CHAPTER 1
INTRODUCTION TO THE FLAVIVIRUSES, WITH EMPHASIS ON JAPANESE ENCEPHALITIS VIRUS

INTRODUCTION

The family Flaviviridae consists of more than 73 members, most of which are important human pathogens. Using polyclonal antisera, flaviviruses have been classified into 8 antigenic complexes based on cross neutralization. The members in a particular serocomplex share common biological properties (Table 1.1). Japanese encephalitis virus (JEV) is the prototype of the JE antigenic complex.

JEV is the leading cause of viral encephalitis in Asia (1). There are 35,000-50,000 Japanese encephalitis (JE) cases reported annually in the Asian continent, mostly in children younger than 10 years, with a high case-fatality rate (25-30%) (2). Epidemics in India have been increasing since the early 1970’s (3). In 2005, JE killed more than 1200 children during an epidemic in the State of Uttar Pradesh, India, and neighbouring Nepal (4). Prophylactic vaccination appears to be the only way of controlling the outbreak of JE in endemic areas, and both live attenuated and inactivated vaccines have proven to be efficacious (5).

The ecological term ‘arbovirus’ is often used to describe the fact that JEV is arthropod borne. JEV is transmitted by mosquitoes in an enzootic cycle involving birds, particularly wading ardeids such as herons and egrets and pigs. Many mosquito species are potential vectors, but culex species such as Culex tritaeniorhynchus and C. vishnui, which breed in rice paddies and other dirty water, are especially important (6). Although many animals can be infected with the virus, only those which develop high viremia are important in the natural cycle. Birds may also be responsible for the spread of JEV to new geographical areas (7). Humans become infected when they are bitten by infected mosquitoes. Although the virus has occasionally been isolated from human peripheral blood, viremia is usually transient and titre low. Humans are therefore considered as dead end hosts from which transmissions do not occur. The ratio of apparent to inapparent infections with JEV is quite low (1:100-1:300) and most human infections are asymptomatic or result in a non-specific flu-like illness (8).

JE is mostly a disease of children and young adults. When epidemics first occurred in new locations, such as in Sri Lanka and Nepal, adults were also affected (7). Unlike smallpox and polio, for which humans are the only host and elimination by vaccination is feasible, the enzootic nature of JEV means that there is no possibility of global eradication. Further, the
The geographical area affected with JEV is expanding, and the 2.8 billion people living in affected areas will continue to be exposed to the virus (9). The development of successful vaccines with alternative strategies is therefore the need of the hour.

Table 1.1: Different viruses among the family Flaviviridae.

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<th>Cluster</th>
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<td>Non vector cluster</td>
<td>Cell Fusing Agent</td>
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<td>Apoi virus</td>
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<td>San Perlita, Jutiapa</td>
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<td></td>
<td>Montana myotis leukoencephalitis, Modoc, Cowborne Ridge, Sal Vieja</td>
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<td></td>
<td>Bukalasa bat, Dakar bat, Rio Bravo</td>
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<td>Carey Island, Phnom Penh bat, Batu cave</td>
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<td>Tick-borne cluster</td>
<td>Gadget gully, Royal Farm, Powassan, Karishi, KFD, Langat, Omsk</td>
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<td></td>
<td>hemorrhagic fever virus, Tick-borne encephalitis virus (far eastern</td>
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<td>subtype, central subtype and Russian spring summer encephalitis</td>
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<td>subtype), Nigish virus</td>
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<td>Kadam, Tyuleniy, Saumarez Reef, Meaban</td>
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<td>Mosquito-borne cluster</td>
<td>Edge Hill, Boubouï, Uganda S, Banzi, Jugra, Saboya, Potiskum</td>
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<td>Sepik, Yellow Fever virus, Sokulkuk, Entebbe bat, Yokose</td>
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<td>Dengue 1 to 4</td>
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<td>Kedougou, Zika, Spondweni</td>
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<td>St. Louis encephalitis virus, Rocio, Ilheus, Tembusu, Ntaya, Israel</td>
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<td>turkey encephalitis, Bagaza, Naranjal, Bussuquara, Aroa, Iguape</td>
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<td>Kokobera, Stratford, Cacipacore, Yaounde, Koutango, Kunjin, West</td>
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**FLAVIVIRUS STRUCTURE**

Flavivirus virions are spherical in shape and small, approximately 500Å in diameter. They contain a positive sense, single stranded RNA genome that is surrounded by an electron-dense nucleocapsid, which is ~30 nm in diameter and predicted to have T=3 icosahedral symmetry (10). The viral capsid protein is further enclosed in a host cell-derived lipid bilayer surrounded by 180 copies of two virally encoded type I membrane proteins: envelope (E) and membrane (M). Intracellular virions contain unprocessed prM protein, which is cleaved to the more mature M protein on release from the infected cell (11).

