.

In case of children, three ml 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 sample was centrifuged and serum was transferred to a tightly stoppered sterile container. The container was sealed with parafilm and labeled properly. 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 using ice box containing ice pack and stored at -70°C until tested.

4.3 Laboratory Tests

4.3.1 IgM-Capture ELISA

The IgM-capture ELISA was performed according to standard protocol of manufacturer. During the testing procedure, the protocol provided by the Panbio diagnostics was strictly followed to achieve high level of accuracy (Appendix- II for detail procedure).

4.3.1.1 Japanese Encephalitis-Dengue IgM Combo ELISA Test

List of chemicals and reagents are given in Appendix- I.

Procedure:

All reagents are equilibrated to room temperature (20-25^{o}C) before commencing assay.

Serum dilution

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 and mixed well.

Preparation of antigen

JE antigen was diluted in the ratio of 1:250 using 10 µl antigens as minimum to antigen diluent. 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^{o}C) until required.

Assay Plate

The required numbers of micro wells were removed from the foil sachet and were inserted into the strip holder. Five micro wells were required for positive control (P), negative control (N) and calibrator (cal) in triplicate. 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. The plate was covered and incubated for 1 hour at 37^{o}C. After incubation, wells were washed six times with diluted wash buffer. The antigen-MAb tracer solution was mixed before transfer. 100 µl of JE antigen-MAb complexes was pipetted into the wells. The plate was covered and incubated for 1 hour at 37 ^{o}C. The wells were washed six times with diluted wash buffer and 100 µl of TMB was pipetted into each well. Timing from the first addition, the plate was incubated at room temperature (20-25 ^{o}C) for 10 minutes. A blue colour was developed. Then 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. The absorbance of each well was read within 30 minutes at a wave length of 450 nm with a reference filter of 630 nm by using Micro plate ELISA Reader Model 700 (Cam Tech. USA).

4.5 Interpretation of the Result

4.5.1 ELISA Result Analysis

A negative result means the virus specific IgM cannot be detected. If a sample is assessed to be positive this means that virus specific IgM has been detected.

Calibration Factor = 0.83 provided in the kit

Cut-off value =Average absorbance of the triplicates of the calibrator x calibration factor

Index value = sample absorbance / cut-off value

Panbio units (PU) of the sample = index value × 10

For positive sample

If PU > 11, then the sample is positive

For negative sample

If PU < 9 then the sample is considered as negative

If PU 9-11 then the sample is considered as equivocal or doubtful

4.6 Statistical Analysis

The collected data was analyzed to find out the age, sex-wise and hospital wise distribution of the cases. The collected data was analyzed using WIN PEPI software (version 7.9, November 24, 2008). Chi-square value and p value was determined to find out whether the findings were statistically significant or not.

CHAPTER- V

5. RESULTS

During the study period a total of 267 serum samples were collected in this study in the year 2007/2008. The samples were collected from Bheri Zonal Hospital, Lumbini Zonal Hospital and Tribhuvan University Teaching Hospital. Out of 267 samples, 242 samples were clinical suspects of acute encephalitis syndrome (AES) and 25 were clinical suspects of viral fever. The serological method enzyme linked immunosorbent assay (ELISA) was performed to detect the anti-JEV IgM antibody against JE in serum.

5.1 Socio-epidemiological Distribution of JE

Genders wise distribution of suspected AES and viral fever cases showed that out of 267 cases 154(57.7%) were male and 113(42.3%) were female. Number of male cases was slightly higher than female cases. The ratio of male cases to female cases was 1.3:1(Table 2).

Table 2: Sex wise Distribution of AES and viral fever Cases

Sex	Total number of Cases	% of total cases
Male	154	57.7
Female	113	42.3
Total	**267**	**100**

Out of 267 cases 242 (90.6%) were clinical suspects of AES and 25 (9.4%) were clinical suspects of viral fever (Table 3).

Table 3: Total number of Suspected AES and Viral Fever Cases

Clinical Diagnosis	Total number of Cases	% of total cases
AES	242	90.6
Viral Fever	25	9.4
Total	**267**	**100**

Hospital wise distribution of suspected AES and viral fever cases showed that out of 267 cases, The highest number of cases 142(53.2%) were recorded from TUTH, Kathmandu followed by BZH, Nepalgunj with 90(33.7%) cases and least number of cases 35(13.1%) were recorded from LZH, Butwal (Table 4).

Table 4: Distribution of AES viral fever cases in different Hospitals

Sample collection site	Total number of cases	% of total cases
Bheri Zonal Hospital	90	33.7
Lumbini Zonal Hospital	35	13.1
TUTH	142	53.2
Total	**267**	**100**

The highest number of cases 159(59.5%) were observed in age group 15-50 years followed by 64(24%) cases from the age group >50 years whereas the least number of cases were from the age group <15 years (Table 5).

Table 5: Age wise distribution of AES and viral fever cases

Age (years)	Total number of cases	% of total cases
Below 15	44	16.5
15-50	159	59.5
Above 50	64	24
Total	**267**	**100**

The laboratory study is based on the serological method for the detection anti- JEV IgM antibodies by IgM capture ELISA

5.2 Serological studies

A total of 267 samples of clinical suspects of AES and viral fever cases were collected from different hospitals and were included in this study for the diagnosis of JE. Out of 267

samples 242 samples were clinical suspects of AES and 25 were clinical suspects of viral fever.

