DISSERTATION

ECOLOGY AND EPIDEMIOLOGY OF JAPANESE ENCEPHALITIS VIRUS IN NEPAL, AND DYNAMICS OF INFECTION WITH THE VIRUS IN BIRDS AND MOSQUITOES

Submitted by

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ABSTRACT

ECOLOGY AND EPIDEMIOLOGY OF JAPANESE ENCEPHALITIS VIRUS IN NEPAL, AND DYNAMICS OF INFECTION WITH THE VIRUS IN BIRDS AND MOSQUITOES

Japanese encephalitis virus (JEV) infection is common in humans and other animals in Asia, including Nepal. Morbidity and mortality due to JEV infection are higher in children than adults, although all age groups of people are vulnerable to JEV, as the areas suitable for rice paddy farming and reservoir host farming are ecologically excellent sites for virus transmission. Several countries currently practice childhood immunization; however, it is limited mostly to city hospitals and frequently does not reach people in rural areas who need it most. The studies reported here had the overall aim to study the ecology and epidemiology of endemic JEV infection in Nepal.

The prevalence of JEV infection in domestic animals is poorly studied at the household scale and is important in assessing the risk of exposure of JEV to humans. Pigs, ducks, and chickens from Rupandehi district of Nepal were tested for antiviral antibody as an index of exposure to JEV, and seroprevalence was characterized for each species at both individual animal and farm level. Additionally, risk factors for JEV exposure to individual species of animal and their farms were assessed. The seroprevalence in pigs, ducks, and chickens was estimated to be 14.7%, 11.8%, and 6.7%, respectively. The farm level seroprevalence of JEV was 31.7%, 31.6%, and 12.8% for farms with pigs, ducks, and chickens, respectively. The major risk factors for JEV infection in these animals were age, locality, practicing household

fermentation, farm size, and location of the farm in the household courtyard. However, the risk factors differed by species of animal.

The incidence of JEV infection in humans is influenced by humans' beliefs, thoughts, and actions, which guides them to different preventive measures. To better understand the influence of these factors, a knowledge, attitude, and practice survey was conducted among 183 households in the Rupandehi district of Nepal to determine whether prior knowledge, current attitudes, and current practices regarding JE/JEV (response variables) guide people in choosing one or the other practices to prevent infection with JEV (outcome of interest). Participants were asked several open- and close-ended questions, and the data were analyzed using univariable and multivariable approaches. The outcomes of interest to which several response variables were analyzed were (i) mosquito population control (removal of stagnant water from surroundings and use of insecticides) and (ii) prevention of mosquito bite (application of insect repellent and using a bed net). Depending on several aspects of knowledge, attitude, and practices, one or the combination of approaches were found to be associated.

Mosquito surveillance for arboviruses is infrequently pursued in Nepal, and the *Culex* species vectors of human pathogens are poorly characterized. A 13-week mosquito sampling in the Rupandehi district of Nepal was carried out at eight different locations to characterize the diversity of *Culex* vectors of JEV, estimate their abundance, blood feeding activity, and to evaluate the influence environmental conditions on those variables. *Culex tritaeniorhynchus* was the most common vector during the course of the study, although 17 additional *Culex* species were detected. Among environmental factors, temperature and precipitation were either positively or negatively correlated with the abundance of different *Culex* vectors.

A final set of studies had the objective to better understanding the phenomenon of genotype displacement for JEV. Since ducks and *Culex quinquefasciatus* mosquitoes are prevalent avian hosts and vectors perpetuating JEV transmission in JE endemic areas, experimental evaluation of virus replication in these species was considered to approximate the natural conditions necessary for studying the role of host, vectors and viral fitness in the JEV genotype displacement context. We evaluated viremia in ducklings infected with three genotype I and three genotype III strains of JEV, and did not detect differences in magnitude or duration of viremia among viruses representing displaced and displacing genotypes. Testing the same six viruses in mosquitoes revealed that the median rates of infection, dissemination and transmission were higher in viruses belonging to genotype I than those representing genotype III, and that the extrinsic incubation period was shorter for the genotype I virus strains. These data suggest that the characteristics of JEV infection of mosquitoes but not of ducklings, may play a role in genotype displacement.

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Japanese encephalitis virus (JEV) is a mosquito-borne virus that causes Japanese encephalitis (JE) disease in humans. It is an RNA virus capable of infecting both vertebrates and insects. The virus is transmitted to humans by the bite of mosquitoes that are infected with the virus by feeding on a viremic mammalian or avian host. It has been one of the most important zoonotic agents circulating in Asia since at least the early 1900s and despite vaccine availability, it has been estimated that approximately 70,000 human cases occur each year in endemic countries (Campbell et al., 2011). In addition, JEV is continuously evolving in its genetic structure and remains as a potential emerging virus worldwide.

1.1.1 History of Japanese encephalitis virus

Japanese encephalitis virus is thought to have emerged from ancestral viruses during the 19^{th} century in the region of the Malay Archipelago and is regarded as a recently emerged virus (Solomon et al., 2003). Historical descriptions of human illness exhibiting clinical manifestations compatible with JE suggest infections occurring as early as 1871 (Solomon et al., 2003; Erlanger et al., 2009). The first definitive report of JEV outbreaks dates to 1924, when an outbreak occurred in Japan (Erlanger et al., 2009; Han et al., 2014). Later, in the 1930s, multiple outbreaks of encephalitis occurred in humans in Japan during the summer months, and the disease was commonly called summer encephalitis of Japan (Taniguchi et al., 1936). The virus was first isolated from the human encephalitic brain in 1935, and since then the virus has been referred as Japanese encephalitis virus (Burke and Leake, 1988). Earlier, in 1933, the virus was isolated

from monkeys in Japan and closely matched with JEV infection in humans (Hayashi et al., 1934 in Taniguchi et al., 1936). Japanese scientists confirmed that the agent causing summer encephalitis of Japan was filterable and transmissible to animals, and demonstrated lesions and histopathologic lesions in mice and monkeys similar to those observed in human patients with encephalitis (Taniguchi et al., 1936). During the summer, an outbreak of JE resulted in 1650 human deaths. Infection of mosquitoes with JEV was confirmed by inoculating into mice the emulsions of *Culex pipiens pallens, Culex tritaeniorhynchus* and *Aedes togoi* that were fed on brain emulsion of mice infected with JEV (Mitamura et al., 1937). In the same study, when they allowed infected mosquitoes to feed on monkeys (*Macacus rhesus*), one of the monkeys developed encephalitis, and JEV was isolated from field caught *Culex* species mosquitoes. This finding was later confirmed by isolating the virus from wild-caught *Culex tritaeniorhynchus* and by experimental infection of *Culex pipiens pallens* and *Culex tritaeniorhynchus* with JEV (Hammon et al., 1949a, b).

After World War II, several researchers from Eastern and Western countries started exploring ecological and transmission studies with JEV. Buescher and Scherer, who belonged to the U.S. Army Medical General Laboratory in Japan, led several important studies and defined the general interaction of JEV and its hosts and vectors (Scherer et al., 1949a, b, c, d; Buescher et al., 1959a, b). Studies by Buescher and colleagues focused in heronries consisting predominantly of herons and egrets surrounded by high human density (about 10,000 people/square mile) and high pig populations (up to 1000 pigs/3-mile radius). The most commonly trapped mosquito vectors within heronries and farms with pigs was *Culex tritaeniorhynchus*. Later, Scherer and coworkers (1959b, d) found that 47% of the herons and egrets, sampled during July-September, tested had either JEV or antibody to JEV, which suggested the role of ardeid birds such as herons

and egrets as reservoir hosts of the virus. Furthermore, Gresser and colleagues (1958a, b) demonstrated experimentally that *Culex tritaeniorhynchus* could acquire the virus from infected birds and transmit to naïve birds through a bite. Additionally, reports of a greater proportion of pregnant pigs aborting and fetal deaths, as well as detection of virus from pigs in areas with high pig population and rapid population turnover, confirmed the role of pigs in JEV transmission (Gresser et al., 1958a, b; Scherer et al., 1959c). The same group of scientists also confirmed that humans and horses are dead-end hosts (Scherer et al., 1959b). Thus, the observational and experimental work of Buescher, Scherer, and Gresser solidified our understanding of the natural cycle of JEV transmission and the potential role of vertebrate animals and vectors in JEV propagation.

1.1.2 Taxonomy of the Virus Family *Flaviviridae*

The family *Flaviviridae* comprises genera *Flavivirus, Hepacivirus, Pegivirus*, and *Pestivirus*, and the *Flavivirus* genus can be sub-categorized into four major groups, namely, mosquito-borne, tick-borne, unknown (no-known) vector, and insect-specific groups. Phylogenetic analysis based on conserved amino acid sequences in the RNA-dependent RNA polymerase provides a genetic basis for classification of the four genera of the *Flaviviridae* family (Figure 1.1). Viruses belonging to the family *Flaviridae* are characterized by monocistronic single-stranded positive sense RNA genomes with no polyadenylate tail. The genus *Flavivirus*, to which Japanese encephalitis virus belongs, comprises small RNA viruses characterized by enveloped, non-segmented, positive sense single-stranded RNA genome of approximately 9-11kb in length (Lindenbach and Rice, 2003). The genus *Flavivirus* includes more than 70 arthropod-borne virus species forming distinct clades, including viruses that infect

mosquitoes and viruses that infect ticks (Kuno et al., 1998). Other medically important viruses of genus *Flavivirus* are dengue virus (DENV), St. Louis encephalitis virus (SLEV), West Nile virus (WNV), Zika virus (ZIKV), and yellow fever virus (YFV).

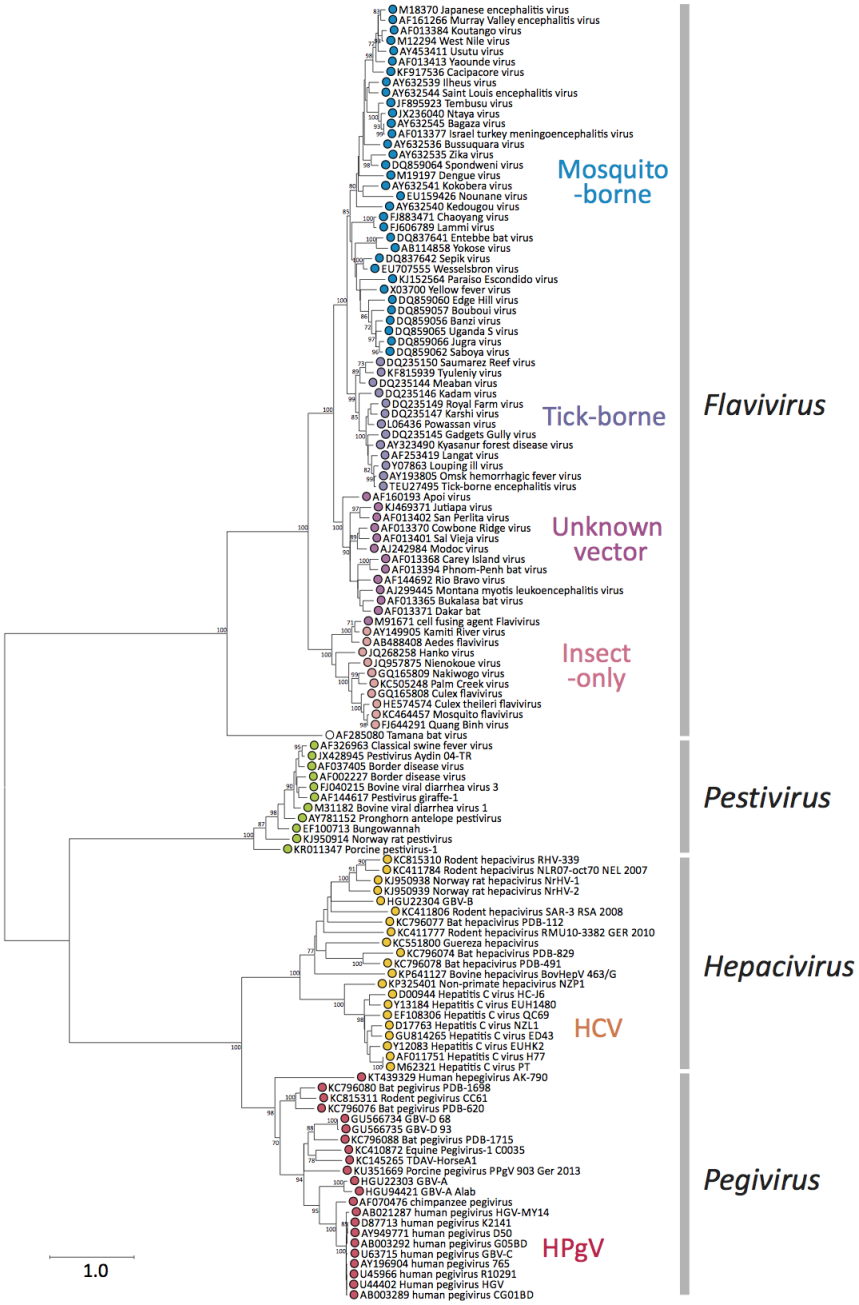


Figure 1.1 Phylogenetic tree of Family *Flaviviridae*
(Source: Simmonds et al., 2017)

1.1.3 Biology of Japanese encephalitis virus

1.1.3.1 Virion and Genome Organization

The JEV virion is morphologically spherical, enveloped and 40-60 nm (~ 510Å) in diameter. The virion capsid has icosahedral symmetry. The RNA of JEV is positive-polarity non-segmented single-stranded RNA of approximately 10-11 kb in size (Lindenbach and Rice, 2003; Unni et al., 2011). Viral RNA found in host cells infected by the JEV contains a single open reading frame (ORF) flanked on either end by shorter 5' non-coding region (NCR) and longer 3' NCR (Figure 1.2). The genome is capped at the 5' end with type I cap (m7GpppAmp) where the highly conserved G nucleotide is followed by the A nucleotide. The 3' end of the genome lacks a terminal polyadenylate tail. The JEV genome is translated as a single polyprotein, which is cleaved by host and viral proteases into structural and non-structural proteins. The structural proteins include capsid (C), envelope (E), and precursor membrane/ mature membrane (prM/M) proteins. The capsid protein forms a nucleocapsid by binding with viral RNA that becomes enveloped by an endoplasmic reticulum-derived membrane containing E and prM. The E protein is the viral hemagglutinin and plays a major role in the entry of the virus into cells (Luca et al., 2011). Besides helping in receptor binding and fusion of the virus to the host cell membrane to promote access into the host cell cytoplasm, the E protein of JEV is a principal determinant of neurovirulence (Wang et al., 2017). The E protein serves as a primary target for neutralizing antibodies and induces protective immunity in addition to flavivirus cross-reactive, non-neutralizing antibodies. The viral RNA encodes 7 non-structural proteins in the infected host cell, designated as NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (reviewed by Chambers et al., 1990; Lindenbach and Rice, 2003; Murray et al., 2008). The NS1 protein is important in viral

RNA replication and is a determinant of neuroinvasion (Melian et al., 2010). NS1 protein also induces antibodies that can contribute to protection against lethal infection in flavivirus-infected vertebrate hosts (Lin et al., 1998; Chung et al., 2006). The N-terminal region of NS3 forms a serine protease complex together with NS2B. The NS2A-B is involved in processing the virus-encoded polyprotein. The C-terminal end of NS3 contains an RNA helicase domain that functions in RNA replication. The largest of all and a highly conserved protein is NS5 which serve as a viral RNA-dependent RNA polymerase. The NCRs form specialized and multifunctional secondary structures that are essential for the replication and translation of the genomic RNA. The sub-genomic RNA derived from the 3'-NCR is also very important for virus replication in host cells and modulates pathogenicity in mammals (Lin et al., 2004).

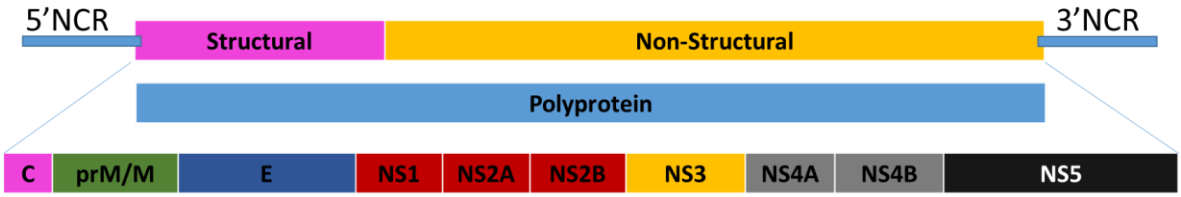


Figure 1.2 Genomic organization of Japanese encephalitis virus
(not to scale)

1.1.3.2 Viral Replication

Replication of JEV occurs in both the phyla Chordata (vertebrates) and Arthropoda (mosquitoes). The site for replication of JEV is host cell cytoplasm (Figure 1.3). Upon attachment to the host cell by binding of the virion with the receptor on the cell surface (step 1), the virion is internalized by receptor-mediated endocytosis (step 2). The acidic pH of endosome induces a conformational change in the viral E glycoprotein that allows fusion of the viral

membrane with the host endosomal membrane (step 3). The viral genome is released from the endosome into the cytoplasm. Translation of genomic RNA occurs next, resulting in the formation of a polyprotein precursor in association with rough endoplasmic reticulum (step 4). The polyprotein is then cleaved into mature viral proteins required for replication and virion assembly. The replication of JEV RNA starts in the replication complex formed with endoplasmic reticulum-derived vesicles (step 5). Early forms of virions start budding from the lumen of endoplasmic reticulum where newly synthesized genomic RNA acquires capsid proteins, prM and E proteins (step 6). Immature virions are transported in the Golgi apparatus through a secretory pathway, and the cleavage of prM to M takes place to yield mature virions (step 7). The mature virions exit the cell into extracellular matrix by exocytosis (step 8). The mature virion is a fusogenic form of the virus, which means the mature virus can bind and fuse with host cells (Yun and Lee, 2014; Wang et al., 2017).

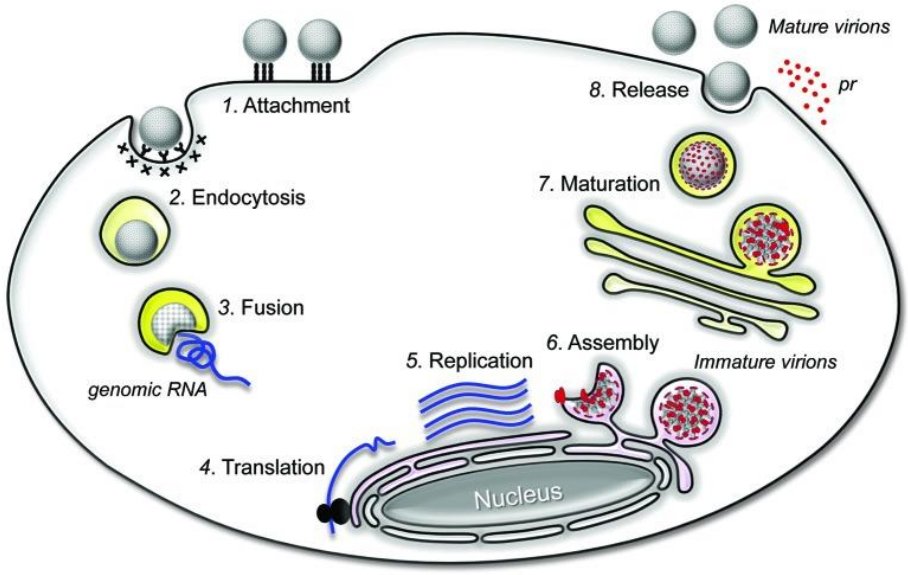


Figure 1.3 Replication cycle of JEV

Source: Yun and Lee, 2014. *Human Vaccines & Immunotherapeutics* 10:2

1.1.3.3 Genetic Variation of Japanese encephalitis virus

Despite the highly conserved nature of JEV among flaviviruses, it is constantly evolving (Chen et al., 1990; Jan et al., 2000; Han et al., 2014). There is considerable genetic variation among JEV strains isolated from geographically distant locations at different time periods. Initially, strains of JEV were grouped based on serological testing (Madrid and Porterfield, 1974; Banerjee, 1986; Kimura-Kuroda and Yasui, 1986) and time of isolation (Kobayashi et al., 1984). The polyclonal sera produced from a given flavivirus infection cross-neutralizes a subset of other flaviviruses, which serves as a basis for classifying the flaviviruses in the JEV serocomplex system (Madrid and Porterfield, 1974). JEV serocomplex constitutes genetically and antigenically related viruses including Alfuy, Koutango, Kokobera, Kunjin, Murray Valley encephalitis, Japanese encephalitis, Stratford, Usutu, West Nile, and St. Louis encephalitis viruses (Poidinger et al., 1996). Later, with the development of nucleotide-based sequence analysis, strains of JEV were grouped according to nucleotide sequence homology (Chen et al., 1990; Chen et al., 1992; Vythilingam et al., 1994; Tsuchie et al., 1997; Huong et al., 1993; Tsuchie et al., 1994). Some earlier studies selected the prM region for the phylogenetic analyses and grouped JEV strains into four genotypes, GI to GIV (Chen et al., 1990; Chen et al., 1992; Huong et al., 1993; Ali and Igarashi, 1997). At least 12% nucleotide divergence in prM region was arbitrarily set to group the isolates into four genotypes (Chen et al., 1990; Han et al., 2014). Later, grouping of JEV strains became based on nucleotide sequence analysis of E region, resulting in classification of JEV into five genotypes - designated GI through GV (Chen et al., 1990; Han et al., 2014; Ni and Barrett, 1995; Paranjpe and Banerjee, 1996; Mangada & Takegami, 1999; Williams et al., 2000; Solomon et al., 2003; Nga et al., 2004; Wang et al., 2007; Jan et al., 2000). Genotype I is further classified into two clades, namely GI-a and GI-b, based on

the time of occurrence of the strains (Han et al., 2014; Schuh et al., 2013; Schuh et al., 2014). The nucleotide sequence is more highly conserved in GI than GIII (Han et al., 2014). The occurrence of genomic variation between genotypes has been thought to enable JEV to become more fit in the ecological niches in which they circulate (Schuh et al., 2014; Sarkar et al., 2012a, Pyke et al., 2001; Schuh et al., 2014; Han et al., 2015; Ma et al., 2003; Mohammed et al., 2011 Nga et al., 2004). Commonly, changes in nucleotide sequences are found in C, prM, E, and NS regions of the JEV viral RNA (Chen et al., 1990; Han et al., 2014; Jan et al., 2000). The capsid-prM region shows the greatest degree of sequence variability (Jan et al., 2000). When the prM region of a fully sequenced virus strain from Japan was compared with 46 JEV isolates from different countries of Asia, the sequence differed by 2-16%. However, the changes in nucleotides did not change the encoded amino acids (silent mutations) (Chen et al., 1990).

Analyses indicated that genotype III consists of most of the virus strains isolated from temperate regions (northern latitudes) while genotypes I and III consist of strains isolated from tropical regions (southern latitudes). Regional variation in genetic pattern and the virulence has been associated with the geography, climatic condition, and the pattern of transmission (Chen et al., 1990; Schuh et al., 2013). Furthermore, sequence of JEV strains also varied from the same geographical region or the country where they were isolated at different time-periods (Chen et al., 1990; Chen et al., 1992; Vythilingam et al., 1994; Tsuchie et al., 1997; Huong et al., 1993; Tsuchie et al., 1994). There is a considerable sequence heterogeneity between strains of JEV isolated from Australia and Asia (Schuh et al., 2014; Sarkar et al., 2012b, Pyke et al., 2001; Schuh et al., 2014; Han et al., 2015; Ma et al., 2003; Mohammed et al., 2011; Nga et al., 2004). Sequence comparison between JEV strains B-2524 and B-9548 isolated in 1985 and JEV strain Nep-1/90 isolated in 1990 from Nepal showed greater high homology (Chen et al., 1990; Ogawa

et al., 1992). In contrast, some investigators have observed a high degree of sequence homology irrespective of the date of collection. JEV isolates collected in Taiwan during 1983-1994 have 93% sequence homology (Jan et al., 2000). Similarly, JEV strains isolated in South Korea at different time-periods also have considerable sequence homology (Ogawa et al., 1992). Genetic comparison of JEV strains suggests there is no consistency in genetic similarity or variability among the strains isolated within the same country or different countries in same time periods or during different time periods.

1.1.4 Geographical Distribution of Japanese encephalitis virus

JEV is distributed in broad areas of Asia, Western Pacific Islands, and northern Australia, collectively including both temperate and tropical regions (Figure 1.4) (Kim et al., 2015; Hanna et al., 1996; Hanna et al., 1999; Paul et al., 1993; Johansen et al., 2000; Han et al., 2014; Erlanger et al., 2009). Recently, there have been reports of autochthonous cases from Italy (Ravanini et al., 2012) and Angola (Simon-Loriere et al., 2017). The geographical range of JEV is expanding and emerging in areas previously thought not suitable for JEV transmission. The wider distribution and perpetuation of JEV in Asia are associated with expanded paddy farming areas, multiple rice farming per year, longer duration flooding in rice fields, increased pig farming, and long distance travel of migratory birds (Marfin and Gubler, 2005; Kuno and Chang, 2005; Simpson et al., 1976; Akiba et al., 2001; Miller et al., 2012; Kim et al., 2015). Commercialization of pig farming and rapid population turnover have supplied abundant numbers of non-immune piglets continuously for multiple and sustained transmission cycles of JEV. The incidence of JEV in northern Australia was thought to be due to an increase in the population of local feral pigs (Mackenzie et al., 2002) and invasion of JEV infected mosquitoes

in northern Australia from Papua New Guinea (Johnassen et al., 2000). The abundance of rice farm breeding JEV vectors, especially *Culex tritaeniorhynchus, Culex vishnui, Culex pseudovishnui,* and *Culex gelidus,* are responsible for both local transmission and wider distribution of JEV in Asia (Soman et al., 1976; Reuben et al., 1971; Rosen, 1986). Furthermore, it is suggested that green revolution in Asia along with intensive pig farming at a larger scale resulted in providing an expanded ecological platform and mosquito breeding terrain for the transmission of JEV in Asia (Kuno and Chang, 2005).

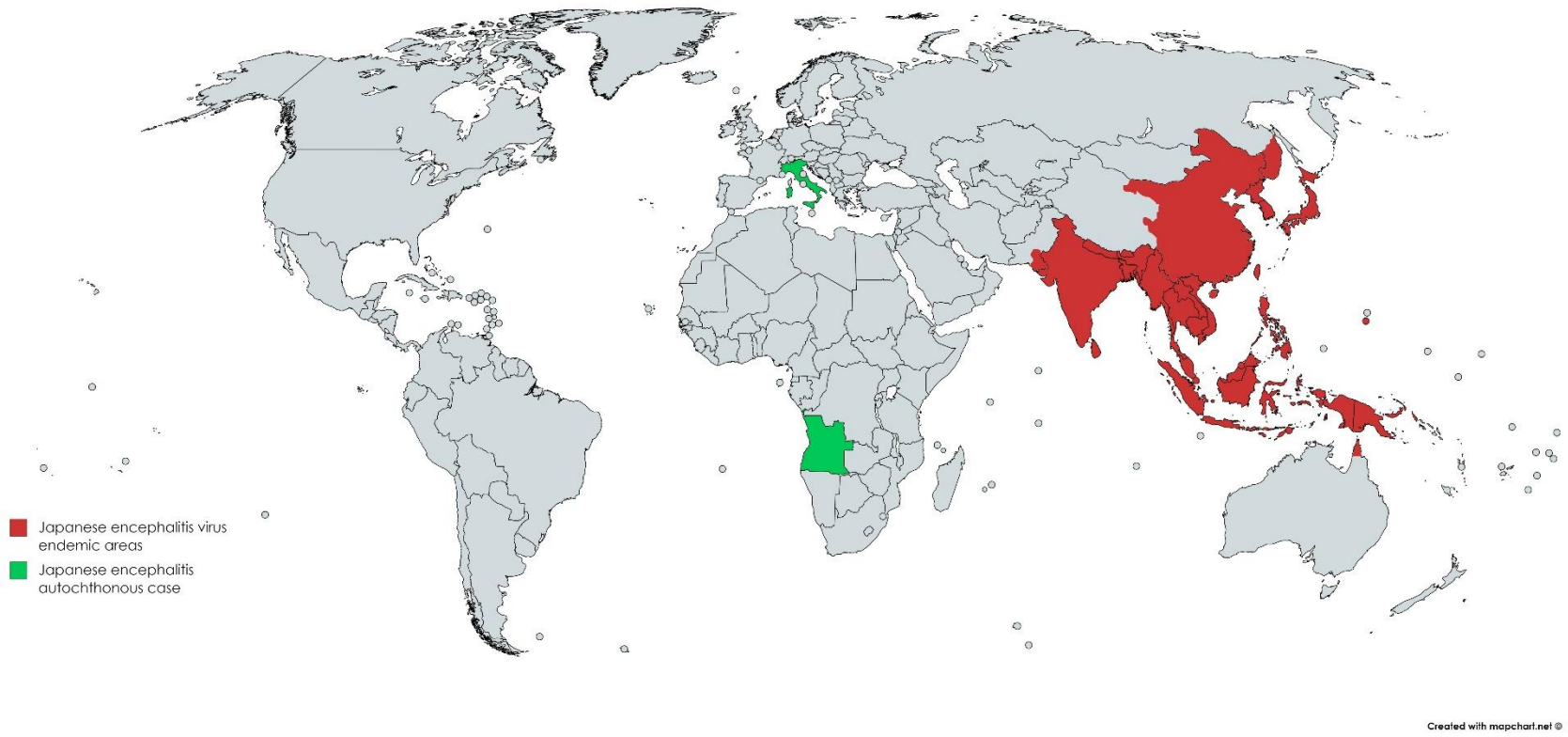


Figure 1.4 Geographical distribution of Japanese encephalitis virus

This graphic was generated using a free online tool https://mapchart.net/detworld.html and is based on information provided by CDC and WHO websites, and review of the literature. Other countries in gray may have cases but have not yet been reported.