The ~10.8 kb viral genome serves as the messenger RNA (mRNA) for translation of all viral proteins, a template during RNA replication, and genetic material packaged within new virus particles. It has one open reading frame (ORF) encoding a single polyprotein that...
has a type I cap structure at its 5’ terminus (m7GpppAmp) (12). Additional methylation of the N2 residue (type II cap) has also been detected in RNA from infected cells (13). RNA from both mosquito-borne and tick-borne flaviviruses lacks a poly (A) tail and contains conserved dinucleotide sequences at the 5’ (AG) and 3’ (CU) termini. The flavivirus ORF is flanked by 5’ and 3’ non-coding regions (NCR) of ~100 nucleotides (ntds) and 400 to 700 ntds, respectively. The sequence of the 5’ NCR is not well conserved between different flaviviruses, although common secondary structures have been found within this region. These structures influence the transcription and/or translation of the viral genome (14).

Figure 1.1: Flavivirus genome structure

Flaviviruses enter host cells by receptor-mediated endocytosis via cellular receptors specific for the viral E protein. The acidic environment of the endosome triggers an irreversible trimerization of the E protein that results in fusion of the viral and cell membranes. After fusion occurs, the nucleocapsid is released into the cytoplasm, the capsid (C) protein and RNA dissociate, and replication of the RNA genome is initiated (15, 16). RNA replication occurs in cytoplasmic replication complexes that are associated with perinuclear membranes. Replication begins with the synthesis of a genome-length minus strand RNA, which then serves as a template for the synthesis of additional plus strand RNA. Replication is asymmetric, with plus strand RNA being produced in excess to minus strand RNA.

Translation is cap dependent and initiates by ribosomal scanning. As most flaviviruses lack a Kozak motif, initiation at the correct AUG may occur by different means, such as secondary structures to enable ribosome pausing at the correct site (17). Translation of the single, long ORF produces a large polyprotein that is co- and post-translationally cleaved into at least 10 proteins. At the amino terminus are the three structural proteins- C, M, and E - that constitute the virus particle. Seven non-structural proteins that are essential for viral replication are encoded by the remainder of the genome. Cleavages between the structural proteins are caused by a host signal peptidase while a virus encoded serine protease is
responsible for cleavages between the non-structural protein junctions (18, 19). The enzyme responsible for NS1-2A cleavage is presently unknown.

After the appropriate proteolytic cleavages, the C protein and viral RNA are localized in the cytoplasm though the C protein remains associated with the endoplasmic reticulum (ER) membrane. The nucleocapsid acquires an envelope by budding into the ER lumen. On the luminal side of the ER, the prM and E proteins form a stable heterodimer within a few minutes of translation. The process of virion assembly is rapid. Progeny virions are thought to assemble by budding into an intracellular membrane compartment, most likely the ER, then transited through the host secretory pathway and released at the cell surface (20).

Initially, immature particles are formed in the lumen of the ER. Subsequently, cleavage of prM occurs in the trans-Golgi network, creating mature, infectious particles. Cleavage of prM by furin and release renders the mature virion ready for acid-catalyzed rearrangements required for productive entry into the host cell.

**Features of the Structural Proteins**

**C Protein**

The C protein is a highly basic protein of ~11 kd (120 amino acids). Charged residues are clustered at the N- and C-termini, separated by an internal hydrophobic region that mediates membrane association (21). The nascent C protein also contains a hydrophobic signal sequence at its carboxyl terminus that translocates prM into the lumen of the ER from its site of synthesis on the surface of the ER. This hydrophobic domain is cleaved from mature C by the viral serine protease. The C protein folds into a compact dimer with each monomer containing four alpha helices that are connected by short loops (22). Helices $\alpha_2$ and $\alpha_4$ of one monomer are anti-parallel to helices $\alpha_2$ and $\alpha_4$ of the adjoining monomer, respectively. It is not yet clear how C protein dimers are organized within nucleocapsids, but interaction with RNA or DNA can induce isolated C protein dimers to assemble into nucleocapsid like particles. It is possible that the rather basic C protein functions like a histone.

**prM/M Glycoprotein**

The glycoprotein precursor of M protein, prM contains two transmembrane helices. It is ~26 kd (165 amino acids) in size, before it is cleaved to yield the pr peptide and the M protein (~75 amino acids). The prM protein might function as a chaperone for folding and assembly of the E protein (23). A major function is to prevent E from undergoing acid-catalyzed rearrangement to the fusogenic form during transit through the secretory pathway.
prM cleavage is delayed until late in infection and coincides with the conversion of immature virus particles to mature virions (24). Following cleavage, prM-E heterodimers dissociate, the pr fragment is released, and E homodimers form.

**E Glycoprotein**

The E glycoprotein is approximately 495 amino acids in size (~53 kd). It is the major protein on the surface of flavivirus virions and contains a cellular receptor-binding site and a fusion peptide. E is synthesized as a type I membrane protein containing 12 conserved cysteines that form disulfide bonds (25). The native form of E folds into an elongated structure rich in β-sheets and form head-to-tail homodimers that lie parallel with the virus envelope. Studies with monoclonal antibodies in the late 1980s suggested three antigenic domains (26), and these were confirmed more recently when the three dimensional structure of the ectodomain (residues 1-395) of the flavivirus tick-borne encephalitis virus (TBEV) was determined by x-ray crystallography (27). The central structural domain I, which contains the N-terminus, is flanked on one side by an elongated dimerization domain (domain II), which projects along the virus surface between the transmembrane regions of the homodimer subunits and contains the fusion peptide at its distal end. On the other side of domain I is domain III, which maintains an immunoglobulin-like fold. Domain III appears to be involved in receptor binding and is a major target of neutralizing antibodies. In mosquito-borne flaviviruses, domain III contains an RGD/RGE sequence, which is the recognition motif for integrin binding (28). The ectodomain of the E protein comprises sets of three parallel dimers that form 30 rafts over the viral surface. During maturation, 60 trimers of prM-E heterodimers that project from the virus surface dissociate and form 90 E homodimers, which lie flat on the virus surface, giving the mature virion its spikeless and smooth surface (16).