5.2.1 Results from IgM capture ELISA

Table 6: Anti-JEV IgM Positivity rate among suspected AES and viral fever cases

Clinical Diagnosis	Number of cases	Positive	Negative	Positive %	% of Total Positive cases	Statistics
AES	242	84	158	34.7	90.3	
Viral fever	25	9	16	36.0	9.7	**χ^2=0.017 p=0.89**
Total	**267**	**93**	**174**	**34.8**	**100**	

Out of total 242 samples from the suspected cases of AES, 84(34.7%) samples had positive result for anti-JEV IgM which accounts for 90.3% of total positive cases and 158(65.3%) samples had negative result for anti-JEV IgM. Out of 25 suspects of viral fever 9 (36%) had positive result for anti-JEV IgM which accounts for 9.7% of total positive cases and 16(64% had negative result. Overall sero-positivity for anti-JEV IgM was 34.8%. Statistically, there is no significant difference between AES and viral fever for the occurrence of JE **($p=0.89$, $\chi^2=0.017$)** (Table 6)**.**

Table 7: Sex wise Positivity of anti-JEV IgM among suspected AES and viral fever cases by IgM capture ELISA

Sex	Number of cases	Positive	Negative	Positive %	% of total Positive cases	Statistics
Male	154	60	94	38.9	64.5	
Female	113	33	80	29.2	35.5	**χ^2=2.73 p=0.098**
Total	**267**	**93**	**174**	**34.8**	**100**	

Among the 154 male cases of AES and viral fever tested, 60(38.9%) showed positive result for anti-JEV IgM antibody, which accounts for 64.5 % of total positive cases and 94 samples showed negative result. Similarly, out of 113 female cases of AES and viral fever, 33 (29.2%) showed positive result for anti-JEV IgM antibody, which accounts for 35.5% of total positive cases and 80 samples showed negative result. Positivity for anti-JE IgM was seen higher in male than female. Statistically, there is no significant difference between male and female for the occurrence of disease **(p=0.098, χ^2 =2.73)** (Table 7).

Table 8: Evaluation of anti-JEV IgM titres of tested samples

S.N.	JE PanBio Units	Number of samples	% of Total Samples
1	>11	93	34.8
2	<9	158	59.2
3	9-11	16	6
Total		**267**	**100**

Out of 267 serum samples 93(34.8%) samples had anti-JEV IgM titres >11 Panbio units, 158(59.2%) samples had anti-JEV IgM titres <9 Panbio units and 16(6.0%) samples had anti-JEV IgM titres in the range of 9-11 Panbio units (Table 8).

Table 9: Hospital wise distribution of positive cases among suspected AES and viral fever cases by IgM capture ELISA

Hospital	Number of samples	Positive	Negative	Positive %	% of Total Positive cases	Statistics
Bheri Zonal Hospital	90	29	61	32.2	31.1	**χ^2=0.47 p=0.78**
Lumbini Zonal Hospital	35	12	23	34.2	12.9	
TUTH	142	52	90	36.6	56	
Total	**267**	**93**	**174**	**34.8**	**100**	

Hospital wise sero-positivity was observed highest in TUTH (36.6%) which constituted 56% of total positive cases followed by LZH (34.2%) which constituted 12.9% of total positive cases and least in BZH (32.2%) which constituted 31.1% of total positive cases. Similarly, highest number of positive cases was observed in TUTH (52) followed by BZH (29) and least number of positive cases were observed in LZH (12). Statistically there is no significant difference between hospitals and the occurrence of the disease **($p=0.78, \chi^2=0.47$)** (Table 9).

Table 10: Age wise distribution of positive cases among suspected AES and viral fever cases by IgM capture ELISA

Age (years)	Number of cases	Positive	Negative	Positive %	% of total Positive cases	Statistics
Below 15	44	13	31	29.5	14	
15-50	159	64	95	40.2	68.8	$\chi^2=5.32$ $p=0.07$
Above 50	64	16	48	25.0	17.2	
Total	**267**	**93**	**174**	**34.8**	**100**	

Age wise positivity for anti-JEV IgM was observed highest in age group 15-50 years (40.2%) which constituted 68.8% of total positive cases followed by age group below15 years (29.5%) which constitute 14.0% of total positive cases and least in age group above 50 years (25.0%) which constituted 17.2% of total positive cases. Similarly, highest number of positive cases was seen in age group 15-50 years (159) followed by age group above 50 years (64) and least number of positive cases was seen in the age group below 15 years (44). Statistically, there is no significant difference between age and the occurrence of disease **($p=0.07$, $\chi^2=5.32$)** (Table 10).

CHAPTER-VI

6. DISUSSION AND CONCLUSION

6.1 Discussion

JE is one of the most important forms of viral encephalitis worldwide, causing a serious public health problem in Nepal and Southeast Asia. In Nepal, the majority of JE cases occur in the lowland plains or Terai ecological region that borders India during and after monsoon season from May to October (Partridge et. al., 2007). JE is generally diagnosed on the basis of clinical symptoms in the rural areas of Nepal and Asia. There are various techniques available to diagnose JE. The present study was conducted using IgM capture Enzyme Linked Immunosorbent Assay (MAC ELISA). The MAC ELISA is sensitive, specific and does not have a cross reactivity with other flaviviruses and has been proved to be a reliable serological method for JE diagnosis (Bundo and Igarashi, 1985; Cuzzubbo et al., 1999).

The present study was carried out during a period from August 2007 to August 2008 in Everest International Clinic and Research Centre (EICRC), Kathmandu.

The viral fever cases had more sero-positivity than AES cases. This shows that diagnosis in viral fever cases may be the mild febrile manifestation of JE. Statistically, there is no significant difference between AES and viral fever for the occurrence of the JE (**$p=0.017$; $\chi^2=0.2$**). This study is in contrary to the similar kind of study done by Khanal (2008) which demonstrated more sero-positivity in AES.

The overall sero-positivity rate of this study was 34.8% which is quite higher in comparison with national figure. In the year 2006, out of 1316 laboratory examined samples only 285(21.6%) were positive for JE (EDCD, 2005/2006). The positivity difference between the previous reports and present finding could be due to small sample size and localized nature of study in contrary to wide coverage of surveillance samples. The sero-positivity of this study was is in accordance with some of the previous findings

from Nepal. Studies carried out by Dumre in 2006, Bista and Shrestha in 2005, Pandey et al., in 2003 and Sherchand et al., in 2001 had more or less similar positivity rate. However, the present finding does not correlate with 13.2 % positivity reported by Kubo et al. (1993), 15.4 % by Kubo et al. (1996) and 13.3 % using HI and 22 % using neutralization test by Ogawa et al. (1992). The present study shows quite higher positivity rate than the above reports which could be due to variation in geographical distribution and the techniques used.

