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Review Article

Japanese Encephalitis Virus in Pakistan: An Update and Way Forward

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Abstract

Japanese encephalitis (JE) is the leading form of mosquito borne viral encephalitis in Asia, with about 100,000 annual cases in which children are more dominant. The causative agent of JE is Japanese encephalitis virus (JEV) which is transmitted by *Culex* mosquitoes particularly *Culex Tritaeniorhynchus*. Mostly JE infections in humans are asymptomatic while severe JEV infection occurred in only few cases characterized by high grade fever, headache, seizures and unusual behavior, lesion and paralysis. The specific treatment of JEV is not available so far but the supportive care is effective. Many antiviral drugs have been investigated, but none of these have shown to improve the effect of JE except minocycline. The objective of gathering information and performing review is to asses JEV emergence possibility in Pakistan and to define some mitigation measure. In this review, the current knowledge of the prevalence and the pathogenesis of the JEV have been summarized. Furthermore, the current scenario of JEV, challenges and possible way forward in Pakistan has been discussed.

Keywords: Japanese Encephalitis Virus (JEV), Viral Encephalitis, Mosquito-borne Disease, Public Health Threat, Disease Surveillance.

INTRODUCTION

The emerging and re-emerging viral infections are frequently causing threat to public particularly health care systems as it raises morbidity and mortality rate (Bankar *et al*., 2022 & Patel *et al*., 2021). Viral encephalitis is one of the emerging viral infection results in inflammation of brain parenchyma cells. It is most prevalent type of encephalitis in young groups (Said & Kang, 2022). Japanese encephalitis (JE) is one of the most important viral encephalitis in humans and rarely in animals. It is a global disease but particularly found in Asia, the Western Pacific countries, and in small regions of Australia (Ashraf *et al*., 2021). The previous estimation of JE cases was in thousand of numbers approximately 68,000 annual cases but recently it is estimated that 100,000 JE cases occurs annually while above 3 billion peoples are at risk of JE, which poses a serious threat to health care financial system (Michaud *et al*., 2022 & Xu *et al*., 2022). Children with an age of 0-15 years are the major JEV affected group with more potential of neurological complications as compared to adult (Sharma *et al*., 2021). The infections caused by JEV are mostly asymptomatic or mild with symptoms like fever and headache while mortality rate of about 30% can be noted in patients suffering from encephalitis (Ashraf *et al*., 2021). The mortality rate of JE cases can approach 25% while neurological damage can be progressed in up to 50% survivors from the disease. The JE characterized as high burden disease globally due to chronic sequel having cognitive dysfunction and neurologic deficit (Oliveira *et al*., 2020). JEV is transmitted between wading birds and pigs by *Culex* mosquitoes particularly *Culex tritaeniorhynchus*. The circulation of JEV is maintained by wading birds through mosquito vector, the wading birds are responsible for the spreading of JEV to new geographic locations by travelling model (Pham *et al*., 2022).

Classification and Genomic Organization

JE is a vector borne disease caused by Japanese encephalitis Virus (JEV) which is an arbovirus belonging to the genus flavivirus and family flaviviridae (Quan *et al*., 2020). The shape of JEV is spherical and diameter is about 50nm. JEV is a single stranded positive sense RNA virus with 11 kb genome size which encodes for single open reading frame (ORF), translates into poly protein that is divided into three structural and seven nonstructural proteins (Mulvey *et al*., 2021). The structural proteins are nucleocapsid protein (C), membrane protein (M) which is non-glycosylated and envelope protein (E) which is glycosylated. The non-structural (NS) proteins are NS1, NS2A, NS2B, NS3,

NS4A, NS4B and NS7 (Tiwari *et al*., 2012). JEV have genetic and antigenic relationship with other arboviruses like West Nile virus (WNV), St. Louis encephalitis virus (SLEV), and Murray Valley encephalitis virus (MVEV) (Mulvey *et al*., 2021).

History & Prevalance

Japanese encephalitis virus was first emerged in the 1870s in Japan and after that, JE outbreak was started to begin. In 1924, major epidemic in Japan occurred where more than 6,000 cases were reported. The first isolate of JEV was detected in 1935 in a brain of encephalitis suffering patient which was designated as Nakayama strain, the prototype JEV strain. In the beginning, the virus was classified in *Togaviridae* family as a group B arbovirus and was called "type B" encephalitis in order to differentiate it with "type A" encephalitis, the sleepy sickness. In 1985, the original term used for JEV was deserted and the virus was classified in the genus flavivirus and family *flavivirdae* (Sarika Tiwari, 2012). Even though, the first isolation of JEV is from Japan but there is an impression that it evolved from African ancestral virus (Mulvey *et al*., 2021).

During late 1960s, the burden of JE in rich countries was reduced due to vaccination and use of pesticides. Though, population growth, pork production and rice cultivation increased the burden of this disease in recent decades. The JE cases reported from Asia shown that the major cause of acute meningoencephalitis (AME) was JEV. In Vietnam, the JEV caused acute meningoencephalitis (AME) in 52% JE cases from 1998-2007. 23% of AME cases were reported in children with age less than 14 years from 2007-2010. In Cambodia, AME cases were reported in children of age less than 15 years. The epidemic of JE has also been reported from the JEV free areas such as Papua New Guinea and Torres Strait region of Australia (Mulvey *et al*., 2021). JEV has a potential to spread into new geographical regions like dengue virus and West Nile virus belonging to flavivirus. The first outbreak of JE was reported in Torres Strait region in 1995 and another was reported in 1998 which result in five human cases in which two were lethal (Van den Hurk *et al*., 2022). JE case was also reported from Angola in 2016 and in 2021from Tiwi Island Australia (Mulvey *et al*., 2021). Furthermore, JEV was identified in a citizen of Northern Territory, Australia in early 2021. In February 2022, the virus was detected in pigs in Northern Territory where virus caused stillbirths, embalmed fetuses and young pigs with neurological illness. In March 2022, the first case of JE in human was reported and by 25 May 2022, 30 confirmed cases were reported throughout the four states of Australia in which five patients lost their lives (Van den Hurk *et al*., 2022). Despite of high burden of JE, the true observation of JE is still lacking in South East Asia due to shortage of diagnostic facilities. Based on clinical diagnosis, it is difficult to access the true burden of JE (Mulvey *et al*., 2021).

Genotypes of JEV

There are five genotypes of JEV based on gene sequencing of envelope gene which are GI, GII, GIII, GIV and GV (Mackenzie *et al*., 2022). The most prevalent genotypes of JEV are GI, GII and GIII which spread in Asia and Australia. The genotypes of JEV have been region wise isolated as GIV restricted to the eastern regions of Indonesia while GV was isolated mainly from Malaysia, Korea and China (Michaud *et al*., 2022). In all genotypes, GV is the eldest and different genotype while GI is the latest genotype. The genotype GI can also be split into GIa and GIb based on phylogenetic analysis and epidemiological basis. The geographic distribution and the disease also differ between genotypes of JEV. GIa, GIII and GIV are endemic in tropical areas while GIb and GIII are epidemic in northern temperate areas. The genotypes of JEV differ in number of aspects including host variation and antigenic heterogeneity, the later one is shown in various immunoassays. There is cross neutralization found between genotypes GIII and GI. The variation in host and efficiency in replication in mosquitoes may also be a cause of genotype substitution from GIII to GIb (Mackenzie *et al*., 2022).

