Preventive strategies for frequent outbreaks of Japanese encephalitis in Northern India

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Japanese encephalitis (JE) remains the most important cause of acute viral encephalitis and continues to spread to hitherto unaffected regions like Indonesia, Pakistan and Australia. Approximately 60% of the world population inhabits JE endemic areas. Despite its restricted range mostly in the developing countries, a high annual incidence of 50,000 cases and about 10,000 deaths has been reported. Disease can be fatal in 25% cases. Magnitude of the problem is even more alarming since the survivors are left with serious long-term neuropsychiatric sequelae. Almost every two years, epidemics of JE occur in Indian subcontinent with a high mortality. JE virus infection results in different disease manifestations in host from mild subclinical febrile illness to clinical infections leading to encephalitis. No antiviral treatment is so far available for JE. The prevention of JE can be achieved by controlling the vector or by immunization regime. The vector control in the rural areas, which are the worst affected ones, is practically almost impossible. Three vaccines that have been implicated against JE include inactivated mouse brain derived, inactivated cell culture derived and cell culture derived live attenuated JE vaccine. But each has its own limitation. Currently, attempts to synthesize recombinant DNA vaccine are being made. New therapeutics are on the way of development like use of minocycline, short interfering RNA, arctigenin, rosmarinic acid, DNAzymes etc. However, the immune mechanisms that lead to JE are complex and need to be elucidated further for the development of therapeutics as well as safe and efficacious JE vaccines.

1. Introduction

Japanese encephalitis is the most important form of epidemic and sporadic encephalitis in the tropical regions of Asia including Japan, China, Taiwan, Korea, Philippines, all of the Southeast Asia and India; however related neurotropic viruses are spread across the global (Solomon 1997). With the advent of molecular virological techniques, it became clear that all the flaviviruses share the common ancestry some 10- 20,000 years ago and are emerging rapidly to fill the ecological niches (Gould *et al* 1997). Countries with the proven epidemics of Japanese encephalitis (JE) are India, Pakistan, Nepal, Sri Lanka, Burma, Laos, Vietnam, Malaysia, Singapore, Philippines, Indonesia, China, maritime Siberia, Korea, and Japan (Vaughn and Hoke 1992). In the past 50 years however; geographic area affected by JE virus (JEV) has expanded. Epidemic activity in Northern India, Central India and Nepal has increased since the early 1970s. Since 1990s the virus has continued to spread in Pakistan (Igarashi *et al* 1994), Nepal (Zimmerman *et al* 1997), and Australia (Hanna *et al* 1996, 1999).

Two epidemiological patterns of JE are recognized. In northern temperate regions of Asia, huge epidemics are reported during summers while an endemic pattern of JE is observed in southern tropical areas, where sporadic cases occur throughout the year with the peak cases of encephalitis during rainy season (Vaughn and Hoke 1992). Various

explanations have been offered to this incongruity. The most likely reason is variation in the temperature pattern. In the south, temperature remains high throughout the year with a constant number of cases each month, however; a sharp rise in temperature (above 20⁰C) during summers in the north corresponds to a sharp rise in the number of encephalitic cases (Solomon *et al* 2000).

2.   Indian scenario

In India, JE is a major pediatric problem and epidemics are reported from many parts. First clinical case of JE in India was observed in 1955 at Vellore (former North Arcot district, Tamil Nadu) (Namachivayam and Umayal 1982). A total of about 65 cases were reported between 1955 and 1966 in South-India (Carey *et al* 1968). Since then many major outbreaks have been reported from different parts, predominantly in the rural areas. In 1973, the first major outbreak occurred in Burdwan and Bankura, the two districts of West Bengal with about 700 cases and 300 deaths (Chakravarty *et al* 1975). Subsequently, another outbreak in the same state occurred in 1976 with 307 cases and 126 deaths (Vaughn and Hoke 1992). Since then, virus is active in almost all parts of India and outbreaks 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 (Kabilan *et al* 2004a). Presently, JE is not only endemic in many areas; it is also spreading to naïve non-endemic areas. JE has emerged as a major public health problem in Kerala (Reuben and Gajanana 1997). Epidemic of JE has occurred in Andhra Pradesh during October-November, 1999 affecting 15 out of 23 districts with 873 cases and 178 deaths (Rao *et al* 2000). At the same time, 3 JE cases were reported for the first time from two villages in Tamil Nadu (Victor *et al* 2000). Later, in July 2003 outbreak occurred in Warangal and Karim Nagar districts of Andhra Pradesh (Das *et al* 2004).