This study is in contrary to some of the previous findings from Nepal. A study carried out in Bheri Zonal Hospital by Bajracharya et al., in 2001, demonstrated a positivity rate of 47.2% which is quite higher than present study. Similarly a study carried out in Bheri Zonal Hospital by Khanal in 2008, demonstrated a positivity rate of 41.4% which is also higher than present study. The difference observed could be due to the reason that these studies were done in the hyper endemic region of Nepal where the positive detection rate could be higher than the national figure but the present study was done in three different hospitals of the country.

In this study the numbers of male cases were higher than female. The present study coincides with the previous studies conducted in Nepal. The study conducted by Bajracharya in 2001, Dumre in 2006 and Khanal in 2008 which demonstrated higher number of male cases than female. Males have higher chance of being bitten by mosquitoes due to their extensive outdoor work where as females are commonly restricted to household works (indoor works).

JE was confirmed in a greater proportion among male (64.5%) than female (35.5%), which is consistent with previous studies conducted in Nepal by Akiba et. al., (2001) and partridge et. al., (2007). The number of JE positive cases in male was higher than in female with the ratio of 1.8:1. This result correlates with the findings of the study conducted by Dumre (2006) where about 58% of the total positive cases were male. This result also correlates well with the findings of the study conducted by Ogawa et al. (1992)

where about 60 % of the total positive cases were male. The higher number of male cases may be probably because of the differential exposure to the vector, because female were more likely to spend the vectors prime feeding hours within the household, whereas male spent more time outside during these hours. Males also have higher chances of being bitten by mosquitoes due to their extensive outdoor activities whereas females are commonly restricted to household works (indoor activities). Females of the endemic region do not roam around the rice fields and jungles during evening time, which males usually do. The sleeping habit of males outdoors and young females indoors in the terai region may be another factor contributing for the higher number of male cases Ogawa et al. (1992). Because of these reasons, males are more exposed to mosquitoes and acquire JEV infection. High mobility of males across the border areas could help to transport the epidemic strains of JEV from India during the outbreak season. The association between the disease and the sex is not statistically significant (**p=0.098; χ^2=2.73**). The finding of present study differs with the results of Bajracharya et al. (2001), Joshi (2004) and Khanal (2008) which stated more positive cases in female than in male. This may be due to the localized nature of study.

The positive result having PanBio units >11, which could be due to the presence of the detectable anti-JEV IgM antibodies in the serum and the individual was recently infected with the JEV. The negative result having PanBio units <9 could be due to the absence of detectable anti-JEV IgM antibodies in the serum samples and the patients might not be infected with JEV and the samples that had PanBio units in the range of 9-11 could be due to equivocal amount of anti-JEV IgM antibodies present in the serum samples.

The highest number of JE positive cases was found in TUTH (52) and least in LZH (12). The sero-positivity against was highest in TUTH (36.6%) followed by LZH (34.2%) and least in BZH (32.2%). Both the number of positive cases and sero-positivity was highest in TUTH than other hospital may be probably due to clinically confirmed referred cases of JE. TUTH is a university teaching hospital having sophisticated treatment facilities and is a tertiary referral centre of the country so many cases were referred for the treatment. This

study is in contrary with the previous studies done in TUTH. The present finding does not correlate with 13.2 % positivity reported by Kubo et al. (1993) and 15.4 % by Kubo et al. (1996). The present study shows higher positivity rate than the above reports which could be due to variation in the techniques used. The sero-positivity for anti-JEV IgM in BZH is in contrary with the previous findings by Bajracharya et al., (2001) and Khanal (2008) 40.8% which stated more seropositivity than the present study. However, the study conducted Pandey et al., in 2003 is consistent with present study. Statistically, there is no significant difference between different hospitals and the occurrence of the disease (**p= 0.47, χ^2 = 0.42**).

The age group 15-50 years (40.2%) were found more affected than the age group <15 years (29.5%). Although, the incidence of JE is high in children, but more sero-positivity was observed in adults this might be due to the effective vaccination in children. The vaccine is not yet administered to adults >15 years in Nepal and the adults are more prone to JEV infection. Statistically, the association between age and the occurrence of disease was found insignificant (**p=0.07, χ^2=5.32**). Due to vaccination among children the incidence of disease in children is decreased and the incidence of the disease has been shifted to the population over-15 year's age group (Joshi et al., 2005).

The present study is in accordance to the previous findings of Bajracharya (2001), Kubo et al., (1996), Joshi (2004) and Khanal (2008) all of which shows high prevalence rate among the adults than children. But the present study is in contrary to previous findings of Nepal. The study of Bista and Banerjee (2000) showed above 40% cases from the age group 5-15 years and the study of Dumre (2006) showed highest number of JE positive cases in age group 5-15 years. This may be due to small sample size in comparison to these studies and the localized nature of study.

The vaccination against JE was conducted in some endemic districts of Nepal from 1999 through 2006. Efficacy of vaccination in Nepal has already been proved by three case-control studies conducted in Nepal regarding JE vaccination (Bista et al., 2001; Ohrr et al., 2005;

Tandan et al., 2007). Therefore, mass immunization for all age group through expanded programme of immunization (EPI) in JE endemic areas should be the most important preventive strategy against JE.

6.2 Conclusion

In 2007/2008, 267 serum specimens were collected from the suspected AES and viral fever patients from three different hospitals and tested for anti-JEV IgM antibody by IgM capture ELISA. The overall seropositivity was **34.8%** which was quite higher than the national figure **(21.6%)**. The males were more affected than females. The adults were more affected than children and highest number of JE was confirmed in Tribhuvan University Teaching Hospital. The regular vaccination program for both human and animals are important for future control of JE. The epidemiological trend of the JE has been changed in Nepal.

