CHAPTER I

1. INTRODUCTION

Japanese encephalitis (JE) is the main cause of viral encephalitis in many countries of Asia. The infection is mosquito-borne and caused by a virus, related to dengue, yellow fever and West Nile viruses. The virus exists in a transmission cycle between mosquitoes and pigs and/or water birds. Humans become infected only incidentally when bitten by an infected mosquito and the disease is predominantly found in rural and peri urban settings. The disease is endemic with seasonal distribution in parts of China, the Russian Federation's south-east, and South and South-east Asia. Estimated annual mortality ranges from 10-15,000 deaths, while the total number of clinical cases may be 50,000. Of these cases, about 50% result in permanent neuropsychiatric sequelae. The spread of JE in new areas has been correlated with agricultural development and intensive rice cultivation supported by irrigation programs (WHO, 2009)

Japanese Encephalitis (JE) is an acute viral infection of the central nervous system. Patients with JE typically present a few days of non- specific febrile illness followed by headache, vomiting and reduced level of the consciousness, often heralded by convulsions and may progress to a serious infection of brain, (encephalitis) (Solomon, 1997). It is the major public health problem of the South east Asia including Nepal(WHO, 2000).

The Japanese encephalitis viruses mostly affect the membranes of the brain, pathologic changes such as perivascular congestion and hemorrhage, may be diffuse or focal but are seen predominantly in the cortical grey and deep grey matter (WHO, 1988). JE usually is severe resulting in fatal outcome in 25% of cases and residual neuropsychiatric squeal in 30% of cases (Burke et.al. 1988).

Japanese encephalitis virus belongs to the genus *flavivirus*, family flaviviridiae and is the causative agent of the JE, a mosquito borne disease transmitted by *Culex* spp. (*Culex tritaeniorhyncus* and *culex vishnui* group) breeding mostly in the flooded rice fields (Kurane, 2002).

It is primarily the disease of the swine, equine and wading birds. The disease accidently affects the human population, though there are many genera and species of

the mosquito vectors believed to be responsible for the disease transmission *Culex tritaeni*orhyncus is the principal vector in Nepal (Gubler et al., 1989; Darsie and Pradhan 1989). It often breeds in the paddy system. Various vertebrate hosts (pigs, birds) are responsible for maintaining the natural virus cycle. Clinical attack rate of the disease is low (1 in 300). JE virus infected persons don't have high titer of viremia and thus are said to be 'dead end hosts'. They don't contribute to the perpetuation of virus transmission (Brooks et al., 2004)

The five World Health Organization (WHO) criteria for laboratory diagnosis of JEV infection include 1) presence of IgM to JEV in cerebrospinal fluid (CSF) or serum; 2) JEV antigen detection in tissues by immunohistochemical analysis; 3) detection of JEV genomic RNA in any sample by nucleic acid detection assays;4) virus isolation from any sample type; or 5) detection of a four-fold change in titer between serologic specimens obtained during acute and convalescent phases of illness as measured by hemagglutination inhibition assay or JEV-specific neutralizing antibody titer as determined by plaque reduction neutralization test (PRNT) (Solomon et.al., 2000; WHO, 2007)

Isolation of JEV is not a sensitive method of laboratory diagnosis in clinical specimen because the low level, transient viremia is cleared soon after onset of illness, In contrast, anti-JEV immunoglobulin M (IgM) is produced soon after infection. Hemagglutination inhibition tests are not as sensitive or specific as the IgM capture ELISA and have cross reactivity with other *flaviviruses.* Plaque Reduction Neutralization test are time consuming and difficult to perform. PCR is primarily used only for the research purpose (MoHs/WHO, 2010).

JEV specific IgM in CSF appears within few days. The detection of JEV specific IgM in CSF by MAC ELISA is reliable diagnostic method for confirmation of JE throughout the disease period, while the detection of IgM in serum sample is reliable method on day 9 or later (Chanama et.al, 2005). Most patients either have antibody at presentation at a health facility or a few days later. CSF is the preferred sample for diagnosis of JE because if anti JE IgM is detected in the CSF this confirms infection of the central nervous system with JEV (WHO, 2007)

Although other diagnostic tools are available, they are slowly being replaced by the Enzyme Linked Immunosorbent Assay. The detection of JEV specific IgM by MAC

ELISA has been accepted as standard for serodiagnosis. The presence of IgM in serum to other *flavivirus* infection, which may not be the cause of encephalitis, is problematic for the diagnosis in areas of south East Asia where co circulation of dengue virus, WNV and JE occurs (Solomon et al., 2000; Tsai 2000)

Antigenic cross- reactivity in *flavivirus*es is common due to the large number of shared epitopes on the viral proteins; it was documented in 1982 that the presence of specific IgM antibodies could be used to diagnose Japanese encephalitis virus infections. The original methods have been modified for use to confirm other *flavivirus* infections, including Dengue which is often used for differential diagnosis in JE confirmatory testing (WHO, 2007)

Several research institution around the worlds have their in house IgM antibody capture ELISA but some of these have shown variable results when used outside the research laboratory and in the field setting (Innis et al., 1995). The conventional IgM capture ELISA have now been replaced by the commercial IgM capture ELISA kits developed by different research institutes and are easy to use and are easily accessible and offer convenience in terms of time, storage of the reagents. They allow standardized and simplified testing at public health laboratories and will expand the laboratory based surveillance of the Japanese encephalitis in the endemic countries.

In Nepal too the surveillance of the Japanese encephalitis has been done since 2004. Twenty four districts have been found to be endemic for the JE. Surveillance in Nepal is laboratory based. The sero- diagnosis of the collected serum and the CSF sample is done by the use of the MAC ELISA developed by the AFRIMS which takes three days. This test method is the one that has been widely adopted in the South East Asia.

The kits developed should be assessed for their diagnostic values and accuracy. Sensitivity, specificity, predictive value positives and predictive value negative, efficiency of the kits of the kits should be predetermined before their use as a diagnostic tool. It is always required to have a diagnostic tool with high sensitivity and high specificity. The kit should be able to distinguish between diseased individual from non diseased ones. The values of the diagnostic kits play an important role in the treatment of the patient. The available commercial kits have been evaluated by the developers as well as the different laboratories across different countries. The kits have been evaluated against the in house developed immunoassay and others. The kits

have shown different ranges of the sensitivities and specificities. Though in house AFRIMS ELSISA has been used at NPHL /other commercial kits are also available for JE diagnosis. This study conducted at the NPHL is concerned with the evaluation of the two commercial ELISA kits, Panbio JE-Dengue IgM Combo ELISA developed by the Inverness Medica, Australia and the XCton JEV CheX ELISA kit developed by XCyton Diagnostics, Pvt. Ltd. Banglore.

CHAPTER II

2. OBJECTIVES

2.1 General objectives

To evaluate the commercially available IgM capture ELISA kits used in diagnosis of JE

2.1 Specific objectives

(a) To find out the sensitivity, specificity, predictive value positive and predictive value negative of the kit.

(b) To find the efficiency of the kit

(c) To check the Cross reactivity of the kits with that of dengue positive serum samples.

(d) To evaluate the kits qualitatively

CHAPTER IV

4. MATERIAL AND METHODS

4.1 Materials

A complete list of equipments, reagents and chemicals required for IgM capture ELISA by the reference method and the test kits have been listed in Appendix I

4.2 Methods

4.2.1 Study design

This study has been designed to evaluate the two commercially available IgM Antibody Capture ELISA test kits used in the diagnosis of the Japanese encephalitis.

4.2.2 Study site

This study was conducted at Japanese Encephalitis National Referral Laboratory, National Public Health Laboratory Teku, Kathmandu, Nepal from April 2010 to December 2010 for a period of 9 months.

4.2.3 Sample

The samples used in the evaluation purpose were the CSF and the serum samples that had been collected at different parts of the country from suspected encephalitic patients and sent to the Japanese encephalitis National Referral Laboratory for serodiagnosis during the surveillance of the JE in 2010 and also those samples that have been collected during the surveillance in the past years and stored at -20^{o} C were also used.

4.2.4 Sample size

A total of 580 samples were used for the evaluation purpose. Of the total samples tested 251 were serum and 329 were CSF. All the serum samples were tested using the Panbio kits and all the CSF samples were tested using the XCyton kit. However 60CSF samples were also tested using the Panbio kit. These samples so tested using the Panbio kit consisted of 30 positive samples and 30 negative samples as tested by the reference method. Similarly, 63 serum samples containing 30 positive and 33 negative by the reference method were also tested using the XCyton kit.

4.3 Sample selection

Samples were selected on a random basis. The samples were selected without any previous knowledge of the sample result, patient name, age, place and patient travel history. But in case of stored samples taken for evaluation purpose the sample type was known but its previous result was unknown.

4.4 Test protocol

In case of the commercial kits the samples were tested using the instruction in the kit inserts. The instructions in the kit insert were developed in the form of the protocol and approved by the laboratory incharge. The protocol is described in appendix III and IV

In case of testing with the reference ELISA method the protocol in the reference laboratory was followed which is as mentioned in the appendix II.

4.5 Evaluation of ELISA test Kits

Two commercially available MAC ELISA assay; Panbio JE-Dengue IgM Combo ELISA and XCyton JEV CheX ELISA were evaluated against the in house AFRIMS ELISA kit as a reference method.

The paramaters used in evaluation purpose can be obtained using the simple table as belows

Table 1: Classification of test result

	Reference test result			Total
		Positive	Negative	
Test Outcome	Positive	True Positive (a)	False Positive (b)	a+b
	Negative	False Negative (c)	True Negative (d)	c+d
Total		a+c	b+d	a+b+ c+d

Different measures used in evaluation purpose are as follows;

Sensitivity

Sensitivity is the ability of a test to identify correctly those who have the disease or condition. It is the proportion of true positive cases having a disease or condition, sensitivity measures the true positive cases among diseased persons. It is expressed in percentage. It is calculated as follows;

$$\textbf{Sensitivity} = \frac{a}{a+c} = \frac{\text{Number of true positives}}{\text{Number of true positives} + \text{Number of false negative}}$$

Specificity

It is the ability of a test to identify correctly those who don't have the disease. It is the proportion of true negative cases among non diseased persons. Specificity is the measures of true negatives among non diseased individuals. It is also expressed in percentage. It is calculated using the following formula.

$$\textbf{Specificity} = \frac{d}{b+d} = \frac{\text{Number of true negatives}}{\text{Number of true negative} + \text{false positive}}$$

Predictive Value Positive

The predictive value positive or precision rate is the proportion of subjects with positive test results who are correctly diagnosed. It is a critical measure of the performance of a diagnostic method, as it reflects the probability that a positive test reflects the underlying condition being tested for. Its value does however depend on the prevalence of the outcome of interest, which may be unknown for a particular target population.

The Predictive Value Positive is defined as

$$\textbf{PVP} = \frac{a}{a+c} = \frac{\text{Number of true positives}}{\text{Number of true positive} + \text{Number of false positive}}$$

Where a "true positive" is the event that the test makes a positive prediction, and the subject has a positive result under the gold reference, and a "false positive" is the event that the test makes a positive prediction, and the subject has a negative result under the gold reference.

If the prevalence, sensitivity, and specificity are known, the predictive value positive can be obtained from the following identity:

PVP = ((sensitivity) (prevalence)) / ((Sensitivity) (prevalence) + (1-specificity (1-prevalence))

Predictive value negative

The predictive value negative is a summary testing used to describe the performance of a diagnostic testing procedure. It is defined as the proportion of subjects with a negative test result who are correctly diagnosed. A high PVN means that when the test yields a negative result, it is uncommon that the result should have been positive.

The Predictive Value Negative is defined as:

$$\textbf{PVN} = \frac{\text{Number of true negative}}{\text{Number of true Negative} + \text{number of false negative}}$$

$$\text{i.e. PVN} = \frac{d}{c+d}$$

Where a "true negative" is the event that the test makes a negative prediction, and the subject has a negative result under the gold reference, and a "false negative" is the event that the test makes a negative prediction, and the subject has a positive result under the gold reference.

If the prevalence, sensitivity, and specificity are known, the predictive value negative can be obtained from the following identity:

$$\textbf{PVN} = \frac{(\text{Specificity})(1-\text{prevalence})}{(\text{specificity})(1-\text{prevalence}) + (1-\text{sensitivity})(\text{prevalence})}$$

Efficiency

It is the percentage of test results that are correctly identified by the test that is true positives and true negatives.

Mathematically,

$$\textbf{Efficiency} = \frac{a+d}{a+b+c+d} \times 100$$

Cross reactivity

Cross reactivity of the kits is calculated to know if the test under use is affected by the presence of the antibody to the other similar *Flavivirus* infection or not. It is expressed in percentage.

$$\%\ \text{Crossreactivity} = \frac{\text{no of sample positive}}{\text{total samples tested}}$$

4.6 Sample processing

All the samples were brought to room temperature and were processed according to the protocol.

4.7 Recording of the test result

All the test result obtained was recorded in the Microsoft Excel 2007 based spread sheet for the further analysis using the available statistical tool.

4.8 Statistical analysis

SPSS tool was used for determining the sensitivity, specificity, predictive value positive, predictive value negative, efficiency values of the kits and also to find the p value for chi-square test at 5% level of significance.

CHAPTER III

3. LITERATURE REVIEW

3.1 Japanese Encephalitis

Japanese Encephalitis (JE) is an acute viral infection of the central nervous system caused by JEV. Patients with JE typically present a few days of non- specific febrile illness followed by headache, vomiting and reduced level of the consciousness, often heralded by convulsions and may progress to a serious infection of the brain (encephalitis) (Solomon, 1997). It is the major public health problem of the South east Asia including Nepal (WHO, 2000).

3.2 JE Virus

The causative organism of the JE is a specific virus named JEV that belongs to the family flaviviridiae and the genus *Flavivirus*. It is one of the 70 members of the genus *Flavivirus*. The agent is antigenetically related to the St. Louis Encephalitis Virus (SLE), Rociovirus, Murray Valley Encephalitis Virus and the West Nile Virus and the several other *Flavivirus*es (Gubler et al., 1989) . It agglutinates the erythrocytes of the chicken pigeon and sheep if conditions of the test are carefully controlled. This virus can be cultivated in either tissue culture (c6/36 cells) or chicken embryos. The term type B encephalitis was originally used to distinguish it from the Van Economos' Encephalitis Lethergica (sleeping sickness known as the type A encephalitis), But the B has since been dropped. In 1933 a filterable agent was transformed from the brain of a fatal case to cause encephalitis in monkey; the prototype Nakayama strain of Japanese encephalitis was isolated from the brain of fatal case in 1935. The virus was later classed as a member of the genus *Flavivirus* (family flaviviridiae) named after the prototype yellow fever virus (Latin; yellow means flavi). Although of no taxonomic significance; the ecological term arbovirus is often used to describe the fact that JEV is insect (arthropod) borne (Solomon, 2000)

3.2.1 Morphology and structure of JEV

Morphologically JEV, the member of genus *Flavivirus* is an enveloped RNA virus containing single stranded positive sensed RNA, 11kb wrapped in a nucleocapsid and surrounded by a glycoprotein which contains the envelope (Solomon, 2003). The

diameter of the envelope is about 50nm. The virus is spherical about 40-50 nm in diameter with a lipid membrane enclosing isometric 30nm nucleocapsid .The single stranded positive sensed RNA genome comprises 3-regions; a short 5' untranslated region(UTR) and a longer 3' UTR and between them a single open reading frame(ORF)(Chamber et al., 1990). Both 5' and 3' UTR flank either end of a single open reading frame. The 5' end the ORF is translated in to three structural proteins; Core (C), Membrane formed by proteolytic cleavage of its precursor protein prM and envelope protein (E). The remaining 3' region codes for seven non-structural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B,AND NS5(Monath et al., 1996). The C protein is highly basic and combines with RNA to form the necleocapsid. PrM is closely associated with E protein and forms a heterodimer and is thought to act as a chaperon impairing its function till the virus is released, the PrM protein is cleaved by a furin like protease to its mature (M) protein form. This allows the formation of the E protein homodimers which are thus activated (Stadler et al., 1997).