Outbreaks of JE has been reported from north-east regions in Lakhimpur district of Assam between July-August, 1989. It affected 90 villages of the district, covering a population of approximately 36,000 and 50% case fatality rate (Vajpayee *et al* 1992). Later, several outbreaks are reported from Assam in 3 consecutive years from August 2000-2002 (Phukan *et al* 2004). Diagnosis was confirmed in 53.7% patients with ratios of 1.8:1 and 1.4:1 for male to female and pediatric to adult patients respectively. Most of the cases were pediatrics at the age of 7 to 12 years (34.2%).

In Northern states, the disease was reported to occur between 1997 and 1981 (Vrati 2000). Gorakhpur region (UP) experienced the most serious outbreak in 1988 with 875 cases (Rathi *et al* 1993). An epidemic of viral encephalitis was reported from July through November 2005 in

Gorakhpur. It was the longest and most severe epidemic in 3 decades; 5,737 persons were affected in 7 districts of eastern Uttar Pradesh, and 1,344 persons died (Parida *et al* 2006). A total of 34 districts were involved (cases 5581, deaths 1593, case fatality rate [CFR] 29). The affected districts decreased to 22 districts in the year 2006 with lower CFR (cases 2075, deaths 476, CFR 23) and in 2007, 24 districts were reported with 2675 cases, 577 deaths and CFR of 22. Gorakhpur, Deoria, Kushinagar, Maharajganj, Basti, Sant Kabir Nagar, Siddharth Nagar, Bahraich, Gonda, Saharanpur and Muzaffar Nagar are the highly sensitive districts of JE.

Although JEV is RNA virus, neither any clinical pattern has changed nor has any mutation in virus taken place. In between we have noticed that the disease manifestations have changed since 2005. The high incidence of encephalitis cases is also attributable to non-JE cases as well with unidentified etiologies and thus the cases can now be classified as JE/ non JE. Among the non-JE cases, enterovirus 89 and 76 were present in the CSF of encephalitis patients from Gorakhpur in the year 2006.

3.   Transmission

3.1   *Mosquitoes*

In a zoonotic cycle, JEV is transmitted by mosquito vectors between wild and domestic birds and pigs. Mosquitoes are vector as well as a crucial intermediate replicative host for the normal enzootic cycle through birds and pigs. Both pigs and birds like heron, ducks, chicks etc. support high viremia and serve as the primary host for virus. Pigs are amplifying hosts with no evident signs of infection. After acquiring the infection from viremic pigs and completing the extrinsic cycle of 14 days, mosquito becomes infectious. In these natural amplifying hosts, however; virus does not produce encephalitis, although abortion occurs in pregnant sows (Guerin and Pozzi 2005). Virus is transmitted to humans by the bite of infected mosquito, which serves as a dead end host due to its short duration and low viremia in man (Scherer *et al* 1959). Humans are the incidental host and not the natural host for JEV infections (Rosen 1986). The life cycle of the virus is illustrated in figure 1.

Despite variation in geographic distribution of the virus, mosquito vector species is relatively constant. Most important mosquito vector in Asia is *Culex tritaeniorhynchus* which breeds in stagnant water like paddy fields or drainage ditches (Innis 1995). Other species are *Culex vishnui* (India), *C. gelidus, C. fuscocephala* (India, Malaysia, Thailand), *C. pipiens.*

3.2   *Other routes*

Besides mosquito, birds also serve to spread the virus to new geographic areas. JEV is transmissible via semen in
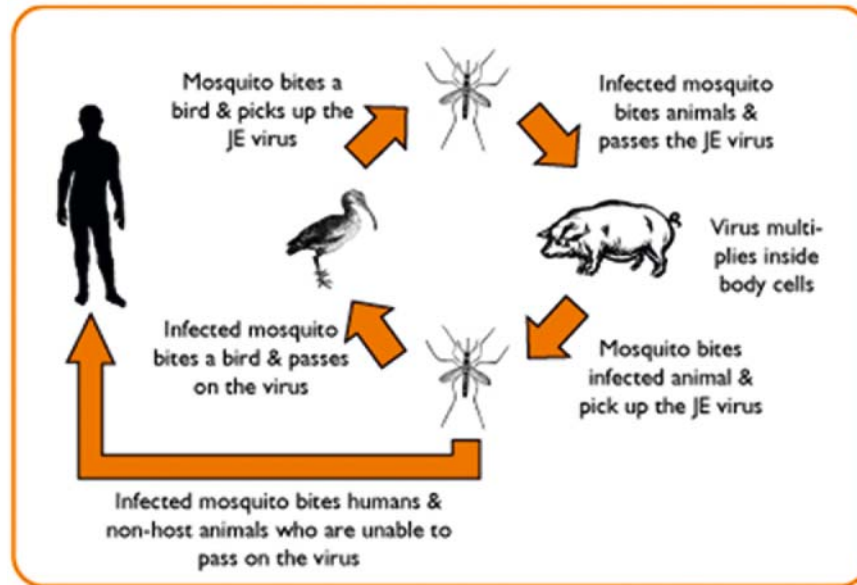
Figure 1. Zoonotic transmission cycle of Japanese encephalitis virus in nature.