JEV TRANSMISSION

The JEV circulates between three different groups of hosts throughout its lifecycle which are amplifying hosts, transferring hosts and dead-end hosts (Figure 1). The primary vector responsible for the transmission of JEV to different host is mosquito vector belongs to the genus *Culex* particularly *Culex tritaeniorhynchus*. The amplifying hosts of JEV are pigs and wading bird which develop high viremia when infected by JEV and further transmit the infection to mosquitoes. The carrier hosts of JEV are bats and migratory birds which play significant role in spreading of JEV to new geographical areas. The dead-end hosts of JEV are Humans and Horses develop JEV infection by chance as they are not the natural hosts of JEV. The level of viremia develop by these hosts is not enough for transmission of JEV to other vulnerable host, however they do develop the critical conditions such as encephalitis and acute neurological disorders (Kumar *et al*., 2022). Recently, the non-vector transmission of JEV between pigs has been noted indicating the possibility of virus transmission in temperate areas with limited mosquito seasons. The virus can be transmitted between pigs by the oro-nasal route while the virus transmission between pigs to human directly is not confirmed. In JE endemic areas with low density of pigs, domesticated birds may be responsible for maintaining the JEV transmission but to a lower extent. The cattle as domestic vertebrates have low level of viremia after JEV infection and therefore, cannot serve as amplifying host in virus transmission cycle and can act only as dead-end host like humans. The dominating domestic birds in low density pig areas produce viremia upon JEV infection therefore may be considered as amplifying hosts of JEV (Lindquist, 2018).

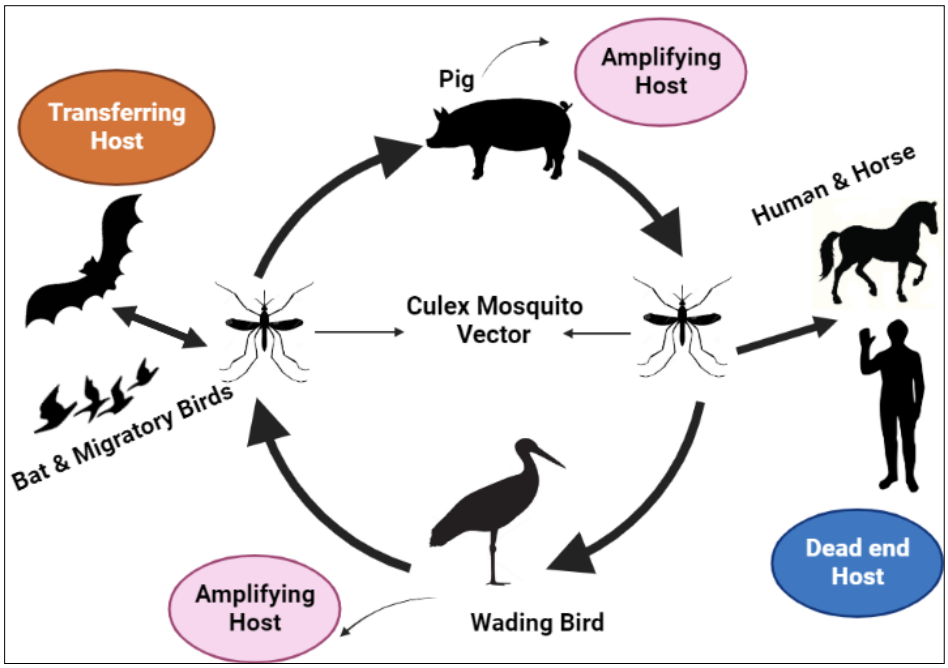

Figure 1: Transmission cycle of JEV

JEV is transmitted by mosquitoes and till now, above 10 mosquito species have been shown competency for the transmission of JEV. Among these mosquitoes, *Culex* mosquitoes including *Culex tritaeniorhynchus*, *Culex gelidus*, *Culex vishnui*, and *Culex annulirostris* are the major competent vector for JEV transmission. In *Culex* mosquito species, *Culex Tritaeniorhynchus* is a top most vector for JEV transmission because of high vulnerability, broad circulation and transmission rate (Park *et al*., 2022). It is a night biting mosquito from evening to morning. The vertical transmission of JEV in this mosquito is of epidemiological significance (Lindquist, 2018). The best environment for the *Culex Tritaeniorhynchus* mosquito is flat flooded regions such as uncultivated rice fields but the existence of this mosquito has been reported in pool water, trench and house hold water storage containers (Mansfield *et al*., 2017).

CLINICAL MENIFESTATION

Initially it was assumed that all cases of encephalitis are caused by JEV due to similarity in signs and symptoms of viral encephalitis with JE, but afterword it has revealed that only 13% cases are caused by JEV. The JE cases may be recognized as the alteration in mental level with high fever and symptoms of confusion leading to coma or talking incapability (Prakash *et al*., 2021).

The incubation period of JEV infection ranges between six to sixteen days. The factors identifying the disease development in infected individuals are unknown but the possible viral factors such as entry route, virus concentration, neuro virulence of the inoculums, and host factors such as health status, age, immunity, and genotypes may be responsible for the development of disease (Tiwari *et al*., 2012). Most of JE infections in humans are asymptomatic or cause mild flu like symptoms which persist for five to fifteen days (Mulvey *et al*., 2021). Less than 1% of the JE cases lead to disease with clinical symptoms which are either mild or neuro invasive, while mortality rate can be as high as 30% in symptomatic patients. The early prevailing symptoms of JEV in children are gastrointestinal pain and vomiting (Sharma *et al*., 2021 & WHO, 2019). Symptomatic severe JEV infection is characterized by high grade fever, headache, seizures and unusual behavior last for 2-4 days after infection. Lesion due to hemorrhage develop in brain and meninges get inflamed, characterized by inflexibility of neck. Paralysis may follow particularly in the upper limbs (Mulvey *et al*., 2021). The clinical symptoms of JE depend on the access of virus to the targeted cells in central nervous system. JEV illness may be mild in case of infection limited to extra neural tissue while infection in nerve cells leads to encephalitis (Tiwari *et al*., 2012). In JE recovered patients, 20-30% experience long lasting neurological squeal, regular seizures and impaired speaking ability (WHO, 2019).