The E protein is a large structural protein consisting of nearly 500 amino acids with two potential glycosylation sites. It is important for the entry of the virus into the host cell and is the major target of humoral immune response. These antigenic domains have been confirmed by X-ray crystallography (Rey et al., 1995). Domain III is the dimerization domain and domain I has a central β barrel and is the hinge domain that links the other two domains. A highly sulfated heparin sulfate molecule has been recently identified as the putative receptor of *Flavivirus* cell entry (Chen et al., 1997). The virus enters the host cell by endocytosis. Subsequent fusion of the lipid membrane of virus with the endosome membrane allows viral RNA to penetrate into the cytoplasm of the infected cell (Chambers et al., 1990) This fusion occurs when the pH of endosome drops and it is thought to be mediated by a conformational change around a putative hinge region(domain I and II of E protein). This results in a barrel shaped fusion peptide at the tip of domain II to insert into the host cell membrane. Heat shock protein 70 has been reported as a possible receptor for JEV (Das et al., 2009).

E gene sequence of *Flavivirus* has been considered responsible for virulence in experimental models. The E protein has a major role in determining the virulence phenotype and a single amino acid substitution may result in loss of virulence or

neuroinvasiveness (Cecilia and Gould, 1991; Ni and Barrett, 1996), whether such differences are important in determining the clinical presentation of JEV human is unknown (Solomon, 2000).

The virus can be inactivated at 56^0C temperature in 30 minutes. It can survive at 0^0C up to three weeks, at -70^0C up to several months, and very long time in 10% serum or milk (Shrestha, 2004)

3.2.2 Replication

JEV enters the host cell by receptor mediated endocytosis. Replication occurs in the cytoplasm. The mode of replication is asymmetric and semi conservative forming the plus strands about 10-100 fold more than the negative strands (Unni et al., 2011)

3.3 Classification of the virus

A comprehensive phylogenetic study was done to establish the genetic relationship among the viruses of the genus *Flavivirus* and to compare the classification based on molecular phylogeny with the existing serologic method. By using a combination of quantitative definitions (bootstrap support level and the pair wise nucleotide sequence identity), the viruses could be classified into clusters, clades, and species. The phylogenetic study revealed for the first time that from the putative ancestor two branches, non-vector and vector-borne virus clusters evolved and from the latter cluster emerged tick-borne and mosquito-borne virus clusters. Provided that the theory of arthropod association being an acquired trait was correct, pair wise nucleotide sequence identity among these three clusters provided supporting data for a borne possibility that the non-vector cluster evolved first, followed by the separation of tick-and mosquito-borne virus clusters in that order. Clades established in their study correlated significantly with existing antigenic complexes (Kuno et al., 1998)

3.3.1 Phylogenetic variation

Phylogenic studies of a number of JEV isolates from different geographic areas using limited nucleotide sequencing in the highly variable PrM gene suggested that there were at least five JEV genotypes. These findings were confirmed using E gene sequencing (Chen et al., 1990, 1992; Ni and Barrett, 1995; Paranjpe and Banerjee, 1996; Williams et al., 2000). Most viruses strains of genotype I were isolated from Northern Thailand, Cambodia and Korea; genotype II from Southern Thailand,

Indonesia, Malaysia and Australia; genotype III from areas of Asia that are largely temperate such as Japan, Korea, China, Taiwan, Philippines ; genotype IV from Indonesia; genotype V from Malaysia (Chen et al., 1990, 1992; Williams et al., 2000; Uchil and Saatchidanandam, 2001).

Most isolates including the Nakayama belong to the genotype III which is the most widely distributed genotype and the only genotype isolated from the Indian subcontinent. Genotype I and III are principally distributed in temperate epidemic areas and genotype II and IV in tropical endemic regions. The ancestor of JEV is probably an Asian virus and may have evolved in past 300 years (Mackenzie et al., 2004). A study in origin and distribution of the JEV in south East Asia revealed that all five genotypes of JEV are found in Indonesia, New Guinea and Malaysia suggesting that JEV originated from its ancestral virus in Indonesia-Malaysia region and evolved there into different genotypes which then spread across Asia.

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

3.4 Epidemiology of JE

3.4.1 Epidemiology in Nepal

Japanese Encephalitis was first confirmed in western part of Nepal in 1978 (Joshi, 1983). It was first reported in the Rupandehi district (Bista and Shrestha, 2005). Since then, Japanese Encephalitis has been confirmed in 54 districts of Nepal. 1777 cases of Japanese Encephalitis has been serologically confirmed in Nepal from 2004-2007. It is important to remember that Japanese Encephalitis is an under reported disease. For every reported JE case, there are approximately 300 asymptomatic cases. (Bista et al., 1999). This means that upwards of 533, 100 people were infected with this disease during 2004-2007, mostly amongst residents of rural areas. Incubation period of Japanese encephalitis varies, but 5-15 days is typical. Japanese Encephalitis has been confirmed in all age groups in Nepal. Almost 50 % of the cases are 15 year or

younger in age. The highest incidence rate is in the age group of 5-15 years. Japanese encephalitis is more common in males than in females, probably due to greater exposure during the mosquitoes feeding hours **(**Akiba et al., 2001; Partridge et al., 2007**).** Almost 60% of the cases occur in males. Japanese Encephalitis has been reported from all eco-regions of the country. The majority of JE cases occur in the Terai. Following mass immunization campaign in endemic districts of Terai in 2006, a decrease in number of reported cases in 2007 and 2008 speak of the quality of the mass immunization campaign. More cases are being reported from Hilly districts of Nepal from 2004. Expansion of surveillance network throughout the country has improved the sensitivity of the system. Mountainous region previously silent have been reporting at least two cases from 2005. Cases are being reported from the hills and mountains (Bhattachann et al., 2009) Occurrence of Japanese Encephalitis now has been confirmed in 54 districts of the country.JE cases have also been reported among the children admitted in the Patan hospital and the Kathmandu valley (Partridge et al., 2007).

The vector *Cx. tritaeniorhyncus* is considered as the principal vector in Nepal. Three different strains of JEV isolated from Nepal are Nep-1/90/B 2524, and B-9548.Conditions in terai regions is most favorable for the breeding of the culex mosquitoes. Although the disease has been reported throughout the year from endemic areas, epidemic occurs during the monsoon season- starting in the April/may period, peaking during August and September, declining in October and leveling off in November (Joshi et al., 2005; EDCD, 2005).

3. 5.2 Epidemiology of JE in world

The second half of 2005 had one of the largest outbreaks of Japanese encephalitis that has occurred in northern India in recent years. Cases were first reported in the state of Uttar Pradesh in July 2005; by November, there had been nearly 5000 cases and 1300 deaths (Solomon, 2006).

On the Korean Peninsula the first JE case were recorded in 1933. On the Chinese mainland, the first cases were documented in 1940. In Philippines, first reports of JE cases occurred in the early 1950s (Barzaga, 1989) Eventually , the JE endemics reached Pakistan (1983) as the further extension in the west and Papua New Guinea

(1995) and northern Australia (Torres Straight) as the furthest south. In parts of south eastern Russia (Primoje Promorsij), a few JE cases have been reported occasionally (eg 2 cases from 1986 to 1990) (Tsai, 1990). JE is potentially endemic to Afghanistan, Bhutan, Brunei, Darusssalam, and the Maldives, but no cases have been reported in these countries for past 30years. According to the WHO JE is endemic to Western Pacific Islands, but cases are rare (Murray et al., 2004). The enzootic cycles on those Pacific Islands might not sustain viral transmission; hence, epidemics occur only after introduction of virus from JE endemic areas. Subtle changes in then spatiotemporal distribution of JEV are difficult to track; thus, the year when a first case of JE in a country is reported does not necessarily correspond with the actual first occurrence of JE in that country.

It is prevalent in Northern Thailand, Uttar-Pradesh, Bihar, Tamil Nadu, and West Bengal states in India, terai regions of Nepal and in Sri-Lanka. Cases have also been reported from Bangladesh Indonesia and Myanmar. The indication of transmission of JE in SEA was from Sri-Lanka when an outbreak was apparently reported in 1948(Tsai , 1997). In India, epidemics of JE were first recognized around Vellore (Shegal, 1989). In 1948 outbreak, 85% donkey sera was JE antibody positive

JE is endemic in parts of China and in Eastern, Southern and Southeastern Asia, and Papua New Guinea (Mackenzie et al., 2004). In the 1990s, JEV spread westward into Pakistan and eastwards into the western Indonesian archipelago, New Guinea and northern Australia (Endy & Nisalak, 2002). The mechanism by which the virus reached the Torres straight in 2000, perhaps through a migratory bird, remains unknown (Solomon, 2003). JE is principally a disease of rural areas in which vector mosquitoes proliferate in close association with birds and pigs. The spread of the disease into non-endemic regions has been correlated with agricultural development and intensive rice cultivation and the breeding of pigs supported by irrigation programs. The disease is currently considered hyperendemic in northern India and southern Nepal, as well as in parts of central and southern India. JE is the most important cause of acute encephalitis in eastern and southern Asia and carries with it a heavy burden of permanent neuropsychiatric sequelae. The figure of 50 000 cases of illness a year probably is an underestimate, because of inadequate surveillance and reporting and because most infections are asymptomatic, with a ratio of symptomatic

to asymptomatic infections that can range from 1 in 25 to probably 1 in 250 infections (Solomon & Winter, 2004). In rural villages, exposure and infection occur at a very early age with half of all cases occurring in children less than 4 years of age. Typical incidence rates in those younger than 19 years range from 10 to 100 per 100 000 population per year (Herwig et al., 2009). Seroprevalence studies indicate nearly universal infection by early adulthood in those areas (Gajanana et al 1995). Transmission of JE is mostly seasonal in temperate areas, but year-round transmission is seen in Indonesia (Vaugh and Duke, 1992) Large outbreaks of JE with clear summer seasonality also are periodically reported on the Indian subcontinent, as illustrated in Uttar Pradash, where 6097 suspected cases, including 1398 deaths, were reported between July 1 and November 10, 2005.

3.5 Epidemic cycle

JEV is transmitted through a zoonotic cycle between mosquitoes, pigs and water birds. Human gets accidentally infected when bitten by an infected mosquitoes and are called as the dead end host. Humans do not participate in the spread of JE because of low level and short-lived viremia.

Animal reservoir

The antibody to the JEV in different animals provides an estimate of degree of exposure and susceptibility of respective species to JEV infection. A high prevalence of JEV antibodies has been documented in pigs, horses and birds and to a lesser extent in cattle, heep, dogs and monkeys. Pigs and ardeid birds are the most important hosts for maintenance, amplification and spread of JEV. Pigs are the main component in the transmission cycle with respect to human infection whereas herons, egrets and other ardeid birds are important maintenance hosts. JEV infected animals and mosquitoes generally remain asymptomatic, although fatal encephalitis occurs in horses and fetal wastage occurs in swine (Burke and Leake, 1988). The domestic animals can get infected but show no evidence of viremia. Rodents appear to be unimportant hosts (Scherer et al., 1959). Amphibians, reptiles and bats can become infected experimentally and the virus can persist. Removing domestic pigs from areas of human habitation may reduce contact between amplifying hosts and vectors (Hurk et al., 2001); it does not eliminate the presence of JEV-infected mosquitoes (Andrew et al., 2008).

Vectors

Many mosquitoes have been evaluated as potential vectors for Japanese encephalitis virus. The *Culex tritaeniorhynchus* mosquito is the main vector to humans in Asia. At least 11 other species have been infected in the laboratory. Many ecologic behavioral features of *Culex tritaeniorhynchus* mosquitoes have been characterized. In most regions, *Culex tritaeniorhynchus* mosquitoes are present in enormous numbers for a short time period each year following periods of heavy rain. Although the *Culex tritaeniorhynchus* mosquito is zoophilic, preferring pigs and birds over man, it bites man with enough frequency to account for transmission of the virus. This mosquito breeds in rice fields some distance from human dwellings but flies to peri domestic areas for blood meals. *Culex tritaeniorhynchus* mosquitoes can fly for a distance of up to 1.5 kilometers and have been found in treetops 43-50 feet above the ground, where virus could be spread among birds.Vertical transmission of JEV has been demonstrated in three different strains of *Culex tritaeniorhynchus* mosquitoes, as well as in *Culex pipiens, Aedes albopictus, Aedes togoi, Culex annulus, Culex quinquefasciatus,*and *Armigeres subalbatus* mosquitoes (Vaugh and Hoke, 1992).

Some hyper endemic districts of Nepal represent the paddy field ecosystem with abundant *Culex* species and amplifying hosts such as the pigs and migratory birds. Because the female *Culex tritaeniorhynchus* is found abundant in rice field ecosystem of the endemic areas during the transmission season, and because JEVisolates have been obtained only from a pool of *Culex tritaeniorhynchus* females, this species is suspected to be the principal vector of JE in Nepal .(Gubler et al., 1989; Darsie and Pradhan). The vector *Culex tritaeniorhynchus* has multiple feeding behavior and feeds upon cattles, pigs, ducks, goats, fowl and humans. (Arunachalam et al., 2005)

Mosquito becomes infective after 14 days of the entry of the JEV from the viremic host.

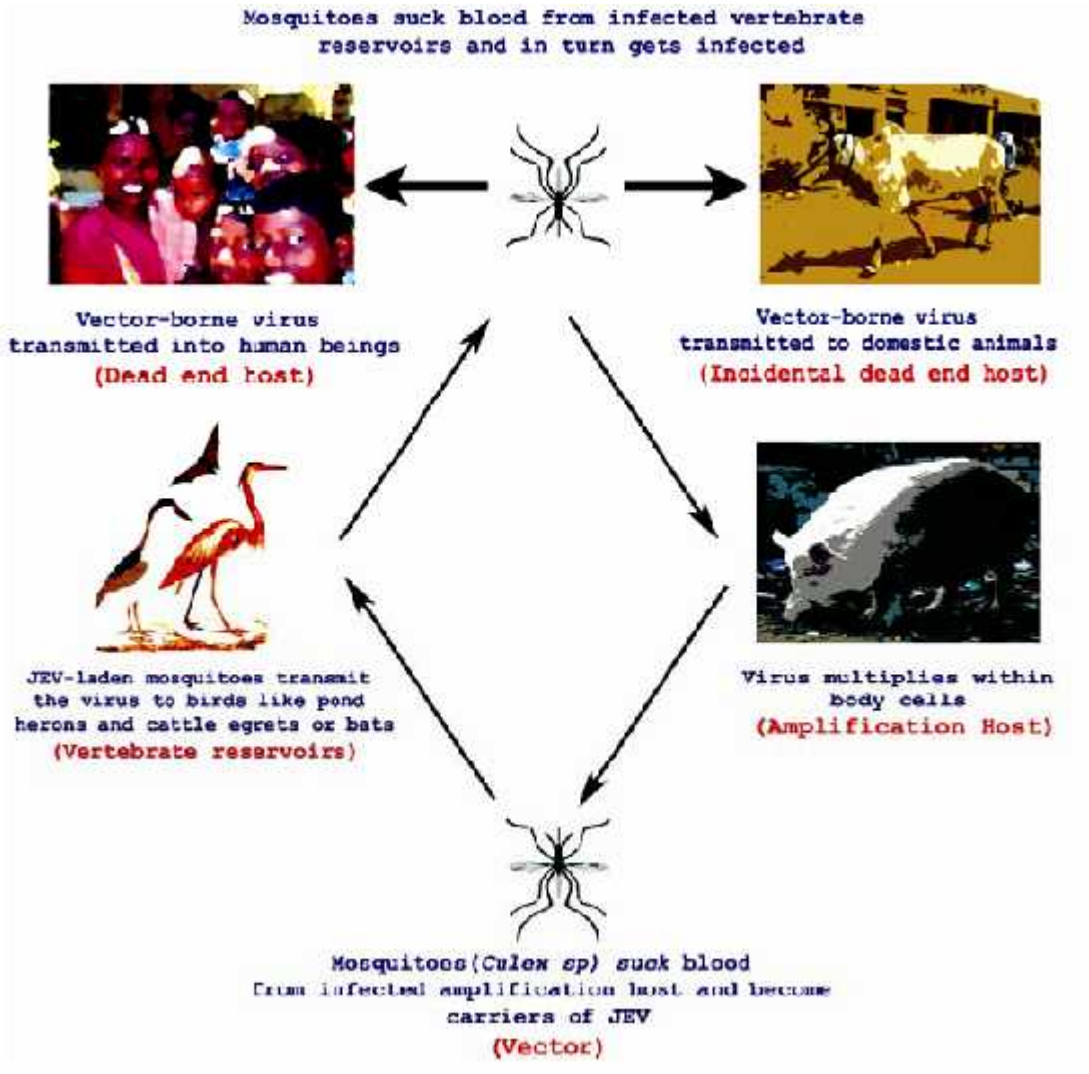
3.5.1 Vertical transmission of JEV

The vertical transmission of JEV refers to transmission of the virus to the next generation of mosquitoes. Vertical transmission probably occurs at oviposition rather than transovarially which might account for the persistence of virus in nature (Rosen et al., 1989). Vertical transmission of JEV has been reported in 3 strains of *Cx.*

tritaeniorrhynchus, Cx. Pipiens, Aedes Albopictus, A. togoi, Cx. annulus, Cx. Quinquefasciatus and *Armigeressubalbatus* mosquitoes.