CHAPTER VII

7. SUMMARY AND RECOMMENDATIONS

7.1 Summary

1. The study was conducted in Everest International Clinic and Research Center and Central Department of Microbiology for a complete year, August 2007 to August 2008.

2. A total of 267 serum samples were collected in this study in the year 2007/ 2008. Out of 267 samples, 242 samples were clinical suspects of AES and 25 were suspects of viral fever.

3. Among the 267 cases of AES and viral fever, 57.7% were male and 42.3% were female.

4. Out of 267 samples 93 positive samples had PanBio units >11, 158 samples had PanBio units <9 and16 samples had PanBio units in the range 9-11.

5. Highest number of AES and viral fever cases was observed in TUTH (142) followed by BZH (90) and least number of cases was observed in LZH (35).

6. Out of total 242 samples from the suspected cases of AES, 84 (34.7%) samples showed positive result by IgM-capture ELISA. Out of 25 suspects of viral fever 9 (36%) samples showed positive result by IgM-capture ELISA. Overall sero-positivity for anti-JEV IgM was 34.8% with IgM capture ELISA.

7. Out of 154 male cases 60(38.9%) showed positive result for anti-JEV IgM by IgM-capture ELISA. similarly among 113 female cases 33(29.2%) showed positive result for anti-JEV IgM by IgM-capture ELISA.

8. Highest number of positive cases was observed in TUTH 52(36.6%) followed by BZH 29 (32.2%) and least number of cases were observed LZH 12 (34.2%) by IgM-capture ELISA.

9. Highest number of positive cases was observed in age group 15-50 year (64) with positivity rate of 36.6% followed by age group above 50 year (16) with positivity rate of 25% and least number of cases were observed in age group below 15 year (13) with positivity rate of 29.5% by IgM-capture ELISA.

7.2 Recommendations

Based on the findings of this study the following recommendations have been made

1. Laboratory testing is limited to few hospitals, but for better laboratory based surveillance, diagnostic facilities (with skilled man power) should be expanded in every hospital in the endemic region of JE.

2. 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 sera samples should be tested for further verification of the test. Similarly, neutralization test such as PRNT should be performed for the confirmation of JE.

3. Continuous surveillance, monitoring and research should be strengthened for JE in the country. Since the JE has been expanding to the new area including Kathmandu valley so, the continuous surveillance and laboratory diagnosis should be done in the valley for implementing future intervention against JE in the non endemic region.

4. Other preventive measure such as use of bed net, mosquito repellant and spraying of insecticides should be implemented through the country to control the disease.

5. Pig must be segregated and reared at least 5km from humans.

REFERENCES

Akiba T, Osaka K, Tang S, Nakayama M, Yamamoto A and Kurane I (2001) Analysis of Japanese encephalitis epidemic in western Nepal in 1997. Epidemiol Infect 126:81-88

Ayukawa R, Fujimoto H, Ayabe M, Shoji H, Matsui R, Iwata Y, Fukuda H, Ochi K, Noda K, Ono Y, Sakai K, Takehisa Y and Yasui K (2004) An unexpected outbreak of Japanese encephalitis in the Chugoku district of Japan, 2002. Jpn J Infect Dis 57:63-66

Bajracharya P (2001) Sero-diagnosis of Japanese encephalitis and malaria and an assessment of public health awareness about the above disease. Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal

Bajracharya P, Sherchand JB and Sharma AP (2001) Japanese encephalitis and malaria: sero-diagnosis and assessment of public health awareness. J Inst Med 24:144-58

Barkham TM, Chung YK, Tang KF, and Ooi EE (2006) The performance of RT-PCR compared with a rapid serological assay for acute dengue fever in a diagnostic laboratory. Trans of the Royal Soc of Trop Med and Hyg 100:142-148

Basnyat B, Zimmerman MD, Shrestha Y, Scott RM, and Endy TP (2001) Persistent Japanese encepahalitis:The need for immunization. J Tra Med 8:270-271

Beech JT, Bainbridge T and Thompson SJ (1997) Incorporation of cells into an ELISA system enhances antigen-driven lymphokine detection. J Immunol Methods 205: 163-168

Bista MB and Banerjee MK (2000) Arboviral encephalitides, including Japanese encephalitis and other Flaviviruses: Analysis of 1999 data and review of literature.

Bista MB and Shrestha JM (2005) Epidemiological situation of Japanese encephalitis in Nepal. J Nep Med Assoc 44:51-56

Bista MB, Banerjee MK, Shin SH, Tandan JB, Kim MH and Sohn YM (2001) Efficacy of single dose SA 14-14-2 vaccine against Japanese encephalitis: a case-control study. Lancet 358:791-795

Bista MB, Bastola SP, Shrestha SB and Gautam P (1999) Japanese encephalitis in Nepal (1993-1997): Epidemiological analysis and review of literature. Epidemiology and Disease Control Division (EDCD), Department of Health services (DoHS), Ministry of Health (MoH), HMG of Nepal and WHO country office Nepal

Brooks GF, Butel JS and Morse SA (2004) Jawetz, Melnic & Adelbug's Medical Microbiology. Mc Graw Hill Company Inc. 23rd Edition 522-523

Bundo K and Igarashi A (1985) Antibody-capture ELISA for detection of Immunoglobulin M antibodies in sera from Japanese encephalitis and Dengue hemorrhagic fever patients. J Virol Methods, 11:15-22

Burke DS, Nisalak A and Ussery MA (1982) ACRIA-antibody capture immunoassay detection of Japanese encephalitis virus Immunoglobulin M and G antibodies in Cerebrospinal fluid. J Clin Microbiol 16:1034-1042

Burke DS, Nisalak A and Ussery MA (1985) Kinetics of IgM and IgG responses to Japanese encephalitis virus in human serum and cerebro-spinal fluid. J Infect Dis 151: 1093-1099

Center for Disease Control and prevention (CDC) yellow book chapter-4 www.cdc.gov.ncidod