**Non-structural (NS) 1 protein**

The NS1 glycoprotein (~46 kd) is translocated into the ER during synthesis and cleaved from E protein by host signal peptidase (29). NS1 is largely retained within infected cells but can localize to the cell surface and is slowly secreted from mammalian cells. The protein contains two or three N-linked glycosylation sites and 12 conserved cysteines that form disulfide bonds. Around 30 minutes after synthesis, NS1 forms highly stable homodimers and acquires an affinity for membranes. NS1 localizes to sites of RNA replication and mutation of the N-linked glycosylation sites in NS1 can lead to dramatic defects in RNA replication and virus production. It elicits a humoral and cellular immune
response in humans and experimental animals, and antibodies against the cell surface form can direct complement-mediated lysis of virus-infected cells (30).

**NS2A and NS2B Proteins**

NS2A is a relatively small (~22 kd) hydrophobic protein. Its N-terminus is generated via NS1–2A cleavage by an unknown ER-resident host enzyme. NS2A is believed to be involved in virus assembly. Dengue virus (DENV) -2 NS2A has also been shown to act as an interferon (IFN) antagonist by inhibiting IFN signalling and specific mutations in Kunjin virus (KUNV) and West Nile virus (WNV) NS2A have been identified that diminish this inhibitory activity and attenuate WNV virulence in mice (31-33).

NS2B is also a small (~14 kd) membrane-associated protein. NS2B forms a stable complex with NS3 and acts as a cofactor for the NS2B-NS3 serine protease (34). The cofactor activity lies in a central peptide that intercalates within the fold of the serine protease domain.

**NS3 Protein**

NS3 is a large (~70 kd) multifunctional protein, containing several activities required for polyprotein processing and RNA replication. The N-terminal third of the protein is the catalytic domain of the NS2B-NS3 serine protease complex (35). In addition to cleaving the NS2A/NS2B, NS2B/NS3, NS3/NS4A, and NS4B/NS5 junctions, the protease generates the C-termini of mature C protein and NS4A, and can cleave at internal sites within NS2A and NS3. RNA-stimulated nucleoside triphosphatase (NTPase) activity and RNA unwinding activity have been demonstrated for flavivirus NS3 proteins (36). The flavivirus NS3 protein also encodes an RNA triphosphatase activity (RTPase) proposed to dephosphorylate the 5’ end of genome RNA before cap addition. All three nucleic acid-modifying activities of NS3 (NTPase, helicase, and RTPase) rely on a common active centre. The NS3 proteins of Langat, DENV-2, and WNV have been shown to induce apoptosis, in some cases through activation of caspase-8 (37-39). Although passive protection in mice by a monoclonal antibody against the non-structural protein NS3 of DENV-1 has been reported, the primary immunologic role of the non-structural proteins, with the exception of NS1, appears to be their function as targets for cytotoxic T cells.

**NS4A and NS4B Proteins**

The NS4A (~16 kd) and NS4B (~27 kd) are small, hydrophobic proteins. A role for NS4A in RNA replication is supported by the co-localization of this protein with replication
complexes (40). Over-expression studies show that regulated NS4A/2K/4B cleavage is necessary for induction of membrane rearrangements by NS4A. NS4B can associate with membranes independently of the 2K signal peptide and appears to be a polytopic membrane protein. NS4B colocalizes with NS3 and viral double stranded RNA (dsRNA) in ER-derived membrane structures presumed to be sites of RNA replication. DENV NS4A and NS4B can also block type I IFN signalling. NS4B has the strongest antagonistic effect.

**NS5 Protein**

NS5 is a large (~103 kd), highly conserved, multifunctional protein with methyltransferase (MTase) and RNA dependent RNA polymerase (RdRP) activities. NS5 forms a complex with NS3 and can stimulate both the NTPase and RTPase activities of NS3 (11). NS5 has been shown to localize to the nucleus. New roles, other than in RNA replication, have recently emerged for flavivirus NS5. DENV-2 NS5 induces IL-8 transcription, and secretion, which may enhance viral spread or disease by recruiting inflammatory cells to the site of infection. In addition, TBEV NS5 blocks signalling of IFN-α/β and IFN-γ by binding to their receptors and inhibiting phosphorylation of both Janus kinases, JAK1 and Tyk2, and hence downstream activation of signal transducer and activator of transcription 1 (STAT1).