1.1.5 Epidemiology and Risk Factors of Japanese encephalitis virus

JEV infection in humans often is mild, with a headache and fever, or often without apparent symptoms. However, approximately 1% of JEV infections in humans result in clinical disease (Campbell et al., 2011) involving rapid onset of high fever, headache, neck stiffness, disorientation, coma, seizures, paralysis, and ultimately death (Giri et al., 2013; Griffiths et al., 2013). There is no consistency in reporting worldwide estimates on morbidity and mortality of JEV infection in published literature, but around 30,000 to 68,000 humans are infected worldwide annually (Tsai, 2000; Campbell et al., 2011) and approximately 10,000 fatalities occur every year in Asia alone (Kuno, 2001). Up to 50% of JEV patients die every year and among those who survive, 22-94% develop long-term or even permanent neurological disability (Campbell et al., 2011; Monath, 2002; Jan et al., 2000; Fischer et al., 2008; Griffiths et al., 2013; Ding et al., 2007; Jacobson et al., 2007; Ooi et al., 2008; Misra et al., 1998; Maha et al., 2009). JE is the leading cause of viral neurological impairment in Asia (Jacobson et al., 2007). Humans of all age groups are susceptible to JEV infection, although children are the predominant victims of JEV infection in areas where childhood immunization is not common (Wierzba et al., 2008; Rayamajhi et al., 2011). True global incidence and burden of JE are largely unknown because of poor documentation, failure to report, variability in in-country diagnostics, misclassification of JE as other encephalitis conditions, and inaccessibility of patients to healthcare facilities (Hecker et al., 2013; Jacobson et al., 2007). Estimation based on 24 JE-endemic countries as of 2011 was that the incidence of JE was 1.8 per 100,000, and that it increased up to 5.4 per 100,000 in children up to 14 years (Campbell et al., 2011). There is no specific antiviral treatment for infections with Japanese encephalitis virus (Akiba et al., 2001). Although vaccines against JEV

and diagnostics are available, they are expensive for people in low and middle-income countries and may contribute to increase in JE cases in JE endemic countries (Jacobson et al., 2007).

The epidemiology of JEV depends on multiple factors that affect vector ecology, reservoir and amplifying host ecology, diversity of farming practices, human population growth, human demography and migration, and seasonality (Bi et al., 2007; Vallee et al., 2009; Liu et al., 2010; Cao et al., 2010; Richards et al., 2010; Sarkar et al., 2012b; Hecker et al., 2013). Vector ecology is affected by environmental temperature, precipitation, relative humidity, and land use. The risk factors of JEV are associated with any single or combined factors that bring humans, other animals, virus, and vectors together for the transmission and perpetuation of the virus. Such factors include deforestation, expansion of agriculture land, animal trade, migration, agriculture and livestock farming in urban areas, environmental destruction for urbanization, and low vaccination coverage (Kuno and Chang, 2005; Akiba et al., 2001; Pandey et al., 2003). The role of seasonality in JEV epidemiology is not fully understood, however it is thought that hibernating vertebrates, overwintering mosquitoes and infected mosquito eggs (Rosen, 1986; Takashima et al., 1988), and possibly ectotherms supplying infectious blood meal to fresh mosquitoes early in the season, and migratory birds and bats (Chen et al., 1990) re-introduces JEV in every season. It is also thought that the JEV is maintained locally in some JEV endemic areas of Asia (Chen et al., 1990).

JE is vaccine-preventable disease, as is evidenced from South Korea by the reduction of human JE cases below 10 per year because of the rolling JEV immunization program in place since the 1970s (Kim et al., 2015). Other countries such as Japan and Taiwan, and including South Korea, have nearly eliminated JE, and China has reduced the number of cases substantially

with mass immunization in humans, pig vaccination, modernization of pig farms, improving living standards, and adopting improved agricultural practices (Marfin and Gubler, 2005; Iragashi, 2002; Erlanger et al., 2009). Despite availability, vaccine cost is paramount, and increases by adding a booster dosage. Therefore, with large families, the cost may often be a large factor in not getting vaccinated. In some JEV endemic countries, adult humans rarely get JEV infection compared to children, and it is thought that the adults get exposure to JEV in their childhood days and develop natural immunity to secondary JEV infection (Solomon et al., 2004). Similarly, paddy farmland has been reduced greatly in Japan and South Korea to prevent humans getting JEV infections (Marfin and Gubler, 2009). Except for China, which had 9% increase in rice farming and production, other countries in Asia such as Nepal, India, Bangladesh have had more than a 20% increase in rice farming and production (Marfin and Gubler, 2005). With this expanded farming and production and breeding ground for rice farm *Culex* vectors, human JE cases will likely increase. Although, currently available anti-JEV vaccines (based on SA-14-14-2 strain and GIII) is protecting against currently circulating GI and GIII in several areas of Asia, emergence of new genotypes might change the epidemiology of the JE and the vaccine currently being used might not be effective against new genotypes (Kim et al., 2015).

The first outbreak of JE in Nepal was reported in 1978 in the Rupandehi district (Hendersen, 1983; Bista et al., 2005; Akiba et al., 2001; Bhattachan et al., 2009). Since then both JEV and antibodies to the virus have been detected in humans and domestic animals from several districts (Pandey et al., 2003; Ogawa et al., 1992), and before 2004, 26,918 JEV infections and 5369 deaths were reported from Nepal (Dumre et al., 2013). JEV is endemic and seasonal in the southern Terai region of Nepal; however recent findings suggest its presence also in higher hills (northern region) (Thakur et al., 2012; Lagarde et al., 2014). An initiative of the Government of

Nepal, with assistance from the World Health Organization, for the surveillance of acute encephalitis syndrome (AES) has been effective in monitoring the incidence of JE cases (Bhattachan et al., 2009). Acute encephalitis syndrome is a condition caused by any pathogen and characterized by acute onset of fever, change in mental status (confusion, disorientation, coma, or difficulty in talking), and seizures. While it is argued that clinical diagnosis of JE is reasonably accurate in Nepal (Pandey et al., 2003), JE is very likely to be misdiagnosed as AES in JE endemic countries where only symptomatic diagnosis is made. Misdiagnosis may be very common in peripheral hospitals of Nepal where trained medical personnel are not available. The epidemiology of JEV changed after 2004 and both the morbidity and mortality due to JEV has reduced significantly to 2040 JE cases and 205 JE deaths as of 2010 (Dumre et al., 2013). This reduction was largely possible due to increased vaccination coverage against JE. Out of 75 districts in Nepal, 62 districts have reported local infection of JEV, including the highland districts (Patridge et al., 2007; Bhattachan et al., 2009; Pant 2009; Impoinvil et al., 2011; Dumre et al., 2013). JEV is the most common viral cause of encephalitis in Nepal, and a greater proportion of AES is due to JEV (Giri et al., 2013; Griffiths et al., 2013). Children and young adults are the frequent victims of AES and JEV cases in Nepal, although 25-40% of JEV confirmed encephalitis cases had been observed in adults over 15 years of age (WHO 2011; Griffiths et al., 2013). Before the vaccination campaign started, large outbreaks of JEV occurred in different southern Terai districts of Nepal (Pandey et al., 2003). Every year over the past 2-3 decades, 200-300 deaths due to JEV were reported between July and October, peaking in August (Pandey et al., 2003; Bista et al., 2001). Studies have reported up to 80% seroprevalence of JE particularly in the western part of Nepal (Akiba et al., 2001). The average incidence ratio of JE in the western part of Nepal was 1.45 cases per 1000 (minimum 0.82/1000 to maximum 1.85/1000)

(Akiba et al., 2001). The prevalence of JEV in humans in Nepal has dramatically decreased with lower case fatality rate due to childhood vaccination program. There has been a substantial reduction in JEV cases in Nepal, with a total of 138 JE cases reported in 2015 from 40 districts and only 11 cases reported in 2016 from 7 districts (WHO, 2016). The hill zones have relatively lower prevalence than the lowland zones of Nepal. Nepal has taken a step ahead in JE surveillance and integrated with the WHO/Immunization Preventable Disease (IPD) network to expand the diagnosis of acute encephalitis syndrome and JEV cases. Vaccination against JEV was first introduced in Nepal in 2006, and in 2009 it was first incorporated in routine childhood immunization programs in 21 JE endemic districts of Nepal, with the addition of 10 more districts in 2011 (WHO, 2016). However, limited vaccine availability in cities prevented wider vaccine coverage, and rural areas with very poor vaccine coverage experience more JE cases (Bista et al., 2001; Tandan et al., 2007). Monthly incidence of JEV cases peaks from May through October (Hendersen, 1983), however the starting month may vary depending on ecological zones of Nepal. JE cases are observed earlier in southern Terai (lowlands), where 61% of the JE cases were reported between May and July, whereas in northern Terai (lowland to highland) a majority (74%) of cases appear in from August to October (Henderson, 1983).

1.1.6 Ecology of Japanese encephalitis virus

1.1.6.1 Transmission Cycle of Japanese encephalitis virus

The natural transmission cycle of JEV involves blood-seeking mosquitoes, especially *Culex* species, vertebrate reservoir hosts, and the competent vertebrate hosts (Scherer et al., 1959; van den Hurk et al., 2009;). Mosquitoes pick up JEV from infected birds, primarily the wetland birds such as egrets and herons (family *Ardeidae*), or pigs (domestic, farmed, or wild)

when they withdraw blood from its host (Figure 1.5). Humans and horses are dead-end hosts because infection does not result in high enough viremia for feeding mosquitoes to be infected. The natural, ecologically relevant hosts of JEV are birds and pigs. Pigs are considered as an amplifying host, and the pig-mosquito cycle occurs independently of the bird-mosquito cycle. Therefore, in areas or countries where pig farming is not common, the mosquito-bird cycle is sufficient for the transmission of JEV to humans. Compared to birds, which play an important role in JEV transmission over long distances, pigs are important for local transmission, including farm to the human household. Such JEV transmission is common in rural areas of Asia where flooded paddy farming is abundant. It is also common in the urban-rural interface, where conditions are favorable for a successful transmission. For successful transmission and maintenance, mosquitoes must be competent to transmit the virus from a reservoir host to another susceptible host. The transmission of JEV can vary within Asia, depending on the areas. It is seasonal in temperate areas, with human cases predominant in summer and fall, while it occurs throughout the year in the subtropics and tropics. Similarly, virus transmission is observed as an epidemic in temperate regions and endemic in sub-tropical and tropical regions of Asia, possibly also due to a regional difference in virulence of JEV (Chen et al., 1990; Burke and Leake, 1988).

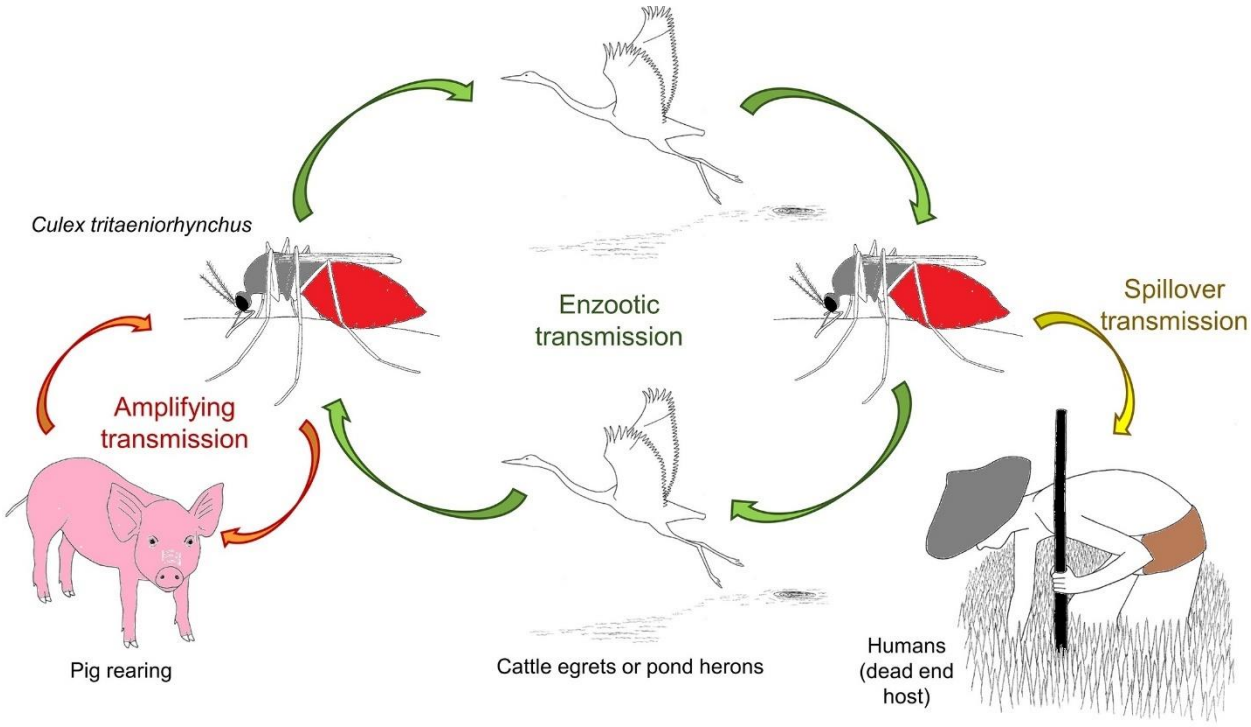


Figure 1.5 Natural transmission cycle of Japanese encephalitis virus

Adapted from Jefferies and Walker, 2015. https://doi.org/10.1371/journal.pntd.0003576.g001

1.1.6.2 Vertebrate Hosts of Japanese encephalitis virus

JEV is a multi-host pathogen and infects both mammals and birds. The higher the genetic variation of a virus, the wider the host range (Kuno and Chang, 2005; Woolhouse et al., 2001) and the vertebrate host range of viruses in the JEV serocomplex is widest among arboviruses (Mattingly, 1960). JEV vectors have been shown to feed on a wide range of vertebrate hosts, including pigs, cattle, or birds (Rueben et al., 1992). Among the susceptible hosts reported to be exposed to JEV, pigs and water birds are essential for the continuation of biological transmission and persistence of JEV, while others are either poorly competent and either contribute only a small fraction to biological transmission or are accidental hosts and do not contribute to a biological transmission. Maternal antibody in piglets wanes with age and is not present several

months after birth, at which time they become fully susceptible to the JEV infection (Hurlbut, 1964; Smith, 1970). Young pigs amplify the virus greater than older pigs (Hurlbut, 1964), so piglets could represent a bigger threat to JEV transmission in areas where pig farming is intense, pigs are not housed in confinement, and competent mosquitoes are present. Secondary infection of pigs with JEV in experimental setting fails to induce sufficient viremia for biting mosquitoes to get an infectious dose (Hurlbut, 1964), and a similar scenario can be predicted in natural condition. Accidental hosts of JEV are "unnatural" vertebrate hosts, which are infected when the interface between infected vectors and the hosts get close enough without any barriers to limit the trophic activity of vectors (Kuno and Chang, 2005). Many times, the ecology of JEV is modified by anthropogenic activities including migration, urban development, deforestation, environmental damage, trade, and animal husbandry (Pandey et al., 2003; Kuno and Chang, 2005). Humans are dead-end hosts of JEV because infected humans do not develop a viremia sufficient to infect feeding mosquito.

1.1.6.3 Vectors of Japanese encephalitis virus

The primary vector of JEV, based on entomological field surveys to detect JEV in Asia, is *Culex tritaeniorhynchus*. It is a paddy farm-breeding mosquito, and is primarily an outdoor dusk and dawn biting mosquito (Kuno and Chang, 2005; Kim et al., 2015). Recent studies have also indicated *Culex tritaeniorhynchus* mosquitoes as endophilic (indoor living and biting). The flight distance of *Culex tritaeniorhynchus* is thought to be at least 1800 meters (Bailey and Gould, 1975). Additional secondary and regional mosquito vectors of JEV include *Culex vishnui, Culex pseudovishnui, Culex bitaeniorhynchus, Culex quinquefasciatus, Culex gelidus, Culex fuscocephala,* and *Culex whitmorei*. Both field and experimental studies in several parts of the

world have shown a wider range of *Culex* species mosquitoes either carrying JEV naturally or competent to transmit the virus. Most of these vectors of JEV share similar ecological requirements for breeding and survival, and thus they are abundant in rural areas where rice farming and animal husbandry are commonly practiced human activities (Kim et al., 2015). JEV vectors, especially *Culex* species mosquitoes, are capable of overwintering, allowing the virus to persist from season to season (Kuno and Chang, 2005: Lee, 1971). Recent findings of either increased number of JE cases in areas with no *Culex tritaeniorhynchus* (Lee et al., 2012), no rice-farming (Chen et al., 2000) or detection of new genotype of JEV in different mosquito vectors, including *Culex bitaeniorhynchus, Culex pipiens, Culex orientalis* in South Korea (Takhampunya et al., 2011, Kim et al., 2015), *Armigeres subalbatus* (Chen et al., 2000) have prompted to study the vector composition in JEV endemic areas of Nepal. Most of the JEV vectors are caught from domestic animal sheds, forests and swamps when it starts getting hot, typically from July to October (Kim et al., 2015). *Culex pipiens* is common in and around human houses and feeds on both birds and mammals (Kim et al., 2015). Inseminated female *Culex* species mosquitoes go through diapause and overwinter in temperate areas (Harlbut, 1949). The diapause and household living is common in *Culex quinquefasciatus* and *Culex pipiens* mosquitoes (Hurlbut, 1949). Hurlbut (1949) demonstrated that JEV infected *Culex quinquefasciatus* remain infected during hibernation and can transmit JEV to suckling mice after hibernation for up to 82 days at 8-13°C, although 14-22% mosquitoes survived in the experiment. In natural conditions, it is more likely that survival of mosquitoes is very low. *Culex quinquefasciatus* mosquitoes can remain infected and able to transmit JEV up to at least 91-days post- inoculation (Hurlbut, 1949). JEV RNA has been detected from field collected *Culex orientalis* and *Culex pipiens* mosquitoes (Kim et al., 2015; Ravanini et al., 2012; Seo et al.,

2013). JEV has been observed in progeny from infected mosquitoes, including *Culex tritaeniorhynchus,* providing evidence for transovarial transmission (Rosen et al., 1978; Rosen et al., 1980).

Availability and density of mosquito vectors are determined by host availability and composition in addition to environmental factors. Mosquitoes can survive without a blood meal but cannot survive without sugar meals from nectars of plants. A blood meal is necessary for their reproduction only, and availability and composition of vertebrate hosts can determine the relative density of mosquitoes in the area (Marquardt, 2004). Female mosquitoes are attracted to carbon dioxide sources and animals emitting carbon dioxide are located by the female mosquitoes as a source of blood meal (Smallegange et al., 2010). Carbon dioxide gas has been used to collect various JEV vectors in the field (Chen et al., 2011).

1.2 Japanese encephalitis virus in Mosquito Vectors

Different vectors of JEV have different abilities to transmit the virus, and the inherent ability of vectors to become infected from an infectious blood meal and successfully transmit the virus through the bite is referred to as vector competence. Similarly, vectors may take variable time in shedding virus in their saliva after having an infectious blood meal, and this interval is known as extrinsic incubation period (EIP). The genetic makeup of the vectors determines the distinction between various mosquito vectors for transmitting JEV. Infection is usually not detrimental to the vector (Kuno and Chang, 2005). Some *Culex* species, mainly ornithophilic types, have shown enhanced attraction to vertebrates with higher body temperature (fever) (Inoue and Kato, 1963; Kuno and Chang, 2005).

JEV was isolated for the first time from field-collected *Culex tritaeniorhynchus* mosquitos in 1936, and the first demonstration of experimental mosquito transmission of JEV was reported in 1938 from Japan (Mitamura et al., 1938). In 1946, experimental transmission of JEV from mosquitoes to mice was reported utilizing mosquitoes from Guam, including *Culex quinquefasciatus, Aedes vexans,* and *Culex jepsoni* (Hodes, 1946). In the same era of elucidating the vector competence of mosquitoes to JEV, laboratory infection and transmission was demonstrated with *Culex tritaeniorhynchus* and *Culex pipiens* var. *pallens,* obtained from Japan (Hammon et al., 1949). Field exploration in different parts of the world revealed many mosquito species carrying JEV, including *Culex vishnui, Culex pseudovishnui, Culex gelidus, Culex pipiens, Culex quinquefasciatus, Culex annulirostris, Culex sitiens, Culex fuscocephala, Armigerous subaltus, Aedes albopictus, Aedes subpictus, Aedes vexans, Mansonia uniformis,* and *Anopheles hyracnus* (Weng et al, 1999; van den Hurk et al., 2003a, b; Weng et al., 2005). Similarly, laboratory experiments also demonstrated that a variety of mosquitoes were able to become infected with JEV and/or able to transmit the virus through their saliva. The vectors of JEV supported by laboratory experiments includes but not limited to *Culex pipiens (var. pallens, pipiens, molestus), Culex (tarsalis, tritaeniorhynchus, annulirostris, sitiens, quinquefasciatus, nigripalpus, restuans, salinarius, stigmatosoma, erythrothorax), Ochlerotatus (vigilax, notoscriptus, normanensis, purpureus, canadensis, cantator, triseriatus, dorsalis, melanimon, sierrensis), Aedes (aegypti, albopictus, vexans), Mansonia (uniformis, septempunctata), Culiseta (melnura, inornata), Psorophora ferox,* and *Coquillettidia perturbans* (Weng et al., 1997; Weng et al., 2000; van den Hurk et al., 2003a, b; Turell et al, 2006a, b). Furthermore, experimental studies with several strains of JEV indicated that *Culex pipiens* and *Culex quinquefasciatus* can transmit various strains of JEV (Turell et al., 2006a, b; Huang et al., 2016). There is

considerable variability in competency relative to the minimum infectious dose of virus in a blood meal, ranging from 1 to 4.5 $\log_{10}$PFU/ml depending on mosquito species (Soman et al., 1977). The EIP for JEV cited in the vector competence experiments ranged from 7-14 days-post infection, and *Culex* species have been shown to have the greatest competence in transmitting the JEV.

1.3 Japanese encephalitis virus in Vertebrate Hosts

The duration of JEV viremia is short, typically 2-4 days, depending on the animal involved. Severe pathogenesis and mortality are not commonly associated with JEV infection in adult mammalian and avian hosts, but piglets often die with JEV infection (Henderson, 1983). Historical experiments have shown mice and monkey as susceptible to JEV infection. *Macacus* monkeys were found to be more susceptible to JEV than *Pitecus* monkeys, and rats, rabbits, and guinea pigs are poor animal models for JEV (Taniguchi et al., 1936). Mice and monkey show histopathological changes in the brain including perivascular infiltration and glial proliferation in the brain. Most animals do not show any symptoms and those who do show fever, paralysis, spasm, tremor, and loss of appetite (Taniguchi et al., 1936). The incubation period of JEV ranges from 4-7 days in mice and 7-14 days in monkeys, and the incubation period becomes shorter than a week when mouse-adapted JEV is experimentally inoculated in monkeys (Taniguchi et al., 1936). Pregnant pigs infected with JEV frequently abort their fetus. Otherwise, they do not show any apparent symptoms (Sazawa et al., 1968).

Wild birds, including house finches, tri-colored blackbirds, English sparrows and, domestic chickens were found to be susceptible to JEV infection (Hammon et al., 1951). Extensive work on host susceptibility to JEV infection revealed that herons (night and pond) and

egrets (plumed, lesser, and cattle) were the predominant hosts infected by JEV in the field (Buescher et al., 1959a, b). The birds in JEV endemic countries have been shown to have high titer viremia and the seroprevalence of JEV in the bird population was also high, suggesting that mosquitoes can become infected and maintain JEV transmission between vertebrate host and vector populations (Takahashi et al., 1976; Soman et al., 1977; Rodrigues et al., 1981). In JE endemic areas of India, serosurveillance studies revealed that household ducks and chickens often had high JEV seroprevalence (Dhanda et al., 1977), demonstrating the importance of household transmission of JEV in rural parts of Asia. Various North American avian species including red-winged blackbird, rock pigeon, European starling, common grackle, house finch, mallard duck, ring-billed gull, cattle egret, and house sparrow are susceptible to JEV infection and the viremia titers in the avian hosts ranged from 2-5 $\log_{10}$PFU/ml (Nemeth et al., 2009; Nemeth et al., 2010; Nemeth et al., 2012). Furthermore, younger birds develop higher viremia, up to 7.5 $\log_{10}$PFU/ml in duckling and 5.5 $\log_{10}$PFU/ml in young chicks than the adult birds (Banerjee and Deshmukh, 1987; Cleton et al., 2014).

The involvement of pigs as reservoir hosts in the JEV transmission cycle was revealed through a series of studies worldwide, including the initial study in Japan. During outbreaks of JEV in Japan, pigs were kept as sentinel animal to check virus circulation (Scherer et al., 1959b). Scherer and colleagues tested the serum samples from pigs from both commercial farms and sentinel pigs, and found many antibody-positive pigs during mid-summer to early winter. Due to the rapid turnover of pig population and short marketable age, the immune pigs get replaced with naïve pigs, preventing the establishment of herd immunity against JEV. Also, pigs were commonly fed upon by known JEV vectors including *Culex tritaeniorhynchus* and efficiently transmitted JEV to naïve pigs under experimental condition (Gresser et al., 1958a). In JEV-

endemic areas of Asia, pigs were found to be the animal most commonly seropositive to JEV (Scherer et al., 1959a; Sazawa et al., 1968; Pant et al., 2006). Taken together, it was concluded that pigs are a natural reservoir host of JEV. A vaccine against JEV was developed utilizing the prM and envelope genes of JEV in recombinant modified vaccinia virus and induced neutralizing antibody (Nam et al., 2002), but the requirement of multiple doses and the cost factor limited its use.

1.4 Immune Response to Japanese encephalitis virus

1.4.1 Immune Response to Japanese encephalitis virus in Vertebrate Host

Most mammalian hosts develop a potent immune response upon natural or experimental infection of JEV. The E protein of JEV serves as a primary target for neutralizing antibodies and induces protective immunity in addition to flavivirus broadly cross-reactive, non-neutralizing antibodies. Polyclonal antisera produced from a given JEV infection cross-neutralizes a subset of other flaviviruses, which serves as a basis for classifying the flaviviruses in JEV serocomplex system (Madrid and Porterfield, 1974). In addition, the prM protein also induces neutralizing antibodies, but most of the time prM protein induces neutralizing antibodies to secondary JEV infection in animals previously infected with JEV. Although it is not common, NS1 protein induces antibodies that can protect against lethal infection. Non-structural proteins are thought not to counter the invading JEV by neutralizing the virus directly, but these proteins may mediate neutralization possibly through complement activation pathway. The strength of interaction between the flavivirus antigen's epitope and the antigen-binding site on the antibody (avidity) determines the neutralizing capacity (Pierson and Diamond, 2009). Low-affinity antibody (IgM) produced in response to primary infection usually neutralize the virus at relatively higher

concentrations than high-affinity antibody (IgG) produced during the secondary immune response. Neutralization of flaviviruses in the host can occur via multiple mechanisms, including virus-to-cell attachment blocking, viral membrane fusion inhibition by stopping conformational change in the E protein, Fc-dependent effector function activation of antibody for virus clearance through complement activation (Pierson and Diamond, 2009). Furthermore, the E protein can facilitate monoclonal antibodies production, and some monoclonal antibodies being more potent than others (Oliphant et al., 2005; Pierson and Diamond, 2009). Studies using mice have indicated that the transfer of either immune serum or spleen cells from mice immune to JEV, protected against JEV experimental challenge (Mathur et al., 1983).

Cross-protection against JEV infection can be elicited from closely related viruses belonging to JEV serocomplex. Studies conducted in both human and other animal populations have clearly demonstrated that challenge with either JEV or other JEV serocomplex flaviviruses following either vaccination or natural infection with one of the JEV serocomplex viruses protected animals from JEV challenge (Hammon et al., 1956; Goverdhan et al., 1992). In the study by Hammon et al., 1956 children who were naturally exposed to St. Louis encephalitis virus (SLEV) were given an inactivated JEV vaccine, which resulted in an increase in neutralizing titers for both SLEV and JEV. In the same study horses receiving anti-JEV vaccine but not exposed to SLEV did not develop neutralizing antibody. In another study, monkeys either immune to JEV or West Nile virus (WNV) were protected from WNV or JEV infection, respectively (Goverdhan et al., 1992). The findings suggest that vaccination does not elicit potent flavivirus cross-reactive antibody responses, but that viral infection does. In addition, pigs challenged with either JEV or WNV and re-challenged with the opposite viruses showed cross-protection (Ilkal et al., 1994). The study by Williams et al., 2001 had a similar result, in which

the pigs that received JEV challenge had elevated antibody response when re-challenged by Murray Valley encephalitis virus (MVEV) or Kunjin virus (KUNV). However, experimental infection with WNV of hamsters immune to a vaccine developed using non-JE serocomplex virus such as yellow fever 17D vaccine did not produce a significant amount of neutralizing antibody level and did not protect all the hamsters (Tesh et al., 2002). Taken together, these studies (Hammon et al., 1956, Goverdhan et al., 1992; Ilkal et al., 1994; Tesh et al., 2002) indicate that the prior infection of a virus from JEV serocomplex cross-protect at least partially from secondary infection with viruses from same JEV serocomplex but not against infection with flaviviruses outside the complex. An implication of these findings is that resident birds in the US that are immune to WNV would likely limit the spread of JEV infection if ever introduced into the United States (Nemeth et al., 2009). However, the protective efficacy of secondary immune response in humans and other animals naturally exposed to JEV serocomplex flaviviruses and duration of immunity is not yet fully understood.

1.4.2 Immune Response to Japanese encephalitis virus in Mosquito Vectors

While vertebrate hosts have both innate and adaptive immunity, mosquitoes only have innate immunity (Fragkoudis et al., 2009). The immune response in mosquito could play a crucial role in the extrinsic incubation period and vector competence by modulating virus replication and mounting an effective immune response against the virus (Blair, 2011). Innate immunity is the immediate response and first line of defense to infectious agents, and it is triggered when pattern recognition receptor (PRR) in the host cell encounters pathogen-associated molecular pattern (PAMP) (Blair and Olson, 2014). Mosquitoes contain several systemic and organ-specific antiviral immune strategies, and RNA interference (RNAi) pathway

is a major innate antiviral response in limiting viral infection, replication, and pathogenesis in mosquitoes. Although the RNAi response controls flavivirus infection, it does not abolish the infection in the vectors, and the virus evades the antiviral response (Sanchez-Vargas et al., 2009; Blair, 2011).

There are three major components of RNAi pathway, the exogenous small interfering (exo-si) RNA pathway, the Piwi-interacting (pi) RNA, and the micro (mi) RNA. The exo-siRNA response in flavivirus-infected mosquitoes is triggered by virus-derived long double-stranded RNA (dsRNA) that is formed during RNA replication in the infected host cell. A dsRNA is formed during virus replication and serves as PAMP, which triggers the PRR in the host cell to activate the RNAi response. Short-interfering RNAs formed from the long dsRNA are the effectors and is an effective and common antiviral immune response in mosquitoes. piRNAs are 24-30nt RNAs generated by "ping-pong" amplification in *Drosophila melanogaster* and act as an antiviral defense but its role in the defense is poorly understood (Chen et al., 2009; Blair and Olson, 2014). Little is known about miRNA in antiviral defense strategy in mosquitoes. Expression of miRNA in WNV-infected *Culex quinquefasciatus* mosquitoes has shown its importance in flavivirus infection of mosquitoes (Skalsky et al., 2010).