Centers for Disease Control and Prevention (CDC) (1993) Inactivated Japanese encephalitis virus vaccine: Recommendations of the advisory committee on immunization practices (ACIP), MMWR 42:1-22

Chambers TJ, Hahn CS, Galler R and Rice CM (1990) Flavivirus genome organization, expression and replication. Annu Rev Microbiol 44:649-688

Chao DY, Davis BR, and Chang GJI (2007) Development of multiplex real-time reverse transcriptase PCR assays for detecting eight medically important flaviviruses in mosquitoes. J Clin Microbiol 45(2):584-589

Chow L, Sun HC and Chen HY (1992) Detection and differentiation of dengue-1 from Japanese encephalitis virus infection by ABC MAC-ELISA. Chung Hua Min Kuo Wei Sheng Wu Chi Mien I Hsueh Tsa Chin 25: 172-80

Cuzzubbo AJ, Endy TP, Vaughn DW, Solomon T, Nisalak A and Kalayanarooj S (1999) Evaluation of a new commercially available immunoglobulin M capture ELISA for diagnosis of Japanese encephalitis infections. J Clin Microbiol 37(11):3738-3741

Desai A, Shankar SK, Ravi V Chandramuki A and Gourie Devi M (1995) Japanese encephalitis virus antigen in the brain and its topographical distribution. Acta Neuropathol (Berlin) 89:368-373

Dumre SP (2006) Sero-epidemiology of Japanese encephalitis Nepal. Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal

Early warning reporting system (EWARS) guidelines (2005) EDCD, Teku, Kathmandu, pp 48-53

Edelman R, Schneider RJ, Vejjajiva A, Pornpibul R and Voodhikul P (1976) Persistence of virus specific IgM and clinical recovery after Japanese encephalitis. Am J Trop Med Hyg 23: 733-738

Emergency and Humanitarian Action (EHA) newsletter (2006) Issue viii, September, pp.5

Endy TP and Nisalak A (2002) Japanese encephalitis virus: ecology and epidemiology. Department of Virology, USAMC-AFRIMS: 12-43

Epidemiology and Disease Control Division (EDCD) (2001) Annual report 2001, Department of Health Services (DoHS), Ministry of Health, Government of Nepal: 42-55

Epidemiology and Disease Control Division (EDCD) (2005) Annual report 2002 and 2003. Department of Health Services (DoHS), Ministry of Health, Government of Nepal: 22-31

Epidemiology and Disease Control Division (EDCD) (2006) Annual report 2004 and 2005. Department of Health Services (DoHS), Ministry of Health, Government of Nepal: 129-131

Epidemiology and Disease Control Division (EDCD) (2007) Annual report 2005 and 2006. Department of Health Services (DoHS), Ministry of Health, Government of Nepal: 135-138

Epidemiology and Disease Control Division (EDCD) (2008) Annual report 2006 and 2007. Department of Health Services (DoHS), Ministry of Health, Government of Nepal: 4, 43-44

Gajanana A, Samuel PP, Thenmozhi V and Rajendran R (1996) An appraisal of some recent diagnostic assays for Japanese encephalitis. Southeast Asian J Trop Med Public Health 23: 673-679

Geraghty CM and McCarthy JS (2004) Japanese encephalitis vaccine: is it being sufficiently used in travelers? Med J Aust 181: 269-270

Gould EA, Solomon T, and Mackenzie JS (2008) Does antiviral therapy have a role in the control of Japanese encephalitis? Antiviral Research 78:140–149

Ha DQ, Huong QVT, Loan KHT, Thong DQ and Deubel V (1995) Current situation of Japanese encephalitis in the south of Vietnam, 1976-1992. Trop Med 36: 202-214

Han XY, Ren QW, Xu ZY, and Tsai TF (1998) Serum and cerebrospinal fluid immunoglobulins M, A and G in Japanese Encephalitis. J Clin Micro 1988, 26(5):976-978.

Hanna J N, Ritchie SA, Phillips DA, Shield J, Bailey MC, Mackenzie JS, Poidinger M, McCall BJ and Mills PJ (1995) An outbreak of Japanese encephalitis in the Torres Strait, Australia. MJA 1996; 165: 256-260

Henderson A (1983) Encephalitis in Nepal: The visitation of the goddess of the forest. J R Army Med Corps 129: 156-162

Henderson A (1984) Immunisation against Japanese encephalitis in Nepal: Experience of 1152 subjects. J R Army Med Corps 130: 188-191

Johnson RT (1987) Pathogenesis of acute viral encephalitis and post- infectious encephalomyelitis. J Infect Dis 155(3): 359-364

Joshi AB, Banjara MR and Wierzba T (2005) Japanese encephalitis in Nepal. Jagadamba Press Pvt. Ltd.

Joshi DD (1983) Incidence of Japanese encephalitis in children: 1978, 1979 and1980 outbreaks. NEPAS J 2: 18-25

Joshi DD (1986) Japanese encephalitis in Nepal. Jap Encephalitis, Haem Fewa Syndrome Bulletin 1:1-5

Joshi YR (2004) Epidemiological study of Japanese encephalitis in Kanchanpur district during outbreak season. Central Department of Zoology, Tribhuwan University, Kathmandu, Nepal

Joshi, DD (2000) Epidemiological profile Japanese encephalitis in Nepal 1996-1997. J Inst Med 22:23-31

Kabilan L (2004) Japanese encephalitis in India: An overview. Indian J Pediatr 71: 609-615

Kalyanarooj S (1995) Japanese encephalitis: Clinical manifestations outcome and management. SEA J Inst Trop Med, 26 Suppl 3

Khanal SR (2008) A comparative study of IgM capture ELISA and particle agglutination assay for the diagnosis of Japanese encephalitis among some Nepalese patients. Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal

Khatri IB, Joshi DD, Pradhan TMS and Pradhan SP (1983) Status of viral encephalitis (Japanese encephalitis) in Nepal. J Nepal Med Assoc 21 (1):97-110

Khatri IB, Joshi, DD, and Pradhan, TMS (1982) Epidemiological study of viral encephalitis in Nepal. Inst Med 4(2):133-144