3.5.1.2. Bird mosquito cycle

Bird mosquito cycle is thought to be important in maintaining and amplifying JEV in the environment. Viremia frequently follows infection of both wild and domestic birds following the mosquito bite. The viral titers in the birds are adequate to infect other mosquitoes. In India, 34.8% of pond herons, egrets and cattle have JEV neutralizing antibodies (Rodrigues et al., 1981). JEV antibody is passively transferred from immune hens as well as from 25% of immune wild herons and egrets to their progeny which are detectable until the third to fifth week of life (Buescher et al., 1959). Once the birds have been infected, they are immune and no longer able to amplify JEV (Scherer et al., 1959; Miyamoto and Nakamura, 1969).



(Source: Dutta et al., 2010)

Fig 1: Transmission cycle of JEV

3.6 Pathogenesis and pathology

3.6.1Pathogenesis

JEV is transmitted to humans via the bite of infected mosquitoes. The virus initially propagates at the site of the bite and in regional lymph nodes. Two cellular characteristics are critical to the pathogenesis:

(1) The M protein, which contains hydrophobic domains that help to anchor the virus onto the host cell, and

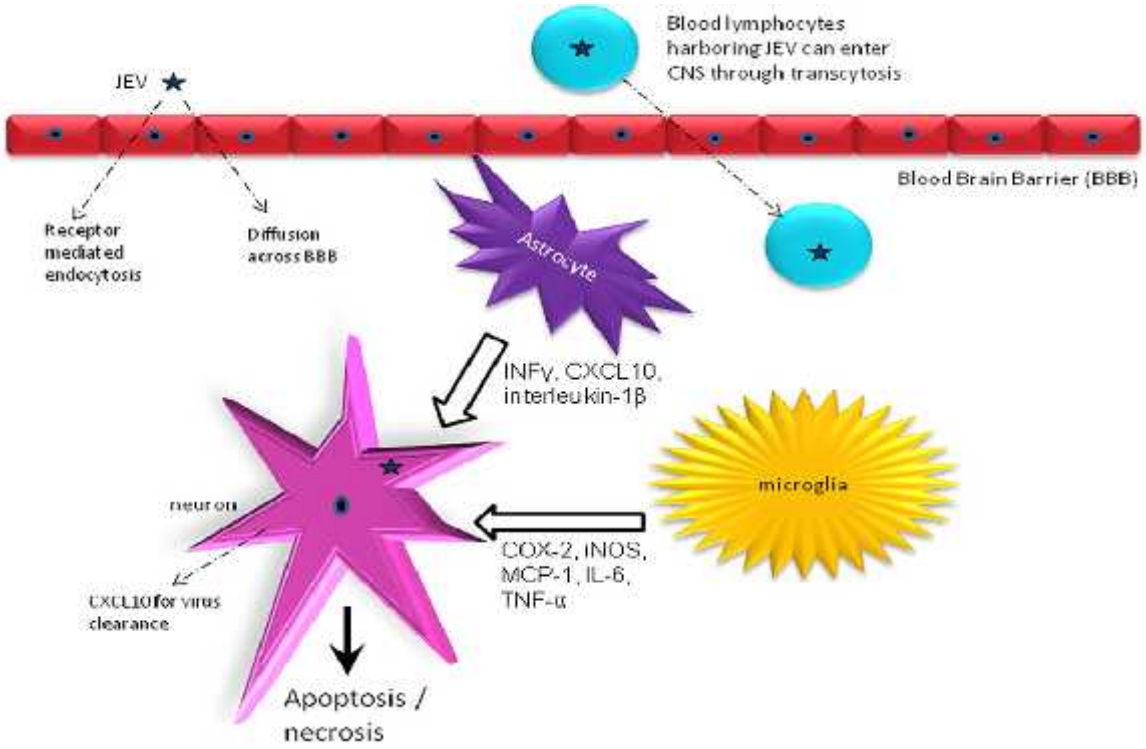
2) The E protein, which is the principal immunogenic feature and which is expressed on the membrane of infected cells. The E protein mediates membrane fusion of the viral envelope and the cellular membrane, promoting viral entry into the host cell.

On a cellular level, after attachment of virus to host cell membrane, local membrane disruption may lead to entry of virus into the cell itself. Subsequently, viremia develops, leading to inflammatory changes in the heart, lungs, liver, and reticuloendothelial system (Jani, 2011).

After the bite of an infected mosquito, the virus amplifies peripherally producing transient viremia before entering in to the central nervous system. The sites of peripheral amplification are dermal tissue and then lymph nodes. The mechanism of entry of JEV across blood brain barrier is not known. Immunohistochemical staining of human postmortem specimens has shown diffuse infection of the brain suggesting a hematogenous mechanism (Johnson et al., 1985). In an experimental model, replication of *Flavivirus*es in the endothelium has been reported and may be a means of crossing the blood brain barrier. In JEV, however, passive transfer across the endothelial cells may be a likely mechanism (Dropulie and Masters, 1990). Other factors which result in breach of blood brain barrier such a meningitis, head injury and neurocysticercosis co-infection may increase the risk of neruoinvasion of JEV. Many studies have reported a disproportionately higher number and more severe JE in those with co-existent neurocysticercosis (Liu et al., 1957; Shankar et al., 1983).

In the central nervous system the JEV virus can replicate only in the neurons. The mechanism of this type has been analyzed. The susceptibility to JEV infection in the rat brain was closely associated with the neuronal immaturity. The immature neurons are more susceptible to JEV infection than mature neurons. The initial specific binding of the virus to cells is one of the reasons for the neurotropism of the JEV. The

treatment of the JEV infection with the neutralizing antibody against the E protein did not inhibit the virus from binding to the cell surface, but strongly inhibited JEV induced cell fusion and internalization of the virus into the host cells. One of the genome regions responsible for neuropathogenesis of the virus was located on the E protein –coding region. The 138th amino acid of the E protein was important for neuropathogenesis expression of the virus. The cell fusion activity of the E protein was closely correlated with neuropathogenesis of the virus (Yasui, 2002).



Source: (Unni et al., 2011)

Fig 2: Neuropathogenesis of JEV

3.6.2 Pathophysiology

Most infections are cleared before the virus can invade the CNS, leading to subclinical disease. In such cases, the infection may not produce symptoms and therefore remains undetected. However, given the neurotropic character of JEV, neurologic invasion can develop, possibly by growth of the virus across vascular endothelial cells, leading to involvement of large areas of the brain, including the thalamus, basal ganglia, brain stem, cerebellum (especially the destruction of the

cerebral, Purkinje cells), hippocampus, and cerebral cortex. Persistent infection and congenital transmission may occur. Higher levels of certain cytokines (interferon-alpha, interleukins 6 and 8) have been associated with an increased mortality risk. The types of response implicate impaired T-helper-cell immunity in patients with severe advanced disease.

Overall, JEV is believed to result in increased CNS pathology because of its direct neurotoxic effects in brain cells and its ability to prevent the development of new cells from neural stem/progenitor cells (NPCs). JEV likely represents the first mosquito-transmitted viral pathogen to affect neural stem cells. These cells can serve important roles in injury recovery; consequently, Japanese encephalitis–induced disruption of neural stem cell growth may be particularly important to further morbidity and mortality (Jani 2011)

3.7 Clinical feature.

Arbovirus infection including JE virus results in nonspecific symptoms necessitating laboratory diagnosis in an individual case. The incubation period of JE is 5-6 days. The severity of clinical manifestations depends upon three variables namely (Rao, 2000)

a) Severity of infection
b) Susceptibility of the host and
c) Location of the agent

3.7.1 Stages of JE

Four stages can be observed in the clinical picture of the JEV.

a) Prodromal stage
b) Acute stage
c) Sub –acute stage
d) Convalescence stage

3.7.2 Prodromal Stage

This stage lasts for 2 to 3 days and has high grade fever with severe headache. Non specific symptoms include malaise, anorexia, nausea and vomiting.

3.7.2.2Acute Stage

In the acute stage, lasting for 3 to 4 days, the patient develops a change in the state of consciousness, which can range from mild clouding to stupor and coma. It is during this phase that patient frequently present for health care. Seizures are common and the patient remains febrile with weakness and stiff neck is frequently seen. Less commonly observed are tumors, abnormal movements and cranial verve involvement. Clinical descriptions from India describe focal neurological deficits as a defining characteristic to differentiate JE from other etiologies. Fatal cases usually deteriorate rapidly at this stage and die.

3.7.2.3 Sub-acute stage The sub acute phase lasts for 7 to 10 days and in uncomplicated cases the fever decreases over a period of 1-2 weeks and neurological sequelae may improve. In severe cases, secondary infections are common during this phase including bladder infection, pneumonia, and bedsores. Close attention by care givers can minimize these problems.

3.7.2.4 Convalescence stage (recovery period, 4-7 weeks) It is prolonged with weakness, lethargy, incoordinations, tremors and neuroses. Weight loss may be severe. Children, who survive slowly, regain the neurological function over several weeks; sequelae are most common in patients who are younger than 10 years at the onset of the disease. Mild case may make complete recovery (Bista and Banarjee, 2000) late developing sequelae have also been described such as optic nerve degeneration and seizures.

3.7.3 Acute Flaccid Paralysis

In 1995 a subgroup of patients infected with Japanese encephalitis virus were identified who presented with a poliomyelitis-like acute flaccid paralysis (Solomon et al., 1998). After a short febrile illness there was a rapid onset of flaccid paralysis in one or more limbs, despite a normal level of consciousness. Weakness occurred more often in the legs than the arms, and was usually asymmetric. Electromyography (EMG) was suggestive of anterior horn cell damage. Flaccid paralysis also occurs in comatose patients with "classic" Japanese encephalitis, being reported in 5% - 20 % (Dickerson et al., 1952). Occasionally respiratory muscle paralysis may be the presenting feature (Tzeng, 1989).

3.7.4 Disability and sequelae

Disability and sequelae have been found in 40 to 70 % of the surviving JE patients. Sequelae fit into four major categories: motor, behavioral, intellectual and other neurological. Motor deficits are common in approximately30% of survivors with significant cognitive and language impairments in 20%. There is also evidence to show that sequelae can develop as well as resolve over time. From a study in Thailand, fine motor disability aggressiveness, uncontrolled emotion/ impulsiveness, and abnormal intelligence were the most common sequelae occurring in greater than 70 % (MoHS/WHO 2010). In a study to assess the outcome and extent of disability following infection with Japanese encephalitis in Indonesia, of 72 children with JE, determination of outcome was possible for 65 (90%). Sixteen died in hospital or before follow-up assessment (25%). Sixteen children (25%) had severe sequelae, indicating their function was impaired enough to likely make them dependent. Five (7%) had moderate sequelae and 12 (18%) had minor sequelae. The remaining 16 children (25%) were considered to have recovered fully (Maha et al., 2009)**.**

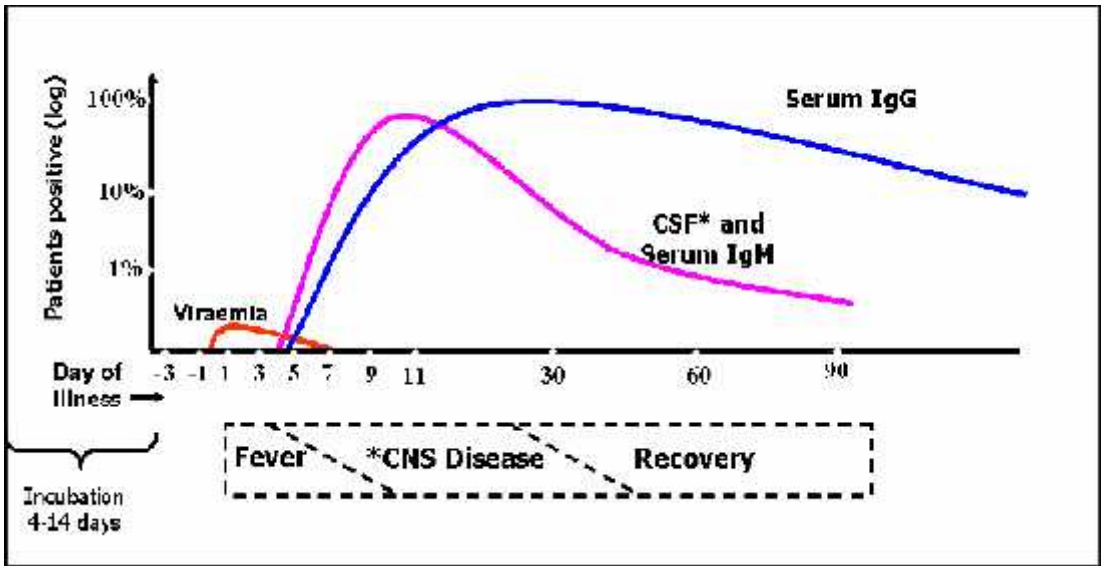
3.8 Immunology of JE

Both humoral and cellular immune responses occur after JEV infection.