Pathogenesis

JEV infection progression is multifactorial these are entry route, virulence of the virus, genetic makeup of the host and the age of the host. When JEV infected mosquito bite a person, the virus replicates in the skin and transfer to local lymph nodes where Langerhans dendritic cells may be responsible for supporting viral replication as reported in other flavivirus including dengue virus and West Nile virus. Initially, the JEV cause temporary viremia in which virus seed in the extra neural tissue followed by virus entry to the central nervous system (CNS), it is most significant perspective in the viral pathogenesis. The crossing of JEV through

blood-brain barrier is not known but through human postmortem studies, it is suggested that JEV can enter through hematogenous route. Moreover, replication in endothelial cells might be an important way of the blood-brain barrier crossing in some flaviviruses. Similarly in case of JEV, the movement across the endothelial cells is most possible mechanism for the crossing of blood-brain barrier (Tiwari *et al*., 2012 & Joe *et al*., 2022). Other risk factors as dementia, stroke, and sepsis have been incriminated the possibility of neuro-invasion. The clinical presentation of JEV infection is exclusively dependent on the CNS attack from the blood. If neural cells are accessible for the JEV, infection can progress to encephalitis while in case of non-neural cells, infection is asymptomatic (Joe *et al*., 2022).

Diagnosis

The JEV was first isolated in 1934 when sample from infected child with encephalitis was inoculated in monkeys and monkey developed encephalitis. Early studies were relied on this method before the development of cell culture techniques (Bharucha *et al*., 2020). JEV has showed the capability to infect and replicate in different cell types including epithelial cells, endothelial cells, central nervous system cells and immune cells. JEV has been isolated different cell lines including human microglial and porcine cell lines, adapted human embryonic Kidney 293 (HEK-293) and human hepatoma derived cell lines (HuH-7, HuH-7.5 and HuH-7.5.1) (Pham *et al*., 2022 & Adetunji *et al*., 2021). Mosquitoes and cell cultures of mosquitoes were used for identification of JEV. Subsequently various serological methods for the detection of JEV were developed including immunofluorescence microscopy, inhibition of heamagglutination, plaque reduction test and ELISA. During the last twenty years, the accessibility of molecular testing has been increased while low viremia restricts the detection of JEV nucleic acid for diagnosis. Other advanced techniques such as near atomic resolution cryo-electron microscopy improve our understanding regarding viral structure, but not for regular detection of JEV (Bharucha *et al*., 2020).

Serological Tests

The primary diagnosis of JEV is based on serology. After JEV infection, antibodies against JEV produced for short time therefore, the detection JEV by direct virus detection methods (nucleic acid amplification and virus isolation) is often ineffective. In most cases of JE, diagnosis is based on serological methods in combination with clinical history of patient (Pham *et al*., 2022). Initially, the heamagglutination inhibition test was commonly used for the detection of JEV by measuring antibodies against JEV. This technique is based on erythrocytes agglutination by JEV envelop protein. Upon JEV infection, antibodies against JEV produced which prevent agglutination of erythrocytes; therefore, it is called heamagglutination-inhibition (Bharucha *et al*., 2020). This technique was easy to use and require minimal training while limitations of this technique were less sensitivity and specificity, low throughput and requirement of paired sera (Pham *et al*., 2022). Subsequently, the plaque reduction neutralization test (PRNT) was developed as high-quality level test for JE diagnosis. This technique is based on comparison of anti-JEV antibodies and antibodies against other flaviviruses in paired acute and convalescent sera. However, it is burdensome, takes a lot of time and demands for high containment level for safe handling of virus. These are certain limitations of this technique (Bharucha *et al*., 2020). It is recommended by World Health Organization (WHO) to test antibodies against JEV in Cerebrospinal fluid (CSF) or serum for JEV diagnosis. The JEV specific antibody (IgM) can be detected in serum by 4 days after onset of disease in 75% JE patients while IgG can be detected in serum by 7 days after onset of disease in 80% JE patients. The Enzyme Linked Immunosorbent Assay (ELISA) was developed during 1980s and it was the most acceptable technique for JEV diagnosis. A competitive ELISA technique can be used for seroprevalence and it does not demand specific anti human antibodies. The recommended technique by WHO for JEV diagnosis was anti-JEV IgM antibody class capture assay (ACCA). Several commercial assays were available which was based on this technique. Other ELISA format such as IgM capture dot ELISA was also developed for early diagnosis of JEV (Pham *et al*., 2022).

Direct Detection of Virus

The amplification of nucleic acid is a rapid platform for diagnosis. The specificity of this technique is very high unlike serological testing where cross reactivity of antibodies exist. However, due to low level of JEV viremia, its utilization in clinical setting is limited. A number of different amplification techniques have been used for JEV diagnosis in humans and pigs including RT-PCR, use of TaqMan and molecular beacon-based approach. The detection of JEV RNA by amplification through RT-PCR is a valuable technique. It is high specific and sensitive and can be used to understand the molecular epidemiology of JEV. Indigenous assays have used different targeted regions which cover conserved regions of JEV genotypes. The most common regions are NS1, NS2, NS3 or E genes in which some differentiate JEV genotypes (Pham *et al*., 2022).

Treatment

There is no treatment available for the JEV infected patients and supportive care has been shown to improve the effect of JE. It is significant to prevent the infection during hospitalization and the patient must be prevented to mosquito bites (Tiwari *et al*., 2012). Previously, there are many antiviral drugs have been investigated against JE including dexamethasone, minocycline, ribavirin, intravenous immunoglobulin (IVIG), interferon and acyclovir but no one has realistically shown to improve the effect of JE except minocycline (Ajibowo *et al*., 2021). Minocycline is a

derivative of tetracycline group of antibiotics decreased JEV titer, prevention of neuronal apoptosis and activation of microglial. Other approaches which successfully inhibited virus infection are synthetic oligonucleotide-based DNAzyme, glucosidase inhibitor and calcium inhibitors (Joe *et al*., 2022). Moreover, many efforts have been made to decrease the inflammatory response and collateral damage in the JEV infected brain which can lead to neuronal cell death (Filgueira & Lannes, 2019).

JEV Prevalence in Pakistan

Neurological illnesses are the most prevalent chronic diseases worldwide. The global burden of neurological diseases is about 13% in which it ranges 4-5% in the lower earning countries and 10-11% in high earning countries (Faruqi *et al*., 2020). Encephalitis is one of the neurological diseases with an influence on public health and also lethal if remain untreated. In 2017 WHO report, the annual prevalence of this disease ranges between 0.0035-0.0075% while mortality rate due to encephalitis was 0.21% . The etiology behind encephalitis is multifactorial but the most notable cause of encephalitis worldwide is viral. The Herpes simplex virus and Japanese encephalitis virus has been reported as a cause of encephalitis in different regions of world (Andleeb *et al*., 2020). JEV is most frequent in Asia, the Western Pacific countries, and in small regions of Australia (Ashraf *et al*., 2021).

Pakistan is a lower income country with high rate of neurological diseases (4-5%) but the data related to occurrence of encephalitis in Pakistan is inadequate. The scarcity of data is may be due to deficiency of diagnostic facilities and unawareness of health issues due to encephalitis. A recent study was conducted in Pakistan which showed 1.9% prevalence of encephalitis and 37.3% mortality rate due to encephalitis in Pakistan (Andleeb *et al*., 2020).