In addition to RNAi, other evolutionarily conserved RNA silencing, innate immune responses in mosquitoes are Toll, immune deficiency factor (Imd), and Janus Kinase (JAK)-signal transduction and activators of transcription (STAT) pathways (Luo et al., 2000; Chen et al., 2015). These pathways are responsible for a non-adaptive, innate immune response to viral infections. Although the information on the role of specific immune machinery of mosquitoes in

Japanese encephalitis virus infection is scarce, mosquito's immune response to other flaviviruses can help us elucidate the general idea in case of JEV.

The Imd pathway is commonly activated when mosquitoes are infected with Gram-negative bacteria (Fragkoudis et al., 2009). However, it has been shown that flavivirus infection also triggers the Imd pathway, for example, in *Aedes aegypti* salivary glands upon dengue virus infection (Luplertlop et al., 2011). Additionally, the Imd pathway is activated in *Anopheles gambiae* when infected by O'nyong'nyong virus (Carissimo et al., 2015). Similarly, the Toll pathway is activated by fungi or Gram-positive bacteria in mosquitoes, but its role in flavivirus infection is virus species-specific (Chen et al., 2015). Dengue virus replication was shown to be enhanced by knocking down the Toll pathway components in *Aedes aegypti* (Xi et al., 2008), but in the case of alphavirus infection in *Aedes albopictus*, Toll pathway does not control the infection (Fragkoudis et al., 2008). The pattern recognition receptor (PRR), once activated, triggers the Imd and Toll signaling cascades pathways. Furthermore, viral infection in mosquitoes also induces another antimicrobial immune pathway, the JAK/STAT pathway, through PRR initiation (Fragkoudis et al., 2009).

1.5 Diagnosis of Japanese encephalitis virus Infection

Like other flavivirus infections in susceptible vertebrate hosts, the time course of JEV infection and antibody production in the host starts with viremia, IgM production and ends with IgG production. Virus isolation and reverse transcription-polymerase chain reaction (RT-PCR) can be utilized for viremic samples to measure the quantity of infectious virus and viral RNA quantity, respectively. Virus isolation is conducted in cell culture system as described in Yamada et al., (2002), but due to the low magnitude and short duration of viremia, isolation of JEV is not

frequently successful. That leaves serologic diagnosis as the most useful way to test patients with suspected JEV. There are several serological methods employed to detect IgM or IgG antibodies for the diagnosis of JEV exposure, including enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody (IFA) test, hemagglutination-inhibition (HI) assay, and plaque reduction neutralization test (PRNT). The HI assay is a classical serodiagnostic used in flavivirus infection, in which total antibodies is measured from paired samples (Nagarkatti and Nagarkatti, 1980). Both IgM and IgG antibodies can measured in HI assay, and it is based on the principle that antibodies developed in response to virus infection inhibit the virus-induced erythrocyte agglutination. The HI assay is non-species specific and can be applied in serosurveillance to test samples from humans and other animals. HI assay used for diagnosis of clinically confirmed JEV cases in Nepal showed result closely matching the IgM ELISA result, 68% and 62%, respectively (Pandey et al., 2003). Though the HI assay does not require control antibodies, the cross-reactivity among the same serocomplex flaviviruses is higher. Similarly, indirect fluorescent antibody (IFA) test detects both IgM and IgG in serum against flaviviruses and can be used for multi-species (Maeda and Maeda, 2013; Foral et al., 2007). Although, the IFA test cannot differentiate acute infection (IgM) from chronic infection (IgG). IFA is simple to use but is somewhat subjective and depends on laboratorian's experience in distinguishing the infected cells versus non-infected cells. Like the IFA test and HI assay, ELISA is another assay based on the principle that complex formed between antigen and antibody linked enzyme immobilized on solid surface imparts measurable product when the substrate binds to the preformed complex. ELISA and PRNT have high specificity for diagnosis of flavivirus in humans and other animals than HI assay and IFA test (Calisher et al., 1989; Maeda and Maeda, 2013).

Due to the requirement of paired samples and the inability to give early diagnosis using the HI assay, ELISA has become very popular, affordable, feasible, and reliable method of diagnosing JEV serologically in humans and other animals. Neutralizing antibody assays of paired serum samples is considered as gold standard for JE diagnosis (Jacobson et al., 2007), but the infrastructure to conduct such assays is not available in many JE-endemic countries. Instead, such countries depend on commercially available or in-house ELISA, which have become a desirable standard for JE diagnostics (Jacobson et al., 2007; Cuzzubbo et al., 1999; Burke et al., 1985). Cerebrospinal fluid and blood samples are obtained from humans to test for anti-JEV specific antibodies utilizing IgM or IgG based ELISA. IgM capture ELISA has been commonly utilized in Nepal for diagnosis of exposure to JEV from human serum and CSF samples. Among many serologic tests for JEV infection, CSF anti-JEV IgM based ELISA have a higher diagnostic accuracy than serum anti-JEV IgM based ELISA (Giri et al., 2013). The cross-reactivity among flaviviruses is well-recognized issue, and therefore many test samples from JE-endemic countries where other flaviviruses such as dengue and West Nile virus is circulating, samples are also tested for these virus exposures (Solomon et al., 2000; Jacobson et al., 2007; Tsai et al., 2000).

1.6 Vaccination against Japanese encephalitis virus

Because there is no specific antiviral therapy for JE patients and environmental management has been an expensive endeavor in JE endemic countries, the most effective alternative currently available to prevent JE infection is vaccination. Prior to the manufacture of live attenuated vaccines, inactivated mouse brain-derived P3 vaccine was used in some countries including China (Marfin and Gubler, 2005). The cost associated with administering multiple

doses of the P3 vaccine and its relatively poor efficacy led to the development of the live-attenuated SA-14-14-2 vaccine (Marfin and Gubler, 2005; Bista et al., 2001). A single dose of SA-14-14-2 given to non-immune children in China, Nepal, and South Korea induced an antibody response in 85-100% children. A booster dose given a year after the first dose resulted in 99.3% protective efficacy compared to 97-98.5% efficacy with single dose of SA-14-14-2 vaccine in preventing JE (Marfin and Gubler, 2005; Halstead and Tsai, 2004; Hennessy et al., 1996; Bista et al., 2001; Ohrr et al., 2005). Protective efficacy up to 5 years after the administration of SA-14-14-2 vaccine has been reported (Tandan et al., 2007). This vaccine has been shown to be effective in preventing JE outbreaks in Nepal (Ohrr et al., 2005). There have not been any reports of safety issues, adverse events or reversal to virulence of the SA-14-14-2 vaccine strain (WHO, 2005). Also, the SA-14-14-2 is relatively less expensive compared to the P3 vaccine. Many countries in Asia, including Nepal, have promoted administration of SA-14-14-2 vaccine and greatly reduced the number of JE cases. Childhood immunization in rice farming and rural areas in JE endemic countries is an important public health intervention for children. In Nepal, during 2014-2015, the JE vaccination average was 52.5% in children under 23 months, and 32 districts out of 75 were covered. However, the national JE vaccination wastage rate was 41% when one dose was administered in the population (DHS Annual Report 2014-15). Although vaccines based on exotic strain are effective for JEV cases in JE endemic areas, it is still questionable if the vaccines would be effective against local strains of JEV in the long run (Henderson, 1983; Das, 1976; Sohn et al., 2008). The protective efficacy of single dose versus multiple doses is currently not confirmed, and is an important issue because a single dose can greatly reduce the cost for both users and producers and can be administered to even larger population at risk.

1.7 Rationale for the Current Study

Japanese encephalitis is an emerging zoonosis in several areas of Asia. With the recent finding of autochthonous cases in Africa and of establishment of known JEV vectors in non-JE endemic countries, JEV poses a global threat. Vector control and confined pig and poultry farming are virtually impossible in resource-poor JE epidemic-prone or endemic countries, which leaves immunization and personal protective measures as the only control and preventive measure to pursue. Use of insecticides is common practice during mosquito season in several countries in Asia, including Nepal, but any discontinuity in insecticide spraying can abrogate that protection. The distribution of insecticidal bed nets to people in endemic districts by the Government of Nepal have not been a successful approach because of the improper use of the nets and the loss of the chemical effect of insecticide-treated bed nets on mosquitoes (Rayamajhi et al., 2007). While human vaccines for JEV are available, the cost factor for the multiple doses required to attain maximum protective efficacy against JEV is a bottleneck for JE prevention campaigns. In addition, vaccinating pigs against JEV is another approach that could be done if vaccine for pigs were commercially available at a price that farmers from JE-endemic countries can afford. Transmission among mammalian, avian and arthropods continues to be a threat despite human vaccinations.

Rupandehi is one of the JE risk areas of Nepal where this disease was first identified and childhood immunization against JEV is practiced. Despite the improvements in immunization rates, every year new JE cases are reported from Rupandehi and other similar districts in the southern lowland of Nepal. Pig farming has increased substantially and, in conjunction with flooded rice paddy farming, creates an excellent environment for JEV transmission. We

hypothesized that JEV is not circulating in domestic animals and that the vectors that are known to be JEV transmitters are not found in the district under study in Nepal. The second hypothesis is that the farmers have limited knowledge of JE and lack the means to control the disease in and around their households.

Historical outbreaks of JEV have involved genotype displacement, in which genotype III was displaced by genotype I, partially in some areas and completely in other areas. The displacement of JEV can be explained to some extent by the nucleotide sequence analysis of strains isolated so far and utilizing *in vitro* assays. However, the question remains as to how the viruses of each genotype replicate in actual ecologically-relevant hosts. *In vitro* assays do not provide a clear picture of virus fitness within the complex system of a living host. Further study in reservoir hosts is required to be able to explain the displacement phenomenon better. The hypothesis to test genotype displacement was that interactions of JEV in mosquitoes and ducks play an important role in fitness advantage for genotype I over genotype III.

CHAPTER 2: SEROPREVALENCE OF JAPANESE ENCEPHALITIS VIRUS IN PIGS, DUCKS, AND CHICKENS IN NEPAL AND CHARACTERIZATION OF RISK FACTORS FOR JAPANESE ENCEPHALITIS VIRUS INFECTION IN DOMESTIC ANIMALS

2.1 Introduction

Japanese encephalitis virus (JEV) is a mosquito-borne virus of the genus *Flavivirus* and family *Flaviviridae,* and the leading cause of arboviral encephalitis in the world (Mackenzie et al., 2004). The virus is zoonotic and transmitted by mosquitoes, primarily *Culex* species, infected by the virus obtained while blood feeding on mammals and birds in their viremic stage (Williams et al., 2001; van den Hurk et al., 2009). Among many *Culex* species, *Culex tritaeniorhynchus, Culex vishnui,* and *Culex pseudovishnui* are primary vectors for transmitting JEV to humans and reservoir hosts. *Culex tritaeniorhynchus* is common in rice farming areas, as it prefers to breed in the stagnant water of rice paddy fields (Hammon et al., 1949; Buescher et al., 1959b; Erlanger et al., 2009). Pigs and birds of the family *Ardeidae* are common reservoir hosts of the virus (Buescher et al., 1959a; Cleton et al., 2014; Nemeth et al., 2012). Humans are dead-end hosts for JEV, but infection causes substantial morbidity and mortality. Although human vaccines are available, JEV continues to cause 50,000-60,000 cases of encephalitis worldwide every year, affecting predominantly children (Campbell et al., 2011; Mackenzie et al., 2004). Infection in pigs is usually subclinical, however infection in pregnant sows can result in abortion and infected neonatal piglets may die (Hurlbut, 1964; Henderson, 1983; Mackenzie et al., 2004). Serological studies in field and experimental infections have indicated that domestic pigs and domestic birds, including chickens and ducks, can be infected and can serve as potential reservoir hosts of JEV

(Nemeth et al., 2012; Buescher et al., 1959a; Thakur et al., 2012; van den Hurk et al., 2009). Domestic livestock thereby pose a threat of JEV outbreaks in farming communities.

Japanese encephalitis virus is common in Asia, where paddy farming, pig husbandry, and mosquitoes are common, and the distribution of the virus has extended from Asia to Australasia (Miller et al., 2012). Nepal is a JEV-endemic country, with vast paddy-farming land and mixed livestock husbandry that includes chickens, ducks, pigs, goats, cattle, and buffalo. The conventional animal farming practice in Nepal is of the free-ranging type. JEV was first reported in humans in Nepal in 1982, in Rupandehi district, and later reported from several other districts including those in lowlands, mid-hills and lower altitudinal regions of the high hills (Joshi, 1983; Dhakal et al., 2014; Impoinvil et al., 2011; Rayamajhi et al., 2007; Thakur et al., 2012). Despite the endemic nature of JEV in Nepal, few studies of the animal reservoirs have been reported. The aim of the work reported here was to characterize the frequency of previous exposure to JEV in pigs, ducks, and chickens in Rupandehi district of Nepal and characterize risk factors for exposure of those animals to JEV.

2.2 Materials and Methods

2.2.1 Ethical Consideration

Animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Colorado State University (protocol number 14-4849A). Permission to perform domestic animal studies in Nepal was obtained from Nepal Veterinary Council. Informed consent was obtained from owners of domestic animals before sampling blood, and a registered veterinarian of Nepal (author) collected all blood samples.

2.2.2 Study Design, Study Population, and Sample Collection

A cross-sectional study was designed to estimate the species-specific and farm level seroprevalence of anti-JEV antibody in the Rupandehi district of Nepal. In 1978, Rupandehi district was the first district in Nepal from which a JEV outbreak was reported, and JE cases have been reported in that district in every subsequent year. JE vaccination coverage among children in Rupandehi is 94% (DHS Annual Report 2014-2015). From July 2014 to October 2014, households having at least one of the species of interest (pig, duck, and chicken) were selected on a convenience basis. The geographical distribution of sampling sites for each animal species sampled is shown in Figure 1.2. Convenience factors included accessibility and availability of time, resources, and domestic animals in the households. The reference population (target population) for our cross-sectional study was pigs, ducks, and chickens in the Rupandehi district and the districts of Nepal closely matching with Rupandehi districts of Nepal in terms of environment, topography, and agriculture. The study units (study population) were pigs, ducks, and chickens associated with households of the Rupandehi district. A sample size required for each domestic animal species was calculated based on the animal level expected prevalence of 30% (pig), 20% (duck), and 15% (chicken), with 5% margin of error and 95% confidence of detecting anti-JEV antibodies. The obtained sample sizes were 323 (pig), 246 (duck), and 196 (chicken), however, domestic animals were over-sampled and final sample sizes were 339 (pig), 288 (duck), and 209 (chicken). A multi-stage sampling with sampling size from each farm was proportionate (20%) to the farm size whenever possible, assuming within-household variation was small. Briefly, households with pigs, ducks or chickens were selected based on convenience, and then a subset of pigs, ducks, and/or chickens from each household were sampled. Only those domestic animals were selected to sample that were easy to approach and handle. Only pigs older

than four months were included in the study. Blood samples from pigs (5ml), chickens (2ml), and ducks (2ml) were obtained by jugular venipuncture under aseptic conditions. Serum was separated in the field using a portable centrifuge, stored temporarily at 4°C, then transported to Kathmandu and stored at -20°C until assay.

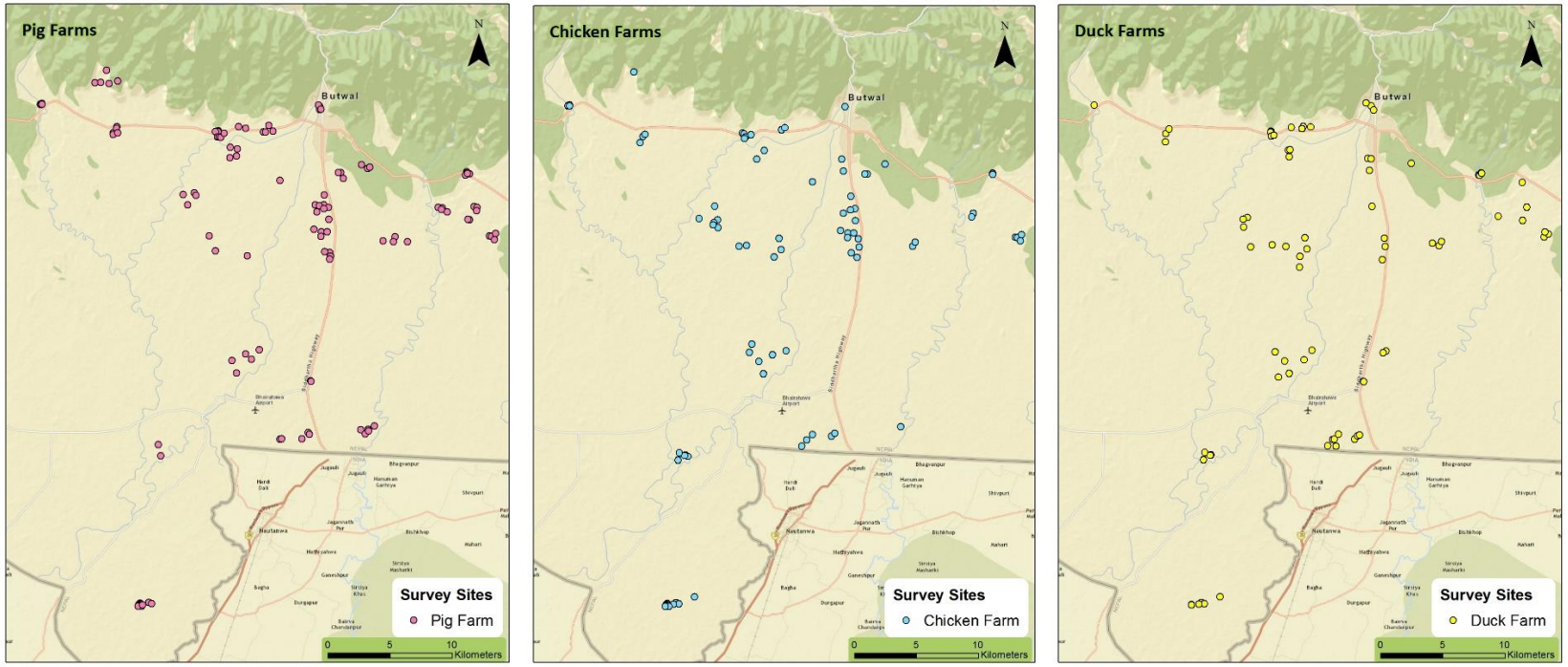


Figure 2.1 Survey Sites of Pigs, Chickens and Ducks in Rupandehi District of Nepal

2.2.3 Data Collection

Data were collected for each domestic animal species and their farms using a standardized questionnaire for their owners. Individual animal sex, age, and the breed were obtained. Breed information of chickens and ducks were not recorded due to difficulty in differentiating their breeds since most of the ducks and chickens were locally and indiscriminately bred. Prior abortion history was recorded only when abortion occurred in last two years in the same pig farm. Similarly, prior disease (morbidity and mortality) in the chicken or duck farms was recorded only when the disease was observed within last two years of data collection. Demographic information such as locality, free range, farm size, household fermentation practice, individual domestic animal or farm distance to paddy field, individual domestic animal or animal farm in the household courtyard, domestic animal composition, domestic animal in household courtyard, prior JEV vaccine, prior classical swine fever vaccine, and prior other vaccines were collected. Fermentation process using organic matter and plant products emits carbon dioxide (Barnett, 2003) and which attract mosquitoes and could therefore influence transmission of arboviruses such as JEV (Smallegange et al., 2010). GIS data were obtained by using a handheld GPS set (Etrex10, Garmin, KS) for both individual domestic animals and their farms.

2.2.4 Serologic Testing

Antibodies to JEV were assayed using an indirect fluorescent antibody (IFA) test. Slides for this assay were initially prepared at Colorado State University. Drained monolayers of Vero cells were inoculated with the P3 strain of JEV at a MOI of 0.01, incubated for 1 hour at 37°C, and re-fed with medium. Substantial cytopathic effect occurred three days later and cells were

collected by scraping from the flasks; non-infected Vero cells were collected similarly. Infected and non-infected cells were collected by centrifugation and re-suspended in PBS. After adjustment of cell density, the cells were then pipetted into wells of Teflon-coated spot slides (Figure 1.2) in such a way that each slide contained a row of infected and a row of non-infected cells. After air drying, the slides were fixed in 70% acetone for 30 minutes, dried and stored in the refrigerator. Slides were transported to the Central Veterinary Laboratory (CVL) of the Government of Nepal in Kathmandu, where the IFA assay was conducted.

Sera from pigs, ducks and chickens were diluted 1:100 in PBS [1.24gm/L Na_2HPO_4, 0.18gm/L $NaH_2PO_4 \cdot H_2O$ and 8.5gm/L NaCl] and each diluted serum sample was pipetted into one well of JEV-infected and one well of non-infected cells. The slides were incubated for 60 minutes at 37°C, washed 2-3 times in PBS, rinsed in water and dried. The slides were then exposed for 60 minutes at 37°C to an FITC anti-species conjugate, washed, and dried as before. The slides were mounted using 90% glycerol in PBS and observed with a fluorescent microscope (Olympus BX60). When sera from pigs, ducks, and chickens were tested for evidence of exposure of JEV by IFA test, the positive test sera looked bright, and the negative test sera looked dull under fluorescent microscopy (Figure 1. 3 a, b). This assay system was tested in a blinded manner at Colorado State University using known positive and negative pig sera and was found more than 90% accurate in identifying the true antibody status of test sera.

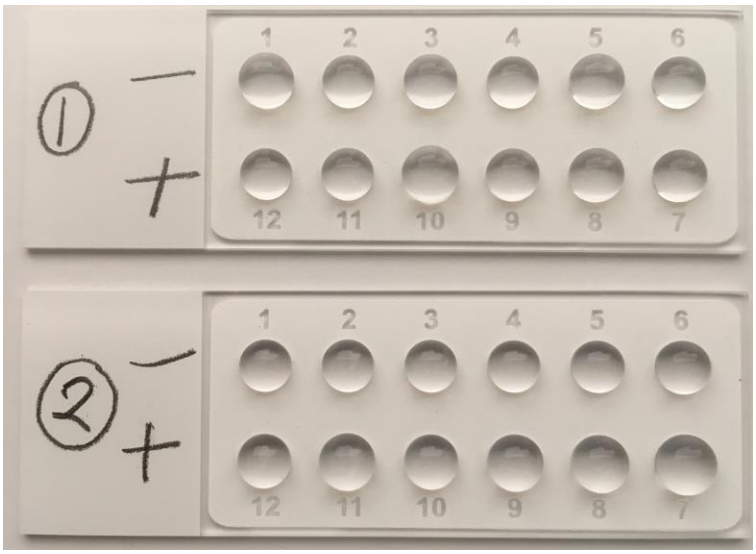
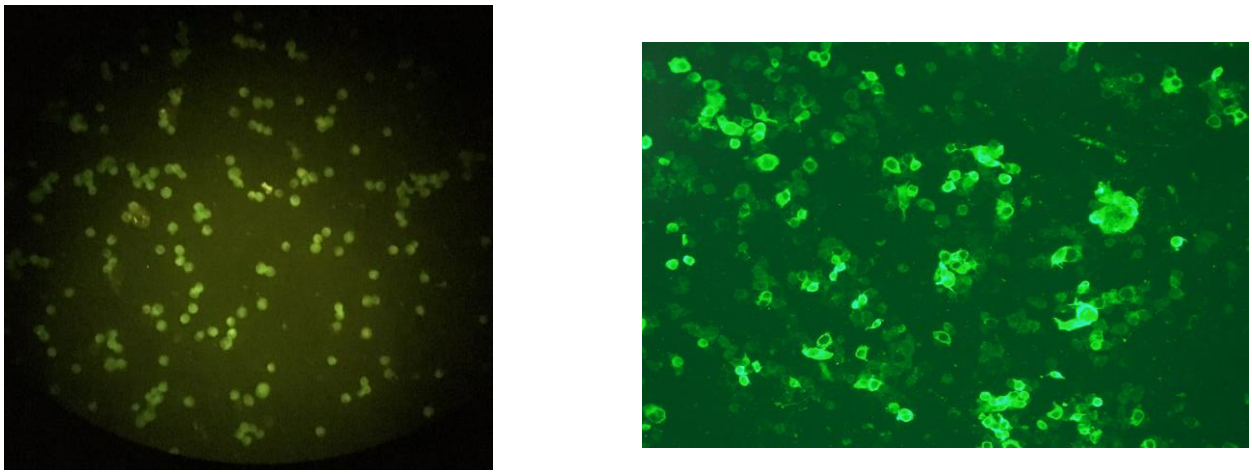


Figure 2.2 Spot Slides with Diluted Serum Samples



(a) Negative pig serum

(b) Anti-JEV antibody positive pig serum

Figure 2.3 Slides under fluorescent microscope

2.2.5 Data Analysis

Serum samples were tested once. The IFA test result for each serum sample was categorized as 1=true negative (no fluorescence); 2=questionable (difficult to evaluate between

negative and moderate); 3=moderate (clearly positive but does not look strong); and 4=strong positive (very clear difference between infected and non-infected cells). For data analysis, categories 1 and 2 were considered negative and categories 3 and 4 were classified as positive. The association between serological status of each domestic animal and their demographic characteristics were assessed using Chi-square test of univariate association with selected risk factors. Odds ratios along with their 95% confidence interval were calculated. The associations were regarded statistically significant when $p < 0.05$. Data analysis was done with SAS (SAS Institute INC, Cary, NC). The spatial distribution of the selected farms/households was plotted on a map using ArcGIS 10.4 (ESRI, Redlands, CA).

2.3 Results

2.3.1 Demographic Characteristics

A total of 183 households were visited, and serum samples were collected from 339 pigs, 288 ducks, and 209 chickens, along with their respective demographic data (Table 2.1). None of the animals had received JEV vaccines. A total of 120 farms with pigs, 79 farms with ducks and 86 farms with chickens were sampled (Table 2.2).

Table 2.1 Species-specific individual domestic animal characteristics

Variables	Pig	Duck	Chicken
Sex			
Male	165 (48.67%)	154 (53.5%)	119 (56.94%)
Female	174 (51.33%)	134 (46.53%)	90 (43.06%)
Median age (range) (months)	8 (5-18)	ND	ND
Age (months)			
Up to 8	224 (66.08%)	ND	ND
9-above	115 (33.92%)		
Breed			

Indigenous	25 (7.37%)	ND	ND
Exotic or mixed	314 (92.63%)		
Farm size			
Up to 15 (pig); Up to 8 (chicken and duck)	140 (41.3%)	90 (31.25%)	80 (38.28%)
16-above (pig); 9-above (chicken and duck)	199 (58.7%)	198 (68.75%)	129 (61.72%)
Locality			
Rural	230 (67.85%)	183 (63.54%)	164 (78.47%)
Urban	109 (32.15%)	105 (36.46%)	45 (21.53%)
Household practicing fermentation			
Yes	196 (57.85%)	110 (38.19%)	78 (37.32%)
No	143 (42.18%)	178 (61.81%)	131 (62.68%)
Animal to paddy field distance			
<500m	230 (67.85%)	183 (63.54%)	164 (78.47%)
>500m	109 (32.15%)	105 (36.46%)	45 (21.53%)
Animal in household courtyard/backyard			
Yes	284 (83.78%)	212 (73.61%)	152 (72.73%)
No	55 (16.22%)	76 (26.39%)	57 (27.27%)
Living with other animals			
Yes	186 (54.87%)	119 (41.32%)	30 (14.35%)
No	153 (45.13%)	169 (58.68%)	179 (85.65%)
Prior anti-JEV vaccine			
Yes	0	0	0
No	339 (100%)	288 (100%)	209 (100%)
Prior anti-CSFV vaccine			
Yes	148 (43.7%)	NA	NA
No	191 (56.3%)		
Prior other vaccines			
Yes	0	0	0
No	339 (100%)	288 (100%)	209 (100%)
Free range			
Yes	35 (10.32%)	288 (100%)	209 (100%)
No	304 (89.68%)	0	0

ND: No data; NA: Not applicable

Table 2.2 Species specific-farm level characteristics

Variables	Pig	Duck	Chicken
Locality			
Rural	78 (65%)	59 (74.7%)	61 (70.9%)
Urban	42 (35%)	20 (25.3%)	25 (29.1%)
Household practicing fermentation			
Yes	68 (56.7%)	32 (40.5%)	33 (38.4%)
No	52 (43.3%)	47 (59.5%)	53 (61.6%)
Animal farm to paddy field distance			
<500m	78 (65%)	59 (74.7%)	61 (70.9%)
>500m	42 (35%)	20 (25.3%)	25 (29.1%)
Farm-history of abortion			
Yes	19 (15.8%)	NA	NA
No	101 (84.2%)		
Farm practicing free range			
Yes	12 (10%)	78 (98.7%)	86 (100%)
No	108 (90%)	1 (1.3%)	0
Median farm size, (Range)	11 (4-56)	8 (2-21)	7 (3-29)
Farm size			
Up to 15 (pig); Up to 8 (duck and chicken)	81 (67.5%)	46 (58.2%)	55 (63.9%)
16-above (pig); 9-above (duck and chicken)	39 (32.5%)	33 (41.8%)	31 (36.1%)
Median age to market/slaughter (Range), months	9 (2-36)	8 (5-12)	8 (5-18)
Median farming duration (Range), years	3 (1-10)	3 (0.5-10)	3 (0.8-10)
Farm in household courtyard/backyard			
Yes	105 (87.5%)	71 (89.9%)	78 (90.7%)
No	15 (12.5%)	8 (10.1%)	8 (9.3%)
domestic animal's entry into household at night	0	17 (21.5%)	34 (39.5%)
Yes	120 (100 %)	62 (78.5%)	52 (60.5%)
No			
Household domestic animal composition			
Single species	54 (45%)	29 (36.7%)	10 (11.6%)
Multiple species	66 (55%)	50 (63.3%)	76 (88.4%)

NA: Not applicable

2.3.2 Species-specific Seroprevalence

Initially, sera from pigs, ducks, and chickens were qualitatively classified into four categories (Table 2.3). For the simplicity, the strong and the moderate positive samples were grouped as positive, and the questionable and the negative samples were grouped as negative serum samples of all domestic animals sampled (Table 2.4).