**JEV WORLD SCENARIO**

JEV has a wide distribution in Asia. Reported cases of JE range from India, Nepal, Pakistan, Sri Lanka, Bangladesh, Burma (Myanmar), Laos, Cambodia, Vietnam, Thailand, Malaysia, Brunei, Singapore, Taiwan, Philippines, Indonesia, China, maritime Siberia, Korea and Japan, to Papua New Guinea and Australia (3). Two epidemiological patterns of JE are recognized. In southern tropical areas, the disease is endemic with occurrence of sporadic cases throughout the year. In temperate zones and the subtropics, outbreaks have a marked seasonal pattern, usually occurring during the rainy season (41).

Earliest studies on JEV strain variance were reported by Chen et al. who sequenced 240 ntds from the prM gene of 58 isolates obtained from diverse geographic origins and, based on 12% ntd divergence, divided the viruses into 4 genotypes (42). The E gene has mainly been used recently for phylogenetic analysis (42-45). Uchil and Satchidanandan (2001) used 107 E gene sequences available for isolates from different geographic locations worldwide to reveal five distinct genotypes of JEV. Four of these groups broadly matched the four genotypes that were proposed earlier using the prM gene region, while a fifth genotype, containing the lone 1952 isolate from Singapore, the Muar isolate, was singled out (46).
All genotypes form a single JEV serotype, despite differences in nucleotide sequence among virus strains. Genotype I (GI) includes isolates from northern Thailand, Cambodia, and Korea, genotype II (GII) includes isolates from southern Thailand, Malaysia, Indonesia, and Northern Australia, genotype III (GIII) includes isolates from mostly temperate regions of Asia, including Japan, China, India, Nepal, Sri Lanka, Taiwan, Phillipines and Vietnam, and genotype IV (GIV) includes isolates from Indonesia, mostly those isolated in 1980-1981. Majority of the viruses studied to date fall into GIII, suggesting that this genotype has spread the most widely (41).

Of the JEV genotypes, GIV was the basal group in all trees, suggesting that it represents the most ancient lineage, which branched off before GI, II, and III. Taken together these observations suggest that JEV originated in the Indonesia-Malaysia region from an ancestral virus common to JEV and Murray Valley encephalitis virus (MVEV).
Though it had been recognized since the 1870s, the first large epidemic of JE was recognized in 1924 in Japan (47). The first isolate of JEV, and the prototype strain, was isolated in 1935 in Tokyo from a human brain and is designated the Nakayama strain. Analysis of JEV strains isolated in Tokyo and Oita using the prM region suggested that GIII was the major genotype of JEV circulating in Tokyo and Oita until 1991, while those isolated after 1994 belonged to GI showing a close genetic relationship with those from Korea and Malaysia (48).

The incidence in Japan, South Korea, and Taiwan has declined dramatically since the 1980s, due to the widespread use of vaccine in children and other preventive measures. Although the annual incidence of JE in China is also declining as a result of childhood immunization, cases are still being reported every year.

JE has been recognized in China since 1940, with the virus being isolated in 1949 (Beijing and P3 strains). The recognition of JE as a public health problem occurred in 1966, when 40,000 cases of encephalitis were reported. JEV is active in all of the provinces in China except for Tibet, Xinjiang and Qinghai provinces, and nearly 80% of globally reported cases occur in China (49). The analysis of 44 isolates from mosquito pools in Taiwan from 1983 to 1994 demonstrated the circulation of JEV GIII forming three distinct clusters (50).

Recent JEV isolates from China were divided into two genotypes, GI and GIII; GI JEV strains have been isolated in China since 1979, while GIII JEV strains have been isolated since the 1940s. JEV strains isolated in Shanghai in 2001 belonged to GI and those isolated in Fujian Province in 2002 belonged to GIII (49, 51). It is believed that JEV belonging to GI was introduced to China in recent years and GI and GIII co-circulate in China at present.

Solomon et al. (41) suggested that JEV GI was transported from the South-East Asian region, especially Indonesia and Malaysia, to the north. The introduction of JEV belonging to GI was also reported recently in Japan (52), Vietnam (45) and Korea (53).

Since 1969, cases of JE have been reported every year in Thailand with 2,413 cases being reported in 1980. Although a vector control programme was initiated in 1970, cases of encephalitis continued, leading to a national vaccination programme in 1989. The north and north-eastern provinces of Thailand have seasonal JE epidemics which peak during May to September, while the central and southern regions are less affected and have a year round transmission of the virus.

Similar north-south disease patterns are observed in other countries (e.g., Vietnam), whereas major epidemics are rare in countries such as Indonesia and the Phillipines that have no subtropical region.
Malaysia appears to have 3 genotypes of JEV circulating over the past 35 years. Viruses isolated from Selangor in 1993, Kuala Lumpur in 1970 and the island of Sarawak in 1968 and 1994 were of GII while viruses isolated from Perak in 1994 grouped into a subset of GI (54). In addition, the Muar strain, which is distinct from all other JEV strains, was isolated in Singapore, an island just south of the Malaysian mainland.

Three cases of encephalitis, two of them fatal, were reported from Badu, an island in the central Torres Strait of Northern Australia in the spring of 1995 (55). Two strains of the virus, FU and NO, were isolated from the sera of Badu residents. Serological testing for prior infection suggested that this was the first incursion of the JEV into the Torres Strait.