pigs which become asymptomatic with high level of viremia (Guerin and Pozzi 2005). In both JE infected humans (Chaturvedi *et al* 1980) and in experimentally infected pregnant mice (Mathur *et al* 1981, 1982), transplacental infection occurs and this mode of transmission results in abortion of the embryos.

4. Seasonal pattern

Two epidemiological patterns of JE infection occur on the basis of difference in the seasonal pattern. In the tropical areas, endemic cases with sporadic cases throughout the year while in temperate areas, seasonal incidence during the monsoon/rainy season is marked from the month of July to September. In India, Karnataka state experiences two epidemics each year, a severe form from April to July and a milder one from September to December along with the rest of India (Vaughn and Hoke 1992).

Precipitation and temperature are two important determinants of vector density that decide the disease burden. At cooler temperatures, virus transmission rate gets reduced. The reason being prolonged mosquito larvae development and longer extrinsic incubation duration at lower temperatures.

5. High risk groups

Children and young adults are the mainly affected ones. Age distribution of the disease varies with the region. Attack rates in children (3–15 year age groups) are 5–10 times higher than adults (<15 year age). Children and elderly, as well as those with debilitating chronic illness or immunosuppression are statistically at greater risk of disease, with symptomatology ranging from malaise to meningoencephalitis with seizures and death (King *et al* 2007).

The attack rate decreases with age in the heavily affected populations due to the presence of neutralizing antibodies as a result of natural exposure and subclinical infections in such individuals. However; in relatively non-endemic areas where the virus is introduced for the first time, individuals of all the age groups are the victim (King *et al* 2007). In Gorakhpur (UP), adults JE cases have come up (Kar *et al* 1992).

6. Clinical spectrum

JEV infections are mostly asymptomatic in about 90% of the cases. An estimated ratio of symptomatic to asymptomatic infection has been 1 to 25–1000 (average of 1:300). Both viral and host factors are believed to be responsible for this low ratio of symptomatic to asymptomatic infections. Viral factors include route, titer and neurovirulence of the inoculum while host factors may be age, genetic constitution, general health or pre-existing immunity (Grossberg and Scherer 1966). Infection with JEV is mainly manifested as non-specific febrile illness or aseptic meningitis. Meningo-encephalitis is the most important and serious form of JEV infection resulting in death in 5–35% cases. About 50-60% of the survivors suffer from serious long-term neurologic sequelae manifested as convulsions, tremors, paralysis, ataxia, memory loss, impaired cognition,

behavioural disturbance and other such symptoms (Halstead and Jacobson 2003).

There is an incubation period of 4-14 days in humans during JEV infection and patients are presented with few days of fever including coryza, diarrhea and rigors (Solomon 1997). Headache, vomiting and reduced levels of consciousness is followed by convulsions. A spontaneous recovery is observed in a large proportion of patients, termed as abortive encephalitis. In some patients, aseptic meningitis with no encephalopathic features may appear (Solomon *et al* 2000). Convulsions occur more frequently in children in upto 85% cases than in adult patients i.e. 10% (Kumar *et al* 1990). Other important features of JE patients are flat dull mask like faces with wide unblinking eyes, tremors, and cogwheel rigidity. Poliomyelitis like acute flaccid paralysis (AFP) is also reported. About 30% of cases developed encephalitis with reduced level of consciousness. Legs are more affected than the arms (Solomon *et al* 1998).

The clinical picture of this infection has four stages: prodromal, acute, subacute and convalescent.

(i) Prodromal stage:
- Lasts for 2–3 days.
- Characterized by an abrupt onset of high fever accompanied by headache, with non-specific symptoms including malaise, anorexia, nausea and vomiting.