Pakistan has a history of encephalitis and JEV or other related arboviruses were considered the cause of this neurologic disease. Initially, West Nile virus (WNT) was the only reported arthropod-borne virus circulating in the northeastern region of Pakistan (Hayes *et al*., 1982). When the serious epidemic of JE occurred in the neighboring country India, Pakistan was considered free of JEV as no case was reported but mosquito vector for the transmission of JEV was present in Pakistan (Umenai *et al*., 1985 & Reisen, 1978). According to World Health Organization (WHO), the endemic of JEV expand from east to west such as from western pacific islands to southern region (Sindh Province) of Pakistan (Fatima *et al*., 2020). After that, many cases of encephalitis's were detected in and around the southern region of Pakistan (Karachi) and there was an assumption that JE cases may be found among them (Ahmed, 1980; Takasu *et al*., 1986 & Sugamata *et al*., 1986). The acute encephalitis was the critical health issue in Karachi, Pakistan due to high fatality and critical sequelae and therefore, JEV and other related arboviruses were suspected as causative agents (Igarashi *et al*., 1994). Studies regarding JE prevalence in Pakistan are available (**Table 1**). The sero-epidemiological studies detected high prevalence of JE among healthy individuals while studies in domestic animals showed high level of antibodies against WNT than JEV (Sugamata *et al*., 1987 & Takashima, 1991). The incidence of JE in Pakistan was not evident until 1982. In 1986, a study about JEV prevalence was reported where serum and CFS samples were collected from 1971-1983 and July 1983-March 1984. The antibody titer against JEV was estimated by heamagglutination inhibition test (HI). Among 19 cases of acute encephalitis, 26% samples were associated with the high level of serum antibodies against JEV in which one of them had high titer for CSF antibodies against JEV (Takasu *et al*., 1986). In 1986, another sero-epidemiological study on viral encephalitis was reported in Karachi where antibodies against JEV were identified in 56% samples (Sugamata *et al*., 1986). A sero-epidemiological study on West Nile Virus (WNV) infection was reported in 1988 from Karachi, Pakistan. The study conducted on serum samples collected in 1983 and 1985 from healthy Pakistani individuals. All the samples were tested for the existence of antibodies to WNV and JEV by neutralization test (NT) and heamagglutination inhibition test (HI). Among 156 tested samples, 78 samples were positive for WNV and or JEV in which 12% had high antibodies titer against JEV than WNV (Sugamata *et al*., 1988). Another clinical study conducted on acute encephalitis cases from January to July 1992 in Karachi where the study conducted on the cerebrospinal fluid (CSF) of encephalitis patients. The study revealed that the causative agents of acute encephalitis were WNV and JEV and the techniques used for the identification of both viruses were RT PCR and IgM ELISA. The JEV was detected 4% samples by RT- PCR while 18% samples showed IgM antibodies against both JEV and WNV. Overall, the study showed that JE caused only the small fraction of acute encephalitis in Karachi while West Nile virus was the most frequent cause of acute encephalitis (Igarashi *et al*., 1994). 1n 2016, a cross-sectional, surveillance was conducted in which three arboviruses (DENV, WNV and JEV) were identified as a cause of undifferentiated fever in different regions of Sindh, Pakistan. The technique used for the detection of WNV and JEV was IgM ELISA. The study revealed JEV exposure in 7.73% patients (Khan *et al*., 2016). The level of JEV in native equines has also been identified by ELISA and Microneutralization tests (MNTs) in the two provinces (Khyber Pakhtunkhwa and Punjab) of Pakistan. The most frequently identified virus was WNT (55.4%) while in 9.12% inconclusive samples, antibodies against both WNV and JEV were detected by Micro Neutralization Tests MNTs (Zohaib *et al*., 2015). In 2018, a cross-sectional study was conducted to determine which arbovirus (DENV, WNV and JEV) was responsible for causing undifferentiated febrile disease in Province of Sindh, Pakistan. ELISA results of the study

showed that in 105 positive samples of West Nile Virus (WNV), 75.23% samples were IgM positive for JEV while Plaque Reduction Neutralization Test (PRNT) results showed that 16.1% samples had higher level of neutralizing activity for JEV than WNV (Khan *et al*., 2018). A study conducted in southern regions of Pakistan where sera and CSF samples were collected from April 2015-January 2018. The objective of this study was to determine JEV as a cause of acute encephalitis seroprevalence determined by ELISA and PRNT. The results showed that 8 samples (five CSF and three serum samples), six were positive and two were equivocal for JEV IgM antibodies (Fatima *et al*., 2020). One more study conducted in southern region of Pakistan, sera of dengue negative patients were tested for other arboviruses by ELISA. The detected viruses were Chikangunya Virus (CHIKV), Zika Virus (ZIKV), West Nile virus (WNV) and JEV. The results showed that 4.3% samples were positive IgM antibodies positive for JEV (Imtiaz *et al*., 2020). A recent study conducted in Karachi where IgG and IgM antibodies were detected against WNV and JEV by ELISA in serum of patients with acute undifferentiated illness. The patients included in this study were negative for both Dengue virus (DENV) and Chikungunya virus (CHIKV). The results showed that 43% samples were positive for JEV, in which 42% were positive for only IgM while 16% for both IgM and IgG (Guhar *et al*., 2022).