Concurrent with a further incursion of JEV in the Torres Strait in 1998, a fisherman contracted the infection in southwest Cape York in northern Queensland, the first human case on mainland Australia (56). A comparison of the nucleotide sequences in the prM and E gene of the 1995 and 1998 isolates with other JE viruses showed that they were related to GII viruses and most similar to 1981 isolates from Java.

Three isolates of JEV were obtained from mosquitoes in the Western Province of Papua New Guinea (PNG) or Irian Jaya. The PNG isolates belonged to GII and shared ~99.2% identity with isolates from humans and mosquitoes from the Torres Strait. Indeed, all Australian and PNG isolates share an eleven base deletion in the 3’ UTR immediately downstream of the ORF stop codon, suggesting that PNG is the source of incursions of JEV into Australia.

The sudden appearance of JEV on Cape York Peninsula in 2004, 6 years after the 1998 outbreak suggests that it either dispersed into the region from infected areas to the north or that JEV has been present in cryptic cycles since 1998. However, the 1998 and 2004 viruses have been shown by RNA sequencing to be distinct, with the 1998 virus belonging to GII, and all isolates since 2000 belonging to GI. Thus, the 2004 event represents a relatively new incursion of JEV into the mainland of Australia (57).

**JEV IN INDIA**

In India, JE was first recognized in 1955 when cases of encephalitis from North Arcot district and the neighbouring districts of Tamil Nadu and Andhra Pradesh, admitted to Christian Medical College Hospital, Vellore were serologically diagnosed as JEV infection. In 1958, this virus was isolated from wild-caught mosquitoes in the same area and isolated from brain tissue of human cases (58). The first major outbreak of JE involving more than
700 cases and 300 deaths occurred in Burdwan and Bankura districts of West Bengal in 1973, followed by a second outbreak in 1976 (59).

In India, there is a rise of JE incidence and outbreaks have occurred in 25 states. Majority have been reported from the states of Bihar, Uttar Pradesh, Assam, Manipur, Andhra Pradesh, Karnataka, Madhya Pradesh, Maharashtra, Tamil Nadu, Haryana, Kerala, West Bengal, Orissa and Union territories of Goa and Pondicherry (60). JE was also recognized in the Tarai region of Nepal in 1978.

Seasonal patterns of JE incidences in different parts of India depend on the local agrarian ecology, which is again dependent on the annual rainfall pattern. JE outbreaks in south India are mainly centred during the months of August to December, except in the Mandya district, Karnataka, where a second epidemic spell occurs during April to May. In West Bengal, epidemics occur between May and October. In Bihar and Uttar Pradesh, September to December is the period when epidemics are mainly reported, and in Assam, it is between July to September (61).

The state of Uttar Pradesh has seen a constant focus of JEV activity every year. The second half of 2005 saw one of the largest outbreaks of JE that has occurred in northern India in recent years (4). Cases were first reported in the state of Uttar Pradesh in July 2005. By the end of 2005, there had been more than 6000 cases and 1500 deaths, as well as outbreaks in neighboring Nepal. It was the longest and most severe epidemic in 3 decades. Rural populations between the ages of 3 months and 15 years were affected and the overall case-fatality ratio was 23%.

Uchil and Satchidanandam analyzed 8 independent isolates of JEV from various parts of India and compared them with global E-gene sequences that also included 2 strains each from Nepal and Sri Lanka (46). This analysis grouped JEV strains from the Indian subcontinent together and also demarcated the Indian strains into three different clusters within GIII. An arbitrary cut-off of 3.4% divergence was set to define these three groups, the first group designated as Vellore group consisted of two Vellore strains P20 (isolated in 1958 from human brain), and G8924 (isolated in 1956 from mosquito) plus a strain 782219 from Tamil Nadu (isolated in 1978). The Sri Lankan strain, 691004 (isolated in 1969 from human brain) which was placed with the Nakayama strain from Japan, was 2.9% divergent from the Vellore group, reflecting the geographic proximity of Sri Lanka to south India. The second group designated as Bankura group comprised four Indian strains, which included strains from northern India (GP78 from Gorakhpur, isolated in 1978 and 733913 from Bankura isolated in 1973) and western India (826309 from Goa isolated in 1982). In addition, this
group contained a strain H49778 from Sri Lanka (isolated in 1987). The third group, designated as Nepal group, was formed by an Indian strain 7812474 from Assam (isolated in 1978 from human brain) and the B2524 strain from Nepal (isolated in 1985). The Assam strain 7812474 could be placed either in the Bankura or Nepal groups, based on the 3.4% divergence cut. Comparison of JEV strains isolated in China, Japan, and India demonstrated that the viruses had a number of amino acid substitutions within the viral E protein (62).