(ii) Acute stage:
- Lasts for 3–4 days.
- Changes in the level of consciousness ranging from mild clouding to stupors, semicoma or coma.
- Generalized or focal convulsions are common with neck stiffness and weakness of extremities.
- In this stage, fatal cases progresses rapidly and die.

(iii) Subacute stage:
- Lasts for 7–10 days.
- Fever decreases with improved neurologic sequelae in uncomplicated cases.
- Secondary bacterial infections are common in severe cases.

(iv) Convalescent stage:
- Lasts for 4–7 weeks.
- Complete recovery in mild cases.
- Severe cases improve but left with neurological deficits.

7. Preventive measures

The prevention of JE can be achieved by controlling the vector or by an immunization regime. To control the vector population, spraying of an appropriate insecticide should be carried out in the resting places of mosquitoes. Thermal fogging with ultra low volume insecticides such as pyrethrum or malathion has been recommended for the prevention of local transmission during epidemics, particularly in peri-urban areas with marshes. However; the vastness of breeding places makes larvicidal measures currently impracticable. Effective measures undertaken in some countries to prevent or inhibit larval development include novel water management and irrigation practices such as periodic lowering of the water level, intermittent irrigation, and constant flow systems. Vector control alone cannot be relied upon to prevent JE since it is practically almost impossible to control mosquito density in the rural areas which are the worst affected areas due to poor socio-economic conditions (Tiroumourougane *et al* 2002). Therefore, the need is large scale immunization of susceptible human population to prevent this deadly disease. Mass scale vaccination can significantly reduce the disease burden and incidence.

8. Current vaccines against JE

Three types of JE vaccine are currently in use: mouse-brain derived inactivated, cell-culture derived inactivated and cell-culture derived live attenuated JE vaccine. Formalin inactivated vaccines are the safe and effective against JEV for at least 30 years (Tsai 1999). Mouse-brain inactivated vaccine is the most widely produced and internationally distributed. The efficacy and the strain from which these are produced are given in table 1.

8.1 *Mouse-brain derived inactivated JE vaccine*

Mouse-brain derived inactivated vaccine is the only WHO approved vaccine against JE. It is produced from Nakayama strain and Beijing-1 strain, the later strain is in use of late due to its high cross-reactivity among the JEV strain. Vaccine produced from the original Nakayama strain, is manufactured in Japan and licensed in 1954. It is available internationally under the Biken label (Vaughn and Hoke 1992). This vaccine is also independently produced in China, India, Thailand and Taiwan. Central Research Institute, Kasauli is the manufacturer in India.

Table 1. Vaccines against Japanese encephalitis

Vaccine	Strain	Efficacy
Inactivated mouse brain	Nakayama Beijing-1	91%
Inactivated primary hamster kidney cells	P-3	85%
Live attenuated primary hamster kidney cells	SA 14-14-2	>95%

It is available in lyophilized form in which gelatin and sodium glutamate is used as stabilizers and thimerosal as preservative (Bharati and Vrati 2006). An efficacy of 91% is reported in a study from Thailand with >65,000 children (Hoke *et al* 1988).

Although safe and effective, this vaccine has some common side effects like erythema, swelling, tenderness, fever, headache, malaise and dizziness (Plesner 2003). Further due to its high production cost, lack of long term immunity and adverse allergic reactions, this vaccine is not practical to administer in the poor rural areas, where the vaccine is urgently needed. All these difficulties have led to the development of improved vaccines.

8.2 *Cell-culture derived inactivated JE vaccine*

In China, an inactivated vaccine produced in primary hamster kidney (PHK) cell culture was developed and is in use since 1967. It is produced from the Beijing-P3 strain. It has relatively less side effects and is easy to manufacture. In an extensive randomized field trial in China, its efficacy was found to range between 76-90% (Tsai *et al* 1999).

In the last decade, Vero cell-culture based inactivated vaccine using various local JEV isolates has also been developed and undergoing clinical trials. A vero-cell culture derived formalin inactivated vaccine is being developed using an attenuated SA14-14-2 strain and it induced high titers neutralizing antibodies in mice after two injections (Srivastava *et al* 2001). Recently, vero cell-culture derived formaldehyde inactivated JE vaccine using P 20778 (Indian isolate) has been developed, which generated high titers of anti-JEV antibodies in mice and sera from immunized mice efficiently neutralized different JEV strains with different efficacies (Appaiahgari and Vrati 2004).