Kubo T, Rai SK, Rawal S and Yamano T (1993) Serological study of Japanese encephalitis in Nepal. SEA J Trop Med Public Health 24(4):756-760

Kubo T, Rai SK, Rawal S, Pokhrel BM, Shrestha HG and Prasai BR (1996) Changing seroepidemiological pattern of Japanese encephalitis virus infection in Nepal. J Inst Med 18: 1-9

Kumar R (1999). Viral encephalitis of public health significance in India: current status. Indian J Pediatr 66(1): 73-83

Kumar R, Mathur A and Kumar A (1990) Clinical features and prognostic indicators of Japanese encephalitis in children in Lucknow (India).Indian J Med Res 91:321-327

Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ and Vorndam VA (1992) Rapid detection and typing of dengue viruses from clinical samples using reverse transcriptase-polymerase chain reaction. J Clin Microbiol 30: 545-551

Mackenzie JS (2005) Emerging zoonotic encephalitis virus: lesson from Southeast Asia and Oceania. J Neurovirol 11:434-440

Meiyu F, Hnosheng C and Cuihua C (1997) Detection of flaviviruses by reverse transcriptase-polymerase chain reaction with the universal primer set. Microbiol Immunol 41: 209-213

Ministry of Agriculture and Co-operatives (2008) Selected indicators of Nepalese agriculture and population. Government of Nepal.

Ministry of Health (MoH)/Environmental Health Project (EHP)/United States Agency for International Development (USAID) (2003) An analysis of base line survey data on Japanese encephalitis: What can we learn about making interventions more effective? Ministry of Health (MoH)/Environmental Health Project (EHP)/United States Agency for International Development (USAID)

Nga PT, Phuong LK, Nam VS, Yen NT, Tien TV and Lien HP (1995) Transmission of Japanese encephalitis virus in Gia Luong district, Ha Bac Province, Vietnam after JE vaccination, 1993-1994. Trop Med 37: 129-134

Ogawa S, Shrestha MP, Rai SK, Parajuli MB, Rai JN, Ghimire SC, Hirai K, Nagata K, Tamura T, Isegawa Y, Okuno Y and Ueda S (1992) Serological and virological studies of Japanese encephalitis in the Terai region of Nepal. SEA J Trop Med Public Health 23: 37-43

Ohrr H, Tandan JB, Sohn YM, Shin SH, Pradhan DP and Halstead SB (2005) Effect of single dose of SA 14-14-2 vaccine 1 year after immunization in Nepalese children with Japanese encephalitis: a case-control study. Lancet 366: 1375-1378

Oya A and Kurae (2007) Japanese encephalitis for a reference to international traveler. J of tra med 14(4):259-268

Panbio Diagnostics 532 Seventeen Miles Rocks Rd. Sinnamou Park, Brisbane, QLD 4073 Australia, www.panbio.com

Pandey B, Yamamoto A, Morita K, Kurosawa Y, Rai SK and Adhikari S (2003) Serodiagnosis of Japanese encephalitis among Nepalese patients by particle agglutination assay. Epidemiol Infect 131: 881-885

Pant GR (2006) A serological survey of pig, horses and ducks in Nepal for evidence of infection with Japanese encephalitis virus. Ann NY Acad Sci 1081:124-129

Pant GR, Lunt RA, Rootes CL, and Daniels PW (2006) Serological evidence for Japanese encephalitis and West Nile viruses in domestic animals of Nepal. Comp Immunol Microbiol Infect Dis 29:166-175

Parajuli MB, Joshi DD, Pradhan SP, Chamling M and Joshi AB (1992) Incidence of Japanese encephalitis during 1989 in Nepal. J Nepal Med Assoc 30: 7-14

Parida MM, Santoshi SR, Dash PK, Tripathi NK, Saxena P, Ambuj S, Sahni AK, Rao PVL and Morita K (2006) Development and evaluation of reverse transcription-loop-mediated isothermal amplification assay for rapid and real-time detection of Japanese encephalitis virus. J Clin Microbiol 44(11): 4172-4178

Park K (2002) Park's Textbook of Preventive and Social Medicine 17th edition M/s Banarsidas Bhanot Publishers, India: 218-219

Partridge J, Ghimire P, Sedai T, Bista MB and Banerjee M (2007) Endemic Japanese encephalitis in the Kathmandu valley. Am J Trop Med Hyg 77(6):1146-1149

Pradhan S (1981) Role of mosquitoes in the transmission of Japanese encephalitis. Seminar in viral encephalitis, Siddhartha Jaycees Souvenir 23rd Jestha, 6-8

Rai SK, Sharma CM, Bhandari RK, Nagata K and Okuno Y (1987) Serological study of Japanese encephalitis in Kathmandu, Nepal. J. Inst. Med. 9:259-264

Rajanasuphot S, Charoensook O, Srijaggrawalwong A, Pothipanya S, Panthumachinda B and Ungchusak K (1992) Japanese encephalitis antibody prevalence survey in northern Thailand. Mosq Borne Dis Bull 9: 111-118

Rao JS, Misra SP, Patanayak SK, Rao TVV, Gupta RKD and Thapar BR (2000) Japanese encephalitis epidemic in Anantapur district, Andhra Pradesh (October-November, 1999). J Communicable Dis 32:306-312

Rayamajhi A, Singh R, Prasad R, Khanal B, and Singhi S (2007) Study of Japanese encephalitis and other viral encephalitis in Nepali children. Pediat Inter 49:978-984

Regmi DN and Joshi DD (1985) Epidemiological surveillance report on Japanese encephalitis from 1978-1984. Epidemiology and Disease Control Division (EDCD), Department of Health Services (DoHS), Ministry of Health and Population (MoHP), Government of Nepal

Reuben R and Gajanana A (1997) Japanese encephalitis in India. Indian J Pediatr 64(2): 243-251