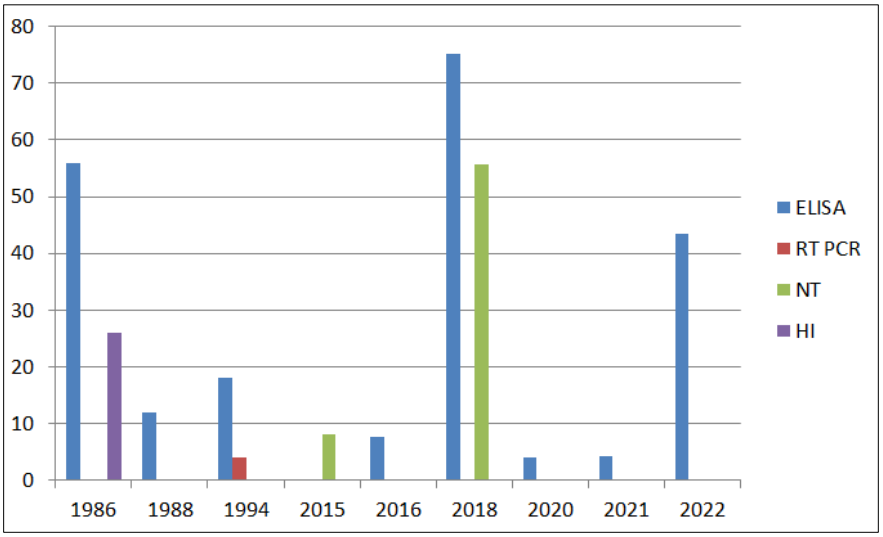

Figure 2: Prevalence of JEV in Pakistan by laboratory testing during 1986-2022

Table 1: Prevalence of JEV in Pakistan during 1986-2022

Location	Reported Year	Sample Collection Year	Diagnosis	Reference
Karachi	1986	July 1983-March 1984	HI & ELISA	(Takasu *et al*., 1986) (Sugamata *et al*., 1986)
Karachi	1988	1983 & 1985	NT	(Sugamata *et al*., 1988)
Karachi	1994	Sep1988-Dec 1992	RT PCR & ELISA	(Igarashi *et al*., 1994)
KPK & Punjab	2015	2012-2013	ELISA & MNTs	(Zohaib *et al*., 2015)
Sindh	2016	May–October 2015	ELISA	(Khan *et al*., 2016)
Sindh	2018	May 2015-Dec 2016	ELISA & PRNT	(Khan *et al*., 2018)
Karachi	2020	April 2015–January 2018	ELISA	(Fatima *et al*., 2020)
Karachi	2021	April 2015-Dec 2016	ELISA	(Imtiaz *et al*., 2020)
Karachi	2022	2015-2019	ELISA	(Guhar *et al*., 2022)

CHALLENGES REGARDING JEV IN PAKISTAN

Pakistan is in continuous exposure with Arbovirus outbreaks and epidemics due to many factors which are favorable environment for mosquito vector, deprived economic condition of society, and unhygienic surroundings (Guhar *et al*., 2022). Moreover, recent incidents of Arboviral infections (WNV, DENV and CHIKV) have been reported from Pakistan (Imran *et al*., 2022). The occurrence of mosquito vector *Culex* (competitive vector for the transmission of JEV) is high as compared to *Aedes* and *Anopheles*. Currently, Pakistan is not endemic for JEV but variation in agriculture tradition and climate, traveling patterns of birds and shifts of population can enhance the risk of JEV outbreak in future. Even though the JEV was not confirmed in patients suffering from of acute encephalitis in two urban hospitals in Karachi, the presence of mosquito vector (*Culex tritaeniorhynchus*) and related species and large production of rice in the area may increase the risk (Fatima et al., 2020; Karthika *et al*., 2018; Samy *et al*., 2018; Khaliq *et al*., 2019 & Brookes *et al*., 1989).

Pakistan is endemic with the vector (*Culex*) responsible for the transmission of JEV. The favorable environment and poor hygienic condition are helpful for the proliferation and spreading of vectors which can lead to the epidemic outbreak of JEV in Pakistan (Fatima *et al*., 2020). As the vector (*Culex*) is present in Pakistan, the prevalence of JEV is also possible but the true epidemiology of JEV in Pakistan is unknown due to lack of active surveillance system for Arbovirus infection at national level. In Pakistan, the information regarding JEV is very limited, the risk assessment for the epidemic of JEV and other flavivirus is not known and also the surveillance system regarding acute encephalitis is not present (Guhar *et al*., 2022). The proper mitigation strategies regarding JEV are also not available in Pakistan. The most studies regarding JEV in Pakistan have been conducted in southern region of Pakistan which limited the JEV prevalence to specific region. The data available in Pakistan regarding JEV is limited and not a true and complete representative for overall Pakistan.

The errors in diagnostics, especially missed diagnosis commonly result from incompetency towards correct diagnosis (Kämmer *et al*., 2021). The co-circulation of DENV with WNV and/or JEVin Pakistan is common which result in misdiagnosis due to sharing of similar symptoms. These viruses usually cause flu-like disease characterized by headache, myalgia, fever, maculopapular rash and gastrointestinal disturbance. About 1% of WNV/JEV infected patients might express neuro-invasive disease. Clinical symptoms like altered mental status, headache, GI distress, limb weakness, and motor neuron disease pattern with flaccid paralysis are helpful for the diagnosis of CNS infection with WNV and JEV. The differential clinical symptoms for JEV infected patient are nausea, thrombocytopenia but still confirmation is required by diagnostic testing for correct diagnosis (Khan *et al*., 2018). Pakistan is endemic for both DENV and CHIKV and therefore it is very challenging to differentiate JEV with other circulating flaviviruses (DENV & CHIKV) due to similarity in symptoms and sharing of similar ecological conditions, seasonality and mosquito vectors (Guhar *et al*., 2022).

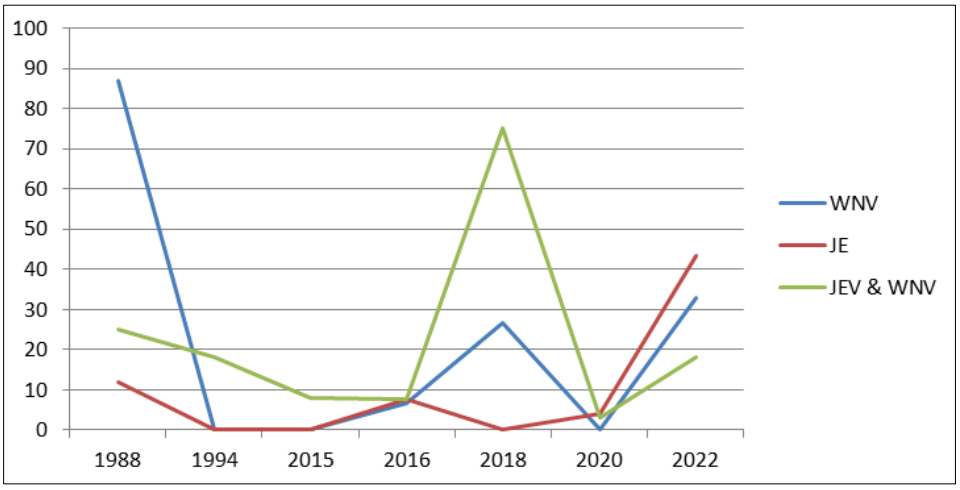

Figure 3: Co-circulation of Japanese Encephalitis Virus (JEV) with West Nile Virus (WNV) in Pakistan

The JEV diagnosis is difficult due the probability of infection with other flaviviruses co-circulating in Pakistan. The studies regarding JEV prevalence have been reported in Pakistan (Table 1) but mostly studies are based on serological test in which cross reactivity is the major issue making difficult to differentiate JEV with other flaviviruses (Figure 3). Cross-reactivity of JEV with WNV has been reported in 25% JEV positive Pakistani military personal in the early 2000s, therefore an accurate identification of infection could not be demonstrated (Bryan *et al*., 1996). In another study, cross-reactivity between the WNV and JEV by ELISA assays was documented in up to 13% samples and to confirm the actual disease agents, confirmation was performed through Plaque Reduction Neutralization Test (PRNT) (Khan *et al*., 2016). The circulation of both WNV and JEV had been reported from Pakistan and cross reactivity about these viruses has also been documented therefore, other technique Microneutralization tests (MNTs) has been utilized for the identification of circulating flaviviruses (Zohaib *et al*., 2015). Khan *et al*., reported significant cross-reactivity of WNV IgM antibodies with JEV in 71% patients with acute febrile illness in southern regions of Pakistan (Khan *et al*., 2018). In 2020, Fatima et al also reported substantial cross reactivity between JEV, DENV, and WNV IgM antibodies (Fatima *et al*., 2020). The cross reactivity between flaviviruses (DENV, JEV and WNV) in serological tests is a major issue and therefore there is a requirement of another test which is plaque reduction assay to confirm the prevalence of JEV. This type of system in lab and medical services is totally lacking in Pakistan (Guhar *et al*., 2022).