Figure 1.3: Japanese encephalitis affected areas in India

CLINICAL FEATURES

Most JEV infections are asymptomatic, or cause a non-specific febrile illness, which may include coryza, diarrhea and rigors. In the full-blown encephalitic form, onset is rapid, beginning with a 2 to 4 day prodrome of headache, fever, chills, anorexia, nausea and vomiting, dizziness and drowsiness. These symptoms are followed by nuchal rigidity, photophobia, altered consciousness, and hyper-excitability (63).
In more severe cases, patients present with meningoencephalitis, though some present with aseptic meningitis, or a recently described polio-like acute flaccid paralysis (64) suggesting infection of lower motor neurons. The classical description of JEV infection includes a “Parkinsonian” syndrome of dull flat “mask- like” faces with wide unblinking eyes, tremor, generalised hypertonia, and cogwheel rigidity. Other abnormalities include cranial nerve palsy, tremulous eye movements, and coarse tremors of the extremities, involuntary movements, generalized and localized paresis, in-coordination, and pathological reflexes. Convulsions occur frequently in JE, and have been reported in up to 85% of children, (65) and 10% of adults (66). Repeated convulsions and status epilepticus are associated with a poor prognosis and death usually occurs on the fifth to ninth day or during a more protracted course with cardiopulmonary complications.

Pathological and imaging studies have shown that some of the clinical features reflect the anatomical sites of damage, such as the basal ganglia or the anterior horns of the spinal cord (67). The leptomeninges are seen to be normal, or slightly hazy and histological examination shows an inflammatory infiltrate. In JE, the brain parenchyma is congested with focal petechiae or hemorrhage in the gray matter. Blotchy necrolytic zones are seen when patients die more than 7 days into the illness (68). These are distributed through the thalamus, basal ganglia, midbrain, cerebellum, brain stem and gray matter of the cerebral cortex, and provide the anatomical correlates for many of the clinical signs seen in JE, including Parkinsonism, tremors and brainstem signs. The white matter usually appears normal in JE. In some patients, the gray matter of the spinal cord is confluent and discolored, resembling that of poliomyelitis (69) and accounting for the flaccid paralysis. Microscopically there is perivascular cuffing, with infiltration of inflammatory cells (T cells and macrophages) into the parenchyma, and neuronophagia of infected cells by glial cells leading to the formation of glial nodules (68). In patients who die rapidly from JE, there may be no histological sign of inflammation, but immuno-histochemical studies reveal viral antigen in morphologically normal neurons (70). Single photon emission tomography (SPECT) studies carried out acutely may show hyper-perfusion in the thalamus and putamen (71). In both comatose and conscious JEV infected patients, nerve conduction studies demonstrate markedly reduced motor amplitudes, and electromyography shows a chronic partial denervation.

OUTCOME

About 30% of patients admitted to hospital with JE die, and around half of the survivors have severe neurological sequelae. Sequelae are more frequent in patients whose
acute disease is severe, prolonged, associated with coma and localizing neurological signs. A mixture of upper and lower motor neuron weakness, cerebellar and extrapyramidal signs are commonly observed (72), as are fixed flexion deformities of the arms, and hyper-extension of the legs with ‘equine feet’. Magnetic resonance imaging in patients with sequelae has revealed abnormal intensity in the thalamus, globus pallidus, hippocampus, and substantia nigra. Twenty percent of patients have severe cognitive and language impairment, and 20% have further convulsions (73). Even those with an apparently good recovery may have more subtle sequelae, such as learning difficulties and behavioural problems.

TREATMENT

There are currently no specific antiviral therapies for JE or WNE, and treatment is supportive. Complications of infection such as seizures and raised intra-cranial pressure need to be identified and treated. For many years corticosteroids were given for JE, but a double blind randomized placebo-controlled trial of dexamethasone failed to show any benefit (74). A number of compounds have shown some efficacy against JEV in vitro or in animal models, including isoquinolones, anti-JEV monoclonal antibodies, nucleoside analogues, Concanavalin A, nitric oxide, ribavirin and interferon alpha (IFN-α). However, a randomized double-blind study of IFN-α2a carried out among 112 Thai children hospitalized with suspected JE showed no difference in outcome between treatment and placebo groups (75). A furanaphthoquinone derivative, FNQ3, and more recently, suramin, an anti-trypanosomal drug, and diethyldithlocarbamate also showed some antiviral activity in vitro (76). The antibiotic minocycline was found to completely cure JEV infection in mice when administered one day post infection (PI) (77).

JEV VACCINES

The control of JE is of prime importance, especially in developing countries like India where clinical incidences of encephalitis reach staggering numbers. Added to this are the severe neuropsychiatric sequelae experienced by survivors, most of whom are children. Although vector control is theoretically the most effective means of preventing arbovirus infections, realistically it is only achievable on a small-scale in countries with infrastructures that are well developed. Antivirals are being developed, but they will be fairly expensive and therefore will initially benefit patients in developed countries. A safe and effective vaccine is currently the best strategy to prevent and control JEV infections. However, vaccines should be produced at prices that developing countries can afford.
**Existing Vaccines**

a) First generation vaccines against JEV have been available in Japan since the 1930s. The Japanese mouse brain derived, formalin inactivated vaccine produced from the prototype Nakayama strain is available internationally under the Biken label (Research Foundation for Microbial Diseases, Osaka University), and a vaccine made from the Beijing-1 strain, which shows greater geographical cross-reactivity, is also used in Japan. This is currently the only WHO approved vaccine against JEV and is also produced in Korea (Green Cross). A US-FDA approved Biken vaccine is commercially distributed in the USA by Aventis-Pasteur under the trade name JE-VAX. In India, the Central Research Institute, Kasauli, manufactures the mouse brain derived JE vaccine (78). Although used by travellers and in rich Asian countries, the Biken vaccine’s cost and complex production have meant it has not been used widely in many countries that need it. Furthermore, vaccine-related neurological side effects have been reported, either due to the gelatin stabilizer used or residual neural tissue content in the vaccine. These safety concerns led to the suspension of manufacture of the vaccine and routine immunization by Japan in May 2005. Supplies will probably be exhausted in the next 1–2 years.