Saito M, Sunagawa T, Makino Y, Tadano M, Hasegawa H and Kanemura K (1991) Three Japanese encephalitis cases in Okinawa, Japan. SEA J Trop Med Public Health 30: 277-279

Sapkal GN, Wairagar NS, Ayachit VM, Bondre VP, and Gore MM (2007) Detection and isolation of Japanese encephalitis virus from blood clots collected during acute phase of infection. A. J Trop Med Hyg 77(6):1139-1145

Sherchand JB, Pandey BD, Haruki K, and Jimba M (2001) sero-diagnosis of Japanese encephalitis and dengue virus infection from clinically suspected patients of Nepal. J Inst Med 23:25-31

Singh A, Kulshreshtha R and Mathur A (2000) An enzyme immunoassay for detection of Japanese encephalitis virus-induced chemotactic cytokine. J Biosci 25(1): 47-54

Solomon T, Kneen R, Dung NM, Khanh VC and Thuy TTN (1998) Poliomyelitis-like illness due to Japanese encephalitis virus. Lancet 351:1094-1097

Solomon T (1997) Viral encephalitis in Southeast Asia. Neurological Infections and Epidemiology 2:191-199

Solomon T (2003) Recent advances in Japanese encephalitis. J Neuro Virol 9:274-283

Solomon T and How O M (2003) West Nile encephalitis. BMJ, 326:865-869

Solomon T, Dung NM, Kneen R, Gainsborough M, Vaughn DW and Khanh VT (2000) Neurological aspects of tropical disease: Japanese encephalitis. J Neurol Neurosurg Psychiatry 68: 405-415

Solomon T, Dung NM, Kneen R, Thao LTT, Gainsborough M, Nisalak A, Day NPJ, Kirkham FJ, Vaughn DW, Smith S and White NJ (2002) Seizures and raised intracranial pressure in Vietnamese patients with Japanese encephalitis. Brain 125:1084-1093

Solomon T, Ni H, Beasley DWC, Ekkelenkamp M, Cardosa MJ and Barrett ADT (2003) Origin and evolution of Japanese encephalitis virus in Southeast Asia. J Virol 77(5): 3091-3098

Solomon T, Thao LTT, Dung NM, Kneen R, Hung NT, Nisalak A, Vaughn DW, Farrar J, Hien TT, White NJ and Cardosa MJ (1998) Rapid diagnosis of Japanese encephalitis

by using an Immunoglobulin M Dot Enzyme Immunoassay. J Clin Microbiol 36(7): 2030-2034

Tandan JB, Ohrr H, Sohn YM, Yoksan S, Ji M, Nam CM, and Halsted SB (2007) Single dose of SA 14-14-2 vaccine provides long-term protection against Japanese encephalitis: A case–control study in Nepalese children 5 years after immunization. Vaccine 25:5041-5045

Tiroumourougane SV, Raghava P and Srinivasan S (2002) Japanese viral encephalitis. Postgrad Med J 78: 205-215

Toriniwa H and Komiya T (2006) Rapid detection and quantification of Japanese encephalitis virus by real-time reverse transcription loop-mediated isothermal amplification. Microbial. Immunol. 50(5):379-387

Tsai TF (1990) Japanese encephalitis vaccines. Arbovirus disease branch, CDC, Fort Collins, Colorado, NCID, www.cdc.gov/search.htm

Tsai TF (1992) Arboviral infections: general considerations for prevention, diagnosis and treatment in travelers. Semin Pediatr Infect Dis 3: 62-69

Tsai TF, Chang GJJ, and Yu YX (1994) Japanese encephalitis vaccines. In: Plotkin SA, Mortimer EAJ, eds. Vaccines. Philadelphia: WB Saunders 672-709

Vaughn DW and Hoke CH (1992) The epidemiology of Japanese encephalitis: prospects for prevention. Epidemiol Rev 14: 197-221

Wierzba TF, Ghimire P, Malla S, Banerjee MK, Shrestha S, Khanal B, Sedai TR and Gibbons RV (2008) Laboratory-based Japanese encephalitis surveillance in Nepal and the implications for a national immunization strategy. Am J Trop Med Hyg 78(6):1002-1006

World Health Organization (1998) Japanese encephalitis vaccines WHO position paper www.who.int/docstore/wer/pdf/1998/wcr7344pdf

World Health Organization (2006) Introduction of Japanese Encephalitis Vaccine in the South-East Asia Region Operations Guidelines (with focus on SA 14-14-2 JE Vaccine). Pp:1-54

World Health Organization (2007) Manual for the Laboratory Diagnosis of Japanese Encephalitis. Pp: 1-52

Wu YC, Huang YS, Chien LJ, Lin TL, Yues YY and Tseng WL (1999) The epidemiology of Japanese encephalitis in Taiwan during 1966-1977. Am J Trop Med Hyg 61: 78-84

Yamamoto A, Nakayama M, Tashiro M, Ogawa T and Kurane I (2000) Hydroxyapatite-coated nylon beads as a new reagent to develop a particle agglutination assay system for detecting Japanese encephalitis virus-specific human antibodies Journal of Clinical virology 19:195-204

Yang DK, Kweon CH, Kim BH, Lim SI, Kim SH, Kwon JH, and Han HR (2004) TaqMan reverse transcription polymerase chain reaction for the detection of Japanese encephalitis virus. J. Vet. Sci. 5(4):345–351

Zimmerman M, Scott R Vaughn D, Rajbhandari S, Nisalak A and Shrestha M (1997) Short report: an outbreak of Japanese encephalitis in Kathmandu, Nepal. Am J Trop Med Hyg 57: 283-284

APPENDIX-I

Materials

Equipments, chemicals and reagents available at Everest International Clinic and Research Centre, Kathmandu were used during this study. The reagents for IgM capture ELISA was used from Panbio diagnostics, Brisbane, Australia.