3.8.1 Humoral immunity

Humoral immunity plays an important role in JEV infection. Antiviral antibodies have been reported as an important arsenal to deal with the arbovirus induced encephalitis. Cell free virus is usually cleared by antibodies through neutralization of the virus infectivity and phagocytic clearance of the virions (Griffin 1995). The effectiveness of virus-specific antibodies in vivo reflects their capacity to inhibit virus entry and spread through several mechanisms, including the direct neutralisation of virus infection (Pierson and Diamond 2009). Following primary infection (first JEV (infection), a rapid and potent IgM response occurs in serum and CSF within days. By the seventh, all the patients have raised IgM titres (Burke et al., 1985). In case of neuronal infection, antibodies are supposed to act at the surface of infected neurons to alter intracellular replication of viruses in order to follow the noncytolytic mechanism (Griffin 1995). After infection most patients produce IgM, both in serum and cerebrospinal fluid (CSF). The presence of JEV specific IgM antibodies in the serum or CSF is necessary for laboratory confirmation of JEV infection (Solomon et al.,

2008). JEV specific IgM has been used for clinical diagnosis of JEV infected patients through IgM capture enzyme linked immunosorbent assay (MAC ELISA). Class switching to IgG occurs few days after the onset of the symptoms (Burke et al., 1985). However, if a person has been infected with DENV prior to JEV Infection, high titers of IgGs have been reported (Innis et al., 1985) due to the presence of cross reactivity of JEV with other *Flavivirus*. NS1 specific antibodies are detected in sera of JE patients and such sera have shown to have complement mediated lysis activity against JEV in JEV infected BHK-21 cells (Krishna et al., 2009). Passive transfer of monoclonal antibody (mAb) against JEV has shown to protect mice from JEV infection (Kimurakuroda and Yasui 1988; Zhang et al., 1989). E glycoprotein is the major target of neutralizing antibodies in the host. Various studies have proved the efficiency of different epitopes of E protein in eliciting an immune response in the host (Verma et al., 2009; Feng et al., 2007). Recombinant E protein has also shown to be immunogenic (Xu et al., 2010)



Source: (Solomon et al, 2003)

Fig 3: Schematic antibody responses in JE infection

Antibodies to JEV proably protect the host by restricting viral replication during the virmic phase before the virus crosses the blood brain barrier (Hammon and Sather, 1973). Evidence from other *Flavivirus* infection suggests that it may limit the damage during established encephalitis by neutralizing the extracellular virus and facilitating the lysis of infected cells by antibody dependent cytotoxicity (Carmenaga et al.,

1974). In surviving patients, immunoglobulin class switching occurs; IgM declines and IgG starts rising and by 30 days most patients have serum IgG against JEV. Asymptomatic infection is also associated with raised IgM in the serum but not in the CSF (Burke et al., 1985). This secondary pattern of antibody activation is characterized by an early rise of IgG with a subsequent slow rises of IgM (Solomon 2000).

3.8.2 Cell Mediated Immunity

The cellular immune response seems to contribute to the prevention of disease during acute infection by restricting viral replication before the central nervous system is invaded. Athymic nude mice have increased susceptibility to JEV infection (Yu et al., 1985). Transfer of splenic cells from the mice immunized with live attenuated virus conveys immunity to infection (Jia et al., 1992). JEV replication was inhibited in the brain of animals that were adoptively transfused with JEV specific T lymphocytes (Desai et al., 1997)

With other human virus infections, including influenza, HIV Epstein- Barr Virus, and dengue cytotoxic T lymphocytes might be important in the control and possibly the clearance of the JEV (Bukowski et al., 1989; Mc Michael 1994). JE virus induces human peripheral blood monocytes to secrete a chemotactic cytokine [human macrophage derived factor (hMDF)] that causes chemotaxis of neutrophils (Singh et al., 2000). Je virus may be degraded by neutrophils. Ability of neutrophils to degrade JEV, via triggering of respiratory burst and generation of toxic radicals, has been investigated, JE or JEV induced macrophage derived factor (MDF) induces increase in intracellular signals with generation of superoxide anion via activation of cytosolic NADPH and subsquent formation of hydrogen peroxide with maximum activity of day 7 post infection (Srivastava et al., 1999). From biochemical analysis of impact of nitric oxide on JEV replication in cell culture by Lin YLin 1997, NO was found to profoundly inhibit viral RNA synthesis, viral protein accumulation and virus release from infected cells. NO may play crucial role in innate immunity of host to restrict the initial stage of JE virus infection in CNS. Immunization with inactivated JE vaccine induces T-cell activation invivo (Aihara et al., 2000). These studies reflect the protective role of Cell Mediated Immunity (CMI) in JE

Preliminary experimental evidence is in agreement with this: T Lymphocytes responses were characterized in seven convalescent patients with Japanese encephalitis and 10 vaccine recipients'. Japanese encephalitis virus specific T cell proliferation (including $CD4^+$and $CD8^+$) was demonstrated in both groups. JEV specific and *Flavivirus* cross reactive $CD4^+$ T lymphocyte response which recognize E protein in an HLA restricted manner were recently demonstrated in two vaccine recipient (Solomon, 2000).

3.8.3 Cytokines and chemokines

Cytokines are the soluble factors that play role against viral infections as a part of immune response. JEV infection activates microglia, which in turn produces proinflammatory cytokines like COX-2, iNOS, MCP-1, IL-6 and TNF-a (Ghoshal et al., 2007). This leads to bystander death of neurons in CNS where neurons die due to the effect of cytokines produced by microglia rather than by JEV infection itself (Thongtan et al., 2010). Different regions of the brains express variable levels of the proinflammatory cytokines. Hippocampus, the region of the brain associated with memory and learning, has the highest amount of these proinflammatory cytokines (Ghoshal et al., 2007). Tumor necrosis factor receptor (TNFR-1) complex is activated during JEV infection, specifically in the neurons, initiating the apoptotic cascade through the p38 mitogen activated protein kinase (MAPK) and c-Jun NTerminal Kinase (pJNK) pathways(Swarup et al., 2007). It leads to mitochondria mediated apoptosis in the neurons. Infected NPSCs secrete INF-γ and IL-6, which activate the microglia and astrocytes. They also produce TNF-α and CCL-2 (Kumar et al., 2004). Chemokines RANTES, IP-10 and IL-8 get induced in CNS following JEV infection (Chen et al., 2004; Singh et al., 2000) JEV-induced RANTES production by astrocytes and microglia contributes to recruitment of immune cells (Chen et al., 2004)

Activated microglia and astrocytes secrete chemotactic cytokines which attract the inflammatory cells. The chemotactic cytokines are named as chemikines and play an important role during inflammatory response observed within the central nervous system in JE (Winter et al., 2004). Cytokines such as INF α/β, INF –α and TNF- α have the potential to trigger activation of intracellular antiviral pathways after they bind to specific receptors on the surface of the infected cells. Other cytokines such as

IL-1 α/β, IL-2, IL-6, IL-12, IL -13 and IL-18 are believed to contribute to the antiviral response. It has been shown that various proinflammatory mediators like INF α, TNF α,MIF, IL-8, IL -6, RANTES, Cox -2, IL-1β and MCP-1 are elevated during JE infection (Burke et al., 1985; Singh et al., 2000; Winter et al., 2004; Ghoshal et al., 2007). In JE patients CSF IL-6 and IL -1B and RANTES were significantly higher in patients compared to controls (Babu et al., 2006). In another large study on JE, CSF TNF α, IL- 8 and IL-6 were higher in non-survivors compared to survivors (Winter et al., 2004).

3.9 Cross reactivity among *Flavivirus*

Different *Flavivirus*es share antigenic epitopes, which elicit antibodies commonly referred to as cross-reacting antibodies. These *Flavivirus* cross-reactive responses can confound the interpretation of serological tests, and it is often impossible to determine with certainty the infecting virus without resorting to performing neutralization tests (Cardosa et al., 2002).

In Southeast Asia, dengue viruses often co-circulate with other *Flavivirus*es such as JEV, and due to the presence of shared antigenic epitopes it is often difficult to use serological methods to distinguish between previous infections by these *Flavivirus*es. Cross-reactivity among *Flavivirus* has been a diagnostic challenge, especially for members of the JE serocomplex in which differentiation is often difficult even using the neutralization test, recognized to have the highest specificity among the currently available serological tests (Williams et al., 2000; McLean et al., 2002), mainly because these virus antigens contain the same highly conserved immunodominant E glycoprotein epitopes responsible for eliciting large proportion of cross-reactive serum antibodies during viral infection. Virus-specific and conserved B-cell epitope on NS1 protein of JEV have been identified that are useful in the diagnosis without cross reactivity (Wang et .al., 2009).

3.10 Diagnosis of JE

3. 10.1Clinical diagnosis

The WHO recommended case definition for suspect Japanese Encephalitis is:

Clinically, a case of acute encephalitis syndrome is defined as a person of any age, at any time of year with the acute onset of fever and a change in mental status (including symptoms such as confusion, disorientation, coma, or inability to talk) AND/OR new onset of seizures (excluding simple febrile seizures). Other early clinical findings may include an increase in irritability, somnolence or abnormal behavior greater than that seen with usual febrile illness (WHO, 2006).

The clinical symptomatology of all viral encephalitis is similar and therefore clinical diagnosis at best can only be an educated guess and is made by the association of encephalitis and some symptoms and signs with possible viruses (Rao, 2000).

In JE, there may be peripheral leucocytosis and hyponatremia. The typical CSF findings include moderate pleocytosis (10–100per cubic mm), mild protein rise (50–200 mg/dl) and normal glucose. Usually there is lymphocytic pleocytosis but in the early stage polymorphs may predominate. Very rarely CSF may be acellular (Mishra and Kalita 2010).

Investigation are made by Cranial CT scan or MRI, Electroencephalography (EEG), Nerve conduction and evoked potential studies, Single photon emission computed tomography (SPECT).

Presence of anti JE IgM in CSF indicates confirmed JE while its presence in serum indicates probable JE

Case classification

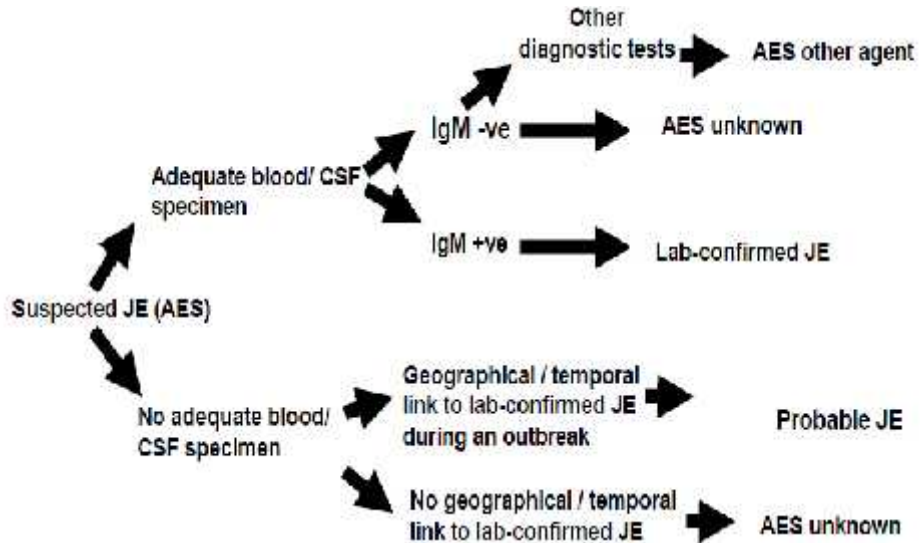
Suspected case: A case that meets the clinical case definition for AES.

Laboratory-confirmed JE: A suspected case that has been laboratory-confirmed as JE.

Probable JE: A suspected case that occurs in close geographical and temporal relationship to a laboratory-confirmed case of JE, in the context of an outbreak.

“Acute encephalitis syndrome” – other agent: A suspected case in which diagnostic testing is performed and an etiological agent other than JE virus is identified.

"Acute encephalitis syndrome" – unknown: A suspected case in which no diagnostic testing is performed or in which testing was performed but no etiological agent was identified or in which the test results were indeterminate.



(**Source**: WHO 2007)

Fig 4: Final classification scheme of AES cases

3. 10.2 Virus Cultivation

Isolation of virus from a clinical specimen is generally considered a rare occurrence (Shope and Sather, 1979) proably because of the low viral titres, rapid production of neutralizing antibodies, frequent freezing and thawing of clinical materials, and the logistic difficulty in transport of specimens in the developing countries (Mohanrao et al., 1988; Leake et al., 1996).lack of skilled man power and virus culture laboratories are also major hindrances to viral isolation.

All arboviruses, including JE virus, are high-risk pathogens, all attempts at virus isolation must take account of the risks and appropriate laboratory biosafety practices should be observed. As a minimum, laboratory biosafety level 3 (BSL-3) equirements

should be in place. (WHO Biosafety Manual, Third edition, World Health Organization, 2004), and staff should be vaccinated against JE.

Isolation of JE virus from routine clinical samples is very challenging but may occasionally be successful from CSF or from brain tissue samples of fatal cases. After isolation, virus can be confirmed and identified using: appropriate polyclonal or monoclonal antibodies, by indirect immunofluorescence, by RT-PCR using JEV specific primers, or by nucleotide sequencing.

JE virus can be isolated by a variety of methods, the classic being intracranial inoculation of clinical specimen in suckling mice. Normally, 1 or 2 day old suckling mice (*Musmusculus*) are inoculated intra-cranially with 0.002ml of suspension of clinical material. Mice are observed twice a day for the first sign of encephalitis (failure to eat as evidenced by a lack of milk in the stomach, color change, wasting, runting or tremors), which occur approximately 3-10 days after inoculation. Confirmation of JE virus is performed by staining the brain with either a polyclonal or monoclonal antibody specific for JE conjugated with a fluorescent tag.

Various cell cultures such as primary chick, duck embryo cells, and the cell- lines of Vero, LLCMK2, C6/36 and API cells are more often used to isolate the JEV. Recently, sensitive mosquito inoculation techniques have been described for the isolation of JEV (Gajanana et. Al., 1995).

3. 10.3. Serological diagnosis.

Serological tests are useful to determine the prevalence of infection in an animal population, the geographical distribution of the virus, and the degree of antibody production in vaccinated individuals. If serology is to be used for the diagnosis of the disease in individual it should be remembered that individuals in an endemic area may have been inapparently infected with the virus or may have been immunized with a vaccine. Antibody assay is a useful technique for determining the prevalence of infection in a population, and also for diagnosing JE in diseased individuals. The assay methods include virus neutralization (VN), haemagglutination inhibition (HI), complement fixation (CF), and enzyme-linked immunosorbent assay (ELISA). Diagnosis requires a significant rise in antibody titer in paired sera collected during the acute and convalescent phases. The specificity of each serological test should also

be considered. An ELISA for antibodies to a nonstructural protein (NS1) of JEV can be used to differentiate antibodies following natural infection from those induced by inactivated vaccines. The presence of antibody to these other *Flaviviruses* cans make serological diagnosis of Japanese encephalitis difficult. There is some cross reactivity with other *Flaviviruse*s on all the tests; the plaque reduction VN test is the most specific, especially if a 90% neutralization threshold is used. Antibodies begin to appear soon after onset, but only about 70-75% of patients have IgM antibody in specimens collected up to 4 days after onset. However all patients will have antibody 7-10 days after onset (WHO 2007).

a. Plaque Reduction Neutralization Titre Assay

It is possible to confirm JE ELISA results using the sensitive plaque reduction neutralizationTitre assay (PRNT) method to differentiate JE antibody from other *Flavivirus*es. 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. This assay is considered more sensitive than ELISA for differentiating between different *Flavivirus*es. However PRNT is time-consuming to perform, has a long incubation period and is labor 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.

In 1959,Henderson and Taylor developed a method to detect plaques of arboviruses using an agar overlay stained with neutral red (Henderson and Tayor 1959).This technique allowed an easy in-vitro assay to directly detect and measure the infectivity of a virus preparation or stock as Plaque forming units. Standard JE serotype –specific monkey sera are utilized in every PRNT assay as positive controls as well as negative controls that form the baseline plaque count.

b. Hemagglutination Inhibition.