WHY PAKISTAN IS AT RISK FOR JEV?

Pakistan is geographically important country as it is situated in the convergence of South Asia, Central Asia and West Asia. Pakistan has major cargo port which

enables most import and export hence, there is a risk of spreading diseases during trading between neighboring countries (Imran *et al*., 2022).

The circulation of mosquitoes in different regions is the important factor for the epidemic of disease. The mosquito species responsible for the transmission of JEV has been identified in Pakistan. A study conducted in Khyber Pakhtoon Khaw KPK identified four genera of culex in which Culex. tritaeniorhynchus was one of the dominant species (Wajiha *et al*., 2017). As the mosquito vector is prevalent, JEV epidemics can hit Pakistan in future. There are many factors responsible for the widespread of mosquito vectors:

The change of climate is a growing issue worldwide. It is affecting every zone of the world but Asia and Africa are more vulnerable for this. The influences of climate change enhance the number of vectors and spreading potential of many infectious diseases (Jabeen *et al*., 2022). Pakistan experiencing a major climate change issue. Pakistan is facing extensive variation in climate, the temperature rises through which winter is not cold as before (Fahad & Wang, 2020). According to The Fifth Assessment Report (AR5) of the Intergovernmental Panel on Climate Change (IPCC) for the region of South Asia, warming is expected to be above the global mean and climate change will influence the glaciers' melting rate and precipitation patterns. In Pakistan, the annual mean temperature is likely to increase by 3°C to 5°C by the end of this century. The future scenario of Pakistan regarding climate change intimated that Pakistan is likely to experience increased variability of river flows due to increased variability of precipitation and the melting of glaciers (Jabeen *et al*., 2022).

The association of temperature with the mosquito borne disease (JE) is very strong. The increase in temperature enhances the JEV replication in mosquito vector, indicating that global warming will raise the competence of mosquito which will ultimately increase the transmission of JEV (Liu *et al*., 2023). The maximum range of transmission temperature of JEV mosquito is 14-18 ^{0}C while minimum range is 35-40 ^{0}C (Jabeen *et al*., 2022). The activities of mosquito vector increase as the temperature become 26-29 ^{0}C (Mordecai *et al*., 2017). At high temperatures (30-32 ^{0}C), there is a decrease in the external incubation period, adult female mosquito digest blood more rapidly and development period become limited which result in the production of large numbers of vectors. Moreover, the higher rainfall has been associated with many vector borne diseases including JEV (Jabeen *et al*., 2022). The average temperature of Pakistan is different with respect to locations. The annual area average temperature of Pakistan in 2022 from different regions ranges from 15-28^{0}C, high temperature is usually associated with the southern areas of Pakistan while northern areas have low temperature. The annual area weighted rainfall pattern of Pakistan in 2022 recorded as ranges between 221mm-851mm (Department, 2022). Both the temperature and rainfall pattern of Pakistan is most suitable for the proliferation of mosquito vector which will further enhance in future and ultimately the vector borne disease burden including JE.

Urbanization and colonization are the outcomes of economic growth. But numerous problems are linked with urbanization including pollution in environment, damage of ecosystem, and overcrowding. Urbanization effects transmission of vector borne disease both directly and indirectly (Imran *et al*., 2022). Pakistan is one the highest urbanizing nation in South Asia with a standard yearly growth rate of 2.7%. The highest rate of urbanization in Pakistan is due to the migration of peoples from rural to urban cities (Hassan, 2021). The considerable movement for industrialization from rural to urban areas induced the growth of vectors responsible for the transmission of Dengue virus (*Aedes aegypti* and *Aedes albopictus*) which increased the threat of disease epidemic (Bostan *et al*., 2017). The poor socioeconomic condition is also a significant risk factor. As the highest urbanization of Pakistan is supporting for the proliferation of dengue mosquito vectors, it can also support JEV mosquito vector and may be responsible for the JEV epidemic in future.

The travelling and the transportation of products can be a source of vector-borne disease transmission. The mosquito vector (*A. aegypti*) responsible for the transmission of Dengue was first appeared in Africa and then transmitted worldwide. It is suggested that *A. aegypti* was entered in new world due to unpleasant atmosphere of Africa together with slave trade and then transmit to Indian and Pacific Ocean (Kraemer *et al*., 2015). Similarly, it is derived the introduction of Dengue vector in Pakistan was taken place from India by the transport of tires (Khan *et al*., 2016). There is no implement of control measures or health criteria for international movements in Pakistan which can lead to the further spread of the mosquito vector or the disease.

The awful unhygienic conditions further stimulate the proliferation of mosquito vector by providing the ideal environment. The hygienic conditions in Pakistan are very poor; Pakistan is in the list of top 10 countries in the world with shortage of clean water. More than seventy million people in Pakistan have not appropriate bathrooms (Imran *et al*., 2022). The Karachi city provides ideal conditions for the proliferation and spread of *Culex* spp. The survival and breeding of dengue mosquito vector in sewage water is reported which reflect that larva propagation of JEV vector can increases in this region due to large quantity of sewage water (Khan *et al*., 2018 & Hai *et al*., 2021). These unhealthy environments intensify the spreading of mosquito vector which are responsible for outbreak of

disease. All these factors including humid environment and unhygienic conditions lead to the propagation of mosquito vector in Pakistan (Rauf *et al*., 2017).

High Risk Regions around Pakistan for JEV Transmission

Pakistan is situated at an extremely remarkable geo-strategic region around the globe. Pakistan is not JEV prevalent country but in its neighboring countries India and China, where lots of JEV cases have been reported (Chen *et al*., 2021; Joe *et al*., 2022; Huanyu *et al*., 2009; Paulraj *et al*., 2022 & Tiwari *et al*., 2012) (Figure 4). JEV was brought into China in the 1930s. After that, JE was prevalent in China and many JE cases were reported from 2001-2020. China became JE endemic country due natural environment, farming civilization, high population and sociodemographic representation. The south eastern region of China has high temperature and rainfall with large plain area and low elevation which provide ideal environment for the proliferation of mosquito vector and ultimately, the JEV. These areas of China are also suitable for human living and hence majority (96%) of Chinese population is residing in these areas. The existence of mosquito vector and the amplification host are common as these regions are loaded with water networks and rice cultivation has dominated which is the most important breeding site of with *Culex (Cx.) tritaeniorhynchu*. All these factors are responsible for the high number of JE cases occurred in China (Chen *et al*., 2021). JEV is the major health issue in India where it was first identified in 1952. After that, many JEV cases were reported from different regions of India. The JEV has affected 24 states of India in which 75 percent cases have been reported solely from Uttar Pradesh. The JEV prevalence resulted in serious illness with up to 50% mortality rate of JEV cases (Rajaiah *et al*., 2022).