A. Equipments & supplies

Adjustable micropipettes
Aluminium foils (Hindalco, India)
Beakers (100 ml, 500 ml and 1000 ml)
Cotton
Disposable gloves
Disposable pipettes tips (5-1000µl)
Disposable syringes (3 ml and 5 ml) (Lifeline, Everest Med Pvt. Ltd., Nepal)
Electronic Scale (QHAS Corporation, USA)
ELISA plate reader model 700 (Cam Tech USA)
Freezer (-20°C)
Incubator
Liquid household bleach for inactivating clinical specimens
Measuring cylinders (50 ml, 100 ml, 500 ml and 1000 ml)
Micropipettes (Gilson)
Micropipettes tips (Gilson)
Paper towels
Parafilm
Reagent bottles (500 ml)
Refrigerator (2-8°C) (Sanyo)
Stirring rod
Test tube racks (3 x 10 holes)
Test tubes
Timer
Tip boxes
Vortex shaker (Genie)
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)

APPENDIX 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 vein puncture 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.

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

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.

APPENDIX VI

Flow chart Methodology

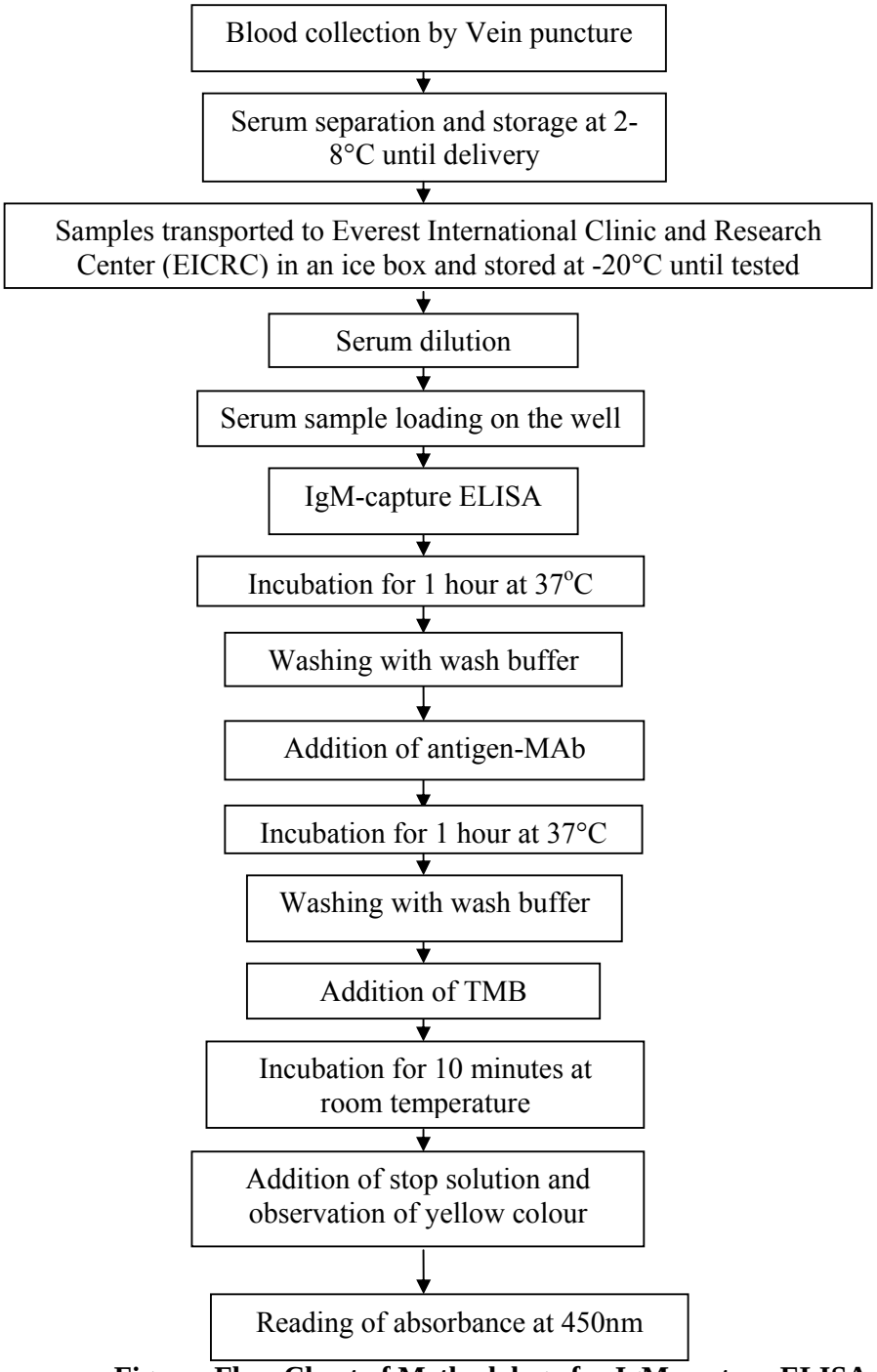


Figure: Flow Chart of Methodology for IgM capture ELISA

APPENDIX VII

JAPANESE ENCEPHALITIS CASE DETAILS FORM

Bed No: ……

Name of the Hospital/Primary Health center: ……

Full Name of the Patient: ……..…….....

Age…….. Sex…….

Name of the Father's/Husband's:……

Address: District ……..…… ……VDC/Municipality: ……

Ward No…… Village…… Tole……

Travel history of patient's within 14 days before of fever onset: ☐ Yes ☐ No

If yes Place……..

History of JE Vaccine: ☐ Yes ☐ No ☐ Unknown

Date of Hospitalization: ……..

Date of fever onset: …….

Clinical Findings (if present, check the box): ☐ Yes ☐ No

Fever:	☐	Disorientation:	☐
Headache:	☐	Unconsciousness:	☐
Neck Rigidity:	☐	Loss of Coordination:	☐
Convulsion:	☐		

Provisional Diagnosis (From Physician) ……...

APPENDIX VII

Statistical Analysis

The data were statistically analyzed by Chi square (χ^2) test and P value determination with the help of a computer based program, WIN PEPI 6 (Compare2 Version 1.96-January 1997)**.**