The HI test is widely used for the diagnosis of Japanese encephalitis, but has cross-reactivity with other *Flavivirus*es. For this test, the sera must first be treated with acetone or kaolin, and then adsorbed with homotypic RBCs to remove any nonspecific haemagglutinins in the test sera. The RBCs of geese or of 1- day-old

chickens are used at the optimum pH. The optimal pH is dependent on the JEV strain used. The test should be conducted with the treated sera and 8 units of standard antigen; this is commercially available in some countries.

c. Enzyme Linked Immunosorbent Assay

Both IgM and IgG antibodies are detected against the JE either from the CSF or from the serum samples collected from an encephalitic patient. In 19820s IgM and IgG ELISAs were developed which have become the accepted standard for diagnosis of the JE (Solomon et al., 1998; Burke et al., 1982). The IgG antibody shows cross reactivity with other flaviviurs and the detection of the specific anti JEV IgM antibody in the CSF and the serum sample is a useful technique. IgM antibody is detected using the MAC ELISA. This method makes use of the JEV antigens derived from the mouse brain or those from the culture system.

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 modification of the ,MAC ELISA that have been used in antibody detection. MAC ELISA in standard format is very sensitive in the diagnosis of arboviral infection including the members of *Flavivirus.* (Martin et al., 2002)

3.10.4 Western blot

It is a technique that utilizes the characteristic of proteins to travel through the polyacrylamide gel, and to transfer onto nitrocellulose sheets when an electrical current is passed through the gel. Separation of proteins occurs as they travel through a gel at different speeds depending upon their molecular weight and charge. The result, when applied to proteins derived from JE, is a spread of differing structural and non structural proteins components across a gel, predominantly E, NS1 ad pre-M. Human sera is applied on the proteins transferred on to nitrocellulose paper after protein separation and specific protein is recognized as the human antibody specific to JE protein will bind; an antihuman antibody conjugate system is tensed to detect binding of human antibody. The result is a band showing the presence of antibody to those proteins. This assay has limited diagnostic utility as JE and Dengue virus

proteins are highly cross-reactive and western blot is unable to distinguish the two infections.

3.10.5 Antigen detection

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

3.10.6 RT-PCR

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.

3.11 IgM Antibody Capture ELISA (MAC ELISA)

a. IgM assay

Although antigenic cross- reactivity in *Flavivirus*es is common due to the large number of shared epitopes on the viral proteins, it was documented in 1982 that the presence of specific IgM antibodies could be used to diagnose Japanese encephalitis virus infections. The original methods have been modified for use to confirm other *Flavivirus* infections, including Dengue which is often used for differential diagnosis in JE confirmatory testing.

The IgM diagnostic assay is based on the principle of IgM capture. Several JE research laboratories have developed their own in-house assays but these are generally not available to a wider market. Currently there are only a small number of commercial assays available. One commercial IgM assay currently uses separate JE and Dengue antigen wells to help differentiate between recent JE and Dengue infections.

The procedure in fig 5 describes a generic outline for the detection of IgM antibodies to Japanese Encephalitis virus in human sera and CSF using an antibody capture technique.

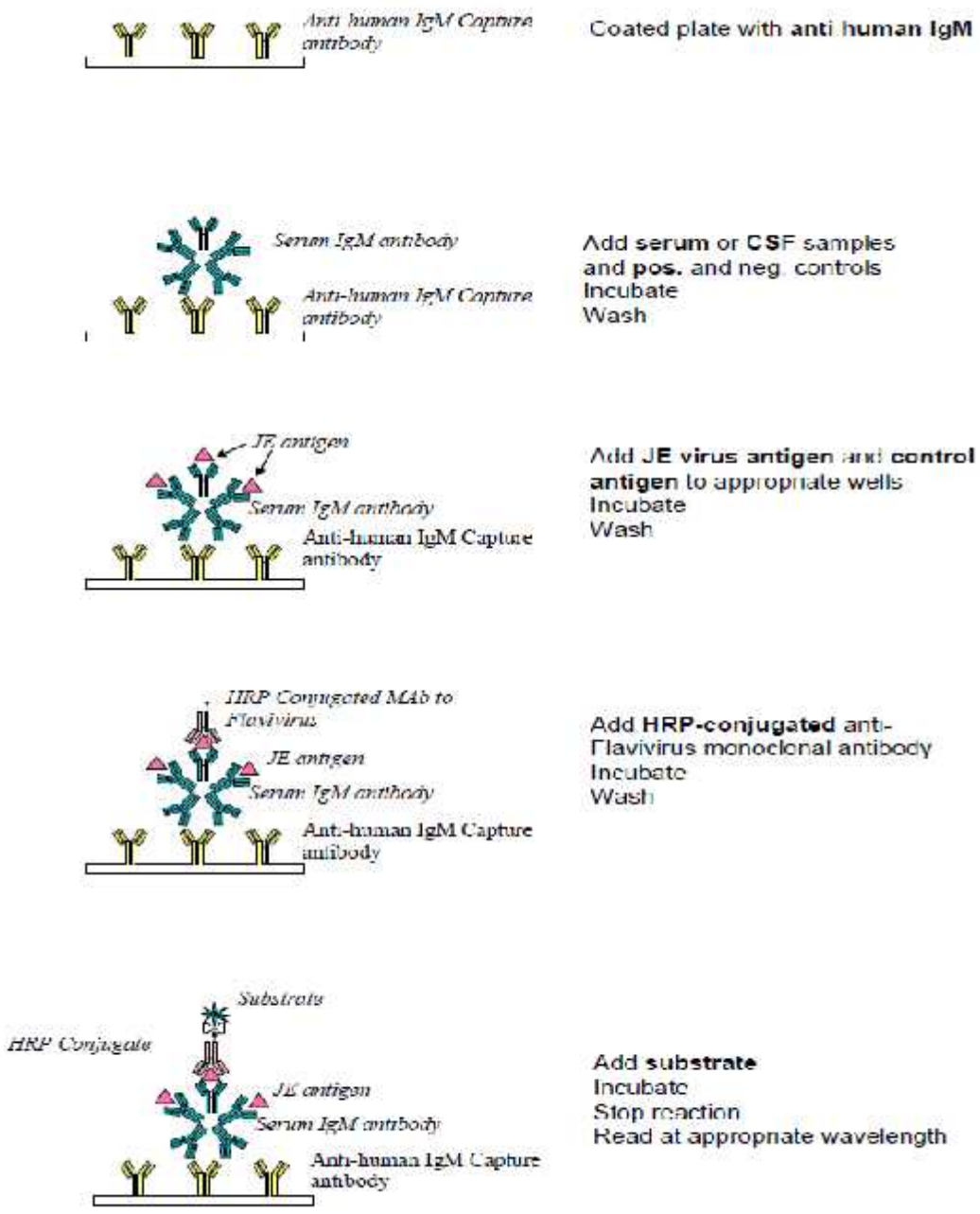
b. IgM Test principle

IgM antibody in the patient's serum or CSF is bound to anti-human IgM antibody adsorbed into a solid phase, usually in a microtitre plate. This step is non virus-specific and eliminates competition with IgG;

• The plate is then washed, removing other immunoglobulins and serum proteins;

• JE antigen is then added and allowed to bind to any JE-specific IgM present;

• After washing, bound JE antigen is detected using anti-JE monoclonal antibody, following which a detector system with chromogen substrate reveals the presence or absence of JE IgM in the test sample.



Source: (WHO 2007**)**

Figure 5: Generalised principle of the MAC ELISA

3.12 Prevention control and treatment.

The control of JE is based essentially on three interventions: mosquito control, avoiding human exposure to mosquitoes and immunization. Mosquito control has been very difficult to achieve in rural settings and avoidance of exposure is difficult as *Culex* mosquitoes bite during day time.

Human vaccination is the only method that has proved effective for controlling JE disease. Other methods, such as mosquito control and pig control, have had little success.

3. 12.1Control of vector

The use of pesticides to control Japanese encephalitis virus vectors has generally been effective only in limited areas for a limited amount of time and at great cost. Ultra-low volume fenitrothion delivered from fixedwing aircraft was effective in reducing *Culex tritaeniorhynchus* adult mosquito populations by nearly 80 percent over a 4 day period in Korea (Self et al., 1973). The use of the herbicides/ larvacides CNP (p-nitro-phenyl 2,4,6 trichloro-phenyl-p, nitrophenyl ether) and nitrofen (2,4-dichlorophenyl-p, nitrophenyl ether) are credited with decreasing the numbers of *Culex tritaeniorhynchus* mosquitoes in the Kyoto city area, Japan, in the early 1970s (Maeda et al., 1978)**.**

Spraying is generally used only for outbreak suppression or at the beginning of the rainy season in villages with repeated epidemics (Phanthumachindra, 1989). Keeping people from mosquitoes by distancing housing from rice fields and pigsties, as well as the use of netting, can help but is impractical (Wada, 1989).

3. 12.2 Prevention of mosquito bites

Personal precautions should be taken by residents of endemic regions, and travelers to these areas, to avoid mosquito bites. These precautions include

1) Minimizing outdoor exposure at dusk and dawn and during overcast days,
2) Sleeping in screened quarters or under mosquito netting,
3) Keeping pig or cattle pens away from housing and avoiding the animals at dusk,
4) Wearing clothing leaving a minimum of bare skin, and

5) Using insect repellents with at least 30 percent active ingredient DEET (N,N diethyl- meta-tolumide) on exposed skin surfaces (Vaugh and Hoke, 1992).
Gurung et al., in2003 reported that the mosquito-net non users are at 2.6 times greater risk of developing JE in comparison to that of net users.

3. 12.3 JE awareness programs

Awareness in the local people about the disease, its mode of transmission and season, the age group it affects and the outcomes after infection along with the preventive measures that can be applied in order to be safe is the best way of prevention. Mass educating people through mass education, advertisement via radio, television newspapers help in reducing the infection. People should be informed of vaccines, good husbandry, protection from bites, use of mosquito net and others.

3. 12.4 Vaccines

Immunization is the only effective method for sustainable control. Routine immunization of school age children is currently in use in Korea, Japan, China, Thailand and Taiwan. The introduction of the JE vaccine into the Expanded Program of Immunization has helped curb the disease in countries like Thailand, Vietnam, Sri Lanka and China (Tauber & Dewasthaly, 2008).

3. 12.4.1 Inactivated vaccines

Among the currently available vaccines is a formalin-inactivated vaccine derived from mouse brain grown JEV strain Nakayama (Monath, 2002), which still is produced by manufacturers in Korea, Thailand and Vietnam. The vaccine is relatively expensive, requires three doses on days 0, 7 and 30, followed by a booster at 1 year and thereafter at intervals of 3 years. The vaccine can often generate neurological adverse reactions. Another formalin-inactivated JE vaccine is prepared in China using the JEV P3 strain propagated in primary hamster kidney-cell cultures. The vaccine appears to be more immunogenic than that based on the Nakayama strain and can be integrated into the routine childhood immunization schedule but is not distributed outside of China. It is now largely being replaced by the live attenuated vaccine. Several attempts are in progress to prepare inactivated JEV vaccines starting from virus grown in controlled cell line cultures.

3. 12.4.2. Live attenuated vaccines

The live attenuated JE vaccine strain, which was obtained after 11 passages in weaning mice followed by 100 passages in primary hamster kidney cells, has been developed and used in China since 1988. The SA14-14-2 vaccine is a neruoattenuated viral strain that has shown effectiveness in laboratory setting against the P3, Nakayama, 12 Chinese JE field isolates, and JE strains from Thailand, Nepal, Vietnam, Indonesia, India, Japan, and the Philippines (MoH/ WHO, 2010). The vaccine, which is produced by the Chengdu Institute of Biological Products in China, was licensed in recent years in several Asian countries and was extensively used from 2006 to 2008 in mass immunization campaigns in India. Although the product is not WHO prequalified at this time, much investment and efforts have been made to bring the production and quality control to international standards. The vaccine is produced on primary hamster kidney cells, lyophilized, and administered to children at one year of age and again at two years, in annual spring campaigns. Initial observational studies in southern China involving more than 200 000 children had demonstrated the vaccine safety, immunogenicity (99-100% sero conversion rate in non immune subjects) and protective efficacy over 5 years (Monath, 2002). The short-term effectiveness of a single dose of SA14-2-14 was demonstrated in 2001 in a case control study on Nepalese children where an efficacy of 99.3% was reported. A five year follow-up study found the single-dose efficacy was maintained at 96.2%. Another five-year follow up study showed that neutralizing antibody persistence was close to 90% at 4 years and 64% at 5 years after a single-dose of the vaccine in adult volunteers

3. 12.4.3 Chimeric vaccines

A promising approach for a future JE vaccine has been the construction of an YF-JE chimera based on the attenuated 17D YF virus genome, in which the YFV sequences encoding viral structural proteins prM and E were replaced by the corresponding prM and E sequences from JEV strain SA14-2-14. The resulting YF-JE chimeric virus, ChimeriVax-JETM, developed by Acambis and now licensed toS anofi Pasteur, was grown on Vero cells and shown to elicit JEV neutralizing antibodies as well as protection against nasal and intracerebral virus challenge in rhesus monkeys. The vaccine was tested in human adult volunteers in the USA, showing good safety and

immunogenicity,with 94% of the vaccinees in the Phase II trial developing protective neutralizing antibody levels after a single dose.

3. 12.4.4. Live recombinant JEV vaccines

Replication-defective canarypox (ALVAC) and the highly attenuated vaccinia virus strain NYVACwere used as vectors to express the pr-M, E, NS1 and NS2a gene from JEV. The vaccine candidateswere found to be well tolerated but their immunogenicity was too weak, especially in non-vacciniaimmune volunteers, to warrant further development.

3.12.5 Prospects for antiviral treatments

There is no established treatment for the disease, but an understanding of the pathogenesis may point the way toward therapies. Treatment efforts are directed at controlling both the immediate complications of infection, including seizures and increased intracranial pressure, and the longer-term consequences of neurologic impairment, bed sores (Solomon, 2006). A variety of Compounds has shown antiviral activity in vitro and or animal models of infection (Leyssen et al., 2000). Recently, salicylates and non steroidal anti-inflammatory drugs were shown to suppress the in vitro replication of JEV, and prevent apoptosis of infected cells (Chen et al., 2002; Liao et al., 2001). Interferon alpha, produced naturally in response to viral infections, including JEV (Burke and Morrill, 1987), has been the most promising antiviral candidate. In tissue culture, recombinant interferon is effective against JEV and other arbovirus including West Nile virus (Anderson and Rahal, 2002; Harinasuta et al., 1984).

CHAPTER IV

4. MATERIAL AND METHODS

4.1 Materials

A complete list of equipments, reagents and chemicals required for IgM capture ELISA by the reference method and the test kits have been listed in Appendix I

4.2 Methods

4.2.1 Study design

This study has been designed to evaluate the two commercially available IgM Antibody Capture ELISA test kits used in the diagnosis of the Japanese encephalitis.

4.2.2 Study site

This study was conducted at Japanese Encephalitis National Referral Laboratory, National Public Health Laboratory Teku, Kathmandu, Nepal from April 2010 to December 2010 for a period of 9 months.

4.2.3 Sample

The samples used in the evaluation purpose were the CSF and the serum samples that had been collected at different parts of the country from suspected encephalitic patients and sent to the Japanese encephalitis National Referral Laboratory for serodiagnosis during the surveillance of the JE in 2010 and also those samples that have been collected during the surveillance in the past years and stored at -20^{o} C were also used.

4.2.4 Sample size

A total of 580 samples were used for the evaluation purpose. Of the total samples tested 251 were serum and 329 were CSF. All the serum samples were tested using the Panbio kits and all the CSF samples were tested using the XCyton kit. However 60CSF samples were also tested using the Panbio kit. These samples so tested using the Panbio kit consisted of 30 positive samples and 30 negative samples as tested by the reference method. Similarly, 63 serum samples containing 30 positive and 33 negative by the reference method were also tested using the XCyton kit.