b) An inactivated tissue culture derived vaccine, prepared from Beijing P-3 strain of JEV grown in primary hamster kidney (PHK) cells, has been used in China since the late 1960s. Lately this vaccine is increasingly being replaced by the SA 14-14-2 live attenuated JE vaccine, which is cheaper to produce and probably more efficacious.

c) A live JE vaccine produced in PHK cells, involving the stable neuro-attenuated SA 14-14-2 strain of JEV, has been licensed for use in China since 1988. Protective efficacy was approximated at 98% after two doses of the vaccine (79). The live, attenuated SA14-14-2 vaccine has proved to be safe, efficacious, and cheap and more than 200 million doses have been delivered in China since 1988. However, the main hurdle for this vaccine is the PHK substrate, which is not approved by the WHO for human vaccine production. Also, there are concerns that the materials used in the production of the original seed viruses may not have complied with international GMP (good manufacturing practice). However, given the number of doses administered already to Chinese children without apparent harm, and the current disease burden of JE across Asia, it might be argued that while waiting for the development of newer vaccines, the overall benefit of using the current vaccine would outweigh the perceived risk. This vaccine is also being used as part of a major campaign of immunization of children in India and Nepal since 2006. Around 18 million children have so far been vaccinated in India. A case control study in the Bardiya, Banke and Kailali districts of Nepal,
comparing the prevalence of the administration of vaccine in patients with JE hospitalized and in age-sex matched controls, showed that five years after administration of a single dose, the live, attenuated SA 14-14-2 vaccine provided a protective efficacy of 96.2% (80).

Vaccines undergoing clinical trials
a) A new, second-generation, Vero-cell-derived JEV vaccine, containing the purified, inactivated JEV strain SA14-14-2 adjuvanted with aluminium hydroxide (Intercell Biomedical, Livingston, UK) seems to be a promising candidate vaccine. The new vaccine contains no gelatin or mouse proteins and theoretically, fewer hypersensitivity and neurological reactions are expected. 867 adults were included in a multicentre, multinational, observer-blinded, randomised controlled phase III study, which was a non-inferiority comparison of the Vero-cell and mouse-brain vaccines (81). The overall reactivity and immunogenicity profiles were similar or more favourable than for the existing vaccine. Higher titres of neutralising antibodies were achieved with fewer doses (two doses) than the traditional vaccine (three doses). However, given the limited sample size, short duration of follow-up and limited population heterogeneity of pre licensure trials, vigilant post marketing surveillance for safety and efficacy will be necessary.

b) An industrial scale process conforming to the current good manufacturing process (cGMP) criteria has been developed using the Beijing 1 strain of JEV, grown in Vero cells and inactivated. In the preclinical and phase I clinical trials in Japan, the vaccine was found to be safe and its effectiveness to be equivalent to that of the licensed mouse brain derived vaccine (82). However, clinical trials using a larger sample size are required to further validate the safety and efficacy of the vaccine.

c) Live-attenuated 17D Yellow fever (YF) virus is one of the safest and most effective vaccines ever produced. A new generation of chimeric vaccines is being developed in which the immunogenic genes that encode the E and M proteins of pathogenic flaviviruses are exchanged with the equivalent genes in non-pathogenic flaviviruses. ChimeriVax-JE is a chimeric vaccine in which the PrM and E genes of attenuated JEV strain SA14-14-2 were inserted into an infectious clone of the 17D YF vaccine strain. The vaccine replicated efficiently in vitro, and was shown to be safe in mice and non-human primates—being even more attenuated than the original 17D YF strain (83). ChimeriVax-JE was found to generate neutralizing antibodies in 94% of vaccinees in a double-blind Phase II clinical trial carried out on a group of individuals (84). Significantly, immunological memory was also demonstrated in vaccinees in the form of an anamnestic response following challenge with
the licensed JE-VAX. Marketing and distribution agreements of the vaccine, developed by Acambis, with Sanofi Pasteur and Bharat Biotech International Ltd. (Indian subcontinent) have been established. A similar approach, using the same 17D YF virus backbone, is being used to develop chimeric vaccines against WNV and DEN.

Recombinant protein based JEV vaccines

As virus neutralizing antibodies are considered sufficient for protection against JEV infection, the JEV E protein thus has the potential to be used as an immunogen capable of generating protective immunity.