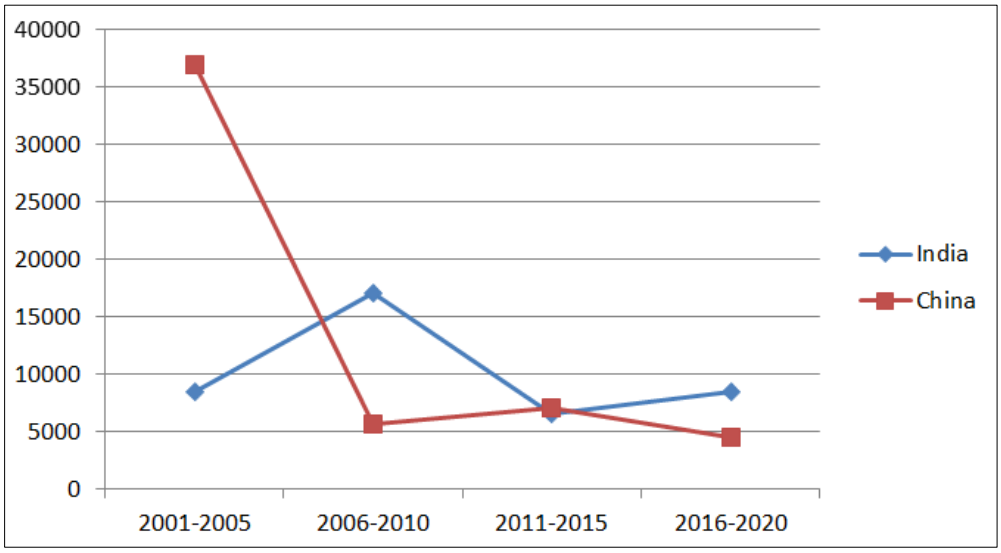

Figure 4: Prevalence of JEV in neighboring countries (India & China)

The JEV cases reported from the southeastern regions of Pakistan while the JEV prevalent countries around Pakistan are India and China which are bordered with the eastern region of Pakistan (Figure 5). The mobility of humans from India or China to Pakistan can inherently associate with the proliferation of JEV in Pakistan. As the travelling, transfer of goods from JEV prevalent areas to Pakistan increases, the chance for spread of JEV and its vector also increases in Pakistan. Therefore, Pakistani Government should take strict action and complete health security plan should be integrated in airports and border regions. The health security plan must be strong enough which can provide timely detection, effective reporting, early and appropriate response and after this the risk of JEV spread can be reduced. The persons or goods will be easily moved from one country to another without any hindrance by implementing the health security plan and by accurate monitoring of travelling and trading. The control measures taken by Government of Pakistan in terms of international movement by implementing strict health criteria will help to achieve global health security by supporting countries to prevent the spread of potential health threats across international borders.

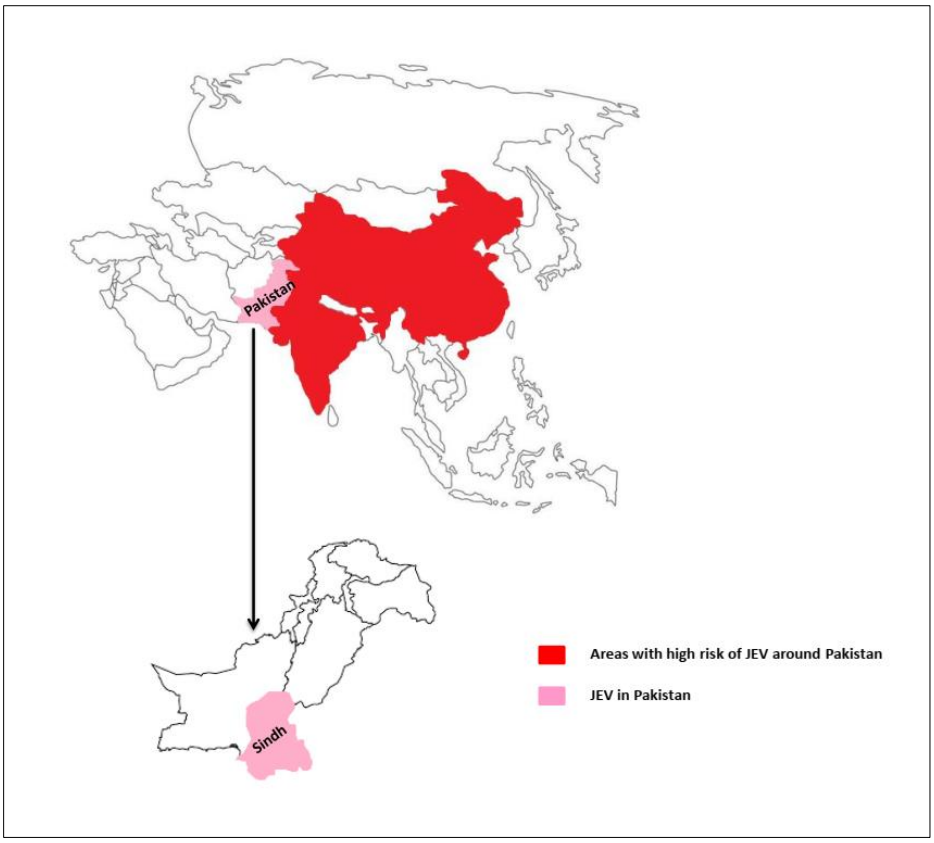

Figure 5: Prevalence of JEV in and around Pakistan

Preventive Measures for JEV Outbreaks in Pakistan

Protective measures are most significant to overcome JEV infection. The infection can be prevented in Pakistan by controlling mosquito vector, strengthen surveillance system, standardization of diagnostic facility and immunization.

Vector Control

Mosquito vector control is the primary prevention to control JEV infection in Pakistan. The most important methods used for the management of mosquito vectors in JEV endemic areas are the larval habitat treatment of rice fields by chemical or mechanical treatment and adult airborne spraying. The insight of public regarding health and environmental effects related to the use of pesticides has significantly influenced the area coverage and the nature of product used for mosquito reduction (Oliveira *et al*., 2020). An integrated approach of mosquito control may be adopted,Mosquito vector can be controlled physically, chemically and biologically (Figure 6).

Physical Control

These methods can be utilized effectively to mitigate mosquito vector in Pakistan. The JEV can also be prevented by avoiding contact with mosquito vector by taking safety measures regarding personal protection. It involves the use of mosquito nets, staying in room with fan or air conditioned, and wearing of full clothing. Awareness programs regarding JEV should be conducted to aware general public about the threat of JEV. Therefore, everyone in Pakistan will become serious to protect own self in terms of mosquito control.