4.3 Sample selection

Samples were selected on a random basis. The samples were selected without any previous knowledge of the sample result, patient name, age, place and patient travel history. But in case of stored samples taken for evaluation purpose the sample type was known but its previous result was unknown.

4.4 Test protocol

In case of the commercial kits the samples were tested using the instruction in the kit inserts. The instructions in the kit insert were developed in the form of the protocol and approved by the laboratory incharge. The protocol is described in appendix III and IV

In case of testing with the reference ELISA method the protocol in the reference laboratory was followed which is as mentioned in the appendix II.

4.5 Evaluation of ELISA test Kits

Two commercially available MAC ELISA assay; Panbio JE-Dengue IgM Combo ELISA and XCyton JEV CheX ELISA were evaluated against the in house AFRIMS ELISA kit as a reference method.

The paramaters used in evaluation purpose can be obtained using the simple table as belows

Table 1: Classification of test result

	Reference test result			Total
		Positive	Negative	
Test outcome	Positive	True Positive (a)	False Positive (b)	a+b
	Negative	False Negative (c)	True Negative (d)	c+d
Total		a+c	b+d	a+b+ c+d

Different measures used in evaluation purpose are as follows;

4.5.1Sensitivity

Sensitivity is the ability of a test to identify correctly those who have the disease or condition. It is the proportion of true positive cases having a disease or condition, sensitivity measures the true positive cases among diseased persons. It is expressed in percentage. It is calculated as follows;

Sensitivity = a

$$\frac{\text{Number of true positives}}{\text{Number of true positives + Number of false negative}}$$

4.5.2 Specificity

It is the ability of a test to identify correctly those who don't have the disease. It is the proportion of true negative cases among non diseased persons. Specificity is the measures of true negatives among non diseased individuals. It is also expressed in percentage. It is calculated using the following formula.

$$\text{Specificity} = \frac{b}{b+d} = \frac{\text{Number of true negatives}}{\text{number of true negative} + \text{false positive}}$$

4.5.3Predictive Value Positive

The predictive value positive or precision rate is the proportion of subjects with positive test results who are correctly diagnosed. It is a critical measure of the performance of a diagnostic method, as it reflects the probability that a positive test reflects the underlying condition being tested for. Its value does however depend on the prevalence of the outcome of interest, which may be unknown for a particular target population.

The Predictive Value Positive is defined as

$$\text{PVP} = \frac{\text{Number of true positives}}{\text{Number of true positives} \mid \text{Number of false positives}} - \frac{\text{Number of true positives}}{\text{Number of positive calls}}$$

Where a "true positive" is the event that the test makes a positive prediction, and the subject has a positive result under the gold reference, and a "false positive" is the event that the test makes a positive prediction, and the subject has a negative result under the gold reference.

If the prevalence, sensitivity, and specificity are known, the predictive value positive can be obtained from the following identity:

$$\mathbf{PVP = ((Sensitivity)(prevalence))/((Sensitivity)(prevalence) + (1 - specificity(1 - prevalence))}$$

4.5.4 Predictive value negative

The predictive value negative is a summary testing used to describe the performance of a diagnostic testing procedure. It is defined as the proportion of subjects with a negative test result who are correctly diagnosed. A high PVN means that when the test yields a negative result, it is uncommon that the result should have been positive.

The Predictive Value Negative is defined as:

$$\mathbf{PVN} = \frac{\mathbf{Number\ of\ true\ negatives}}{\mathbf{Number\ of\ true\ Negatives + number\ of\ false\ negative}} = \frac{\mathbf{Number\ of\ true\ negatives}}{\mathbf{number\ of\ negative\ calls}}$$

i.e.PVN= $\frac{d}{c+d}$

Where a "true negative" is the event that the test makes a negative prediction, and the subject has a negative result under the gold reference, and a "false negative" is the event that the test makes a negative prediction, and the subject has a positive result under the gold reference.

If the prevalence, sensitivity, and specificity are known, the predictive value negative can be obtained from the following identity:

$$\mathbf{PVN} = \frac{\mathbf{(specificity)(1 - prevalence)}}{\mathbf{(specificity)(1 - prevalence) + (1 - sensitivity)(prevalence)}}$$

4.5.5 Efficiency

It is the percentage of test results that are correctly identified by the test that is true positives and true negatives.

Mathematically,

$$\mathbf{efficiency} = \frac{\mathbf{a + d}}{\mathbf{a + b + c + d}} \text{ X } 100$$

4.5.6 Cross reactivity

Cross reactivity of the kits is calculated to know if the test under use is affected by the presence of the antibody to the other similar *Flavivirus* infection or not. It is expressed in percentage.

$$\%\,\text{Crossreactivity} = \frac{\text{no of sample positive}}{\text{total samples tested}}$$

4.6 Sample processing

All the samples were brought to room temperature and were processed according to the protocol.

4.7 Recording of the test result

All the test result obtained was recorded in the Microsoft Excel 2007 based spread sheet for the further analysis using the available statistical tool.

4.8 Statistical analysis

SPSS tool was used for determining the sensitivity, specificity, predictive value positive, predictive value negative, efficiency values of the kits and also to find the p value for chi-square test at 5% level of significance.

CHAPTER V

5. RESULTS

Japanese encephalitis is a vector borne disease causing number of death in the south East Asia. The diagnosis relies mostly on the detection of the specific IgM antibody against the JEV. As cross reaction occurs among the *flavivirus* members a test should be specific enough to distinguish the JE infection from other *flavivirus* infection.

This test was done at the National Public Health Laboratory in order to assess the qualities of the commercial MAC ELISA against the established in house reference AFRIMS ELISA.

5.1Evaluation of Panbio kit using both serum and CSF samples

A total of 311 samples containing 251 serums and 60 CSF were tested using the Panbio kit. The result obtained is as shown in the table below.

Table 2: Comparison of Panbio kit with AFRIMS ELISA using serum and CSF samples

	AFRIMS Result			
Panbio Result	Positive	Negative	Grand Total	statistics
Positive	80	27	107	$\chi 2cal=79.407$
Negative	46	158	204	
Grand total	126	185	N=311	$p<0.05$

The sensitivity, specificity, PVP, PVN and the efficiency of the kit were obtained as 63.4%, 85.4%, 74.7%, 77.4% and 76.5% respectively. The result shows the low diagnostic values when both samples are evaluated.

5. 2 Evaluation of Panbio kit using serum only

The evaluation of the Panbio using the serum samples only and the CSF samples only was also done as to find its fitness in terms of sample type. The following result was obtained while using the serum samples only.

Table 3: Comparison of Panbio kit with AFRIMS ELISA using serum only

	AFRIMS result			
Panbio Result	Positive	Negative	Grand total	statistics
Positive	57	24	81	$\chi 2cal=52.251$
Negative	39	131	170	$p<0.05$
Grand total	96	155	N=251	

Of the total 251 serum samples tested using the Panbio ELISA method against the reference ELISA the Panbio kit showed the sensitivity of 59.3%, specificity of 84.5%, PVP of 70.3%, PVN 77 %, Efficiency of 74.9%.

5. 3: Evaluation of Panbio kit using CSF only

Though the Panbio kit is specifically designed to test the serum samples a panel of 60 serum samples containing 30 positive and 30 negative were also tested and the result obtained is as shown in table below.

Table 4: Comparision of Panbio kit with AFRIMS ELISA using CSF only

	AFRIMS result			
Panbio result	Positive	Negative	Grand total	statistics
Positive	23	3	26	χ2cal=27.149
Negative	7	27	34	
Grand total	30	30	N=60	p<0.05

Using CSF samples for the the Panbio kit showed the Sensitivity, Specificity, PVP, PVN and Efficiency of 76.6%, 90%, 88.4%, 79.4%, and 83.3% respectively. The data indicate that the sensitivity value for this kit using the CSF sample is better than that of the serum sample.

5.4 Evaluation of the XCyton kit using the serum and CSFsamples

The second kit used for the evaluation purpose was the XCyton kit. The kit was also evaluated using both the serum and the CSF samples. The overall performance of the XCyton kit using the 392 samples consisting of 63 serum samples 329 CSF samples is as follows:

Table 5: Comparison of XCyton kit with AFRIMS ELISA using serum and CSF samples

	AFRIMS result			
Panbio result	Positive	Negative	Grand total	statistics
Positive	106	8	114	χ2cal=276.251
Negative	19	259	278	
Grand total	125	267	N=392	p<0.05

From the above table it is clear that the sensitivity is 84.8%, specificity is 97%, PVP is 92.9%, PVN is 93.1% and the efficiency of the kit is 93.1%. Here both the

sensitivity and the specificity of the kit is above 80% with specificity being greater than 90%.

5.5 Evaluation of XCyton using the serum samples only

For evaluation of kit separately for individual sample 63 serum samples were tested using the XCyton kit, the result obtained is as shown in table below

Table 6: Comparison of XCyton kit with AFRIMS ELISA using serum only

	AFRIMS result			
Panbio result	Positive	Negative	Grand total	statistics
Positive	25	0	25	$\chi 2cal=45.592$
Negative	5	33	38	
Grand total	30	33	N=63	$p<0.05$

In using the serum samples for evaluation of the XCyton kit the sensitivity, specificity, PVP, the PVN and the efficiency of the kit were obtained as 83.3%, 100%,100%,86.8%,92% respectively. Here, none of the samples were false positive and of the 30 positives and 33 negative samples by the standard method 25 were true positive and 33were true negative by the test method.

5.6 Evaluation of XCyton kit using CSF only

329 CSF samples were used for the evaluation purpose, the number of the positive and negative test result in comparison to the standard are as depicted in the following table and the performance characteristics have been shown there with.

Table 7: Comparison of XCyton kit with AFRIMS ELISA using CSF only

	AFRIMS result			
Panbio result	Positive	Negative	Grand total	statistics
Positive	81	8	89	$\chi 2cal=229.357$
Negative	14	226	240	
Grand total	95	234	N=329	$p<0.05$

Here of the total 329 CSF containing the 95 positive and 234 negative by the standard method only 81 were true positive and 226 were true negative as shown by the test method .Using the CSF samples the results obtained were sensitivity, specificity, PVP, PVN and efficiency of the kit as 85.2%, 96.5%, 91%, 94.1%, 93.3%.respectively.

In aggregate the performance characteristic of the Panbio was quite lower than that of the XCyton. However, both the tests were statistically significant for the value of chi-square test at 5% level of significance and the P value of less than 0.05

5.7 Comparision of performance characteristics for both kits

Sensitivity, specificity, positive predictive value, negative predictive value, efficiency of both the Panbio kit and the Xcyton kit has been shown in the following table.

Table 9: Overall result for both kits

Kits used	Sample type	Sensitivity (%)	Specificity (%)	PVP (%)	PVN (%)	Efficiency (%)
1. Panbio JE-DengueIgM Combo ELISA	Serum	59.35	84.51	70.37	77.05	74.91
	CSF	76.66	90	88	79.41	83.33
	Both	63.49	85.40	74.76	77.45	76.52
2. XCyton ELISA	Serum	83.33	100	100	86.84	92.06
	CSF	85.26	96.58	91.01	94.16	93.31
	Both	84.8	97	92.98	93.16	93.11

5.8 Cross reactivity of the kits with dengue positive serum samples

The cross reactivity of the kits was assessed in terms of percentage. The cross reactivity of both the kits were determined by using the dengue positive samples that were confirmed positive by SD dengue ELISA Kit.

Of the total 25 dengue positive samples tested using both the commercial kits the cross reactivity percentage was found to be as follows,

Table 8: Cross reactivity of both kits with dengue positive samples

S.N	No of sample tested	Test kit	JE Pos	JE Neg	% Cross reactivity
1	25	Panbio	1	24	4%
2	25	XCyton	8	17	32%

Thus Panbio showed the cross reactivity of 4% while XCyton showed cross reactivity of 32%. It showed that the use of the XCyton could result in cross reaction problems.

5.9 Qualative comparison of the test kits

The test kits were qualitatively compared on the basis of number of samples that can be tested in kit, duration of test, sample types that can be tested, difficulties that may come during the test, volume of samples required and advantages. The qualitative comparison is as on the table below:

Tables 10: Qualitative comparison of the test kits

Features	**Panbio JE-Dengue Combo ELISA**	**XCyton ELISA**
Vol. of sample required	10µL serum.	5 µL serum and 10 µL CSF.
No of maximum samples that can be tested	43 samples in one plate.	88 samples in one plate
Time to complete assay (single plate)	4 hours.	5 hours.
Temperature of storage	2-8^{0}C	2-8^{0}C
Steps involved	Small number of steps	Many steps.
Time interval between steps	Up to one hour incubation period	Short time period between steps.

Advantage	Diagnosis of JE and Dengue possible at Same dilution for both tests	Easy procedure.
Disadvantage	Reagent preparation required. Maximum attention required for the addition of reagent in to the respective JE and Dengue wells.	Preparation of substrate to be done only in provided tube. Reconstitution of streptavidin peroxidase conjugate is cumbersome. JE and dengue cannot be tested at once.

CHAPTER VI

6. DISCUSSION AND CONCLUSION

6.1 DISCUSSION

JE is caused by Japanese encephalitis virus (JEV). JEV is a member of family Flaviviridae, genus *Flavivirus*, transmitted mainly by *Culex* mosquitoes. Except JEV, the Japanese encephalitis virus serocomplex of the family Flaviviridae includes West Nile virus (WNV), Saint Louis encephalitis virus (SLEV) and Murray Valley encephalitis virus (MVEV) (Kuno, 2003).

For surveillance and clinical case management, a high specificity is desirable in JE IgM assay. A false- positive result in the JEV IgM assay may result in the clinician discontinuing further diagnostic testing and not considering management options for other treatable causes of AES such as cerebral malaria and tuberculous meningitis. In contrast, a clinician may still consider JEV infection based on clinical symptoms and timing of specimen collection, despite false – negative result (Ravi et al., 2010).

In many countries where JE is endemic there is almost no systematic, population-based routine surveillance for the disease. Where surveillance does exist, the quality of the system is variable, depending on multiple factors, including timely care-seeking behavior, recognition of symptoms, case finding and reporting, sample collection at the optimal time, and perhaps most importantly, the collection and laboratory testing of appropriate clinical samples because of lack of test reagents. It is absolutely essential, therefore, that easy-to-use, standardized, affordable test kits are available in order to strengthen JE diagnostic and surveillance capacity in resource-poor, endemic settings (Khalikdina et al., 2010)

During the study, a total of 580 samples were used for the evaluation of the kits. Of the 580 samples, 251were serum and the remaining 329 were CSF. All the serum samples were tested using the Panbio kit. Similarly all the CSF samples were tested using the XCyton kit. 60 CSF samples and 63 serum samples were also tested using the Panbio and the XCyton kit respectively. AFRIMS Kit developed was used as the reference ELISA kit. This reference ELISA is the diagnostic method applied in the National Referral Laboratory, NPHL, Nepal.

On testing a total of 311 samples containing 60 CSF and 251 serum using Panbio the sensitivity , specificity, PVP, PVN, efficiency of the kit was found to be 63.4%, 85.4%, 74.7%, 77.4%, and 76.5% respectively. These values obtained using the serum samples only was found to be a little lower as observed with that for both samples. For 251 serum samples the data obtained were 59.3%, 84.5%, and 70.3%, 77%, 74.9% respectively for the sensitivity, specificity, PVP, PVN, efficiency. Similarly the data obtained for the Panbio kit using the CSF sample was 76.6%, 90%, 88.4%, 79.4% and 83.3% respectively for the sensitivity, specificity, PVP, PVN, efficiency. The data obtained for CSF showed that the kit had better performance using the CSF sample although the kit is intended to be tested for the serum samples. The cut off value provided for the serum sample was also used for the CSF samples this might have increased the values for the CSF. More over the performance was assayed using the few CSF samples this might have led to the increased values. The specificity of the kit was above 80% when tested for combined samples types carried out for individual samples but the sensitivity values were a little low. The obtained data for the Panbio kit indicate that the kit has greater ability to correctly distinguish the negative results as negative than its ability to distinguish the proportion of unhealthy individuals. The increased false positive result by this test could result in the misdiagnosis of the patient and thus the treatment. However the test was statistically significant for the values of chi-square test obtained ($P< 0.05$) thus it could be used for the diagnosis purpose.