Table 2.3 Distribution of JE sero-status of livestock species in different IFA subgroups

	Strong Positive	Moderate	Questionable	Negative	Total
Pig	34 (10%)	17 (5%)	83 (24.5%)	205 (60.5%)	339
Duck	9 (3.1%)	25 (8.7%)	58 (20.1%)	196 (68.1%)	288
Chicken	2 (0.9%)	12 (5.7%)	52 (24.8%)	143 (68.4%)	209

Table 2.4 Seroprevalence of anti-JEV antibodies in livestock species

	Pig	Duck	Chicken
Animal level seroprevalence			
proportion (n)	50 (339)	34 (288)	14 (209)
Percentage (95% CI)	14.7% (11-18.5)	11.8% (8-15.5)	6.7% (3.3-10)
Farm level seroprevalence			
proportion (n)	38 (120)	25 (79)	11 (86)
Percentage (95% CI)	31.7% (23.2-40.1)	31.6% (21.2-42.1)	12.8% (5.5-20)

n, Total sample number; CI, confidence interval

2.3.3 Risk Factors for Japanese encephalitis virus Infection in Pigs, Ducks, and Chickens

Based on directed acyclic graphs (DAGs) theory (Greenland et al., 1999) and the ecology of JEV, potential covariates were identified for the outcome of interest (serostatus) for each species, both at individual and farm levels. All 339 pigs, 288 ducks, 209 chickens as well as all 120 pig farms, 79 duck farms, and 86 chicken farms tested for the exposure to JEV were

evaluated for demographic characteristics. Univariable relationships between each predictor variables of pigs and farms with pigs and seropositivity for JEV are described in Tables 2.5 and 2.6. Pig age equal to or lower than eight months were significantly associated with JEV seropositivity ($p=0.001$). Female pigs were 2.3 times more likely to have been exposed with JEV as indicated by being seropositive ($p=0.01$). Similarly, the odds of JEV seropositivity in pigs of rural areas were 2.1 times higher than urban pigs ($p=0.04$). Furthermore, pigs from fermentation practicing household were 2.6 times more likely to be seropositive for JEV than those pigs not exposed to household fermentation ($p=0.004$). Interestingly, the odds of pigs housed away from the household courtyard to be JEV seropositive were 2.1 times greater than those housed in the courtyard. Close proximity to paddy fields was significantly associated with JEV seropositivity in pigs, and the odds of being less than 500 meters closer to paddy field was 2.1 times higher than pigs which are housed more than 500 meters from the paddy field. While considering the farms with pigs, odds of finding JEV seropositivity was 3.7 times greater on farms with a history of abortion ($p=0.007$). Similarly, rural farms with pigs, household fermentation, larger farm size (more than 16 pigs) and closer to paddy field were significantly associated with JEV seropositivity ($p<0.05$) (Table 2.6).

Tables 2.7 and 2.8 display the univariate relationships of predictor variables of ducks and JEV seropositivity and predictor variables of farms with ducks and JEV seropositivity, respectively. Rural ducks, household fermentation, at least 500 meters closer to paddy field, and farm size up to 8 were significantly associated with JEV seropositivity, at an individual level ($p<0.05$). At the farm level, exposure to fermentation at a household level significantly increases the possibility of finding a seropositive duck by 6.5 times. Tables 2.9 and 2.10 show the univariate association of predictor variables of chickens with JEV seropositivity and predictor

variables of farms having chickens with JEV seropositivity. None of the predictor variables of chickens at the individual level was significantly associated with JEV seropositivity. Only farm size equal to or greater than 16 was significantly associated with JEV seropositivity at the farm level.

Table 2.5 Univariable association of select variables of individual pigs with IFA result

Variables	Odds ratio	95% CI	p-value
Age *(months)*			
4 to 8	3.7	1.6-8.4	0.001
9-above	Reference		
Sex			
Male	Reference		
Female	2.4	1.2-4.3	0.01
Breed			
Indigenous	Reference		
Exotic or mixed	2.1	0.5-9.1	0.3
Free range			
Yes	Reference		
No	1.04	0.4-2.8	0.9
Locality			
Rural	2.1	1.1-4.3	0.04
Urban	Reference		
Household fermentation			
Yes	2.6	1.3-5.2	0.004
No	Reference		
Domestic animal in household courtyard			
Yes	Reference		
No	2.1	1.01-4.2	0.04
Paddy field to farm distance			
<500 meters	2.1	1.1-4.3	0.04
>500 meters	Reference		
Household domestic animal composition			
Single species	Reference		
Multiple species	1.1	0.6-1.9	0.8
Farm size			
Up to 15	Reference		
16-above	1.8	0.9-3.4	0.07

Table 2.6 Univariable association of select variables of farms having pigs with IFA result

Variables	Odds ratio	95% CI	p-value
Farm-history of abortion			
Yes	3.8	1.4-10.4	0.007
No	Reference		
Free range			
Yes	1.6	0.5-5.5	0.5
No	Reference		
Locality			
Rural	7.3	2.4-22.6	0.0001
Urban	Reference		
Household fermentation			
Yes	4.3	1.8-10.6	0.0008
No	Reference		
Farm in household courtyard			
Yes	Reference		
No	3.9	1.3-12.0	0.01
Household domestic animal composition			
Single species	1.6	0.7-3.4	0.2
Multiple species	Reference		
Farm size			
1-15	Reference		
16 and above	5.3	2.3-12.1	<0.0001
Paddy field to farm distance			
<500 meters	7.3	2.4-22.6	0.0001
>500 meters	Reference		

Table 2.7 Univariable association of select variables of individual ducks with IFA result

Variables	Odds ratio	95% CI	p-value
Sex			
Male	Reference		
Female	1.8	0.8-4.2	0.1
Locality			
Rural	3.3	1.1-9.8	0.02
Urban	Reference		
Household fermentation			
Yes	7.7	2.8-21.2	<0.0001
No	Reference		
Paddy field to farm distance			
<500 meters	3.3	1.1-9.8	0.02
>500 meters	Reference		
Household domestic animal composition			
Single species	Reference		
Multiple species	1.6	0.6-3.7	0.3
Farm size			
Up to 8	3.1	1.4-7.2	0.005
9-above	Reference		

Table 2.8 Univariable association of select variables of farms having ducks with IFA result

Variables	Odds ratio	95% CI	p-value
Locality			
Rural	2.1	0.5-8.2	0.3
Urban	Reference		
Household fermentation			
Yes	6.5	2.0-20.9	0.0007
No	Reference		
Household domestic animal composition			
Single species	1.01	0.3-2.9	0.9
Multiple species	Reference		
Farm size			
Up to 8	1.3	0.5-3.8	0.6
9- above	Reference		
Paddy field to farm distance			
<500 meters	2.1	0.5-8.2	0.4
>500 meters	Reference		
Domestic animal's entry into household at night	Reference		
Yes	2.8	0.6-13.7	0.2
No			

Table 2.9 Univariable association of select variables of individual chickens with IFA result

Variables	Odds ratio	95% CI	p-value
Sex			
Male	Reference		
Female	1.6	0.5-4.9	0.4
Locality			
Rural	3.5	0.4-27.5	0.3
Urban	Reference		
Household fermentation			
Yes	1.5	0.5-4.6	0.5
No	Reference		
Domestic animal in household courtyard			
Yes	Reference		
No	1.6	0.5-5.4	0.5
Paddy field to farm distance			
<500 meters	3.5	0.4-27.5	0.3
>500 meters	Reference		
Household domestic animal composition			
Single species	1.9	0.5-7.3	0.4
Multiple species	Reference		
Farm size			
Up to 8	Reference		
9-above	3.6	0.8-16.8	0.1

Table 2.10 Univariable association of select variables of farms having chicken with IFA result

Variables	Odds ratio	95% CI	p-value
Locality			
Rural	4.7	0.6-38.9	0.2
Urban	Reference		
Household fermentation			
Yes	1.4	0.4-5.01	0.7
No	Reference		
Farm in household courtyard			
Yes	Reference		
No	5.3	1.05-26.2	0.06
Household animal composition			
Single species	3.6	0.8-16.9	0.1
Multiple species	Reference		
Farm size			
1-15	Reference		
16 and above	5.7	1.5-21.6	0.01
Paddy field to farm distance			
<500 meters	4.7	0.6-38.9	0.2
>500 meters	Reference		
Domestic animal entry into household at night			
Yes	Reference		
No	1.2	0.3-4.3	1.00

A multivariable analysis was done for all significant predictor variables at the individual domestic animal and the farm levels with the outcome of interest. The limitation of the animal level model was that the model was not adjusted for clustering at the farm level. For individual pigs (Table 2.11), lower age group (up to 8 months), female, rural locality, and household fermentation were found to be significantly associated with JEV seropositivity. Distance to rice paddy fields was removed by the statistical program from the multivariable model due to a collinear effect, as rural locality was linearly associated with rice faddy farming (rural locality totally predicted distance to rice paddy fields). Similarly, for farms with pigs (Table 2.12), rural locality, household fermentation, a farm in the household courtyard, and higher farm size were

significantly associated with JEV seropositivity. Surprisingly, the farm history of abortion was not significantly associated with JEV seropositivity. Since there were no significant predictor variables of individual chickens for JEV seropositivity and only one significant predictor variable of the chicken farms for JEV seropositivity, a multivariable model was not appropriate. Among the significant predictor variables of JEV seropositivity for individual ducks (Table 2.13), only household fermentation and smaller farm size (up to 8) were significantly associated with JEV seropositivity ($p<0.05$).

Adding interaction terms of locality, household fermentation, and paddy field distance did not result in statistically significant interaction at the individual pig level. Therefore, the final model based on forward selection included age ($p=0.001$), sex ($p<0.0001$), locality ($p=0.04$), and household fermentation ($p=0.001$), and the Hosmer and Lemeshow Goodness of Fit test for the final model fails to reject null hypothesis ($p=0.6$) and concludes the model is fit. Similarly, adding interaction terms of locality, farm history of abortion, and household fermentation did not result in significant interactions at farms level for pigs. Therefore, the final model for farms with pigs for IFA test include farm size ($p=0.001$), household fermentation ($p<0.001$), locality ($p=0.04$) and farm in the courtyard ($p=0.02$) based on forward selection, and the Hosmer and Lemeshow Goodness of Fit test for the final model fails to reject null hypothesis ($p=0.6$), and concludes the model is fit. Furthermore, for individual ducks adding interaction terms of locality, household fermentation, and farm size, did not result in significant interactions at individual duck level. Therefore, the final model included fermentation at home ($p<0.0001$) and farm size ($p=0.004$) based on forward selection, and the Hosmer and Lemeshow Goodness of Fit test for the final model fails to reject the null hypothesis ($p=0.9$), and concludes the model is fit.

Table 2.11 Multivariable association of significant variables of individual pigs with IFA result

Variables	Adjusted Odds ratio	95% CI	p-value
Age *(months)*			
4 to 8	8.5	3.4-21.3	<0.0001
9 and above	Reference		
Sex			
Male	Reference		
Female	4.4	2.2-9.2	<0.0001
Locality			
Rural	2.3	1.04-5.3	0.03
Urban	Reference		
Household fermentation			
Yes	2.6	1.2-5.7	0.01
No	Reference		
Domestic animal in household courtyard	0.6	0.3-1.5	0.3
Yes	Reference		
No			

Table 2.12 Multivariable association of select variables of farms having pigs with IFA result

Variables	Adjusted Odds ratio	95% CI	p-value
Farm-history of abortion			
Yes	1.9	0.6-6.5	0.3
No	Reference		
Locality			
Rural	4.8	1.3-18.7	0.02
Urban	Reference		
Household fermentation			
Yes	3.9	1.3-11.8	0.01
No	Reference		
Farm in household courtyard			
Yes	0.2	0.04-0.9	0.04
No	Reference		
Farm size			
1-15	0.2	0.06-0.5	0.001
16 and above	Reference		

Table 2.13 Multivariable association of select variables of individual ducks with IFA result

Variables	Odds ratio	95% CI	p-value
Locality			
Rural	1.8	0.5-5.6	0.34
Urban	Reference		
Household fermentation			
Yes	8.3	2.9-24.1	<0.0001
No	Reference		
Farm size			
1- 8	3.7	1.5-9.3	0.004
9 and above	Reference		

2.4 Discussion

In Nepalese households, a JEV transmission interface exists between susceptible humans, competent and amplifying livestock species, and competent vectors. Our study demonstrated seroprevalence of 14.7% for pigs, 11.8% for ducks, and 6.7% for chickens in the Rupandehi district of Nepal. We did not assess the sensitivity and specificity of the IFA test that we established with precision, but as explained in materials and methods section, the test system was 90% accurate to detect the true positive from the true negative serum samples. The IFA test system is a classical qualitative test system and the ability of the test to differentiate infected or exposed from non-infected or unexposed animals depends on laboratorian's experience (personal communication with Dr. Charles Calisher). All the sampled domestic animals were associated with households, meaning that they were either within the household courtyard or nearby but outside of the courtyard. The farms to which they belonged were not large-scale commercial farms. Finding seropositive domestic animals suggests that humans in the same household are exposed to JEV. A high farm-level seroprevalence was also calculated, with 31.7%, 31.6%, and 12.8% in farms with pigs, ducks, and chickens, respectively.

Previous studies of JEV in pigs, buffaloes, chickens, and ducks kept in containment in the eastern part of Nepal showed antibody against JEV in pigs of all ages by the HI test (Henderson et al., 1990). Our study has demonstated seropositivity in pigs in addition to ducks and chickens from both rural and urban areas of Nepal. Of the total pigs that were tested positive, a higher proportion of younger pigs (86%) was found to be seropositive than older pigs (14%) in our study. Since the median age of market or slaughter is nine months for pigs in Nepal, the probability of finding older pigs that are likely to have seroconverted may have been lower. Finding seropositivity in younger pigs indicates recent JEV infection, and our study suggests a high force of infection in domestic animals in Rupandehi district of Nepal. Similarly, female pigs, rural pigs, pigs belonging to farm that is closer to a rice paddy field, pigs belonging to a farm that is exposed to household fermentation was associated with JEV seropositivity. Similarly, for a farm with pigs to be seropositive, the odds were higher in those farms that had a prior history of abortion. A rural location for the pigs or farms was significantly associated with JEV seropositivity. When considering ducks or farms with ducks, household fermentation was significantly associated with the seropositivity. Perhaps, mosquitoes are attracted to carbon dioxide being produced while making alcohol at household, and ducks are getting bitten by mosquitoes carrying JEV. Also, rural ducks close to paddy fields and farm size up to 8 were significantly associated with JEV seropositivity in ducks. Since paddy fields are breeding grounds of most of the JEV vectors, the likelihood of mammalian hosts being bitten by paddy field mosquitoes would be relatively higher. Similarly, chicken farm size above 15 had higher odds of getting infected with JEV. It could be due to large number of chickens attract abundant JEV vectors, and chance of getting infected with JEV in large farms would be relatively greater.

The presence of known reservoir hosts for JEV, including pigs, ducks, and chickens increases the probability of attracting zoophilic mosquitoes to the household, and ultimately humans living in the household can become infected by such mosquitoes. Households in rural areas of Nepal frequently have several species of domestic animals. Keeping competent dometic animals such as pigs, ducks, and chickens in household courtyard farms can be discouraged. Perhaps keeping other dometic animals which do not develop high enough viremia titers to transmit to secondary animal hosts along with competent dometic animals can help serve as to lessen the transmission of JEV (Gould et al., 1964, 1974). Rapid livestock population turnover can be slowed down in household level farms of Nepal, because once seropositive to JEV, the resident domestic animal species can prevent further transmission of pathogen towards the members of the household. The government of Nepal has also advised keeping at least 500 meters distance of a pig farm from human houses. Active immunization with the anti-JEV vaccine is required in rural areas of Nepal where animal farming and paddy farming is common. Families migrating from hills to the southern districts of Nepal are especially at risk because of no previous exposure to any mosquito-borne infections in hills and potential exposure to infectious mosquito bites could in the lowland districts.

CHAPTER 3: KNOWLEDGE, ATTITUDE, AND PRACTICE SURVEY REGARDING JAPANESE ENCEPHALITIS AMONG NEPALESE HOUSEHOLDS WITH LIVESTOCK IN THE COURTYARD

3.1 Introduction

Japanese encephalitis (JE) is an arbovirus-induced inflammatory disease of the brain and a common cause of viral encephalitis in Asia. JEV is an RNA virus belonging to genus *Flavivirus* and family *Flaviviridae.* Mosquitoes of the genus *Culex* are common vectors for the transmission of JEV worldwide, primarily the rice farm-associated *Culex tritaeniorhynchus* mosquito (Mackenzie et al., 2004). Birds, especially of family *Ardeidae*, and pigs are the natural reservoir hosts of JEV, and humans have no role in the subsequent transmission of JEV due to insufficient viremia titers to infect feeding mosquitoes (Nemeth et al., 2012). Transmission of JEV principally exists in tropical and temperate areas where flooding irrigation is practiced in agricultural lands along with pig and/or aquatic bird farming. Furthermore, transmission is observed with the onset of the rainy seasons especially in Southeast Asia, when it is warm and damp. While transmission of JEV may be seen in any month in regions with a tropical climate, it is observed most prominently during summer and autumn seasons in temperate regions of Asia. Outbreaks are often noticed in rural farming areas but also reported in urban areas that are close to integrated rice and livestock farms. The virus is geographically distributed in Southeast Asia and Australasia, and recently reported from some parts of Europe (van den Hurk et al., 2009).

JEV infection in people often is mild, with a headache and fever, or inapparent. However, 1% of JEV infections in humans manifest with clinical disease (Campbell et al., 2011), showing rapid onset of high fever, headache, neck stiffness, disorientation, coma, seizures and

paralysis, and ultimately death. About 20-30% of JE patients die, and among those who survive, 30-50% develop permanent neurological sequelae (Fischer et al., 2008).

Japanese encephalitis was first reported in 1978 in Rupandehi district of Nepal (Joshi, 1983). Due to an increase in frequency and numbers of rice paddy farming and pig farming across different ecological zones, in addition to the expansion of mosquito ranges over the decades, JE has been continuously reported from several lowland districts to highland districts in Nepal. More recently, JEV cases have been observed in people in the high hills who had no prior history of travel to JEV endemic districts of Nepal (Bhattachan, et al. 2009). The overall incidence of JE in Nepal has ranged from 1.0 to 2.8 per 100,000 and the child to adult case frequency ratio has ranged from 4:1 to 5:4 (Campbell et al., 2011). The lack of sufficient and correct knowledge regarding JE, the absence of prevention strategies, and continuous interaction with domestic animals and vectors may contribute to the continuous risk of contracting JE in Nepal. The aim of this study was to assess what aspects of knowledge (K), attitudes (A), and practices (P) of Nepali people raising livestock in their courtyard determine their willingness to use JE preventive approaches.

3.2 Materials and Methods

3.2.1 Ethics Approval

Ethics approval for conducting knowledge, attitude, and practice (KAP) survey was obtained from Institutional Review Board at Colorado State University, Fort Collins, Colorado, USA (Protocol Number: 14-4848H). All the research assistants involved in the study were Nepali veterinary students who had a formal course on ethics and jurisprudence in Agriculture and Forestry University and Tribhuvan University, Nepal. In addition, they were given a short

training by the author in the ethical conduct of human subject research. Furthermore, every participant of the study was briefed about the nature of the project before conducting the KAP survey. Their verbal consent was obtained before enrolling them into the study. The participants participated voluntarily and were informed that they could leave the KAP survey anytime. Personal identifier information (name, telephone number, house number) was not collected from the study participants and responses of the study participants were treated confidentially by assigning them accession numbers.

3.2.2 Study Design and Site

The KAP survey was conducted between July 2014 and October 2014 among the resident farmers in Rupandehi district of far western Nepal (27.6264° N and 83.3789° E) (Figure 3.1). Rupandehi district covers various land types including flat lands and mid-hills along with small to large water bodies. It is the first district of Nepal to have reported JEV outbreak and is adjacent to the Uttar Pradesh state of India to the south and Chure hills to the north. Most the people in the district were associated with agriculture and have very close contact with livestock.

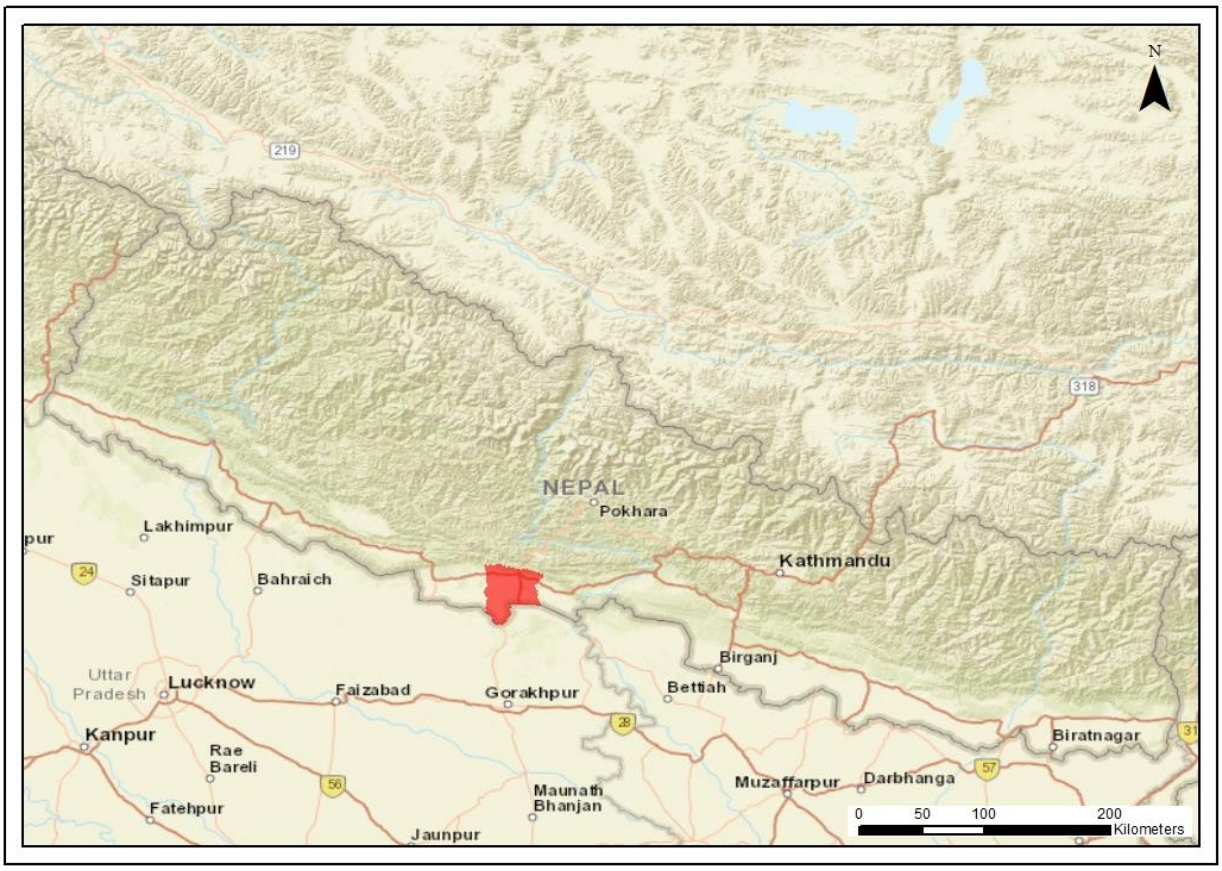


Figure 3.1 Rupandehi district (in red) of Nepal

3.2.3 Knowledge, Attitude, and Practice Survey Settings

The study team conducted 183 KAP surveys, each survey representing a household in the district (Figure 3.2). The KAP surveys were held at individual houses and took an average of 20 minutes, after which participants could interact with the research team. The KAP survey was conducted in native Nepali language and recorded simultaneously in the English language.

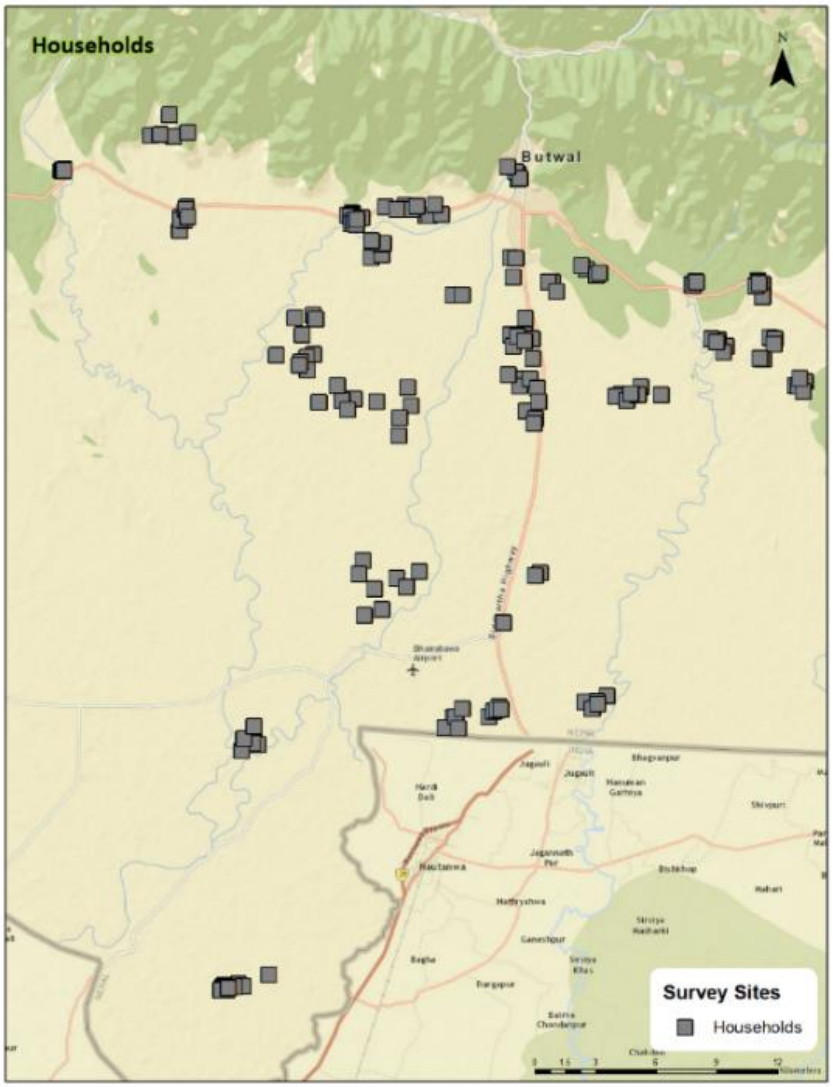


Figure 6 Household survey sites in Rupandehi district

3.2.4 Study Participants

Study participants included males and females directly and indirectly involved in agriculture, irrespective of their age, sex, ethnicity, educational status, and occupation. The inclusion criteria included: resident of the district of study, owning either pigs, ducks or chickens, and willing to participate in the study. The households were chosen based on

availability of people in their houses, farming pigs, ducks, or chickens in their households, and willingness to participate in the survey.

3.2.5 Qualitative and Quantitative Data Collection and Management

Data were collected from the participants using a standardized questionnaire on a paper form, facilitated by trained research assistants. The author briefed participants as to the purpose of the survey, and the research assistants conducted the questionnaire survey without elaborating the questions to the participants. The KAP survey was pre-tested among ten people from the Rupandehi district to ensure its usefulness and appropriateness of questions and interpretation of the responses. The questions were reviewed based on the pre-testing and accordingly revised. The questionnaire comprised the demographics of the KAP survey participants, farming practices, and JE/JEV related open and close-ended questions. The questionnaire was developed using the JE/JEV literature.

3.2.6 Data Analysis

Data were entered in Microsoft Excel from the paper survey forms, checked, and cleaned, and statistical analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA). The demographic characteristics of respondents and their households were summarized using counts and frequencies (%) for categorical variables, and mean ± standard deviation (parametric) and median (range) (non-parametric) for continuous variables. Knowledge, attitude, and practice data were categorical data and summarized using count and frequency (%). Univariate and multivariate analyses were performed with the demographic, knowledge, attitude and practices

variables with the respondents' practices for mosquito bite prevention and mosquito population control.

3.3 Results

3.3.1 Respondent and Household Characteristics

A total of 183 households were surveyed, and one member of each household represented that household in the survey (Table 3.1). Of the total 183 respondents, 77 (42%) were male, and 106 (58%) were female. The mean (± standard deviation, S.D.) age of the respondents was 39.9 ± 11.7 years. Among participants, 14 (7.7%) were 18 years or younger, and 169 (92.3%) were older than 18 years. When asked ethnicity, 19 (10.4%) said they belonged to non-indigenous groups of Nepal (Brahmin, Chettri) and 164 (89.6%) belonged to indigenous groups of Nepal (Tharu, Gurung, Newar, B.K.). Out of 183 households, 131 (71.6%) were in rural areas, and the remainder were in urban areas. The median (range) household size was 5 (2-14). Farming was the primary occupation of 34 (18.6%) of the respondents, and 123 (67.2%) of the respondents had formal education.

Table 3.14 General features of surveyed respondents and households

Variables	Frequency
Sex	
Male	77 (42%)
Female	106 (58%)
Age *(mean ± SD)* (years)	39.9 ± 11.7
Age category (years)	
1 to 18	14 (7.7%)
19 and above	169 (92.3%)
Ethnicity	
Non-indigenous	19 (10.4%)
Indigenous	164 (89.6%)
Locality	
Rural	131 (71.6%)
Urban	52 (28.4%)
Education	
Informal	60 (32.8%)
Formal	123 (67.2%)
Primary occupation of respondent	
Farming	34 (18.6%)
Non-farming	149 (81.4%)
Household size, *median (range)*	5 (2-14)

Similarly, 124 (67.8%) of the respondents responded yes if their family is involved in paddy farming, and almost all practiced animal farming and had domestic animals (Table 3.2).