JEV E protein expressed in an E. coli system:

Peptides comprising a single epitope of E protein ectodomain or the entire domain III were expressed in E coli and seen to elicit neutralizing antibodies in mice (78).

Baculovirus based expression system:

Studies with recombinant baculovirus systems expressing prM and E or E and NS1 proteins were seen to afford protection in mice against challenge with JEV. This system could be further pursued for the development of a protein subunit vaccine against JEV (85).

Particulate JEV immunogens synthesized in cell culture-based expression systems:

Expression of full length E protein together with prM in mammalian cells resulted in the secretion of subviral particles (SVPs) that were highly immunogenic in mice and induced both virus neutralizing antibodies and memory T cells (86).

Recombinant virus based JEV vaccines

Canarypox, vaccinia, and more recently, adenoviruses have been used for the development of recombinant vaccines against JEV (78). In 1991, Konishi and colleagues constructed vaccinia recombinants expressing prM, E and NS1 and found that the structural proteins generated protective responses in mice (87). Subsequently, it was shown that a vaccinia recombinant expressing a C-truncated JEV E protein had enhanced protective efficacy in mice. Safety concerns associated with the vaccinia virus have led to highly attenuated derivatives like NYVAC, the modified Ankara strain (MVA), attenuated canarypox virus (ALVAC). NYVAC and ALVAC vaccines were evaluated in human clinical trials. Although high titres of neutralizing antibodies were elicited in vaccinia - naive human volunteers, humans who had previously been vaccinated with vaccinia did not produce antibody against JEV (88), limiting the vaccine’s potential for future development.
Plasmid based JEV vaccines

DNA vaccines hold considerable promise because of their stability at ambient temperature, reduced opportunity for adventitious virus contamination during production, absence of the risk of disease from a live attenuated virus, and immunogenicity. An important potential advantage of DNA vaccines is the reduction of the immunological interference observed during simultaneous vaccination with multiple attenuated flaviviruses (85).

The first reports of a plasmid DNA vaccine encoding prM and E genes were by Konishi and colleagues. This plasmid was found to elicit JEV-specific cytotoxic T lymphocytes (CTLs), memory B cells and long lasting antibodies in swine. Other studies by Chang et al., Ashok and Rangarajan further demonstrated the immunogenicity of plasmids encoding prM and/or E protein in experiments with mice (78).

Plasmid DNA expressing the JEV NS1 protein has also been shown to elicit protective immunity in mice, generating a strong antibody response exhibiting cytolytic activity against JEV-infected cells in a complement-dependent manner, though no neutralizing activity was detected. In another study, immunization of mice with plasmids encoding NS proteins (NS1, NS2A, NS2B, NS3 or NS5) induced CTL responses that provided only partial protection against lethal JEV challenge, whereas complete protection was observed in mice immunized with the plasmid encoding prM and E proteins (89).

Efforts are now being focused to improve the immunogenicity of potential DNA vaccines. DNA absorbed onto cationic micro-particles or colloidal gold demonstrated an enhanced immunogenic response in mice. A single dose of JE vaccine, when administered with biodegradable poly (gamma-glutamic acid) nanoparticles (gamma-PGA-NPs) as a test adjuvant enhanced the neutralizing anti JEV antibody titer in test mice and all of the immunized mice survived a normally lethal JEV infection. Co-administration of plasmids encoding cytokines like GM-CSF with the JEV-DNA vaccine was also found to enhance antibody titres. In addition, newly developed adjuvants such as vaxfectin have been used to enhance the immunogenicity of JEV-DNA vaccines (78). Although candidate vaccines are tested by their ability to generate neutralizing antibody responses, the role of cellular responses cannot be ruled out in protection. While developing a potential vaccine, it is therefore important to analyze the immune responses that contribute to protection against JEV.
Reasons for caution

Live virus vaccines offer considerable promise in terms of efficacy and cost, but the concern is that recombination can occur within flavivirus species and between dissimilar RNA viruses. Homologous recombination (between strains of the same species) in flaviviruses was firmly established in 1999, and has now been seen in all four DENV serotypes and in JEV and St Louis encephalitis virus (SLEV). Five main problems have been recognized: i) reversion of vaccine strains to increased virulence; ii) development of disease in immuno-compromised individuals; iii) fetal malformation, particularly when the vaccine is given in the first trimester; iv) spread of vaccine strains to unvaccinated persons; and v) the discovery of previously unknown complications (90). Experience with the live-virus 17D YF vaccine is pertinent. In 1994, a strain isolated from a fatal vaccine-associated case of encephalitis was shown to have sequence differences compared with the parent vaccine virus, and was associated with increased virulence for mice and monkeys. Vaccine associated fatal cases have also been reported with live vaccines. It is therefore important that the licensing of live virus vaccines should be approved by an international authority. Dual-infection laboratory studies, including in-vivo experiments between wild-type and vaccine-candidate flaviviruses to assess the likelihood of non-homologous recombination and the host range and virulence of the recombinants should also be carried out. Pre-clinical trials should include elderly patients with special emphasis on measurement of concentrations of virus in the blood (ethical considerations would exclude pregnant women). Other suggestions include case studies of adverse reactions and immunological studies in vaccine recipients with adverse reactions.