8.3 *Cell-culture derived live attenuated JE vaccine*

Live attenuated vaccine appears to offer great prospects for future vaccine development since less virus is needed to mount a satisfactory immune response which makes the vaccine cheaper and fewer doses are required which makes it easy to administer (Solomon *et al* 2000). In 1980s, China developed a live attenuated vaccine named SA 14-14-2 by passaging SA14 strain of JEV in PHK cells. Six amino acid changes in E protein and three in NS genes were associated with attenuation (Xin *et al* 1988; Ni *et al* 1994). In a retrospective case control study, the vaccine efficacy was reported 80% for a single dose and 98% with two doses (Hennessy *et al* 1996). Drawback of this vaccine is the PHK substrate which is not approved by WHO for human vaccine production.

9. Other JE vaccine under development

Several vaccine candidates are still in various stages of development including recombinant protein based vaccines, recombinant virus based/ chimeric vaccine and DNA vaccines.

10. Recombinant protein based JE vaccines

E protein of JEV is important for various functions like receptor binding and fusion and it is capable to induce protective immunity. It is expressed in different expression system in various forms. Its immunogenecity is then tested in animal models, mice. Two expression vectors used for this purpose are *Escherichia coli* (Seif *et al* 1995, 1996; Saini and Vrati 2003; Rauthan *et al* 2004) and baculovirus expression systems (McCown *et al* 1990; Yang *et al* 2005).

11. Recombinant virus-based JE vaccine

Recombinant viruses are important in vaccine development. A number recombinant viruses have been used, each have their own unique feature. Induction of both humoral and cell mediated immune response is the common factor in all the recombinant/ chimeric vaccines. In this strategy, foreign antigen is presented and processed by the host immune system in the way similar to the natural infection. A number of viruses have been used for the production of JE vaccines including poxviruses (Konishi *et al* 1994; Kanesa-Thasan *et al* 2000), adenoviruses (Appaihagari *et al* 2006), yellow fever virus (Guirakhoo *et al* 1999; Monath *et al* 1999, 2003).

12. DNA vaccines

In recent years, plasmid DNA vaccines gained much attention due to their ability to generate a broad range of immune responses, including antibodies induction, generation of CD4 helper and CD8 cytotoxic lymphocytes and in imparting protection against a range of viral infections (Kaur *et al* 2002). These vaccines are cost effective, safe and easy to produce. These vaccines do not interfere with pre-existing antibodies of other flaviviruses or vaccine vector. Here, the DNA encoding a potent immunogen/ its part is placed under a bacterial plasmid under the control of a strong eukaryotic promoter and then administered by different routes like intramuscular or intradermal. There is plasmid endocytosis followed by endogenous antigen production, which allows its presentation by major histocompatibility complex (MHC) class I and leads to production of CD8+ CTL response. Also the uptake of soluble antigen by APC leads to the generation of CD4+ Th response by MHC class II

presentation (Bharati and Vrati 2006). Over the past few years, DNA vaccines against JEV using both structural and non-structural genes have been developed and evaluated in animal models with different efficacies.

Anti-JEV E antibodies play crucial role in imparting protection against JE. For proper intracellular entry and proper confirmation of E protein, PrM is required. For the first time plasmid DNA vaccine against JEV, designated as pcDNA3JEME, was produced in 1998 where both these structural genes were incorporated. It has been observed that DNA vaccines encoding E and PrM genes generated neutralizing (Nt) antibodies, where 70% of mice survived after lethal challenge. Both CTL and B cell responses were mounted and persisted for up to 6 weeks in the spleen cells of immunized mice (Konishi *et al* 1998). Later on a single intramuscular injection was found to provide protection and protected the mice against lethal challenge (Chang *et al* 2000). Immunogenicity of DNA vaccine encoding E without signal peptide was evaluated where E protein remained intracellular. It was noted that protection is imparted against (IC) challenge without the production of antiviral antibodies (Ashok and Rangarajan 1999). Absence of PrM gene can be the probable answer for lack of antiviral antibodies production. Subsequently, immunogenicity of DNA vaccine synthesizing the two forms of E protein i.e. secretory and membrane anchored was tested in mice using intradermal and intramuscular delivery. Approximately 60% protection was observed to be provided by the vaccine irrespective of the route of vaccine delivery and form of E protein (Kaur *et al* 2002).