Table 15 Factors that might increase the risk of mosquito bites and JEV transmission

Variables	Frequency
Family involvement in paddy farming	
Yes	124 (67.8%)
No	59 (32.2%)
Family involvement in animal farming	
Yes	183 (100%)
No	0
Gender involvement in farming	
Male	63 (34.4 %)
Female	120 (65.6 %)
Household alcohol fermentation	
Yes	83 (45.4%)
No	100 (54.6 %)
Dometic animal in household courtyard	
Yes	183 (100%)
No	0
Household domestic animal composition	
Single species	92 (50.3%)
Multiple species	91 (49.7%)
Household to paddy field distance	
Less than 500 meters	131 (71.6%)
More than 500 meters	52 (28.4%)
Window screen in house	
Yes	69 (37.7%)
No	114 (62.3%)
Potential mosquito breeding sites close to house	
Yes	183 (0%)
No	0
Observation of migratory/seasonal birds in the area	
Yes	95 (51.9%)
No	88 (48.1%)

3.3.2 Perceived Knowledge on Mosquito Bites

Outdoor biting by mosquitoes were most commonly reported by the respondents (Table 3.3). When asked specifically about the time of bites over 24 hours, 73.2% and 86.3% of the respondents reported they were bitten mostly in the early evening (5 PM to 7 PM) and late

evening (7 PM to 9 PM), respectively. Very few respondents indicated that they were also bitten during early morning (4 AM to 8 AM), late morning (9 AM to 12 PM), and afternoon (12 PM to 5 PM). Higher mosquito bite frequency after monsoon was reported by a large majority of the respondents. Power outages are common in Nepal, and when posed with the question, "Do you experience increase of mosquito bite during power outages or not?", 79% of respondents reported an increase in mosquito bites during power outages.

Table 16 Knowledge on mosquito bites among respondents

Variables	Frequency
Household mosquito bites	
Indoor	12 (6.6%)
Outdoor	171 (93.4%)
Mosquito bites in a day	
Early morning (4am-8am)	
Yes	45 (24.6%)
No	138 (75.4%)
Late morning (9am-12pm)	
Yes	8 (4.4%)
No	175 (95.6%)
Afternoon (12pm-5pm)	
Yes	11 (6%)
No	172 (94%)
Early evening (5pm-7pm)	
Yes	134 (73.2%)
No	49 (26.8%)
Late evening (7pm-9pm)	
Yes	158 (86.3%)
No	25 (13.7%)
Night (9pm-4am)	
Yes	97 (53%)
No	86 (47 %)
Mosquito bite season	
Pre-monsoon	13 (7.1%)
Post-monsoon	170 (92.9%)
Mosquito bite during power outage	
Yes	145 (79.2%)
No	38 (20.8%)

3.3.3 Perceived Knowledge on Japanese encephalitis and Vaccine

More than 90% of the respondents believed that mosquitos cause diseases (Table 3.4). The incidence of mosquito-borne disease over the past two years in their community was reported to have occurred by 10.4% of the respondents. Roughly 40% of the respondents knew of or had heard of JE disease, but only about half of those correctly knew that JEV is transmitted by the mosquito bite. Only 3 respondents (1.6%) remembered a case of JE in their family in the past at the same location of the household. Only one in five of the respondents knew that there is a vaccine for JE. Regarding their action in seeking healthcare when their family member shows sudden high fever, headache, and vomiting, 80% said they would wait and watch if it subsided before seeking care.

Table 17 Knowledge on Japanese encephalitis and its vaccine

Variables	Frequency
Mosquito cause disease	
Yes	169 (92.4%)
No	14 (7.6%)
Mosquito-borne disease incidence in last 2 years in the community	
Yes	19 (10.4%)
No	164 (89.6%)
Know or heard of JE	
Yes	70 (38.2%)
No	113 (61.8%)
JEV transmission	
Mosquito bite	40 (21.9%)
Other causes or do not know	143 (78.1%)
JE history in the family	
Yes	3 (1.6%)
No	180 (98.4%)
Availability of vaccine for JE	
Yes	40 (21.9%)
No	143 (78.1%)
Family member with sudden high fever, headache, and vomiting	
Wait and watch	145 (79.2%)
Visit hospital immediately	38 (20.8%)

3.3.4 Practices for JE control and Prevention

The most common practice for JE control and prevention in Rupandehi district of Nepal was vaccination, and 57% of respondents knew that vaccination is a measure of prevention from getting the disease (Table 3.5). Most the respondents practice both mosquito population control and vaccination to prevent JEV infection. About 87% of the respondents have received mosquito or mosquito disease control and prevention education. Neither community nor government mosquito control programs are common practices. Use of bed nets and mosquito repellent were commonly practiced by the respondents, but only a minority practiced removal of stagnant water from their surrounding or spraying insecticide.

Table 18 Practices for JE control and prevention

Variables		Frequency
JE prevention		
Mosquito control		20 (10.9%)
Vaccination		105 (57.4%)
Both		58 (31.7%)
Prior JE vaccine in the family		
Yes		8 (4.4%)
No		175 (95.6%)
Mosquito or mosquito disease control education		
Yes		159 (86.9%)
No		24 (13.1%)
Community mosquito control program		
Yes		26 (14.2%)
No		157 (85.8%)
Government mosquito control program		
Yes		50 (27.3%)
No		133 (72.7%)
Mosquito population control		
Remove stagnant water		
	Yes	35 (19.2%)
	No	148 (80.8%)
Insecticide spray		
	Yes	29 (15.8%)
	No	154 (84.2%)
Mosquito bite prevention		
Bed net		
	Yes	173 (94.5%)
	No	10 (5.5%)
Mosquito repellent		
	Yes	86 (47%)
	No	97 (53%)

3.3.5 Attitudes towards JE Preventive/Protective Measures

When asked about their attitudes towards commonly practiced JE preventive or protective measures, a majority of the respondents believed that bed nets are the most affordable protective approach that prevents from mosquito bites (Table 3.6). Less affordable measures of

prevention from getting diseases were vaccination and use of insect repellents. However, when asked about the effectiveness of the control and preventive measures, the least affordable approach was viewed as the most effective measure in prevention and control of the disease. Vaccination was believed to be effective by 66% of the respondents. A majority of respondents believed that community involvement in mosquito control would be sustainable although government operated mosquito program is more active in the district than in other locations within Nepal.

Table 19 Attitude towards JE preventative/protective measures

Variables	Frequency
Affordable practice	
Vaccine	11 (6%)
Bed net	144 (78.7%)
Insect repellent	28 (15.3%)
Effective practice	
Vaccine	121 (66.1%)
Bed net	35 (19.1%)
Insect repellent	14 (7.7%)
Remove stagnant water	13 (7.1%)
Sustainable program	
Government operated	48 (26.2%)
Community operated	135 (73.8%)

3.3.6 Demographics, Knowledge, Attitudes, and Practices related to JE Prevention

Tables 3.7, 3.8, 3.9, and 3.10 display the univariable and multivariable associations of respondents' demographics, knowledge, attitudes, and practices with mosquito population control and bite prevention to prevent from JEV infection. Several factors that could be associated with respondents' current practices for JE prevention were considered, including use of insect repellent, insecticides or bed nets, and removal of stagnant water. We also tracked

attitudes and knowledge about government-funded control measures and educational programs. The time of mosquito bites (early morning, evening, and late evening) was significantly associated with the use of mosquito repellent by both univariable and multivariable analysis ($p< 0.05$) (Figure 3.7). In addition, use of insect repellent by the respondents was significantly associated with the use of bed nets and removal of stagnant water. Similarly, removal of stagnant water from household and its surrounding was significantly associated with the rural locality of the households ($p= 0.01$) (Figure 3.8). Interestingly, those respondents who had prior knowledge of JE, its transmission, vaccine and uses window screen were found to be associated with the willingness of removal of stagnant water from the households and surrounding areas to control mosquito population ($p<0.05$). Our findings suggest that those respondents who correctly knew how the JE is transmitted seems to clear stagnant water from their households and surrounding areas ($p=0.007$). Furthermore, households that have formally educated members seems to know the mosquito population control methods (Figure 3.9). For example, the odds of insecticide spraying was 3.3 times higher in formally educated household with formally educated respondent than households with non-formally educated respondents ($p=0.01$). However, late evening mosquito bites ($p=0.01$) and using bed nets ($p=0.0001$) was the most important and significant factor that could have driven the respondents to use insecticide spraying as a method to control mosquito population. Similarly, respondents believing on vaccine affordability and effectiveness were significantly associated with the use of bed nets as a mosquito bite prevention ($p<0.05$). But, the most important factor that could have helped the respondents to use bed nets were insect repellent ($p=0.03$) and the practice of insecticide spraying ($p=0.0007$).

Table 20 Unadjusted and adjusted odds ratios and 95% confidence intervals (95% CI) of insect repellent as preventive practices by respondent's knowledge, attitude, and practices for JEV

Variables	Univariable (unadjusted)			Multivariable (adjusted)		
	Odds ratio	95% CI	p-value	Odds ratio	95% CI	p-value
Locality						
Rural	1.4	0.7-2.8	0.2			
Urban	Reference					
Sex						
Male	1.1	0.6-1.9	0.8			
Female	Reference					
Age category						
Up to 18	0.8	0.3-2.5	0.7			
19 and above	Reference					
Ethnicity						
Non-Indigenous	0.6	0.2-1.7	0.3			
Indigenous	Reference					
Education						
Non-formal	0.7	0.4-1.4	0.3			
Formal	Reference					
Primary occupation						
Farming	0.6	0.3-1.4	0.2			
Non-farming	Reference					
Household size						
Up to 5	1.0	0.6-1.9	0.9			
More than 5	Reference					
Family involvement in paddy farming						
Yes	2.2	1.2-4.2	0.01	1.9	0.8-4.8	0.1
No	Reference					
Gender involvement in farming						
Male	0.9	0.5-1.7	0.8			
Female	Reference					

Alcohol fermentation in house						
Yes	1.2	0.6-2.1	0.5			
No	Reference					
Domestic animal composition in household						
Single species	0.7	0.4-1.3	0.3			
Multiple species	Reference					
Paddy field distance to household						
Less than 500 meters	1.5	0.7-2.8	0.3			
More than 500 meters	Reference					
Household mosquito bites						
Indoor	2.0	0.9-4.3	0.06			
Outdoor	Reference					
Window screen						
Yes	1.7	0.9-3.1	0.08			
No	Reference					
Migratory bird sighting						
Yes	0.8	0.5-1.5	0.6			
No	Reference					
Mosquito bite season						
Pre-monsoon	1.9	0.6-6	0.3			
Post-monsoon	Reference					
Early morning mosquito bite						
Yes	2.9	1.4-5.9	0.002	3.1	1.2-7.8	0.01
No	Reference					
Late Morning Mosquito bite						
Yes	1.1	0.3-4.7	1.0*			
No	Reference					
Afternoon Mosquito bite						
Yes	1.4	0.4-4.7	0.6			
No	Reference					
Early evening mosquito bite						
Yes	4.4	2.1-9.3	<0.0001	10.1	3.0-33.9	0.0002
No	Reference					

Late evening mosquito bite						
Yes	13.1	2.9-57.2	<0.0001	115.7	5.6 - >999	0.002
No	Reference					
Night time mosquito bite						
Yes	1.9	1.1-3.5	0.02	0.8	0.3-1.9	0.6
No	Reference					
Mosquito bite						
Power outage	0.8	0.4-1.7	0.7			
No difference	Reference					
Mosquito cause disease						
Yes	1.6	0.5-5.2	0.4			
No	Reference					
Mosquito-borne disease in community in last 2 years						
Yes	0.5	0.2-1.3	0.2			
No	Reference					
Know or heard of JE						
Yes	1.5	0.8-2.7	0.2			
No	Reference					
JE transmission						
Mosquito bite	1.5	0.7-3.1	0.2			
Other causes or do not know	Reference					
JE history in the family						
Yes	0.5	0.05-6.3	1.0*			
No	Reference					
Availability of JE vaccine						
Yes	1.02	0.5-2.1	0.9			
No	Reference					
Prior JE vaccine in the family						
Yes	0.4	0.1-1.8	0.2*			
No	Reference					
Mosquito or mosquito disease control education						
Yes	1.1	0.4-2.5	0.9			
No	Reference					

How JE can be prevented							
Mosquito control		2.4	0.9-7.1	0.09			
Vaccination		1.1	0.6-2.1	0.7			
Both		Reference					
Community mosquito control program							
Yes		0.8	0.3-1.8	0.6			
No		Reference					
Government mosquito control program							
Yes		0.7	0.3-1.3	0.2			
No		Reference					
Mosquito control population control or prevent mosquito bites							
Remove stagnant water							
	Yes	18.6	5.4-63.5	<0.0001	101.6	11.1-931	<0.0001
	No	Reference					
Insecticide spray							
	Yes	1.3	0.6-2.7	0.5			
	No	Reference					
Bed net							
	Yes	0.2	0.04-0.9	0.04*	0.4	0.07-2.3	0.3
	No	Reference					
Family member with sudden high fever, headache, and vomiting							
Wait and watch		1.7	0.8-3.5	0.1			
Visit hospital immediately		Reference					
Affordable practice							
Vaccine		1.04	0.3-3.6	0.9			
Bed net		1.2	0.2-4.8	0.8			
Insect repellent		Reference					
Effective practice							
Vaccine		0.7	0.2-2.1	0.5			
Bed net		0.5	0.1-1.7	0.2			
Remove stagnant water		0.6	0.1-2.9	0.5			

Insect repellent	Reference					
Sustainable program						
Government operated	0.8	0.4-1.6	0.6			
Community operated	Reference					

Table 21 Unadjusted and adjusted odds ratios and 95% confidence intervals (95% CI) of stagnant water removal as preventive practices by respondent's knowledge, attitude, and practices for JEV

Variables	Univariable (unadjusted)			Multivariable (adjusted)		
	Odds ratio	95% CI	p-value	Odds ratio	95% CI	p-value
Locality						
Rural	3.7	1.2-11.1	0.01	3.5	0.8-14.8	0.07
Urban	Reference					
Sex						
Male	1.8	0.9-3.9	0.1			
Female	Reference					
Age category						
Up to 18	1.2	0.3-4.4	0.7			
19 and above	Reference					
Ethnicity						
Non-Indigenous	1.6	0.5-4.7	0.4*			
Indigenous	Reference					
Education						
Non-formal	0.5	0.2-1.3	0.2			
Formal	Reference					
Primary occupation						
Farming	1.1	0.4-2.8	0.8			
Non-farming	Reference					
Household size						
Up to 5	1.2	0.5-2.5	0.7			
More than 5	Reference					

Family involvement in paddy farming						
Yes	1.0	0.5-2.3	0.9			
No	Reference					
Gender involvement in farming						
Male	1.2	0.5-2.5	0.7			
Female	Reference					
Alcohol fermentation in house						
Yes	1.2	0.6-2.5	0.6			
No	Reference					
Domestic animal composition in household						
Single species	2.5	1.2-5.6	0.01	3.8	1.2-11.9	0.02
Multiple species	Reference					
Paddy field distance to household						
Less than 500 meters	3.7	1.2-11.1	3.7			
More than 500 meters	Reference					
Household mosquito bites						
Indoor	0.6	0.3-1.5	0.3			
Outdoor	Reference					
Window screen						
Yes	2.3	1.1-4.9	0.02	2.8	0.9-7.7	0.05
No	Reference					
Migratory bird sighting						
Yes	1.7	0.8-3.7	0.1			
No	Reference					
Mosquito bite season						
Pre-monsoon	2.9	0.9-9.5	0.1*			
Post-monsoon	Reference					
Early morning mosquito bite						
Yes	2.1	0.9-4.7	0.05			
No	Reference					
Late Morning Mosquito bite						
Yes	1.4	0.3-7.4	0.6*			
No	Reference					

Afternoon Mosquito bite						
Yes	0.9	0.2-4.5	1.0*			
No	Reference					
Early evening mosquito bite						
Yes	1.1	0.5-2.5	0.8			
No	Reference					
Late evening mosquito bite						
Yes	1.3	0.4-4	0.8*			
No	Reference					
Night time mosquito bite						
Yes	0.9	0.4-1.9	0.8			
No	Reference					
Mosquito bite						
Power outage	0.5	0.2-0.9	0.02	0.3	0.09-1.1	0.06
No difference	Reference					
Mosquito cause disease						
Yes	3.3	0.4-25.9	0.5*			
No	Reference					
Mosquito-borne disease in community in last 2 years						
Yes	0.8	0.2-2.8	1.0*			
No	Reference					
Know or heard of JE						
Yes	2.6	1.2-5.5	0.01	1.5	0.4-6.1	0.5
No	Reference					
JE transmission						
Mosquito bite	4.3	1.9-9.6	0.0001	7.7	1.7-34.6	0.007
Other causes or do not know	Reference					
JE history in the family						
Yes	†	-	-			
No	Reference					
Availability of JE vaccine						
Yes	2.6	1.2-5.9	0.01	1.9	0.6-6.1	0.3
No	Reference					

Prior JE vaccine in the family							
Yes		2.7	0.6-11.8	0.2*			
No		Reference					
Mosquito or mosquito disease control education							
Yes		1.2	0.4-3.8	1.0*			
No		Reference					
How JE can be prevented							
Mosquito control		2.5	0.8-7.6	0.1			
Vaccination		0.6	0.3-1.5	0.3			
Both		Reference					
Community mosquito control program							
Yes		2.1	0.8-5.4	0.1*			
No		Reference					
Government mosquito control program							
Yes		1.7	0.8-3.8	0.1			
No		Reference					
Mosquito control population control or prevent mosquito bites							
Repellent							
	Yes	18.5	5.4-63.5	<0.0001	44.2	9.2-212.8	<0.0001
	No	Reference					
Insecticide spray							
	Yes	1.8	0.7-4.5	0.2			
	No	Reference					
Bed net							
	Yes	0.3	0.1-1.2	0.1*			
	No	Reference					
Family member with sudden high fever, headache, and vomiting							
Wait and watch		0.8	0.3-2.1	0.7			
Visit hospital immediately		Reference					
Affordable practice							
Vaccine		2.5	0.3-20.5	0.4			

Bed net	2.1	0.2-21.1	0.5
Insect repellent	Reference		
Effective practice			
Vaccine	0.7	0.2-3.1	0.7
Bed net	0.5	0.1-2.7	0.5
Remove stagnant water	1.3	0.2-7.5	0.7
Insect repellent	Reference		
Sustainable program			
Government operated	1.2	0.5-2.6	0.7
Community operated	Reference		

Table 22 Unadjusted and adjusted odds ratios and 95% confidence intervals (95% CI) of insecticide spray as preventive practices by respondent's knowledge, attitude, and practices for JEV

Variables	Univariable (unadjusted)			Multivariable (adjusted)		
	Odds ratio	95% CI	p-value	Odds ratio	95% CI	p-value
Locality						
Rural	0.7	0.3-1.6	0.4			
Urban	Reference					
Sex						
Male	2.2	1.05-4.9	0.04	1.6	0.6-4.7	0.3
Female	Reference					
Age category						
Up to 18	0.3	0.04-3.1	0.7*			
19 and above	Reference					
Ethnicity						
Non-Indigenous	2.1	0.7	6.3*			
Indigenous	Reference					
Education						
Non-formal	0.3	0.1-0.8	0.01	0.5	0.1-1.8	0.3
Formal	Reference					

Primary occupation						
Farming	0.6	0.2-2.0	0.4			
Non-farming	Reference					
Household size						
Up to 5	1.1	0.5-2.5	0.8			
More than 5	Reference					
Family involvement in paddy farming						
Yes	1.1	0.4-2.5	0.9			
No	Reference					
Gender involvement in farming						
Male	1.0	0.4-2.3	0.9			
Female	Reference					
Alcohol fermentation in house						
Yes	0.6	0.2-1.3	0.2			
No	Reference					
Domestic animal composition in household						
Single species	1.7	0.8-3.9	0.2			
Multiple species	Reference					
Paddy field distance to household						
Less than 500 meters	0.7	0.3-1.6	0.4			
More than 500 meters	Reference					
Household mosquito bites						
Indoor	0.4	0.2-0.9	0.02	0.5	0.2-1.5	0.2
Outdoor	Reference					
Window screen						
Yes	1.9	0.9-4.4	0.08			
No	Reference					
Migratory bird sighting						
Yes	0.9	0.4-2.2	0.9			
No	Reference					
Mosquito bite season						
Pre-monsoon	2.5	0.7-9.0	0.1*			
Post-monsoon	Reference					

Early morning mosquito bite						
Yes	0.9	0.4-2.4	0.9			
No	Reference					
Late Morning Mosquito bite						
Yes	1.8	0.3-9.5	0.6*			
No	Reference					
Afternoon Mosquito bite						
Yes	1.2	0.2-5.8	0.7*			
No	Reference					
Early evening mosquito bite						
Yes	0.9	0.4-2.3	0.9			
No	Reference					
Late evening mosquito bite						
Yes	0.3	0.1-0.8	0.03*	0.2	0.08-0.7	0.01
No	Reference					
Night time mosquito bite						
Yes	0.7	0.3-1.5	0.3			
No	Reference					
Mosquito bite						
Power outage	1.0	0.4-2.7	0.9			
No difference	Reference					
Mosquito cause disease						
Yes	0.4	0.1-1.5	0.2*			
No	Reference					
Mosquito-borne disease in community in last 2 years						
Yes	0.6	0.1-2.7	0.7*			
No	Reference					
Know or heard of JE						
Yes	1.6	0.7-3.6	0.2			
No	Reference					
JE transmission						
Mosquito bite	1.4	0.6-3.6	0.4			
Other causes or do not know	Reference					

JE history in the family							
Yes		2.7	0.2-30.9	0.4*			
No		Reference					
Availability of JE vaccine							
Yes		2.6	1.1-6.2	0.02	2.4	0.8-6.9	0.1
No		Reference					
Prior JE vaccine in the family							
Yes		3.4	0.7-15.2	0.1*			
No		Reference					
Mosquito or mosquito disease control education							
Yes		0.4	0.1-1.0	0.07*			
No		Reference					
How JE can be prevented							
Mosquito control		0.9	0.2-5.2	0.9			
Vaccination		2.2	0.8-5.7	0.1			
Both		Reference					
Community mosquito control program							
Yes		2.3	0.8-6.0	0.1*			
No		Reference					
Government mosquito control program							
Yes		2.5	1.1-5.8	0.02	1.1	0.4-3.1	0.8
No		Reference					
Mosquito control population control or prevent mosquito bites							
Remove stagnant water							
	Yes	1.8	0.7-4.5	0.2			
	No	Reference					
Repellent							
	Yes	1.3	0.5-2.7	0.5			
	No	Reference					
Bed net							
	Yes	0.01	0.001-0.1	<0.0001*	0.01	0.001-0.1	0.0001
	No	Reference					

Family member with sudden high fever, headache, and vomiting			
Wait and watch	1.0	0.4-2.7	0.9
Visit hospital immediately	Reference		
Affordable practice			
Vaccine	0.2	0.1-1.1	0.06*
Bed net	0.3	0.1-1.8	0.2*
Insect repellent	Reference		
Effective practice			
Vaccine	2.2	0.3-18.2	0.4*
Bed net	2.0	0.2-18.9	0.5*
Remove stagnant water	4.8	0.4-50.1	0.2*
Insect repellent	Reference		
Sustainable program			
Government operated	1.9	0.8-4.4	0.1
Community operated	Reference		

Table 23 Unadjusted and adjusted odds ratios and 95% confidence intervals (95% CI) of bed net as preventive practices by respondent's knowledge, attitude, and practices for JEV

Variables	Univariable (unadjusted)			Multivariable (adjusted)		
	Odds ratio	95% CI	p-value	Odds ratio	95% CI	p-value
Locality						
Rural	0.6	0.1-2.9	0.7*			
Urban	Reference					
Sex						
Male	0.7	0.2-2.5	0.6*			
Female	Reference					
Age category						
Up to 18	0.7	0.1-6.2	0.5*			
19 and above	Reference					

Ethnicity			
Non-Indigenous	1.0	0.1-8.7	1.0*
Indigenous	Reference		
Education			
Non-formal	4.6	0.5-37.6	0.1*
Formal	Reference		
Primary occupation			
Farming	2.1	0.2-17.3	0.7*
Non-farming	Reference		
Household size			
Up to 5	1.5	0.4-5.5	0.5*
More than 5	Reference		
Family involvement in paddy farming			
Yes	0.8	0.2-3.5	1.0*
No	Reference		
Gender involvement in farming			
Male	1.2	0.3-4.9	1.0*
Female	Reference		
Alcohol fermentation in house			
Yes	1.2	0.3-4.6	1.0*
No	Reference		
Domestic animal composition in household			
Single species	0.4	0.1-1.6	0.3*
Multiple species	Reference		
Paddy field distance to household			
Less than 500 meters	0.6	0.1-2.9	0.7*
More than 500 meters	Reference		
Household mosquito bites			
Indoor	1.8	0.4-7.4	0.4*
Outdoor	Reference		
Window screen			
Yes	0.4	0.1-1.4	0.2*
No	Reference		

Migratory bird sighting			
Yes	0.4	0.1-1.7	0.3*
No	Reference		
Mosquito bite season			
Pre-monsoon	0.7	0.1-5.7	0.5*
Post-monsoon	Reference		
Early morning mosquito bite			
Yes	0.5	0.1-1.7	0.3*
No	Reference		
Late Morning Mosquito bite			
Yes	†	-	-
No	Reference		
Afternoon Mosquito bite			
Yes	†	-	-
No	Reference		
Early evening mosquito bite			
Yes	0.7	0.1-3.2	1.0*
No	Reference		
Late evening mosquito bite			
Yes	0.7	0.1-5.7	1.0*
No	Reference		
Night time mosquito bite			
Yes	0.3	0.1-1.3	0.1*
No	Reference		
Mosquito bite			
Power outage	1.7	0.4-6.8	0.4*
No difference	Reference		
Mosquito cause disease			
Yes	†	-	-
No	Reference		
Mosquito-borne disease in community in last 2 years			
Yes			
No	1.0	0.1-8.7	1.0*

	Reference					
Know or heard of JE						
Yes	0.9	0.3-3.4	1.0*			
No	Reference					
JE transmission						
Mosquito bite	1.1	0.2-5.5	1.0*			
Other causes or do not know	Reference					
JE history in the family						
Yes	†	-	-			
No	Reference					
Availability of JE vaccine						
Yes	0.4	0.1-1.5	0.2*			
No	Reference					
Prior JE vaccine in the family						
Yes	0.1	0.02-0.8	0.06*			
No	Reference					
Mosquito or mosquito disease control education						
Yes	0.7	0.08-5.9	1.0*			
No	Reference					
How JE can be prevented						
Mosquito control	0.2	0.01-1.8	0.1*			
Vaccination	0.2	0.02-2.0	0.2*			
Both	Reference					
Community mosquito control program						
Yes	0.6	0.1-3.2	0.6*			
No	Reference					
Government mosquito control program						
Yes	0.1	0.03-0.6	0.004*	0.1	0.01-0.8	0.03
No	Reference					
Mosquito control population control or prevent mosquito bites						
Remove stagnant water						
Yes	0.3	0.08-1.2	0.1*			

Repellent	No	Reference					
	Yes	0.2	0.04-0.9	0.04*	0.1	0.01-0.8	0.03
Insecticide spray	No	Reference					
	Yes	0.01	0.002-0.1	<0.0001*	0.01	<0.001-0.1	0.0007
Family member with sudden high fever, headache, and vomiting	No	Reference					
Wait and watch		0.9	0.1-4.6	1.0*			
Visit hospital immediately		Reference					
Affordable practice							
Vaccine		10.4	1.5-70.6	0.01*	25.6	0.6-999	0.9
Bed net		1.0	0.2-6.2	0.9*			
Insect repellent		Reference					
Effective practice							
Vaccine		5.2	1.1-23.8	0.03*	0.001	0.001-999	0.9
Bed net		9.3	0.8-98.5	0.06*			
Remove stagnant water		Reference					
Insect repellent		†	-	-			
Sustainable program							
Government operated		1.4	0.3-7.1	1.0*			
Community operated		Reference					

Fisher's Exact Test odds ratio *
Odds ratio was not calculated due to zero cell †

3.4 Discussion

To our knowledge, this KAP survey is the first study conducted in Nepal to explore potential risk factors for JEV infection and the perceived factors that help human behavior to choose one or the other measures of mosquito bite prevention and mosquito population control. Survey participants from different parts of the district were representative of the district of study as well the other endemic districts of Nepal.

Through the survey, we were able to show that paddy farming, early morning mosquito bites, early evening mosquito bites, late evening mosquito bites, and night-time mosquito bites frame humans' practice to utilize insect repellent as a preventive measure to mosquito bites. Also, the people who tended to use bed nets and remove stagnant water from their surroundings also use insect repellent to protect from the bites of mosquitoes. Paddy farming close to households increases mosquito bites, consistent with a report Dhakal and colleagues, (2014), and their utilization of protective measures is consistent with a report by Dhimal et al., (2016). Living in a rural locality, where both domestic animals and paddy farming are common, determined a majority of the respondents to remove stagnant water so that mosquitoes would not breed near households. Although our study was able to show statistically that single domestic animal species composition at a household level was a significant predictor to decide to utilize an environmental approach such as removal of stagnant water, we think it has no biological relevance. Window screens have been well-studied intervention measures globally to prevent from mosquito bites inside the household. We found that people who put screens in their windows also tend to remove stagnant water from surroundings, as a double approach to control mosquito population while preventing their indoor bites. This may not be important for *Culex tritaeniorhynchus,* which are outdoor biters but could be important for other mosquito species

that bites indoor and transmit pathogens like *Plasmodium* and dengue viruses. Our study explored whether people perceived excessive mosquito bites during power outages in Nepal, and the majority of people indicated affirmatively. This could be true, as it is common in Nepal to come out of their houses when there is no power supply and that is the time when they get bitten by mosquitoes.

Participants of the study seemed to be informed about JE and transmission of JEV, which could be due to several awareness programs on television, radio, newspaper, or word of mouth. Pre-existing knowledge may have guided people to remove stagnant water from their surroundings to prevent mosquito bites. Interestingly, we found a disparity in gender regarding what preventive measure to use, with males favoring insecticide use. Formally educated people tend to be thoughtful, and chose insecticide spraying to stop population growth of mosquitoes. Also, outdoor mosquito bites were found to be significantly associated with the practice of spraying insecticides to curb the population of mosquitoes. Similarly, late evening mosquito bites was significant determinant to use insect repellent or insecticides spraying to prevent from getting mosquito infections. The common approaches to preventing mosquito-borne infection in Nepal followed by the government are human vaccination, media awareness, insecticides spraying and bed nets distribution.

With regards to the bed net utilization, the participants of Rupandehi district did not seem to rely on government supply of bed nets as the odds of using bed nets was 10 times higher in people who did not get government support in bed nets. Bed nets also seems to be common in people who have not been using insect repellents and not spraying insecticides. Insect repellent and insecticide spray are also favored while using a bed net. The reason could be the reduced effectiveness of bed net and to prevent mosquitoes to enter, and therefore people might be using

insect repellent while still using a bed net. Similarly, though vaccine is an affordable approach for a smaller number of participants, it is significantly associated with using bed nets. Perhaps those who can afford vaccines believe that vaccine is an effective approach. The findings of this study may contribute to government and allied veterinary and public agencies of Nepal in designing of control strategies of JE in community-suitable approaches.