In the evaluation of the another commercial MAC ELISA i.e. the XCyton JEV CheX using 329 CSF and 63 serum samples the overall performance values obtained were 84.8%, 97%, 92.9%, 93.1% and 93.1% respectively for sensitivity , specificity, PVP, PVN, efficiency. These values using 329 CSF only were obtained as 85.26%, 96.58%, 91 %, 94.1%, and 93.3% respectively and those using 63 serum only were 83.3%, 100%, 100%, 86.5%, and 92 % respectively. The values obtained were good with both samples types. The sensitivity values ranged from 83% to 85% while the specificity values ranged from 95% to 100%, the PVP, PVN, values obtained were also significant ranging from (91-100) %. The efficiency of the kit was found to be greater than 92% for any sample type. Thus this test could be used for the diagnosis of both the serum and the CSF of the AES patients. The kit showed greater performance values with CSF than that of the serum; however the performance values as obtained

with the serum were also good. Statistically the values obtained using the XCyton kits were highly significant with p values of less than 0.05

From the study it was observed that the performance characteristics for the XCyton kit were better than that of the Panbio kit. The XCyton kit showed much improved performance values in comparison to the values for the Panbio. Though the antigen type in the Panbio is not known, the improved values in case of the XCyton kit can be attributed to the antigen type used in the kit. The kit makes use of the cell derived antigen of the Indian prototypes of the JEV. It can be assumed that the prototypes in Nepal can be closely related those in India and hence better results for the XCyton kit.

Isolation of JEV is not a sensitive method of laboratory diagnosis in clinical specimens because the low-level, transient viremia is cleared soon after onset of illness. In contrast, anti- JEV immunoglobulin M (IgM) is produced soon after infection and is detectable in 90% of cases in cerebrospinal fluid (CSF) by 4 days and in serum by 7–9 days following the development of clinical illness (Burke et al 1985; Chanama et al., 2005; Solomon, 2003). The JEV-specific IgM antibody capture enzyme-linked immunosorbant assay (MAC ELISA) has become the first-line serological assay recommended by the World Health Organization (WHO) to diagnose acute JEV infections (WHO, 2007).

An ideal field-based diagnostic test should meet the ASSURED criteria: affordable to diagnostic laboratories with limited resources, sensitive, specific, user-friendly, rapid, equipment- free, and delivered to those who need it. The JEV IgM ELISA format has the potential to fulfill most of these criteria. The JEV IgM ELISA is relatively sensitive, with detection of JEV IgM in 95% of patients within 5 days of illness onset (Robinson et, al., 2010).

Evaluation of kits has been done from time to time. It is essential to carry out the evaluation for better diagnosis of the diseases condition and implication of appropriate prevention strategies. In a study carried out by Lewthwaite et al for the evaluation of the Panbio and the XCyton kit ; the sensitivity and specificity values obtained for the XCyton kit using the CSF samples were 77.8 % and 97.3% respectively. Their findings correlated well with the present study in case of the specificity while the sensitivity was higher than in present study. Similarly the

sensitivity and specificity for serum samples for Panbio kit was 72.5% and 97.5% which were both higher than in the present study.

Both the kits are based on a standard ELISA format and results calculated, although the reagents and components vary among them. The Panbio kit contains JE and DEN recombinant antigens produced in an insect cell expression system. Whether the antigen is a complete envelope protein or not is proprietary information; however, antigen conformation has been shown to affect reactivity to IgM. The DENV and JEV Mab conjugates used in the Panbio kit also have not been disclosed, but the antigenic sites that they recognize and their avidity may factor into the sensitivity of the assay. The XCyton JEV CheX kit contains inactivated cell-culture JEV antigen from the JEV Indian prototype (NIVP20778, 1956, Vellore, India). The Mab conjugate used in the XCyton kit was developed against the Indian prototype as well. Thus, the XCyton kit might be expected to have the highest sensitivity in this group of specimens from India and surrounding nations, as the JEV Indian prototype is probably antigenically the closest to the JEV strain presently circulating there. The reactivity of the JEV Mab has not been determined and it is possible that the Mab has high specificity but low avidity for the JE antigen, which could account for the low sensitivity.

Using AFRIMS ELISA as the reference method the evaluation of these two kits was also done previously in NPHL where the sensitivity values for the Panbio were found to be ranging from 71-80% and the specificity values ranging from 95-97% and these values obtained for the XCyton kits were 93% and 89% (Khalakdina et .al., 2010). The values for the XCyton were comparable with the present study while the values for the Panbio were much higher.

The values obtained by Jacobson et al in 2007 using the serum sample to find the performance characteristic of the Panbio, XCyton, had shown that the sensitivity and the specificity values for Panbio were above the values obtained in this study while the specificity values correlated well with this study. However in their study also the performance of XCyton was greater than that of the Panbio as has been found in the present study.

The values obtained for both the kits using the CSF samples correlate well with the findings by Ravi et al in2010. The sensitivity and the specificity values of 76.6%, 90% respectively for Panbio and 85.2, 96.5% respectively for XCyton in this study are similar to the values of 65 to 80% sensitivity of Panbio in their study but the specificity value of 95% in their study is greater than the present finding. However for XCyton the specificity values are close i.e. 97.5% in their study while the sensitivity calculated here is less than calculated by them. Cuzzoboo et. al., in 1999 also had evaluated the Panbio kit using serum however their values were higher than obtained here.

Very low sensitivity values for the Panbio and XCyton using the serum and CSF samples were obtained by Robinson et al in 2010. Their values for sensitivity were very low in comparison to the present study. They found the sensitivity of the kits ranging from 17-53% for CSF samples and 20-57% for the serum samples. The Panbio kit had overall sensitivity of 33%(20% for CSF and 39% for serum)and the sensitivity of the XCyton was 19%(17% for CSF and 20% for serum).however the kits had excellent specificity values of 98.8%(99.5% for CSF and 98.2% for serum)for Panbio and 97.2% (97.4% for CSF and 97.0% for serum) for XCyton. On comparing their finding s with this study, sensitivity value calculated in this study is greater than their study; while the specificity value for Panbio is less than that calculated by them in case of Panbio while that for XCyton is almost similar.

Cross reactivity study against the dengue positive serum samples showed that the Panbio had lower cross reactivity of 4% in comparison to 32% for the XCyton kit. This indicates that the XCyton kit may give false data if JE and dengue co-circulate in some endemic regions. The cross reactivity among the different flavivirus is due to the sharing of the common envelope protein epitope. Dengue virus and the JEV virus share some antigens in common and the antibodies raised against the epitope of one virus type reacts with the epitope of another virus type showing certain degree of cross reactivity.

Although both sensitivity and specificity are important, one must consider the scope of testing to be done. In diagnostic and surveillance activities, specificity of assays is of primary importance as some etiologies of meningo-encephalitis have available treatment and/ or vaccines for prevention. If assays have low specificity and samples

are falsely identified as JEV infections there are several potential consequences. These consequences may arise because of a lack of further testing after the JEV diagnosis is made. This would preclude patients infected with treatable diseases, such as bacterial meningitis, from receiving needed treatment. In addition, false diagnosis of JEV can cause overestimation of disease burden leading to extensive and possibly ineffective vaccination campaigns, which can be quite costly and may put unnecessary burdens on already weak public health systems

Sensitivity of the assay can also present a problem in patients with encephalitis who present to the hospital soon after onset of illness, which may be before the rise of the neutralizing antibodies, or even IgM, to detectable levels. The IgM ELISA may have low sensitivity in this population of acutely ill patients.

6.2 CONCLUSION

Out of 580 total samples tested using two commercial MAC ELISA the performance values for the XCyton ELISA was found to be better in comparison to that of the Panbio ELISA. The values of sensitivity, specificity, PVP, PVN, and efficiency of the Panbio kit using both the serum and the CSF sample was found to be was 63.4%, 85.4%, 74.7%, 77.4%, and 76.5% respectively. These values obtained using the serum samples only was found to be a little lower as observed with that of both samples. For 251 serum samples the data obtained were 59.3%, 84.5%, and 70.3%, 77% 74.9% respectively for the sensitivity, specificity, PVP, PVN, efficiency. Similarly the data obtained for the Panbio kit using the CSF sample was 76.6%, 90%, 88.4%, 79.4% and 83.3% respectively for the sensitivity, specificity, PVP, PVN, efficiency. For XCyton JEV CheX using 329 CSF and 63 serum samples the overall performance values obtained were 84.8%, 97%, 92.9%, 93.16% and 93.1% respectively for sensitivity, specificity, PVP, PVN, efficiency. These values using 329 CSF only were obtained as 85.2%, 96.5%, 91%, 94.1%, and 93.3% respectively and those using 63 serum only were 83.3%, 100%, 100%, 86.5%, and 92% respectively. The percentage cross reactivity against dengue positive serum samples was higher in XCyton (32%) than for the Panbio kit (4%).In all the performance of XCyton was found better than that of Panbio ELISA kit. As for both kits at 5% level of signifance for the chi-square test the p value was found to be below 0.05 both kits could; however be used for the diagnosis purpose

CHAPTER VI

6. DISCUSSION AND CONCLUSION

6.1 DISCUSSION

JE is caused by Japanese encephalitis virus (JEV). JEV is a member of family Flaviviridae, genus *Flavivirus*, transmitted mainly by *Culex* mosquitoes. Except JEV, the Japanese encephalitis virus serocomplex of the family Flaviviridae includes West Nile virus (WNV), Saint Louis encephalitis virus (SLEV) and Murray Valley encephalitis virus (MVEV) (Kuno, 2003).

For surveillance and clinical case management, a high specificity is desirable in JE IgM assay. A false- positive result in the JEV IgM assay may result in the clinician discontinuing further diagnostic testing and not considering management options for other treatable causes of AES such as cerebral malaria and tuberculous meningitis. In contrast, a clinician may still consider JEV infection based on clinical symptoms and timing of specimen collection, despite false – negative result (Ravi et al., 2010).

In many countries where JE is endemic there is almost no systematic, population-based routine surveillance for the disease. Where surveillance does exist, the quality of the system is variable, depending on multiple factors, including timely care-seeking behavior, recognition of symptoms, case finding and reporting, sample collection at the optimal time, and perhaps most importantly, the collection and laboratory testing of appropriate clinical samples because of lack of test reagents. It is absolutely essential, therefore, that easy-to-use, standardized, affordable test kits are available in order to strengthen JE diagnostic and surveillance capacity in resource-poor, endemic settings (Khalikdina et al., 2010)

During the study, a total of 580 samples were used for the evaluation of the kits. Of the 580 samples, 251were serum and the remaining 329 were CSF. All the serum samples were tested using the Panbio kit. Similarly all the CSF samples were tested using the XCyton kit. 60 CSF samples and 63 serum samples were also tested using the Panbio and the XCyton kit respectively. AFRIMS Kit developed was used as the reference ELISA kit. This reference ELISA is the diagnostic method applied in the National Referral Laboratory, NPHL, Nepal.

On testing a total of 311 samples containing 60 CSF and 251 serum using Panbio the sensitivity , specificity, PVP, PVN, efficiency of the kit was found to be 63.4%, 85.4%, 74.7%, 77.4%, and 76.5% respectively. These values obtained using the serum samples only was found to be a little lower as observed with that for both samples. For 251 serum samples the data obtained were 59.3%, 84.5%, and 70.3%, 77%, 74.9% respectively for the sensitivity, specificity, PVP, PVN, efficiency. Similarly the data obtained for the Panbio kit using the CSF sample was 76.6%, 90%, 88.4%, 79.4% and 83.3% respectively for the sensitivity, specificity, PVP, PVN, efficiency. The data obtained for CSF showed that the kit had better performance using the CSF sample although the kit is intended to be tested for the serum samples. The cut off value provided for the serum sample was also used for the CSF samples this might have increased the values for the CSF. More over the performance was assayed using the few CSF samples this might have led to the increased values. The specificity of the kit was above 80% when tested for combined samples types carried out for individual samples but the sensitivity values were a little low. The obtained data for the Panbio kit indicate that the kit has greater ability to correctly distinguish the negative results as negative than its ability to distinguish the proportion of unhealthy individuals. The increased false positive result by this test could result in the misdiagnosis of the patient and thus the treatment. However the test was statistically significant for the values of chi-square test obtained ($P< 0.05$) thus it could be used for the diagnosis purpose.

In the evaluation of the another commercial MAC ELISA i.e. the XCyton JEV CheX using 329 CSF and 63 serum samples the overall performance values obtained were 84.8%, 97%, 92.9%, 93.1% and 93.1% respectively for sensitivity , specificity, PVP, PVN, efficiency. These values using 329 CSF only were obtained as 85.26%, 96.58%, 91 %, 94.1%, and 93.3% respectively and those using 63 serum only were 83.3%, 100%, 100%, 86.5%, and 92 % respectively. The values obtained were good with both samples types. The sensitivity values ranged from 83% to 85% while the specificity values ranged from 95% to 100%, the PVP, PVN, values obtained were also significant ranging from (91-100) %. The efficiency of the kit was found to be greater than 92% for any sample type. Thus this test could be used for the diagnosis of both the serum and the CSF of the AES patients. The kit showed greater performance values with CSF than that of the serum; however the performance values as obtained

with the serum were also good. Statistically the values obtained using the XCyton kits were highly significant with p values of less than 0.05

From the study it was observed that the performance characteristics for the XCyton kit were better than that of the Panbio kit. The XCyton kit showed much improved performance values in comparison to the values for the Panbio. Though the antigen type in the Panbio is not known, the improved values in case of the XCyton kit can be attributed to the antigen type used in the kit. The kit makes use of the cell derived antigen of the Indian prototypes of the JEV. It can be assumed that the prototypes in Nepal can be closely related those in India and hence better results for the XCyton kit.

Isolation of JEV is not a sensitive method of laboratory diagnosis in clinical specimens because the low-level, transient viremia is cleared soon after onset of illness. In contrast, anti- JEV immunoglobulin M (IgM) is produced soon after infection and is detectable in 90% of cases in cerebrospinal fluid (CSF) by 4 days and in serum by 7–9 days following the development of clinical illness (Burke et al 1985; Chanama et al., 2005; Solomon, 2003). The JEV-specific IgM antibody capture enzyme-linked immunosorbant assay (MAC ELISA) has become the first-line serological assay recommended by the World Health Organization (WHO) to diagnose acute JEV infections (WHO, 2007).

An ideal field-based diagnostic test should meet the ASSURED criteria: affordable to diagnostic laboratories with limited resources, sensitive, specific, user-friendly, rapid, equipment- free, and delivered to those who need it. The JEV IgM ELISA format has the potential to fulfill most of these criteria. The JEV IgM ELISA is relatively sensitive, with detection of JEV IgM in 95% of patients within 5 days of illness onset (Robinson et, al., 2010).

Evaluation of kits has been done from time to time. It is essential to carry out the evaluation for better diagnosis of the diseases condition and implication of appropriate prevention strategies. In a study carried out by Lewthwaite et al for the evaluation of the Panbio and the XCyton kit ; the sensitivity and specificity values obtained for the XCyton kit using the CSF samples were 77.8 % and 97.3% respectively. Their findings correlated well with the present study in case of the specificity while the sensitivity was higher than in present study. Similarly the

sensitivity and specificity for serum samples for Panbio kit was 72.5% and 97.5% which were both higher than in the present study.