Among the non-structural (NS) proteins of JEV, NS-1 is expressed on the surface of the infected cells and is extracellularly secreted. DNA vaccines encoding NS-1 gene has been shown to protect the mice against JEV by imparting a strong antibody response with cytolytic activity without antiviral antibodies (Lin *et al* 1998). In another study, mice were immunized with a plasmid DNA encoding the PrM and E proteins of JEV along with plasmid DNA encoding NS1, NS2A, NS2B, NS3 or NS5, it was found that DNA with NS proteins provided partial protection than with PrM and E where neutralizing antibodies are formed (Konishi *et al* 2003). These antibodies prevented the viral spread from periphery into brain. This further confirms that for protective immunity neutralizing antibodies are most important whose production depends on the presence of CD4+ and not CD8+ T cells (Pan *et al* 2001). NS-3 protein has been reported as a dominant CD4+ as well as CD8+ T cell-antigen (Kumar *et al* 2004a, b). However, role of T cells needs detailed investigations.

Further attempts to improve immunogenecity of plasmid DNA vaccines are made. Firstly use of plasmid DNA in particulate form showed enhanced immunogenicity in mice (Kaur *et al* 2004). In another approach, co-administration of DNA vaccine with plasmid encoding cytokines like IL-12 (Chen *et al* 2001), IL-2 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Bharati *et al* 2005) were tested in mice for improved immunogenicity. It was reported that co-administration of pIL-12 suppressed the protective immunity provided by DNA vaccine encoding E protein and using IFN-γ gene-disrupted mice, it was shown that the suppressive activity of IL-12 plasmid was dependent upon endogenous production of IFN-γ. On the contrary, intra-dermal co-administration of plasmid DNA vaccine and plasmid encoding GM-CSF (pGM-CSF) in mice, using the gene gun, enhanced anti-JEV antibody titers resulting in an increased level of protection in mice against lethal JEV challenge. Vaxfectin, used as an adjuvant has been shown to improve the efficacy of DNA vaccine in animal model (Nukuzuma *et al* 2003). Very recently, biodegradable poly(gamma-glutamic acid) nanoparticles (gamma-PGA-NPs) were used as a test adjuvant and found as a novel and safe adjuvant for JE vaccine (Okamoto *et al* 2008).

13. Disease management

Japanese encephalitis is a complex disease with a high mortality and disability. Currently there is no specific treatment for the disease. *In vitro* utility of isoquinolone compounds (Takegami *et al* 1998) and monoclonal antibodies in animal models (Zhang *et al* 1989; Kimura-Kiroda and Yasui 1988; Gupta *et al* 2003) have been demonstrated. Recently treatment with salicylates and non-steroidal anti-inflammatory drugs has been found to suppress the *in vitro* JEV replication and prevent apoptosis of the virus infected cells (Chen *et al* 2002; Liao *et al* 2001). Treatment with glycoprotein, interferon-α is the most promising anti-viral candidate. It is produced naturally in CSF of the patients with JEV infection (Burke and Morill 1987). It has *in vitro* efficacy against JEV (Harinasuta *et al* 1984). Satisfactory results have been observed when recombinant IFN-α has been administered in open trial to few Thai patients (Harinasuta *et al* 1985). However; in a completed double-blind placebo-controlled trial, no improved outcome in JE patients has been observed with IFNα-2a treatment in 112 Vietnamese children with suspected JE, 87 of whom had serologically confirmed infections (Solomon *et al* 2003), however IFN-α delayed the time to death. Other therapeutics under development includes use of si RNA, minocycline, arctigenin and DNAzyme etc. An RNA interference (RNAi)-based intervention has shown that siRNA against cd loop-coding sequence in domain II of the viral Envelope protein can be effectively used as broad spectrum antiviral against encephalitis (Kumar *et al* 2006). Rosmarinic acid (RA) is also an efficient antiviral that reduced JE mortality in mice by reducing viral load along with proinflammatory cytokines (Swarup *et al* 2007). DNAzymes (Dzs) that cleave the RNA sequence of the 3′-NCR of JEV genome *in vitro*

has also been tested and found to inhibit virus replication in mouse brain (Appaiahgari and Vrati 2007). Recently, therapeutic efficacy of a plant lignan, arctigenin is shown both *in vitro* and *in vivo*. It has been reported that arctigenin reduces the virus replication in brain along with reducing neuronal death and secondary inflammation and oxidative stress resulting from microglial activation (Swarup *et al* 2008). However, the immune mechanisms that lead to JE are complex and needs further elucidations for the development of therapeutics as well as safe and efficacious vaccines.

14. Conclusion

There is no specific treatment for the disease, early symptomatic management is important. Thus, what is needed today is high vaccine coverage along with a strong and active surveillance system. Though the ultimate objective of surveillance is prevention of disease occurrence, the immediate objective is detection of early warning signals for any potential JE outbreak and initiate timely effective control measures.

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