CHAPTER 4: VARIABILITY IN ABUNDANCE AND DIVERSITY OF JAPANESE ENCEPHALITIS VIRUS VECTORS IN WESTERN NEPAL

4.1 Introduction

Japanese encephalitis virus (JEV) is a mosquito-borne virus of genus *Flavivirus* and family *Flaviviridae.* It is an RNA virus and causes encephalitis in humans and horses. Humans acquire JEV infection when an infectious mosquito probes or feeds on them. Although a vaccine for humans is available, JEV continues to cause 50,000-60,000 cases worldwide every year, affecting predominantly children (Mackenzie et al., 2004). The natural transmission cycle of JEV include birds (family *Ardeidae)* and pigs as common reservoir hosts, humans as spillover and transient hosts, and *Culex* mosquitoes as competent vectors. Among many *Culex* species, *Culex tritaeniorhynchus* is a commonly known primary vector for transmitting JEV to humans and other animals (van den Hurk, et al., 2009). *Culex tritaeniorhynchus* is the dominant *Culex* mosquito in areas where paddy farms and livestock are abundant (Endy and Nisalak, 2002). However, there are other Culex vectors that have either experimentally transmitted JEV to mammalian hosts or carried JEV naturally. For example, *Culex vishnui, Culex pseudovishnui, Culex quinquefasciatus, Culex gelidus*, and many others (van den Hurk, et al., 2009; Kim et al., 2015; Takhampunya et al., 2011; Ravanini et al., 2012; Seo et al., 2013).

Nepal is one of the Asian countries having endemic JE, and a large fraction of encephalitis cases are due to JEV infections (Robertson et al., 2013). The temporal pattern of human Japanese encephalitis (JE) cases in Nepal are associated with rainfall and start to rise following the onset of monsoon season (usually July), and persist until the start of winter (usually October) (Impoinvil et al., 2011). Due to the close interface of humans with rice paddy

fields and reservoir livestock species, JEV outbreaks are seasonally observed in the lowlands to the mid-hills of Nepal. Recently, JE cases have also been observed in people from the high hills who had no prior history of travel to JEV endemic districts of Nepal (Bhattachan, et al 2009).

The ecology of JEV in terms of its vector has not been characterized in Nepal. Past studies have focused on serosurveillance and social surveys on JE, and speculated that the dominant vector of JEV transmission in Nepal is *Culex tritaeniorhynchus.* Our objective was to characterize the abundance and diversity of *Culex* vectors as they play an important role in JEV transmission. The abundance and diversity of vectors is an important part of arboviral disease surveillance and control programs for preventing outbreaks in human population (Barker et al., 2010). In addition, knowing the pattern and distribution of vector species potentially responsible for transmitting JEV can substantially contribute to predicting the course of disease epidemiology (Smith et al., 2014). While continuous sampling of vertebrates and testing them for exposure to JEV can be expensive, labor intensive, and ethically complex, mosquito surveillance is a relatively straightforward method for arbovirus surveillance. The aims of the study presented here were to document the abundance and diversity of JEV vectors in Nepal and to seek associations between *Culex* species composition and environmental variables.

4.2 Materials and Methods

4.2.1 Study Area and Mosquito Collection

The study was conducted in Rupandehi district (27.6264° N and 83.3789° E) (Figure 4.1). This district is unique in Nepal, constituting vast lowlands (less than 300 meters above sea level), highlands and several national interest wetlands, and is inhabited by varied ethnic and multi-cultural groups of people. It also was the first district of Nepal to have reported outbreaks

of JE. The district is located between India to the south and the Chure hills to the north. A majority of people in the district are associated with agriculture and have a very close association with livestock. The district has a monsoon climate (180-225 cm precipitation), 80-90% relative humidity and belongs to the sub-tropical zone. The general climate of Rupandehi is composed of 4 seasons. Spring starts in March and lasts until May, which is warm (22°C) with rain showers. Summer is monsoon season in Nepal when rain showers become intense, temperature can reach up to 34°C, farmers start planting rice in the paddy fields, and vegetation becomes lush green. Autumn starts in September and remains until November, when it is cool and temperature is 25°C. Winter is cold and extends from December to February, when the temperature can reach 20°C. The district is adjacent to Indian regions where JE epidemics occur every year (Joshi et al., 2004).

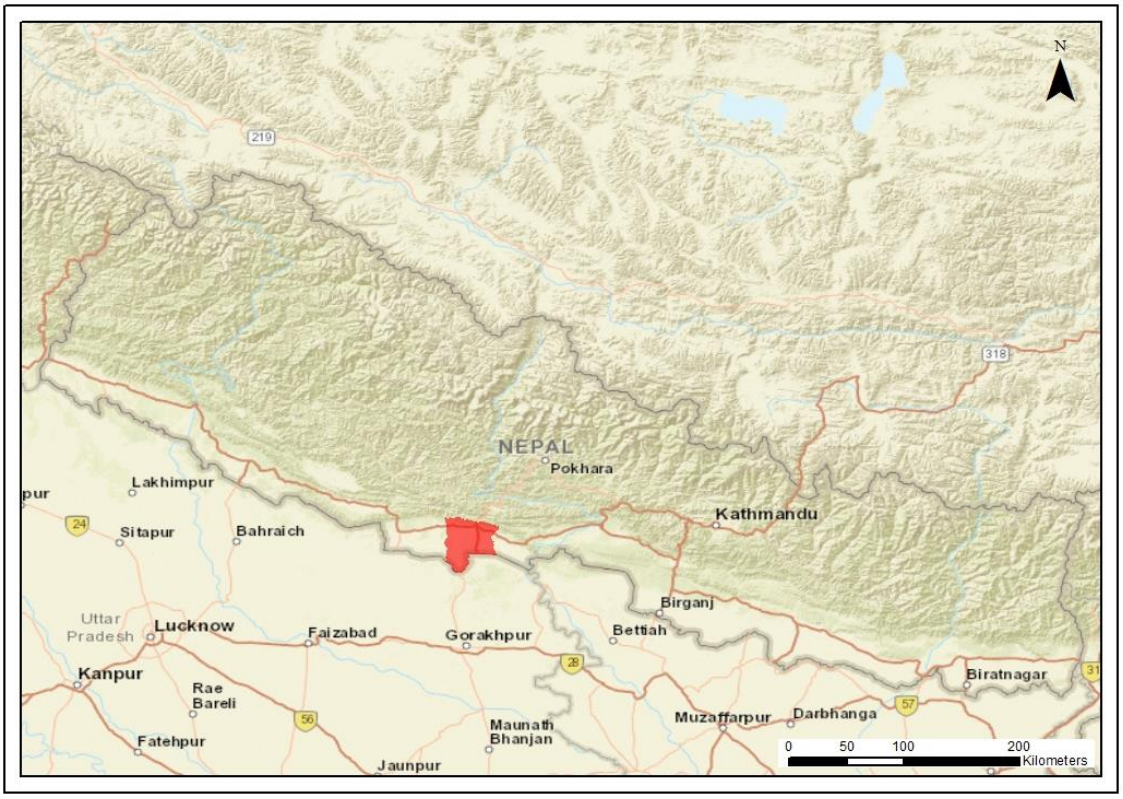


Figure 7 Rupandehi district (in red) in Nepal

Mosquitoes were trapped twice every week (total 13 weeks) across Rupandehi district from July 2014 to October 2014. CDC miniature light traps (John W. Hock Company, Gainesville, FL) were placed at 8 different locations in Rupandehi district (Figure 4.2), and remained at the same location for the entire mosquito collection period. The traps were suspended approximately 1.5 meter above the ground. Sites of the traps were recorded with a hand-held global positioning system (GPS) receiver (Etrex10, Garmin, KS) and mapped using ArcGIS 10.4 program (ESRI, Redlands, CA). The traps were placed and turned on at 1600-1700 hours and collected the next morning at 0700-0900 hours. Each site had one trap located in areas close to a paddy field, human households, farms with pigs, ducks or chickens, or cow sheds (Table 4.1). Collected mosquitoes were immediately placed into an insulated box with cold packs before anesthetizing them with chloroform and speciating them under a stereo-microscope (AmScope, Irvine, CA). Only undamaged *Culex* species mosquitoes were saved and they were speciated based on morphology following the keys developed by Darsie and Pradhan, (1990), Darsie et al., (1993), and Das, (2013). Blood-fed mosquitoes were counted from each collection site for each species of *Culex* mosquitoes. Abundance was calculated as number of female mosquitoes per species of *Culex* with or without blood in their gut. The speciated mosquitoes were frozen in RNAlater for future investigation.

District specific climate data (daily minimum temperature, daily maximum temperature, weekly average rainfall, daily dew point temperature, and daily relative humidity) for the entire mosquito collection period of 2014 was obtained from the NASA Prediction of Worldwide Energy Resource (POWER) on Climatology Resource for Agroclimatology website

(power.larc.nasa.gov). The data was retrieved from the website using latitude and longitude information of the district, 27.32 and 83.28, respectively.

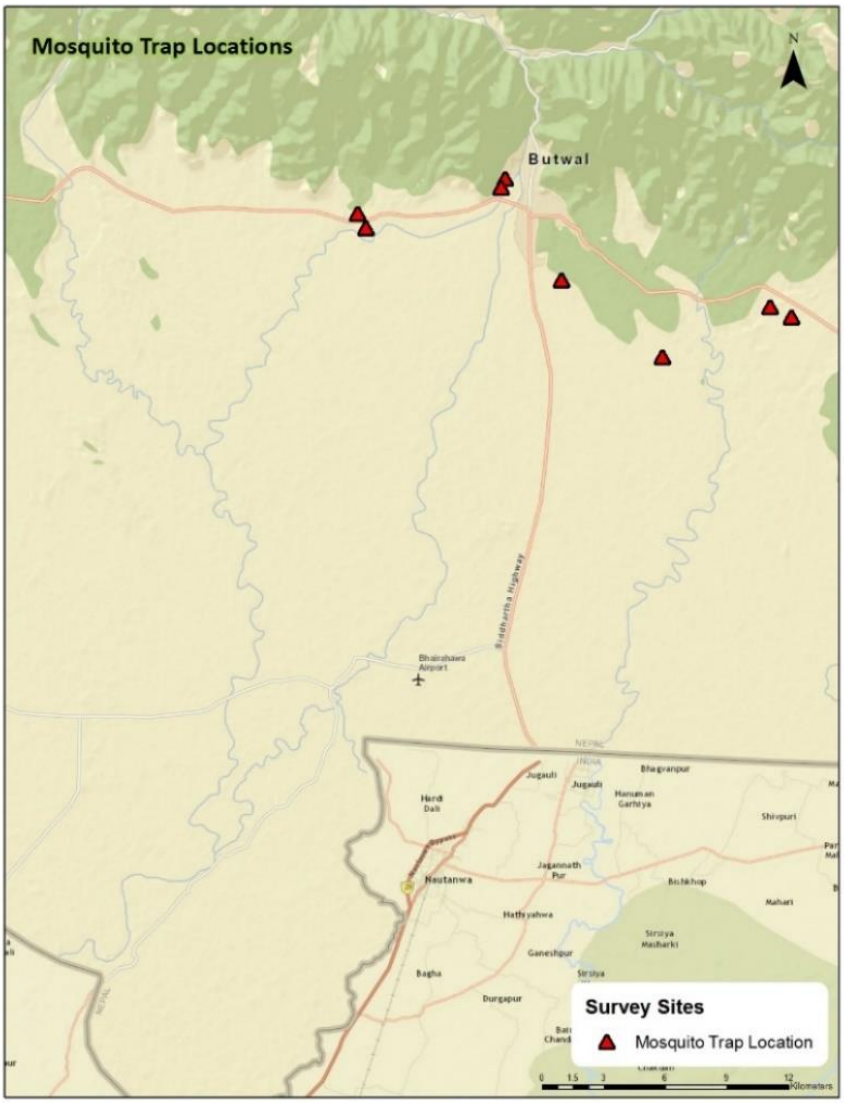


Figure 8 Mosquito trap locations in Rupandehi district of Nepal

Table 24 Trap location characteristics

Trap #	Latitude	Longitude	Elevation (m)	Location	Host composition	Habitat	Trap site
1	27.68610	83.38947	135	Tamnagar	Pig	Paddy field, ditches	Pig farm
2	27.68055	83.39317	133	Tamnagar	Human, goat, pig, duck	Paddy field, water bodies, human house	Interface
3	27.69962	83.45410	174	Jeetgadhi	Human, pig, cow, buffalo, chicken	Paddy field, ditches, human house	Interface
4	27.69635	83.45239	173	Jeetgadhi	Pig	Paddy field, river, forest, bush, ditches	Pig farm
5	27.66025	83.47874	152	Shankarnagar	Pig	Paddy field, ditches	Pig farm
6	27.63068	83.52277	128	Shankarnagar	Human, pig, chicken	Paddy field, ditches, human house	Interface
7	27.64996	83.57007	134	Devdaha	Human, duck, chicken	Paddy field, ditches, human house	Interface
8	27.64608	83.57934	140	Devdaha	Pig	Paddy field, ditches	Pig farm

4.2.2 Data Analysis

Data were entered in Microsoft Excel (Microsoft, Seattle, WA) by week of collection, genus, species, blood meal status, and sites where mosquito traps were set. Spearman correlation was applied to assess association between mosquito abundance and environmental variables. All statistical analyses were carried out using the SAS 9.2 statistical package (SAS Institute, Cary, NC) and results were considered significant when $p<0.05$. Graphs were prepared using GraphPad Prism 7.0 (La Jolla, CA).

4.3 Results

4.3.1 Trap Site and Mosquito Collection

Mosquito traps were placed in 8 different latitudes and longitudes at varying elevations (Table 4.1). Each of the localities, Tamnagar, Jeetgadhi, Shankarnagar, and Devdaha, received two traps. The domestic vertebrate host composition within 500 meters of the traps was recorded composed of pigs, goats, cows, buffaloes, chickens, ducks, and humans. The habitat of trap placement included paddy fields, human houses, irrigation channels, domestic animal houses, and stagnant water in ditches. Out of 8 traps, trap # 1, 4, 5, and 8 was placed close to farms with pigs, and the rest of the traps were placed at an interface of paddy fields, human houses, irrigation channels, domestic animal houses, and stagnant water in ditches.

A total of 26,824 identifiable female mosquitoes representing 18 different *Culex* species were identified, and a total of 8,646 of the total had a blood meal (Figure 4.3 and Table 4.2). *Culex tritaeniorhynchus*, *Cx. vishnui*, *Cx. psuedovishnui*, and *Cx. whitei* were predominant vectors, and *Cx. tritaeniorhynchus* was the most abundant species. Among the collected and

identified mosquitoes, 32% of the total mosquitoes had consumed blood meals and. *Cx. tritaeniorhynchus, Cx. vishnui, Cx. pseudovishnui, Cx. whitei, Cx. sinensis* and *Cx. fuscocephala* showed higher blood fed status (31-35%) than the other *Culex* species mosquitoes.

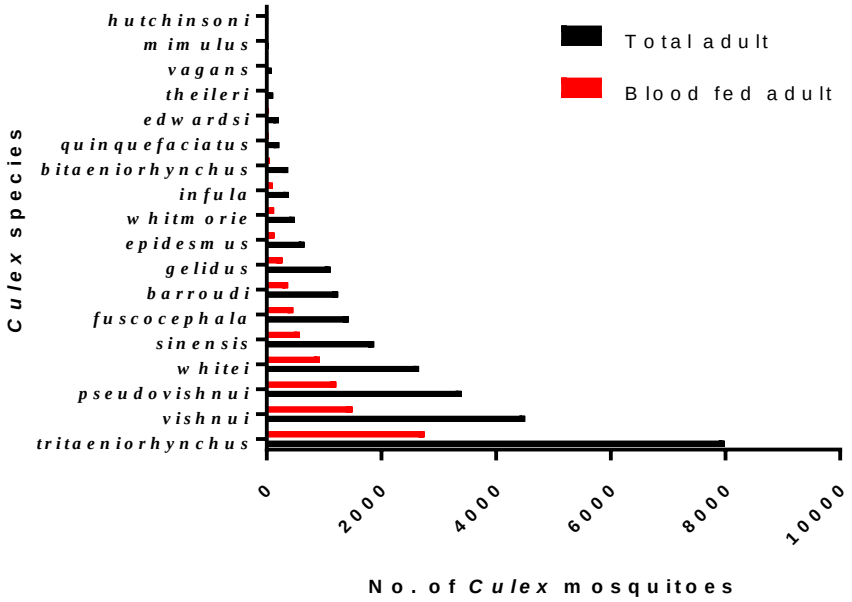


Figure 9 *Culex* species composition

Table 25 *Culex* mosquito composition and blood feeding status

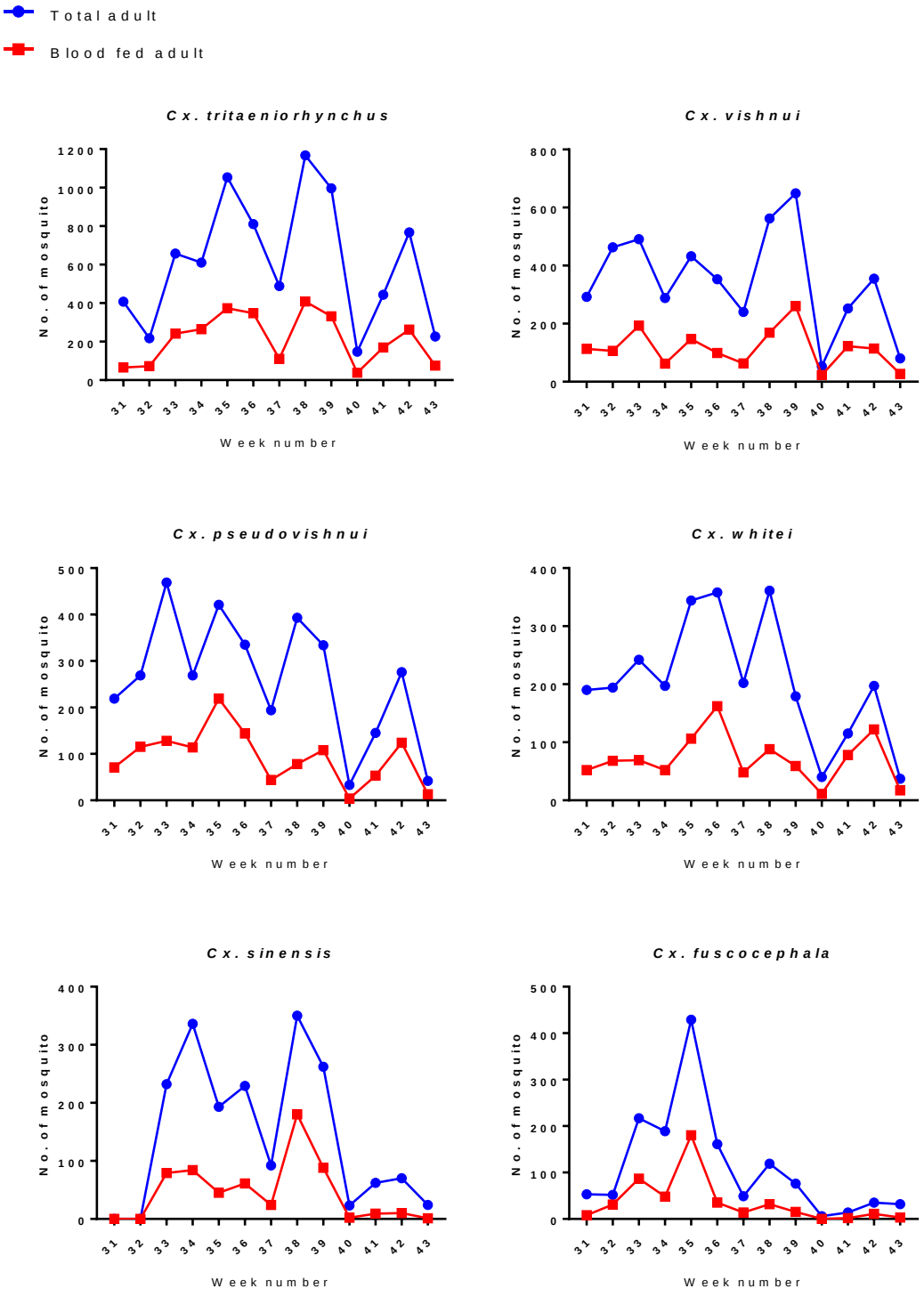
Culex species	Count (% of total mosquito collected)	Blood fed (% of species-specific total)
tritaeniorhynchus	7988 (29.8)	2755 (34.5)
vishnui	4510 (16.8)	1498 (33.2)
pseudovishnui	3399 (12.7)	1215 (35.7)
whitei	2656 (9.9)	932 (35.1)
sinensis	1873(7.0)	583 (31.1)
fuscocephala	1432 (5.3)	466 (32.5)
barraudi	1248 (4.7)	373 (29.9)
gelidus	1112 (4.1)	273 (24.6)
epidesmus	657 (2.4)	136 (20.7)
whitmorie	491 (1.8)	130 (26.5)
infula	385 (1.4)	109 (28.3)
bitaeniorhynchus	373 (1.4)	57 (15.3)
quinquefaciatus	219 (0.8)	34 (15.5)
edwardsi	215 (0.8)	42 (19.5)
theileri	116 (0.4)	21 (18.1)
vagans	87 (0.3)	19 (21.8)
mimulus	37 (0.1)	0 (0)
hutchinsoni	26 (0.1)	3 (11.5)
Total	26824 (100)	8646 (32.2)

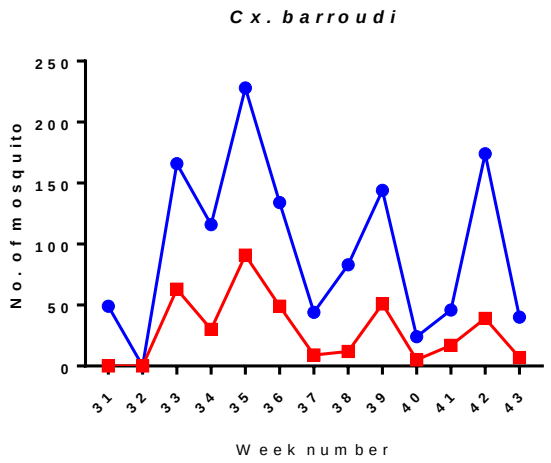
4.3.2 Weekly Trend of *Culex* species

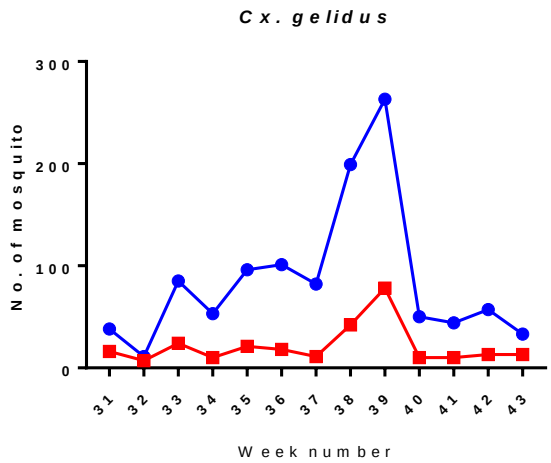
A varying trend in mosquito abundance by species was observed during study period (Figure 4.4). *Cx. tritaeniorhynchus* and *Cx. vishnui* seemed to peak during mid-August to mid-October but *Cx. pseudovishnui* seemed to be active till early September. Other *Culex* species were mostly trapped at higher abundance during middle August to middle of September. However, rise and fall of *Culex tritaeniorhynchus, Cx. vishnui, Cx. pseudovishnui, Cx. whitei, Cx. fuscocephala, Cx. sinensis,* and *Cx. barraudi* abundance occurred at the same times throughout the study period (Figure 5.5a). Variation in the abundance was observed among all 18 species of *Culex* mosquitoes. Similarly, blood fed adult mosquitoes were higher for *Culex tritaeniorhynchus, Cx. pseudovishnui, Cx. vishnui, Cx. fuscocephala, Cx. whitei,* and *Cx.*

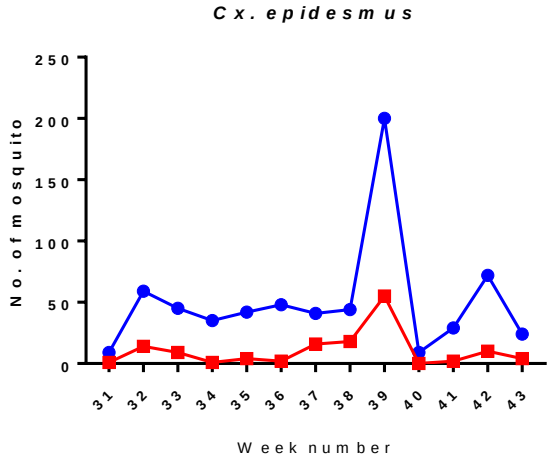
barraudi from weeks 31-43 (Figure 5.5b). *Cx. tritaeniorhynchus* was the species with the highest fraction of engorged females.

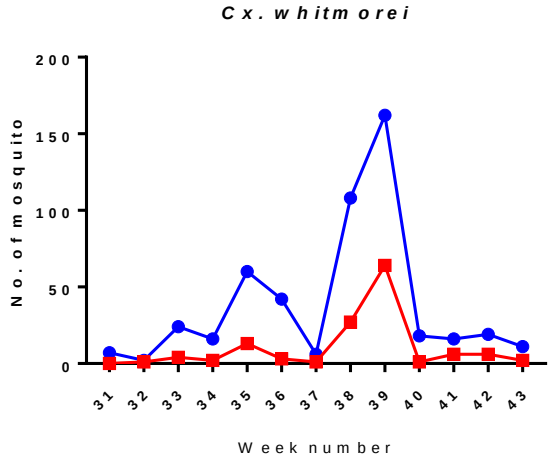
Blood feeding was highly active from week 33 to week 39.and the blood feeding activity of the predominant vector, *Cx. tritaeniorhynchus*, was observed greater from week 34 (mid-August) to 42 (mid-October) (Figure 5.5b). For *Cx. vishnui* and *Cx. sinensis*, most of the engorged mosquitoes were collected in weeks 38 to 39 (second-half of September). Most of the common *Culex* species mosquito feeding was during weeks 35-36 (last week of August to first week of September).