Both the kits are based on a standard ELISA format and results calculated, although the reagents and components vary among them. The Panbio kit contains JE and DEN recombinant antigens produced in an insect cell expression system. Whether the antigen is a complete envelope protein or not is proprietary information; however, antigen conformation has been shown to affect reactivity to IgM. The DENV and JEV Mab conjugates used in the Panbio kit also have not been disclosed, but the antigenic sites that they recognize and their avidity may factor into the sensitivity of the assay. The XCyton JEV CheX kit contains inactivated cell-culture JEV antigen from the JEV Indian prototype (NIVP20778, 1956, Vellore, India). The Mab conjugate used in the XCyton kit was developed against the Indian prototype as well. Thus, the XCyton kit might be expected to have the highest sensitivity in this group of specimens from India and surrounding nations, as the JEV Indian prototype is probably antigenically the closest to the JEV strain presently circulating there. The reactivity of the JEV Mab has not been determined and it is possible that the Mab has high specificity but low avidity for the JE antigen, which could account for the low sensitivity.

Using AFRIMS ELISA as the reference method the evaluation of these two kits was also done previously in NPHL where the sensitivity values for the Panbio were found to be ranging from 71-80% and the specificity values ranging from 95-97% and these values obtained for the XCyton kits were 93% and 89% (Khalakdina et .al., 2010). The values for the XCyton were comparable with the present study while the values for the Panbio were much higher.

The values obtained by Jacobson et al in 2007 using the serum sample to find the performance characteristic of the Panbio, XCyton, had shown that the sensitivity and the specificity values for Panbio were above the values obtained in this study while the specificity values correlated well with this study. However in their study also the performance of XCyton was greater than that of the Panbio as has been found in the present study.

The values obtained for both the kits using the CSF samples correlate well with the findings by Ravi et al in2010. The sensitivity and the specificity values of 76.6%, 90% respectively for Panbio and 85.2, 96.5% respectively for XCyton in this study are similar to the values of 65 to 80% sensitivity of Panbio in their study but the specificity value of 95% in their study is greater than the present finding. However for XCyton the specificity values are close i.e. 97.5% in their study while the sensitivity calculated here is less than calculated by them. Cuzzoboo et. al., in 1999 also had evaluated the Panbio kit using serum however their values were higher than obtained here.

Very low sensitivity values for the Panbio and XCyton using the serum and CSF samples were obtained by Robinson et al in 2010. Their values for sensitivity were very low in comparison to the present study. They found the sensitivity of the kits ranging from 17-53% for CSF samples and 20-57% for the serum samples. The Panbio kit had overall sensitivity of 33%(20% for CSF and 39% for serum)and the sensitivity of the XCyton was 19%(17% for CSF and 20% for serum).however the kits had excellent specificity values of 98.8%(99.5% for CSF and 98.2% for serum)for Panbio and 97.2% (97.4% for CSF and 97.0% for serum) for XCyton. On comparing their finding s with this study, sensitivity value calculated in this study is greater than their study; while the specificity value for Panbio is less than that calculated by them in case of Panbio while that for XCyton is almost similar.

Cross reactivity study against the dengue positive serum samples showed that the Panbio had lower cross reactivity of 4% in comparison to 32% for the XCyton kit. This indicates that the XCyton kit may give false data if JE and dengue co-circulate in some endemic regions. The cross reactivity among the different flavivirus is due to the sharing of the common envelope protein epitope. Dengue virus and the JEV virus share some antigens in common and the antibodies raised against the epitope of one virus type reacts with the epitope of another virus type showing certain degree of cross reactivity.

Although both sensitivity and specificity are important, one must consider the scope of testing to be done. In diagnostic and surveillance activities, specificity of assays is of primary importance as some etiologies of meningo-encephalitis have available treatment and/ or vaccines for prevention. If assays have low specificity and samples

are falsely identified as JEV infections there are several potential consequences. These consequences may arise because of a lack of further testing after the JEV diagnosis is made. This would preclude patients infected with treatable diseases, such as bacterial meningitis, from receiving needed treatment. In addition, false diagnosis of JEV can cause overestimation of disease burden leading to extensive and possibly ineffective vaccination campaigns, which can be quite costly and may put unnecessary burdens on already weak public health systems

Sensitivity of the assay can also present a problem in patients with encephalitis who present to the hospital soon after onset of illness, which may be before the rise of the neutralizing antibodies, or even IgM, to detectable levels. The IgM ELISA may have low sensitivity in this population of acutely ill patients.

6.2 CONCLUSION

Out of 580 total samples tested using two commercial MAC ELISA the performance values for the XCyton ELISA was found to be better in comparison to that of the Panbio ELISA. The values of sensitivity, specificity, PVP, PVN, and efficiency of the Panbio kit using both the serum and the CSF sample was found to be was 63.4%, 85.4%, 74.7%, 77.4%, and 76.5% respectively. These values obtained using the serum samples only was found to be a little lower as observed with that of both samples. For 251 serum samples the data obtained were 59.3%, 84.5%, and 70.3%, 77% 74.9% respectively for the sensitivity, specificity, PVP, PVN, efficiency. Similarly the data obtained for the Panbio kit using the CSF sample was 76.6%, 90%, 88.4%, 79.4% and 83.3% respectively for the sensitivity, specificity, PVP, PVN, efficiency. For XCyton JEV CheX using 329 CSF and 63 serum samples the overall performance values obtained were 84.8%, 97%, 92.9%, 93.16% and 93.1% respectively for sensitivity, specificity, PVP, PVN, efficiency. These values using 329 CSF only were obtained as 85.2%, 96.5%, 91%, 94.1%, and 93.3% respectively and those using 63 serum only were 83.3%, 100%, 100%, 86.5%, and 92% respectively. The percentage cross reactivity against dengue positive serum samples was higher in XCyton (32%) than for the Panbio kit (4%).In all the performance of XCyton was found better than that of Panbio ELISA kit. As for both kits at 5% level of signifance for the chi-square test the p value was found to be below 0.05 both kits could; however be used for the diagnosis purpose

CHAPTER VIII

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APPENDIX I

MATERIALS

Equipments, reagents, chemicals and other supplies available at NPHL were used during the study period.

A. Equipments

Analytical balance (Kroy and Company)

Automatic ELISA plate washer (Stat Fax 2600)

Centrifuge (Gemmy Industrial Corp., PLC Series)

Digital Electric balance (Scalteec, 0.00001g readability)

Distillation plant (Aquatron, A 4000 D)

ELISA plate reader (Humareader, Human, Germany)

Freezer,-20^{o}C (Thermo Elecronic Corporation)

Freezer,-70^{o}C (EURO WARM, Mod.1-5 408.000MKDK)

Hot air oven (Memmert Germany)

Incubator (Sakura, Tokyo, Japan)

pH metre

Printer (EPSON)

Refrigerator (2-8 ^{o}C) (Videocon, India)

Stirrer (HANNA Instruments, HI 322)

Timer (United Kingdom)

Vortex mixture (Germany Industrial Corp., VM 300)

Water bath (Narang Scientific Works Pvt. Ltd., India)

B. Supplies

Adjustable pipette filler and dispenser

Aluminium foils (Hindalco, India)

Beakers (100ml, 500 ml, 1000 ml and 2000ml) (Pyrex, USA)

Buffer tank (2-4 liter capacity)

Cotton

Disposable gloves (Kanam Latex Industries, India)

Disposable Linbro/Titertek E.I.A Microtitration plate, 96flat bottom wells (uncoated) (ICN Biomedicas Inc.)

Disposable plastic pipettes (1ml, 5ml, and 10ml) (Falcon, Becton Dickinson, USA)

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

Eppendorf tube

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

Micro pipette 100-1000µl (Human, Germany)

Micro pipette 50-200 µl (Human, Germany)

Micro pipette5-50 µl (Human, Germany)

Micro pipette tips (to fit above) (Human, Germany)

Multichannel pipette (8 channels) 25-100 µl (Human, Germany)

Paper towels

Parafin (American National Can TM)

Plastic box for humified reaction chamber (15 X 30cm)

Reagent bottles (500 ml and 1000 ml)

Reagent reservoir trays

Samples vials storing boxes or Cryo- boxes (10 X 10 vials capacity)

Serum vials (2ml)

Stirring rod

Test tube racks (4 X10 holes)

Test tubes (Khan Tubes)

Tips boxes (Corning, UK)

Volumetric flasks (250ml, 500ml, 1000ml, 2000ml) (Pyrex, USA)

Washing bottles

C. Chemicals and reagents

Bovine serum albumin (BSA) Fractio V ppowder (Sigma)

Buffer solutions (pH 4, 7 and 10)

Citric acid ($C_6H_8O_7$) (Qualigens Fine Chemicals, India)

Concentrated sulphuric acid (H_2SO_4) (Qualigens Fine Chemicals, India)

Distilled water

Goat anti- human IgM (KPL USA)

Human anti-flavivirus IgG-HRP (Horse Redox Perocidase) conjugate

Hydrogen peroxide (H_2O_2) (Qualigens Fine Chemicals, India)

JE antigens

JE negative control (NHS)

Normal Human Serum (NHS)

OPD (O-phenylenediamine dihydrochloride) granules

Potassium chloride (KCl) (Hi Media Laboratories Ltd., India)

Potassium phosphate monobasic anhydrous (KH_2PO_4) (Hi Media Laboratories Ltd., India)

Sodium bicarbonate ($NaHCO_3$) (Qualigens Fine Chemicals, India)

Sodium carbonate ($Na_2CO_{3)}$ (Merck Ltd., India)

Sodium chloride (NaCl) (Hi Media Laboratories Ltd., India)

Sodium hydroxide

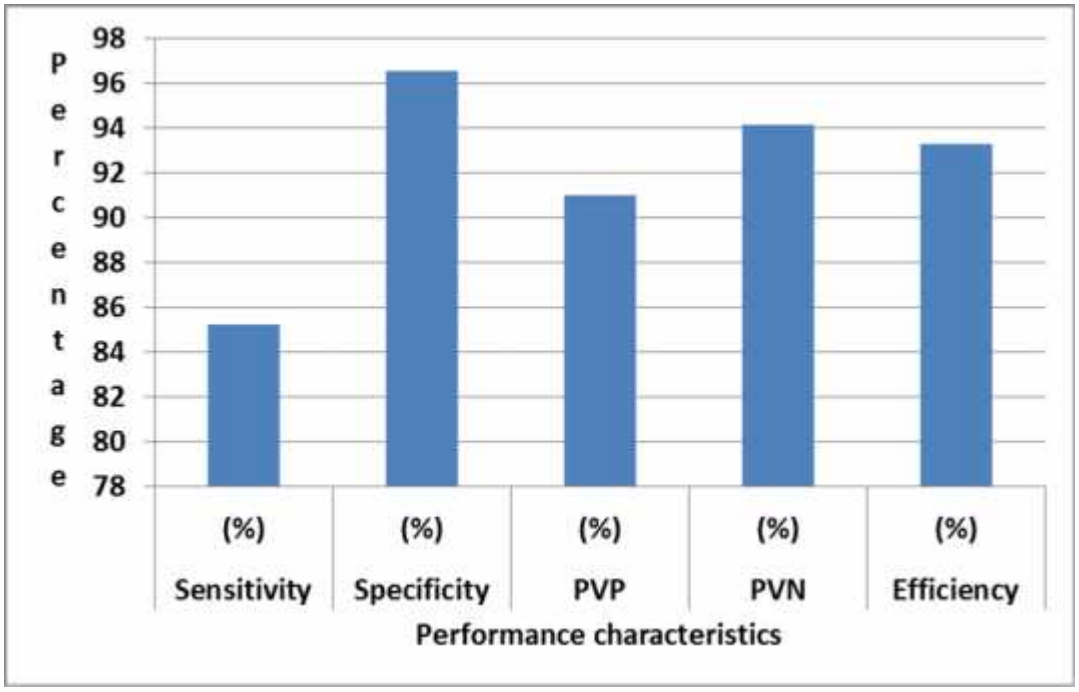
Sodium Hypochlorite

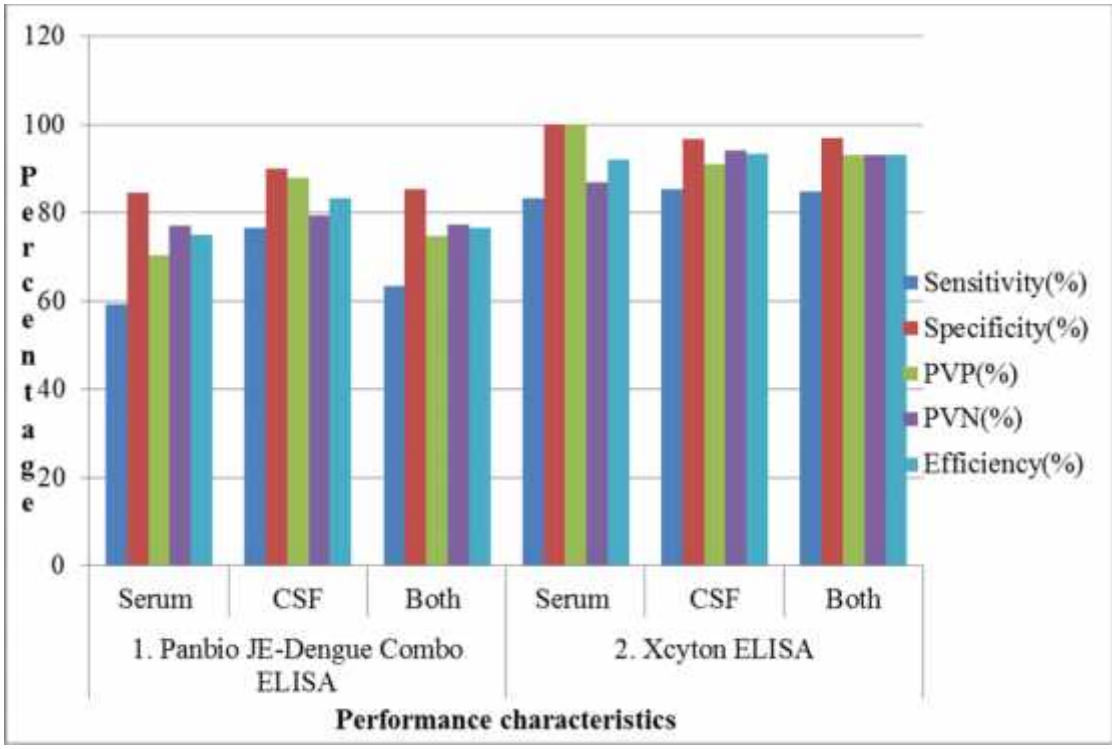
Sodium phosphate dibasic anhydrous (Na_2HPO_4) (Hopkin and William, UK)

Spirit and ethanol (Bengal Chemicals and Pharmaceuticals Ltd., India)

Tween 20 (Polyoxy- ethylene- sorban-monolaurate) (Loba Cheme Pvt. Ltd., India)

Weak anti-JEV IgM (Positive control)


Percentage
98
96
94
92
90
88
86
84
82
80
78
(%) Sensitivity
(%) Specificity
(%) PVP
(%) PVN
(%) Efficiency
Performance characteristics


120
100
80
60
40
20
0
Percentage
Sensitivity(%)
Specificity(%)
PVP(%)
PVN(%)
Efficiency(%)
Serum
CSF
Both
Serum
CSF
Both
1. Panbio JE-Dengue Combo ELISA
2. Xcyton ELISA
Performance characteristics