Chemical Control

The mosquito borne disease can be controlled chemically by using insecticides impregnated nets spraying of insecticides and mosquito repellent (Onen *et al*., 2023). The most conventional insecticides used for the reduction of mosquito borne diseases are pyrethroids, carbamates, neonicotinoids, organophosphates and chlorinated hydrocarbons (Meier *et al*., 2022). The commonly used insecticide in impregnated nets is pyrethroids which kill or repel the mosquito vector. These insecticides treated nets provide protection at both personal and public level. In spraying of insecticides, both indoor residual and space spraying methods are used. Mosquito repellent are the substances that repel mosquito and inhibit them from biting humans. The long-lasting insect repellent are DEET (N, N-diethyl-m-toluamide or N, N-diethyl-3-methylbenzamide) and IR3535 (3-(N-Butyl-N-acetyl)-amino propionic acid) which are synthetic insect repellent while mosquito repellent from natural sources are recommended to insure environmental safety (Onen *et al*., 2023).

Biological Control

On the other hand, biological control measures such as bacteria have been found to be effective against mosquito borne diseases. The treatment of *Culex* mosquito larva with *Bacillus thuringiensis* can be used as environment friendly method in the biological control

actions against *Culex* mosquito (Ahmed *et al*., 2017). Similarly, *Bacillus sphaericus* has also been used to control *Culex* mosquito. But the resistance of *Culex* mosquito against toxins of *Bacillus thuringiensis* and *Bacillus sphaericus* has also been found. Another biological control approach is Wolbachia endosymbiotic bacteria which infect mosquitoes and causes cytoplasmic incompatibility and production of non-viable progeny. It naturally infects *Culex* mosquito and therefore it can be used to control *Culex* mosquito (Padmanaban *et al*., 2022).

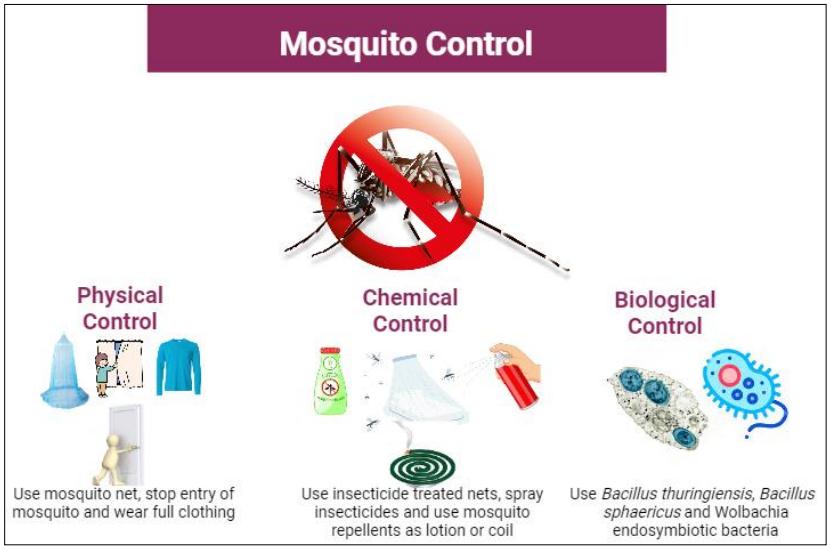

Figure 6: Integrated Approach of JEV Vector Control

Strengthen Surveillance System

Surveillance may be an essential tool to overcome the future outbreak of JEV in Pakistan. There is an urgent and dire need to strengthen our surveillance system both at local and national level for effective implementation of prevention and control measures regarding JEV. The routine practice of case reporting is very necessary for active surveillance system. The surveillance regarding larva and pupa surveys in larger areas are required to determine the clear picture of different mosquito species. The most studies regarding JEV in Pakistan have been conducted in southern region of Pakistan; potential transmission of JEV in other areas should be investigated to collect more comprehensive surveillance data. Moreover, the prevalence of JEV in domestic animals should be determined which will demonstrate the requirement to device an integrated vector control strategy. The surveillance studies must be designed to monitor the vector-based transmission but human to human transmission possibility must not be neglected. The public health surveillance team should be organized in different regions of Pakistan to timely collect, analyze data related to JEV which will provide effective management against future JEV epidemic.

Surveillance of JEV and Standardization of Diagnostic Facility

The development active surveillance system is a basic requirement to control upcoming JEV epidemic and the correct active and passive surveillance is based on accurate diagnosis. Therefore, it is necessary to standardize the diagnostic techniques and confirmation assays such as PRNT which can make easy to differentiate JEV with other flaviviruses actively co circulating in Pakistan. As the incidents of other arboviruses (DENV & WNV) have been reported along with past evidence of JEV in Pakistan, antibodies against all these viruses should be tested in both CSF and serum samples of patient with acute encephalitis. Indigenous standard immunodiagnostic methods need to be followed for JEV detection as a first time of diagnosis along with DENV and WNV in all febrile illness or acute encephalitis cases. The detection of WNV and JEV in serum and CSF samples by molecular method is usually unhelpful due to low viral titer. The gold standard method for the detection of JEV is IgM detection followed by PRNT which truly validate the existing viral infection (Fatima *et al*., 2020). JEV virus culture must be performed from serum and CSF samples of acute encephalitis and febrile illness cases followed by confirmatory PCR, antibody neutralization and sequencing of cultured JEV virus. Another aspect regarding JEV surveillance is differential diagnosis, any patient with acute encephalitis should be diagnosed differentially because viruses (JEV, DENV and WNV) don't show differences in clinical presentation in patient with acute encephalitis. The development of organized and authentic labs is necessary for the identification of JEV in Pakistan. These labs must be equipped with both nucleic acid amplification and sequencing techniques for the identification of flaviviruses and other viruses able to cause encephalitis.

IMMUNIZATION

The intervention to control JEV other than vaccination supports less as compared to vaccination.

Therefore, it should be prioritized to vaccinate humans over pigs' vaccinations and control measures against mosquitoes (WHO, 2019). The JEV can be controlled and prevented by means of vaccinations. Several JEV vaccines are available including RS.JEV, CD. JEVAX, JEBIKV, ENCEVAC, IXIARO, JENVAC, IMOJEV, Chimerivax which are in use in many countries and according to WHO, vaccination program should be integrated in national immunization program in areas where JEV is prevalent (Turtle & Driver, 2018). Presently, the vaccination program for JEV is not necessary for southern areas of Pakistan and it is not compulsory for travelers visiting in these areas to get vaccinated. But JEV vaccine can be considered for travelers from high-risk areas such as entomologists or travelers spending long time in agricultural lands (Fatima *et al*., 2020). Travelling should be avoided or safety measures should be taken to keep away from mosquitoes while travelling in JEV endemic areas (WHO, 2019).

CONCLUSION

Japanese Encephalitis Virus has been reported several times in Pakistan. It has potential to emerge as an outbreak here. Constant surveillance through seroprevalence followed by circulating strain identification is must. An integrated approach of JEV vector control need to be adopted.

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