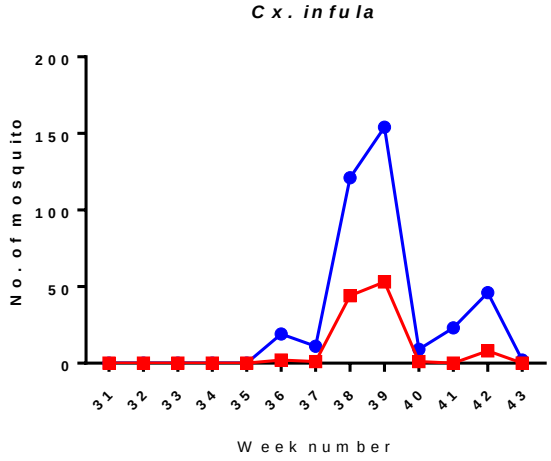

Total adult
Blood fed adult
Cx. tritaeniorhynchus
Cx. vishnui
Cx. pseudovishnui
Cx. whitei
Cx. sinensis
Cx. fuscocephala
No. of mosquito
Week number

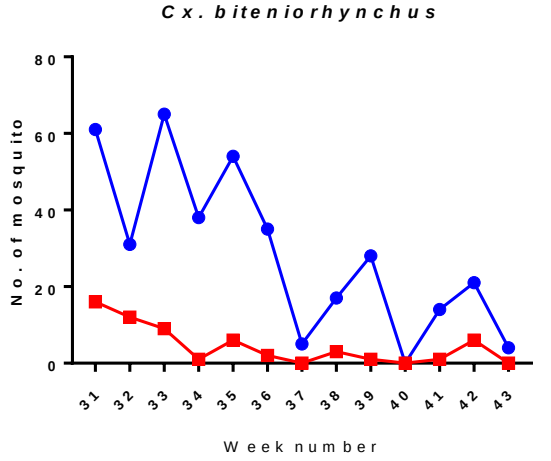

Cx. barroudi
No. of mosquito
250
200
150
100
50
0
31 32 33 34 35 36 37 38 39 40 41 42 43
Week number


Cx. gelidus
No. of mosquito
300
200
100
0
31 32 33 34 35 36 37 38 39 40 41 42 43
Week number


Cx. epidesmus
No. of mosquito
250
200
150
100
50
0
31 32 33 34 35 36 37 38 39 40 41 42 43
Week number


Cx. whitmorei
No. of mosquito
200
150
100
50
0
31 32 33 34 35 36 37 38 39 40 41 42 43
Week number


Cx. infula
No. of mosquito
200
150
100
50
0
31 32 33 34 35 36 37 38 39 40 41 42 43
Week number


Cx. biteniorhynchus
No. of mosquito
80
60
40
20
0
31 32 33 34 35 36 37 38 39 40 41 42 43
Week number

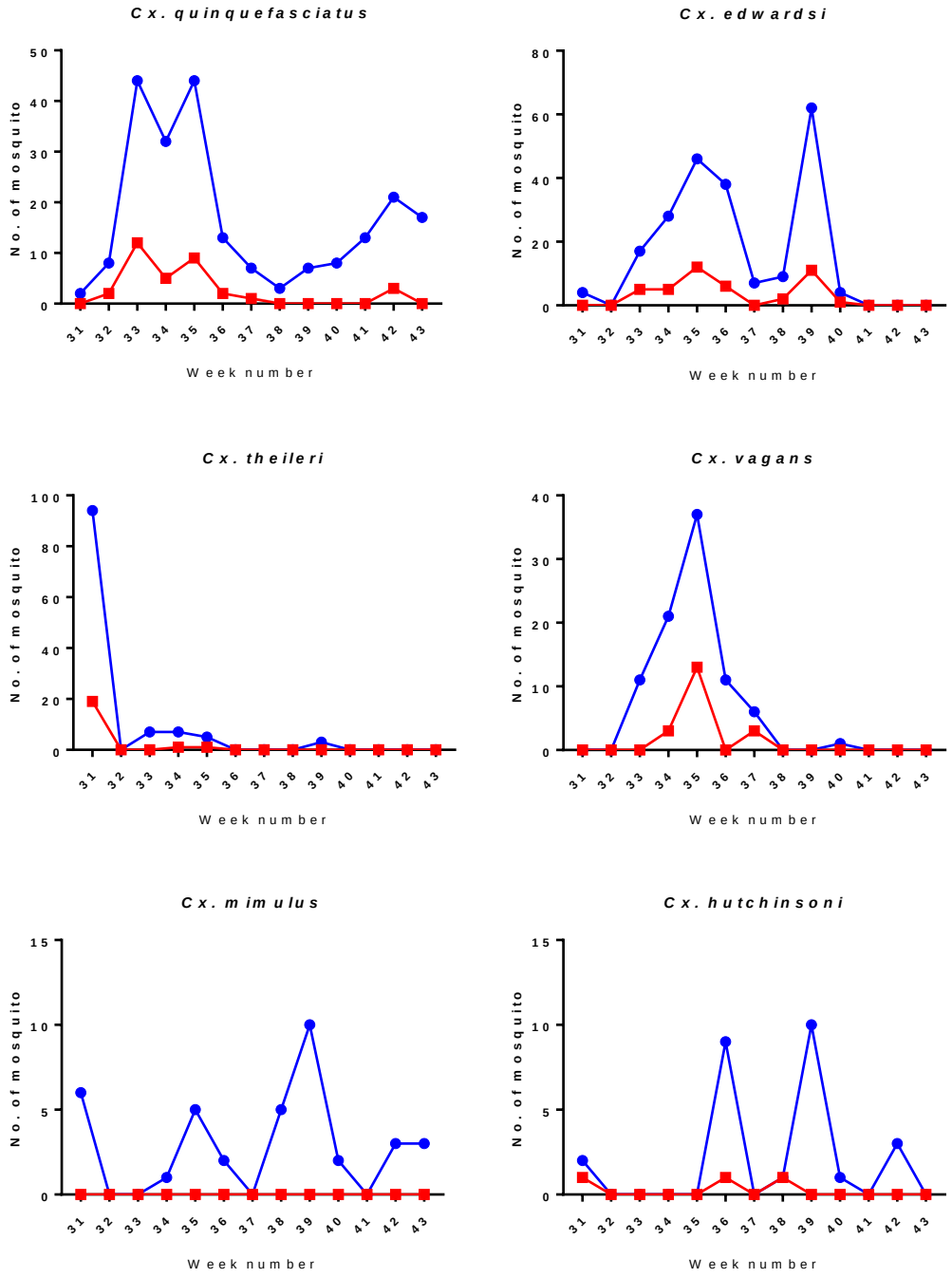
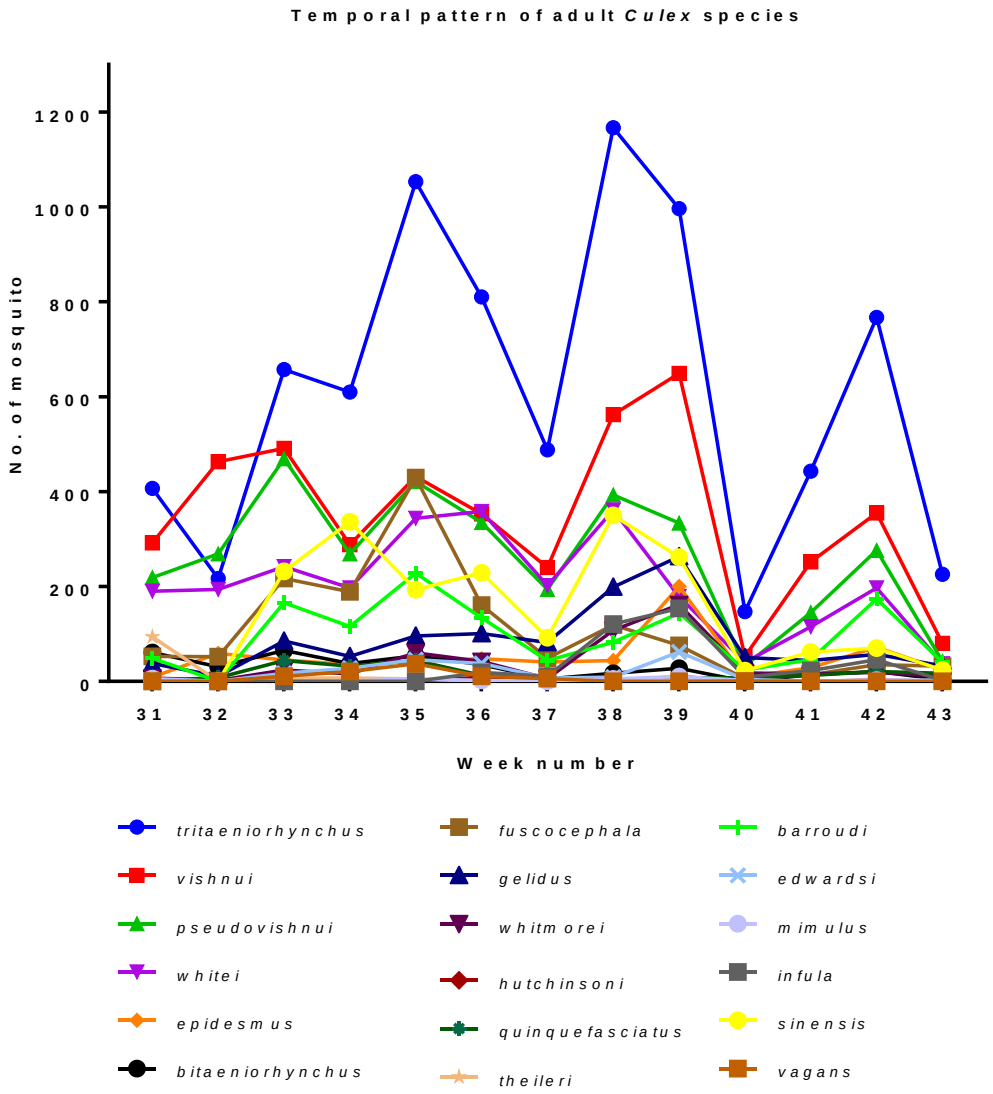


Figure 10 Week-wise abundance of *Culex* species

(a) Temporal pattern of adult *Culex* species



(b) Temporal pattern of adult blood fed *Culex* species

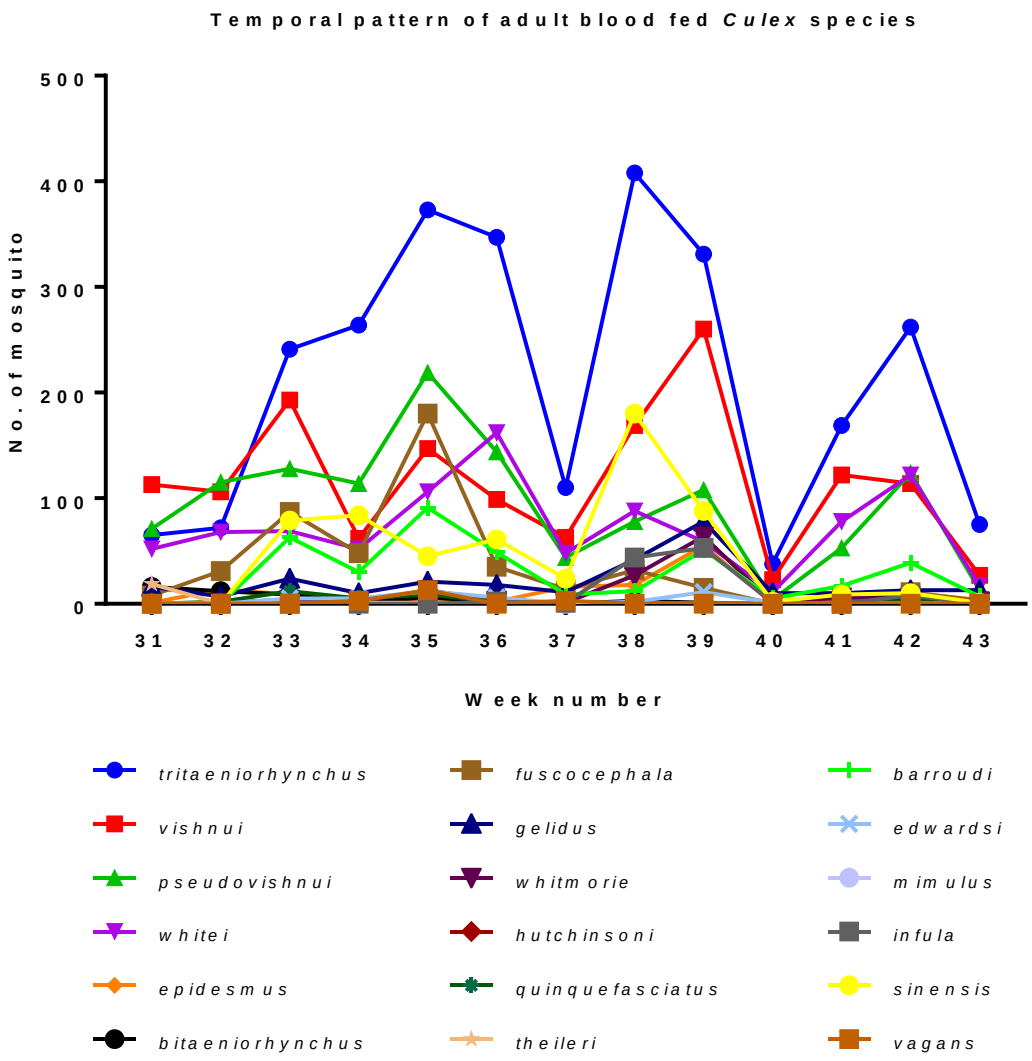


Figure 11 Total adult and blood fed mosquitoes temporal pattern

4.3.3 Comparison of Mosquito and Environmental Data

Figure 4.6 displays environmental data from the study site, including trends of relative humidity (%), dew point temperature (°C), temperature (minimum and maximum, °C), and precipitation (mm/day). The maximum temperature crossed 40°C but remained within the range of 30-40°C and the minimum temperature remained ranged from 20-27°C during the study period. Towards the end of week 43, temperatures fell, and during weeks 31-41 it remained hot during day time and night temperature (minimum temperature) was lower starting from week 38. The dew point temperature remained lower than 25°C, but started to slope down from the week 38. Relative humidity was highest in weeks 36-38 and precipitation was higher in weeks 32-35 and week 37.

Mosquito abundance and diversity data were tested to determine if there was an association with the relative humidity, dew point temperature, minimum temperature, maximum temperature, and precipitation (Table 4.3). Since the distribution of the *Culex* species was not normally distributed, non-parametric measures of correlation (Spearman correlation) were applied. Significant positive correlations were observed between: *Cx. whitei* and precipitation*; Cx. fuscocephala* and *Cx. theileri* and minimum temperature, maximum temperature and precipitation; *Cx. bitaeniorhynchus* and dew point temperature, minimum temperature, maximum temperature, and precipitation; and *Cx. vagans* and minimum temperature and precipitation ($p<0.05$). *Cx. infula* was negatively associated with minimum temperature, maximum temperature and precipitation ($p<0.05$). A significant positive or negative relationship of *Culex* species with the environmental variables were indicated by Spearman's rho values of 0.5-0.8, indicating fair to very good degree of relationship. The association of *Culex* species

abundance with temperature and precipitation variables indicated that fluctuation in temperature and precipitation can affect the abundance of those species.

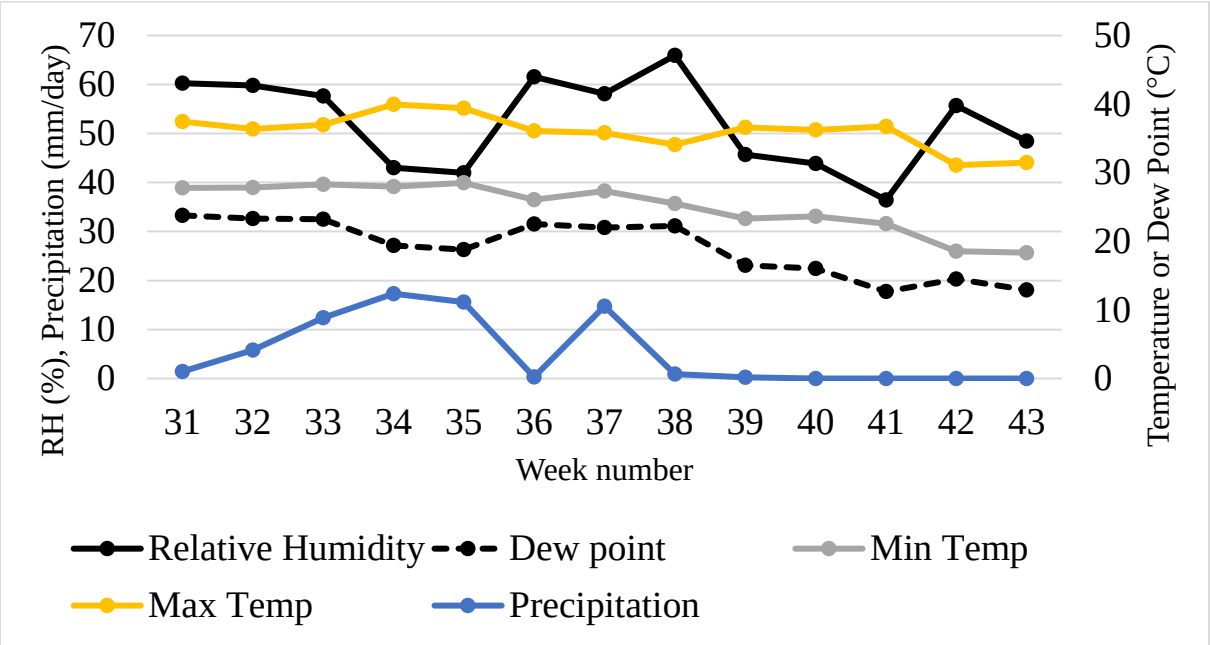


Figure 12 Environmental pattern in Rupandehi district in summer of 2014

Table 26 Association between environmental variables and different *Culex* species

[Spearman correlation coefficients (p-value)]

Culex species	Relative Humidity	Dew Point Temp	Min. Temp	Max. Temp	Precipitation
tritaeniorhynchus	0.1 (0.6)	0.05 (0.8)	0.1 (0.6)	0.01 (0.9)	0.2 (0.4)
vishnui	0.3 (0.2)	0.4 (0.1)	0.2 (0.3)	0.1 (0.6)	0.2 (0.4)
pseudovishnui	0.2 (0.3)	0.4 (0.1)	0.5 (0.07)	0.2 (0.4)	0.4 (0.1)
whitei	0.4 (0.1)	0.5 (0.07)	0.5 (0.05)	0.02 (0.9)	0.5 (0.04)
sinensis	0.02 (0.9)	0.01 (0.9)	0.1 (0.5)	0.1 (0.7)	0.3 (0.2)
fuscocephala	0.1 (0.6)	0.5 (0.05)	0.7 (0.002)	0.5 (0.06)	0.7 (0.003)
barraudi	-0.1 (0.6)	-0.01 (0.9)	0.2 (0.4)	0.2 (0.4)	0.2 (0.4)
gelidus	0.1 (0.6)	0.06 (0.8)	0.1 (0.6)	-0.02 (0.9)	0.1 (0.5)
epidesmus	0.2 (0.4)	0.1 (0.6)	0.02 (0.9)	-0.2 (0.4)	0.06 (0.8)
whitmorei	-0.06 (0.8)	-0.1 (0.6)	-0.03 (0.9)	0.01 (0.9)	-0.1 (0.7)
infula	0.09 (0.7)	-0.4 (0.1)	-0.7 (0.005)	-0.5 (0.03)	-0.6 (0.02)
bitaeniorhynchus	0.1 (0.6)	0.6 (0.01)	0.7 (0.003)	0.6 (0.01)	0.6 (0.02)
quinquefasciatus	-0.5 (0.06)	-0.2 (0.3)	0.2 (0.4)	0.2 (0.4)	0.1 (0.5)
edwardsi	-0.02 (0.9)	0.2 (0.4)	0.4 (0.09)	0.4 (0.1)	0.5 (0.07)
theileri	-0.1 (0.6)	0.4 (0.1)	0.6 (0.02)	0.8 (0.0008)	0.5 (0.04)
vagans	-0.2 (0.4)	0.1 (0.5)	0.6 (0.008)	0.4 (0.09)	0.6 (0.01)
mimulus	0.09 (0.7)	-0.05 (0.8)	-0.2 (0.5)	-0.06 (0.9)	-0.1 (0.5)
hutchinsoni	0.3 (0.2)	0.05 (0.8)	-0.3 (0.2)	-0.2 (0.3)	-0.4 (0.1)

4.4 Discussion

The work presented here appears to be the only study of JEV vectors in Nepal conducted during the JEV transmission season. Previous studies on JEV in Nepal have often cited and suspected of *Culex tritaeniorhynchus* as the primary vector based on studies in India (Rayamajhi et al., 2007; Bista and Shrestha, 2005) but there could be other *Culex* species that might also be transmitting the virus.

The trap sites for mosquito collection were appropriately placed in habitats preferred by mosquitoes and a total of 18 *Culex* species were collected, including known JEV vectors. *Culex tritaeniorhynchus*, a dominant vector for JEV, dominated trap collections. Variation in

abundance could be due to the variation in temperature, rainfall, relative humidity, and other unknown factors. Week 34 and onwards is post-monsoon season in Nepal, and it is the time when paddy fields and ditches are full of stagnant water providing mosquitoes with good breeding habitats. A rise in mosquito abundance after week 34 is likely due to emergence of mosquitoes from their breeding grounds.

Temporal patterns for collection of both adult and blood fed *Culex* species revealed a general trend of increasing abundance for all species from week 33 to week 42, although there were some fluctuations. When compared with environmental data of the district, none of the *Culex* species were significantly associated with relative humidity, but some changes in abundance were related to temperature change and associated with minimum and maximum temperature.

A limitation of this study was our inability to conduct virus detection on mosquito pools due to laboratory facility constraints. Nevertheless, the data on *Culex* species abundance over time and in a district with frequent outbreaks of JE should provide a valuable baseline for future studies on JEV vectors in Nepal.

CHAPTER 5: ROLE OF DUCKS AND MOSQUITOES IN JAPANESE ENCEPHALITIS VIRUS GENOTYPE DISPLACEMENT

5.1 Introduction

Japanese encephalitis virus (JEV) belongs to the family *Flaviviridae* and genus *Flavivirus,* and is a single-stranded enveloped positive-sense RNA virus having a genome approximately 11 kb in size (Chambers et al., 1990; Schuh et al., 2013). JEV exists in an enzootic transmission cycle in which the infectious Culicine mosquito, primarily *Culex tritaeniorhynchus,* transmits the virus via a bite to ardeid birds such as herons and egrets (the reservoir hosts) and domestic pigs (the amplifier host) (Lord et al., 2015; Mackenzie et al., 2004). The mosquito-bird and the mosquito-pig cycles are independent of each other, and humans are incidental hosts in either transmission cycle (van den Hurk et al., 2009; Mackenzie et al., 2004). This zoonotic virus is neuroinvasive and neurovirulent in humans and elicits substantial morbidity among all unvaccinated age groups; mortality is seen predominantly in children (Solomon et al., 2003). JEV is the leading cause of arboviral encephalitis in the world and common in regions where paddy fields, pig husbandry, and mosquitoes co-exist (Campbell et al., 2011; Solomon et al., 2003). With an estimated three billion people living in at-risk areas globally, the challenge remains in Africa, Europe, and the Middle East, each of which has populations of the primary mosquito vector of JEV (Solomon et al., 2003; Campbell et al., 2011; Daep et al., 2014; Jefferies and Walker, 2015, Pyke et al., 2001). An effective vaccine for use in humans is commercially available, yet 50,000-60,000 cases of Japanese encephalitis (JE) are reported annually worldwide, likely because of the shortfalls in vaccine supply and cost (Solomon et al., 2003; Lord et al., 2015; Fischer et al., 2010).

Five genotypes of JEV – designated GI through GV- have been characterized as responsible for historical and current outbreaks in endemic regions (Schuh et al., 2013). JEV genotypes have evolved through various strategies to co-circulate and displace each other as has been observed during co-circulation. Most prominently, GI strains came to dominate over GIII strains, and finally displacement of JEV GIII by JEV GI occurred in Asia (Schuh et al., 2014; Sarkar et al., 2012a) Australia (Pyke et al., 2001; Schuh et al., 2014, Han et al., 2015; Ma et al., 2003; Mohammed et al., 2011; Nga et al., 2004). For example, JEV GIII viruses were dominant between 1935 and 1990, but GI viruses began to appear in 1979 and gradually became dominant in most parts of Asia. Co-circulation and genotype/strain displacement is a common phenomenon among flaviviruses. It was observed with West Nile virus (WNV), a closely related flavivirus, when the WNV 02 genotype displaced the WNV NY99 genotype in the USA (Ebel et al., 2004; Moudy et al., 2007). Among the other well studied examples, endemic American genotype of dengue virus type 2 (DENV-2) was displaced by the Southeast Asian genotype of DENV-2 (Armstrong and Rico-Hesse, 2001). Furthermore, strain and clade displacement have also been observed in DENV. A native DENV-3 strain was displaced by an invasive DENV-3 strain in Sri Lanka in the 1980s (Hanley et al., 2008). Similarly, the DENV-2 Asian-American genotype NI-1 clade was replaced by the NI-2B clade in Nicaragua (Quiner et al., 2014). Such displacements have several potential impacts on the international public health infrastructure, including the possibility that currently available vaccines are sub-optimal and that future displacement events could perhaps jeopardize the current vaccination programs. The mechanisms responsible for dominance and displacement of JEV GI over JEV GIII remains poorly understood.

Studies that have tried to address JEV genotype displacement have been based on nucleotide sequence analysis, and have provided important general insights about the displacement of JEV GIII by JEV GI. What has been lacking in previous attempts to explain displacement is the use of ecologically relevant avian hosts and mosquito vectors, which may be important for predicting further shifts in genotype distribution for JEV and could provide valuable insights for control of several arthropod-borne viruses. Due to continued evolution, JEV remains as an emerging worldwide human health threat and disease severity within the human population could be increased by an emergence of new genotypes with altered disease manifestations, transmission potential, and possibly, effects on vaccine efficacy. The study presented here used hosts and the vectors involved in natural JEV ecology, and were designed to enhance our understanding of JEV genotype displacement. Avian hosts are important source of JEV in endemic areas (Rosen, 1986; van den Hurk et al., 2009), and field data suggest ducks are exposed to JEV (Pant, 2006; Simpson et al., 1970). Previous studies involving experimental infection in ducks with JEV suggests that young ducks develop high magnitude viremia titer (up to 7 $\log_{10}$PFU/ml) and that virus titer remain above 4 $\log_{10}$PFU/ml up to 3-4 days post-inoculation (Cleton et al., 2014). Furthermore, it has been reported that domestic ducks infected with JEV can infect biting *Culex* species mosquitoes (Dhanda et al., 1977).

Given the evidence of past displacement of JEV genotypes in several endemic countries, our base hypothesis was that JEV GI viruses have an enhanced ability to replicate in mosquitoes and avian hosts relative to JEV GIII viruses. This hypothesis was tested using recent, low passage isolates of JEV GI and GIII. We attempted to determine the multiplication kinetics of JEV GI and GIII viruses using *Cx. quinquefasciatus* mosquitoes and ducks and to determine the vector competence for the JEV GI and the JEV GIII isolates in *Cx. quinquefasciatus* mosquitoes.

5.2 Materials and Methods

5.2.1 Ethics Approval

The study was approved by the Animal Care and Use Committee of Colorado State University (protocol number: 16-6477A) and was conducted in facility approved by the Association for the Assessment and Accreditation of Laboratory Animal Care, International (AAALAC).

5.2.2 Cells and Viruses

Vero cells (African green monkey kidney cells) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% bovine calf serum (BCS), penicillin (100 U/ml) and streptomycin (50 ug/ml) with 5% CO_2 at 37°C. The strains of JEV utilized in this study are described in Table 5.1. All virus strains were titrated by plaque assay in Vero cells upon arrival using techniques previously described (Cleton et al, 2014).

Table 27 Japanese encephalitis viruses utilized in the study

Strain	Genotype	Country of isolation	Source	Collection year	Passage history	Climate of isolated country	Genbank Accession #	Titer (log_{10} PFU/ml)
KE-093-83	GI	Thailand	Mosquito	1983	Vero1	Tropical	KF192510	6.3
MAR864	GI	Cambodia	*Cx. tritaeniorhynchus*	1967	c6/36#1, Vero1 (NH)	Temperate	D00983	6.6
JE-91	GI	Korea	*Cx. tritaeniorhynchus*	1991	c6/36 #1, Vero1	Temperate	GQ415355	6.8
CH392	GIII	Taiwan	*Cx. tritaeniorhynchus*	1987	Vero1, c6/36#2	Temperate	U44961	8.3
JKT27-087	GIII	Indonesia	Mosquito	1987	(NH) c6/36#1	Temperate	JQ429308	8.0
Sagiyama	GIII	Japan	*Cx. tritaeniorhynchus*	1957	Mosquito? /c6/36#1	Temperate	D00972	7.4

5.2.3 *In Vitro* Replication (Growth Curve)

A multi-step growth curve was performed for all six viruses on Vero cells (passage # 10) at an MOI of 0.01. Briefly, 5 replicates/virus strain were performed for each strain. Before inoculating the cells, two wells of a 6-wells plate were used to count the number of cells using hemocytometer and the average count of cells was used to estimate the quantity of virus to inoculate. After inoculating the cells with viruses, the plates were incubated for two hours of adsorption at 37°C and with 5% CO_2. Following incubation, virus inoculum was removed, cells were washed one time with PBS, and 5ml fresh growth media (DMEM with 10% FBS, penicillin-streptomycin, gentamicin and amphotericin B) was added. At every time points (0 hour, 1 through 8 days), 500ul of supernatant was harvested and replaced with the same amount of fresh growth media. The supernatant was stored in a clean tube with 100ul of FBS at -80°C until assayed. Plaque assay was performed to determine virus titer for each virus at each time point.

5.2.4 Animal Study

Thirty-Two Indian runner ducks (*Anas platyrhynchos domesticus,* Murray McMurray Hatchery, Webster City, IA, USA) were housed in the Animal Disease Laboratory at Colorado State University under biosafety level-3 containment. Ducklings were assigned to six groups, corresponding to the dix virus strains to be tested. An extra ducking was added to groups inoculated with MAR864 and Sagiyama JEV, but was not inoculated and served as contact control. The ducklings were fed standard waterfowl feed and water *ad libitum.* The ducklings were 5-6 days old when inoculated with the viruses. A 100ul blood sample of was withdrawn from each duckling daily and immediately diluted into 450ul of BA-1 medium, yielding serum

concentration of roughly 1:10. Body temperature and weight were measured daily from the day before inoculation through day 7 post-inoculation and on days 10, 14, and 21 post-inoculation. Oral and cloacal swab samples were taken on day 3, 4 and 5-day post-inoculation and immediately added to 450ul of BA-1 medium supplemented with twice the standard concentration of penicillin, streptomycin, gentamicin, amphotericin B, and polymixin B. The samples were immediately stored in -80°C for future assay. All the ducklings were euthanized on 21 days post-inoculation.

5.2.5 Mosquito Study

Cx. quinquefasciatus Say mosquitoes were from a colony maintained at the Arthropod-borne and Infectious Diseases Laboratory at Colorado State University. This colony was established using mosquitoes collected in Sebring County, Florida in 1988. Larvae of the mosquitoes were provided with powdered fish food, and when pupae started emerging they were transferred in a cup with water inside a container covered with a mesh and provided with ad libitum food and sugar cubes. The insectary was maintained at 26-27C, 16:8 light: dark cycle, and 70-80% relative humidity. Female mosquitoes of the F11-F13 generations (5-7 days post-eclosion) were used in this study.

5.2.6 Vector Competence

Vero cells were infected at an MOI of 0.01 with all six strains of JEV GI and GIII in T25 flasks and kept at 37°C, 5% CO2, with growth media. On day 4 after Vero cells were inoculated, the viruses were harvested. Briefly, the supernatant from each flask were collected and centrifuged (10,000xg for 8 minutes at 4°C) to remove cellular debris, and the clarified

supernatant were aliquoted in separate tubes. The aliquoted virus samples were back-titrated in Vero cells, feeding mosquitoes with blood meal, and infectious blood meal titration on the same day of harvest.

Mosquitoes were fed with defibrinated cattle blood mixed with fresh viruses at 1:1 ratio. The blood-virus mixture was pipetted into the loading chamber of a Hemoteck apparatus covered with pork casing (Whole Foods Market, Fort Collins), and the mosquitoes were allowed to feed for 1-2 hours. The temperature and relative humidity of the room where blood feeding was conducted remained at 26°C and 80%, respectively. Blood-engorged mosquitoes for each virus strain were briefly cold anesthetized (4°C) and separated into two groups for assay at 7 and 14 days post-infection. Mosquitoes were incubated at 26°C and 70% relative humidity with *ad libitum* water and sugar cubes.

On days 7 and 14-day post-infection, 60 mosquitoes per virus strain were cold anesthetized (4°C). Legs and wings were removed and transferred into a clean tube containing 250ul mosquito diluent (1X PBS supplemented with 20% heat-inactivated FBS and antibiotics [100 mg/L gentamicin, 200 IU/L penicillin G, 100 mg/L streptomycin, and 5 mg/L amphotericin B]. Additionally, each tube also contained two stainless steel beads for homogenization. The mosquito bodies were placed with their proboscis inserted in a capillary tube containing immersion oil (about 5ul) for at least 30 minutes. After salivation, mosquito bodies were transferred into a separate clean tube with mosquito diluent and beads. The ends of capillary tubes containing immersion oil and saliva were broken off into micro centrifuge tubes containing 100ul mosquito diluent. Mosquito tissues (bodies, and legs+wings) were homogenized at 25 cycles/second for 1 minute in a mixer mill and subsequently centrifuged at 15,000xg for 3

minutes at 4°C. To determine the infection status of mosquito tissues and saliva, 50 ul of each sample was inoculated onto Vero cell in a 96-wells plate and incubated at 37°C. After incubation for an hour, 150ul of DMEM supplemented with 10% FBS was added into each well and the plates were incubated for 24 hours at 37°C. Controls in each assay consisted of duplicate wells inoculated with BA-1 diluent or BA-1 spiked with stock JEV

5.2.7 Virus Titration, Serology, and Indirect Fluorescent Antibody Test

Virus titration for Japanese encephalitis virus in Vero cell infected supernatant, serum, cloacal swab, and oral swab samples of ducks was done using a double overlay Vero cell plaque assay (Cleton et al., 2014). Plaques were counted on one and two days after the second overlay. Virus titers were expressed as plaque forming units (PFU)/mL. The minimum concentration of JEV that could be detecting using this assay was 100 PFU/mL for both swabs and diluted serum samples.

Neutralizing antibody titers in sera were assayed by plaque reduction neutralization test (PRNT) with a 90% neutralization cutoff. Briefly, 2-fold serial dilutions (starting at 1:10) of heat-inactivated serum samples (56°C for 30 min) from ducklings were mixed with equal volume of stock virus (JEV VN strain) diluted to ~200 PFU/mL. After 1 hour of incubation at 37°C and 5% CO_2, the sera-virus mixtures were inoculated into 6-wells plates of confluent Vero cells. An agarose overlay was added after one hour of incubation and after 48 hours of incubation, a second overlay containing neutral red was added. Plaques were counted one day after the second overlay and neutralization titer was expressed as the reciprocal of the highest dilution of the serum that inhibited $\geq$90% of JEV plaques.

Media from the 96-wells plate inoculated with mosquito samples was dumped off after 24 hours; cells were washed once with PBS, fixed with 70% acetone for 1 hour and dried. The cells were stained by addition of 50ul of 1:100 diluted mouse anti-JEV in PBS for 1 hour at 37°C, washed in PBS, then incubated for 1 hour with 50ul of Dylight conjugate of goat anti-mouse Ig (H+L) (1:100 in PBS). The plate was washed again with PBS and observed under the fluorescent microscope. The infection rate was determined as the proportion of mosquito bodies that were found infected to the total number of mosquitoes exposed to the infectious blood meal. Dissemination rate was determined as the proportion of mosquito legs and wings that were found infected to the total number of mosquitoes exposed to the infectious blood meal. Similarly, transmission rate was determined as the proportion of mosquito saliva that we found infected to the total number of mosquito-exposed to the infectious blood meal.

5.2.8 Data Analysis

Mean peak viremia titers for the six groups of ducklings were compared using one-way ANOVA. Similarly, A two-tailed Fischer's exact test was applied to compare the rates of infection, dissemination and transmission in mosquito study. SAS 9.4 (SAS Institute, Cary, NC) was used for all statistical analysis. Graphs were made on GraphPad Prism 7.0 (La Jolla, CA). The statistical significance was declared at $p<0.05$.

5.3 Results

5.3.1 Growth Kinetics *in vitro*

The growth kinetics of all strains of JEV was similar in Vero cells, a mammalian cell type which do not possess functional type-interferon signaling (Figure 5.1). Each of the six

viruses reached at peak titer (7- 8.5 log_{10}PFU/ml) on 2 days post-inoculation and there were no significant differences in peak titer among groups ($p > 0.05$). Virus titer remained similar on days 2-3 post inoculation, then fell to a titer below 4-7 log_{10}PFU/ml on day 5 post-inoculation, which corresponded to the observation of cytopathic effect in the Vero cells.

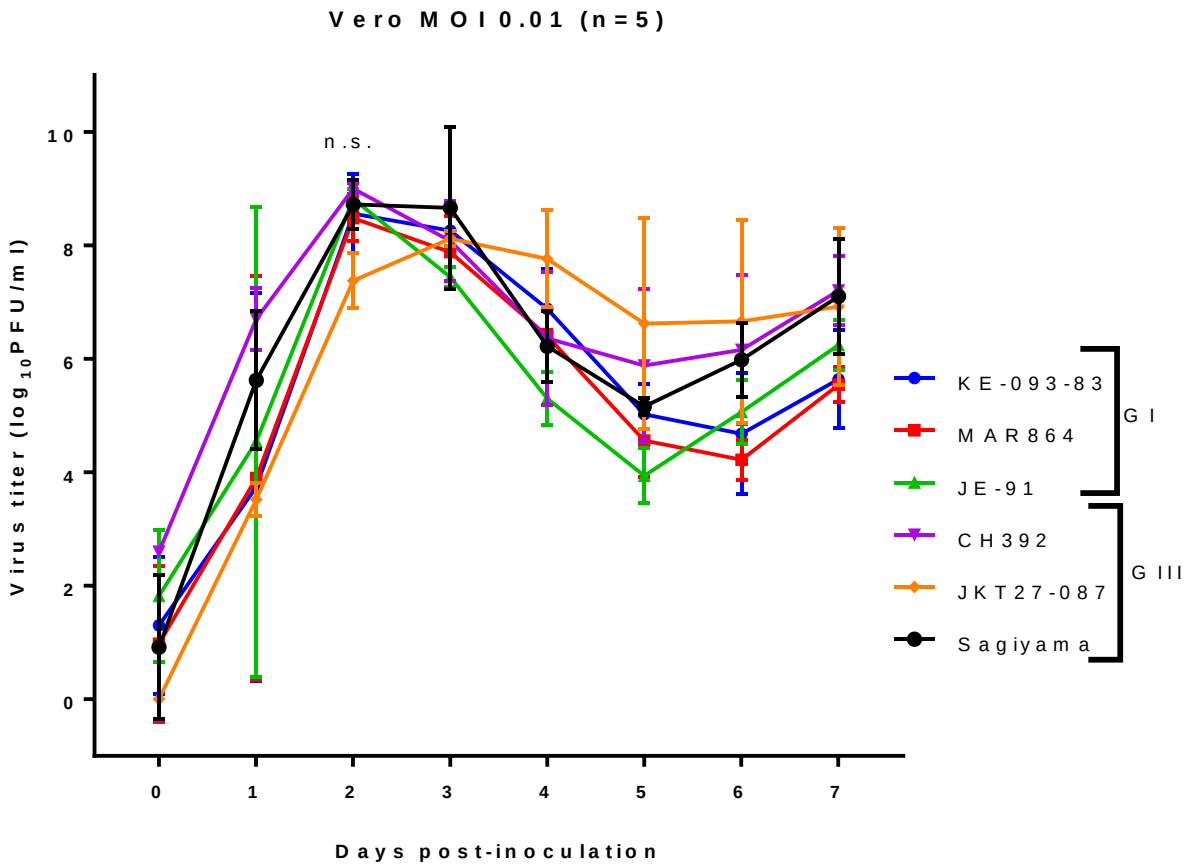


Figure 13 Growth curve of JEV GI and GIII strains in Vero cells
(n.s.= non-significant at peak titer)

5.3.2 Clinical Signs in Ducklings Inoculated with JEV

None of the ducklings inoculated with JEV showed apparent signs of disease or distress. A mild elevation of body temperature (normal body temperature = 104-109°F) at 1 dpi was

observed in ducklings for MAR864, CH392, JKT27-087, and Sagiyama (Figure 5.2). The weight of ducklings increased from around 100 grams to as close as 700 grams in 22 days (Figure 5.3). The body weight of the ducklings did not appear to decrease at all with the age but increased with the age. Although only two control ducklings were present, they remained healthy and gained weight similarly to inoculated ducklings.

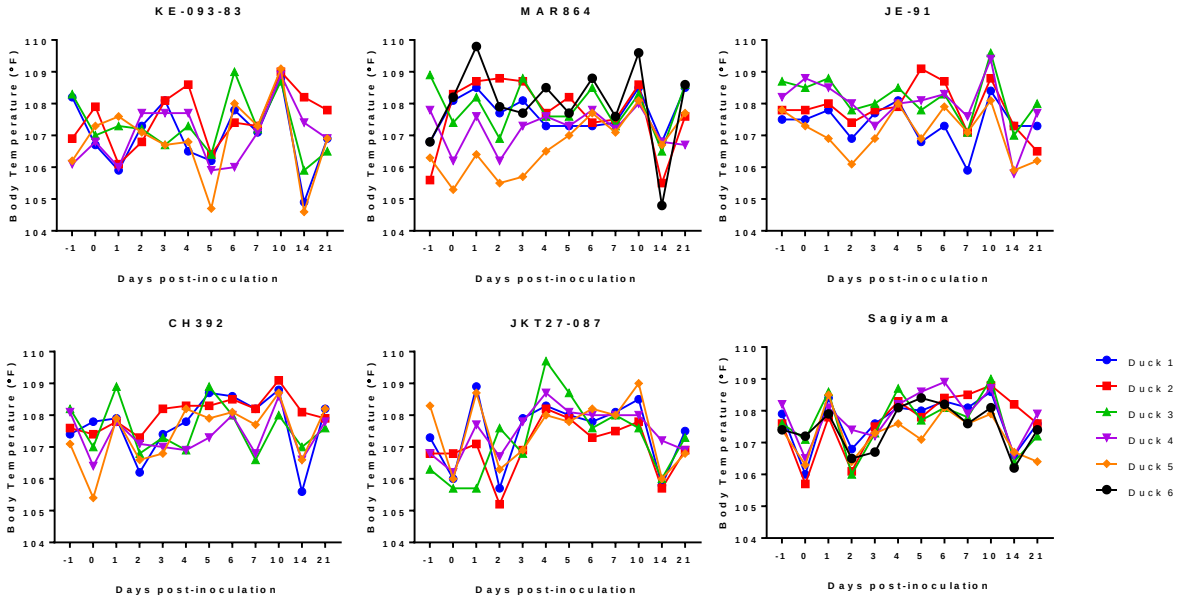


Figure 14 Body temperature in ducklings inoculated with JEV

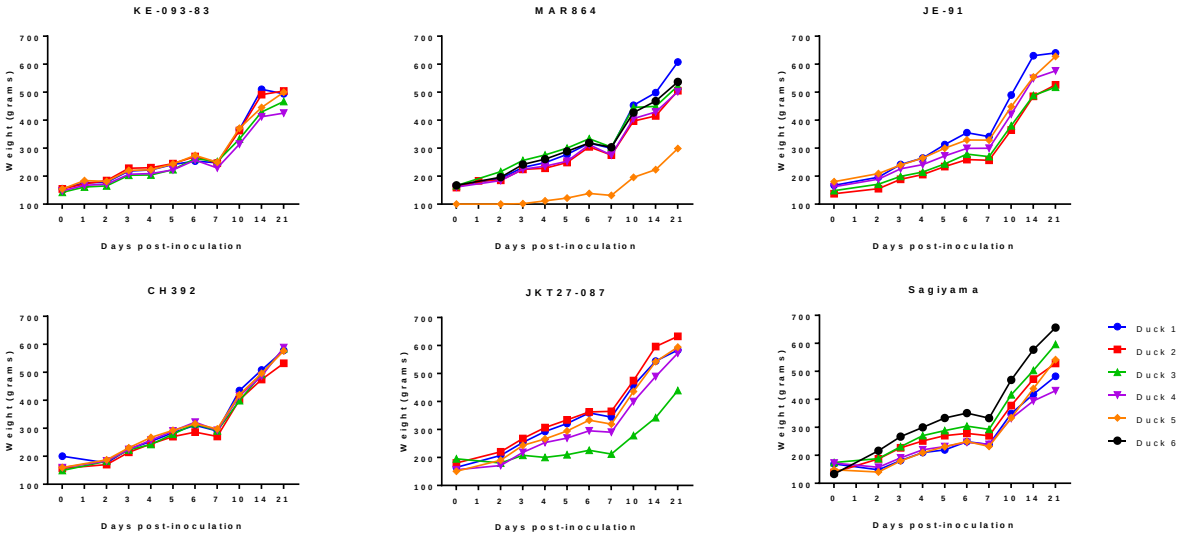


Figure 15 Body weight of ducklings inoculated with JEV

5.3.3 Replication of Japanese encephalitis viruses in Ducklings

The dose of virus inoculated into ducklings ranged from 5.5 to 6.0 $\log_{10}$PFU/ml (Table 5.1). Replication of JEV in ducklings was measured as viremia (Figure 5.4). The peak viremia titers of ducklings in the 6 groups were not significantly different from each other ($P>0.05$, Figure 5.5), nor were the titers different when ducklings inoculated with each strain of JEV were pooled. Virus was not detected in either of the two non-inoculated control ducks. When average of genotype I virus titer was compared with average of genotype III virus titer, the average titer was not statistically different at 1, 2, and 3 dpi ($p>0.05$) (Figure 5.5).

Table 28 Titration of JEV strains given to ducklings

JEV Strain	Genotype	Stock titer (Log_{10}PFU/ml)	Back Titer (Log_{10}PFU/ml)	Delivered dose (PFU/100ml)
KE-093-83	I	6.3	5.7	5.0E+7
MAR864	I	6.6	5.5	5.0E+7
JE-91	I	6.8	5.9	5.0E+7
CH392	III	8.3	5.8	5.0E+7
JKT27-087	III	8.0	5.9	5.0E+7
Sagiyama	III	7.4	6.0	5.0E+7

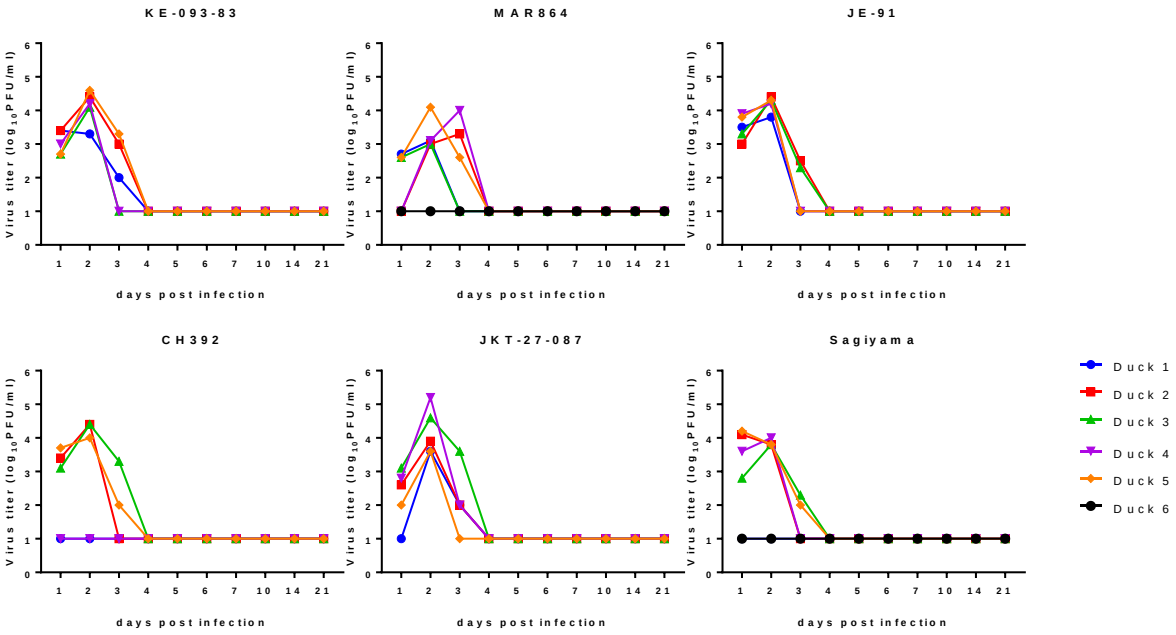


Figure 16 Viremia in ducklings inoculated with JEV

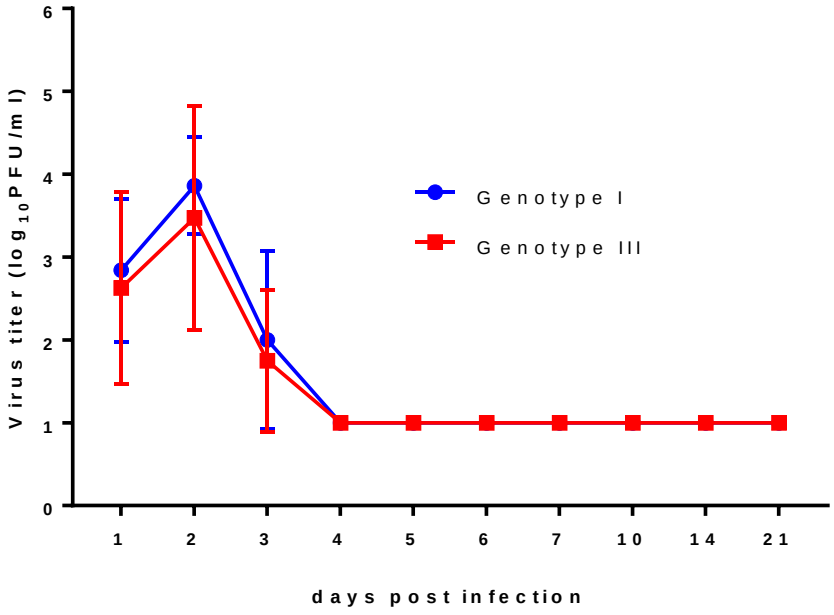


Figure 17 Genotype-specific averaged virus titer in ducklings inoculated with JEV GI and GIII

5.3.4 Antibody Response to Japanese Encephalitis Viruses in Ducklings

Each duckling was seronegative (<10) before inoculation and all but one duckling inoculated with virus had seroconverted by 7 days post-inoculation (Table 5.3). The neutralizing antibody titers continued to increase and all inoculated ducklings were seropositive by 21 days post-inoculation. Neither of the non-inoculated ducklings developed a detectable antibody response by days 21, indicating a lack of contract transmission.

Table 29 Plaque Reduction Neutralization Test antibody titer in ducklings inoculated with JEV (90% cut off)

Virus	Duck	0 dpi	7 dpi	21 dpi
KE-093-83	D1	<10	40	320
	D2	<10	20	320
	D3	<10	40	>=640
	D4	<10	20	80
	D5	<10	80	320
MAR864	D1	<10	160	160
	D2	<10	40	80
	D3	<10	20	160
	D4	<10	10	160
	D5	<10	20	160
Control	D6	<10	<10	<10
JE-91	D1	<10	40	160
	D2	<10	80	80
	D3	<10	80	320
	D4	<10	80	80
	D5	<10	80	160

Virus	Duck	0 dpi	7 dpi	21 dpi
CH391	D1	<10	<10	80
	D2	<10	40	160
	D3	<10	40	>=320
	D4	<10	160	160
	D5	<10	40	80
JKT27-087	D1	<10	40	>=320
	D2	<10	>=320	>=320
	D3	<10	80	>=320
	D4	<10	20	>=320
	D5	<10	40	80
Sagiyama	D1	<10	40	80
	D2	<10	20	>=320
	D3	<10	80	>=320
	D4	<10	160	80
	D5	<10	40	160
Control	D6	<10	<10	<10

5.3.5 Shedding of Japanese Encephalitis Viruses in Ducklings

Virus shedding in oral and cloacal secretions was observed on days 3, 4, and 5 post-inoculation (Figure 6, 7). Relatively higher concentrations of infectious virus were detected in oral samples than cloacal samples. All 5 ducklings in KE-093-83 and JKT27-087 shed virus on 3 dpi, and 4 out of 5 inoculated ducklings in MAR864 and Sagiyama strain group shed infectious virus on 3 dpi. Oral shedding was very low for JE-91 and CH392. Oral shedding on 4 dpi above the limit of detection was common in MAR864 and JKT27-087, however 1-2 ducklings shed infectious virus in the JE-91, CH392 and Sagiyama strain groups at 4 dpi. On 5 dpi, only a single duckling from KE-093-83, MAR864 and JKT27-087 groups shed detectable infectious virus. The virus titers in oral swab samples ranged from 2-5 $\log_{10}$PFU/ml for ducklings inoculated with

both genotype viruses. Compared to oral shedding, cloacal shedding of JEV from ducklings was not common (Figure 5.6). Cloacal shedding was observed on 3 dpi for all six virus strains.

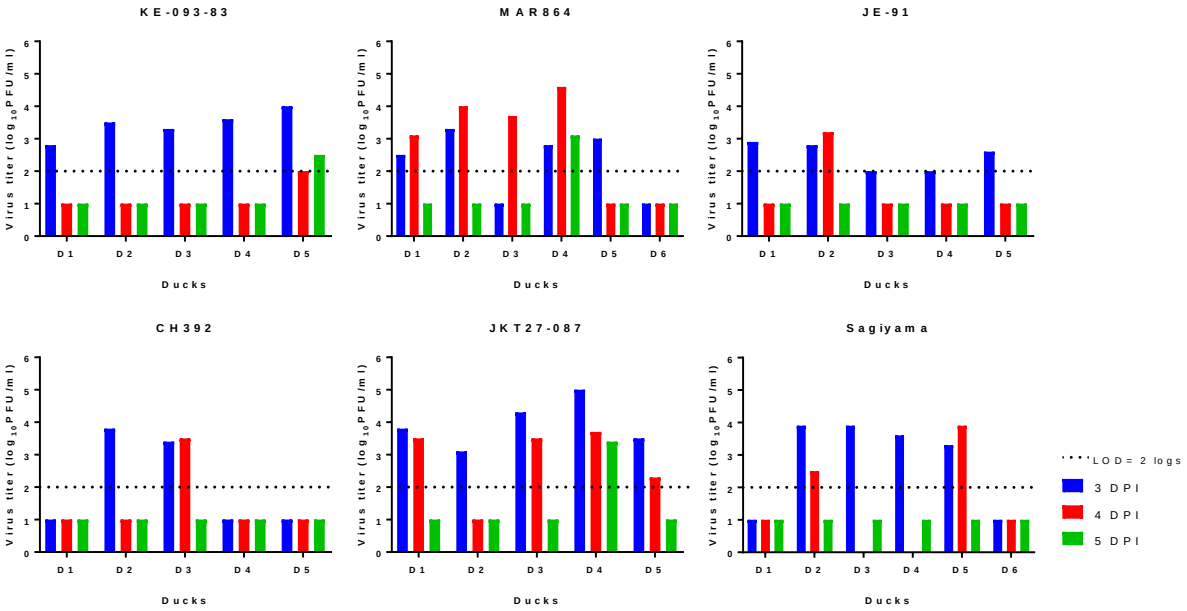


Figure 18 Oral shedding of JEV in ducklings

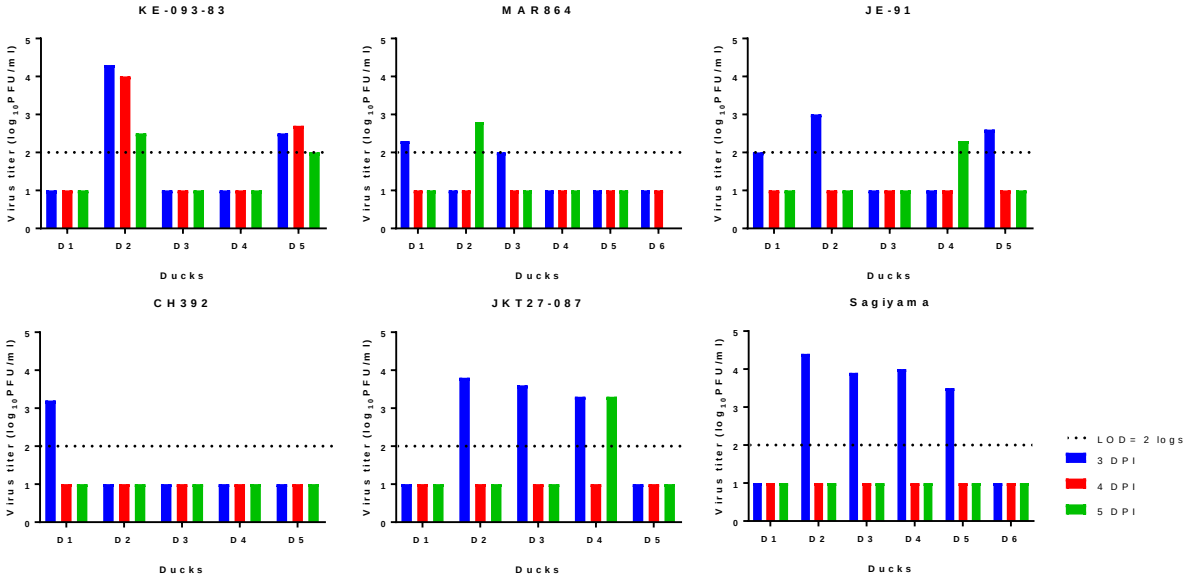


Figure 19 Cloacal shedding of JEV from ducklings

5.3.6 Vector Competence and Extrinsic Incubation Period

Vector competence of *Cx. quinquefasciatus* for JEV GI and GIII viruses was measured among 60 mosquitoes for each virus strain. The amount of virus fed to the mosquitoes for each virus strain ranged from 5.1-6.0 $\log_{10}$PFU/ml of the viruses (Table 5.4). *Culex quinquefasciatus* was found competent to transmit all six strains of JEV, but competence varied between JEV GI and JEV GIII strains (Table 5.5, Figure 5.8, 5.9). In summary, all of the six virus strains were able to infect the mosquitoes, disseminate through legs and wings, and get transmitted through saliva at 14 days post-infection. But, at 7 days post-infection, only GIII virus strains did not get transmitted through saliva of mosquitoes, which indicates longer extrinsic incubation period (EIP) of GIII than GI virus strains in *Culex quinquefasciatus* mosquitoes. When considering median rates, the Figure 5.9 demonstrates that GI is relatively better at infecting mosquitoes, disseminating through body parts of mosquitoes and shed through saliva at an earlier time point.

Table 30 Titration of fresh and blood meal JEV strains

Strain	Genotype	Type	PFU/ml	Log_{10}PFU/ml
KE-093-83	I	Fresh harvest	4100000	6.6
		Blood meal (1:1)	750000	5.9
MAR864	I	Fresh harvest	1300000	6.1
		Blood meal (1:1)	390000	5.6
JE-91	I	Fresh harvest	1400000	6.1
		Blood meal (1:1)	320000	5.5
CH392	III	Fresh harvest	100000	6.0
		Blood meal (1:1)	350000	5.5
JKT27-087	III	Fresh harvest	450000	5.7
		Blood meal (1:1)	140000	5.1
Sagiyama	III	Fresh harvest	1200000	6.1
		Blood meal (1:1)	1000000	6.0

Table 31 Rates of infection, dissemination, transmission at 7 and 14 days post-feeding

Virus strain	DPI	+ Bodies	+ (Legs+ Wings)	+ Saliva
KE-093-83	7	56/60 (93.3%)	18/60 (30%)	1/60 (1.6%)
	14	47/60 (78.3%)	33/60 (55%)	4/60 (6.6%)
MAR864	7	56/60 (93.3%)	21/60 (35%)	8/60 (13.3%)
	14	49/60 (81.6%)	11/60 (18.3%)	5/60 (8.3%)
JE-91	7	47/60 (78.3%)	26/60 (43.3%)	3/60 (5%)
	14	58/60 (96.6%)	43/60 (71.6%)	11/60 (18.3%)
CH392	7	24/60 (40%)	15/60 (25%)	0/60 (0%)
	14	13/60 (21.6%)	10/60 (16.6%)	3/60 (5%)
JKT27-087	7	35/60 (58.3%)	5/60 (8.3%)	0/60 (0%)
	14	42/60 (70%)	7/60 (11.6%)	2/60 (3.3%)
Sagiyama	7	32/60 (53.3%)	19/60 (31.6%)	0/60 (0%)
	14	51/60 (85%)	13/60 (21.6%)	4/60 (6.6%)

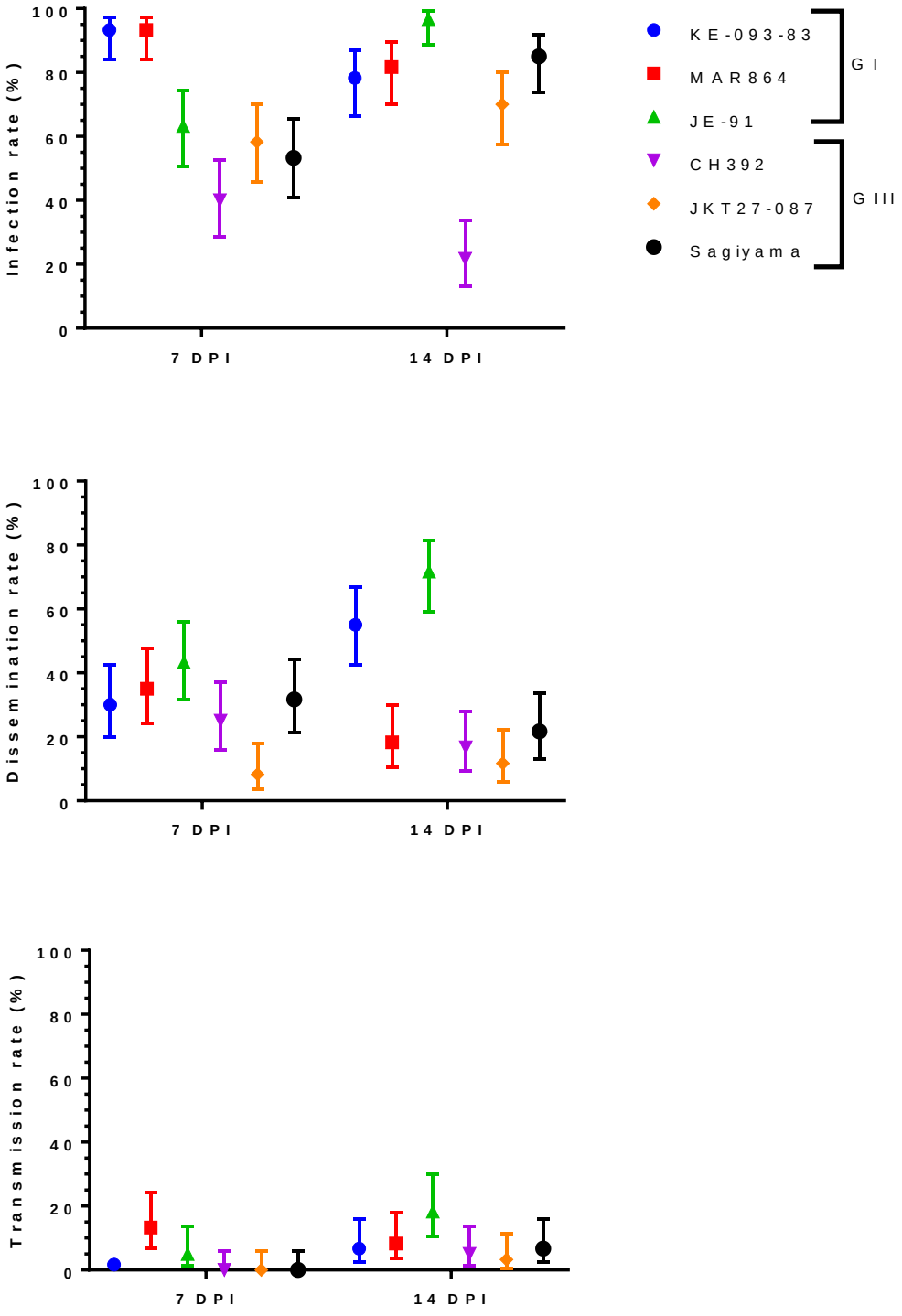


Figure 20 Rates of infection, dissemination, transmission at 7 dpi and 14 dpi for JEV strains

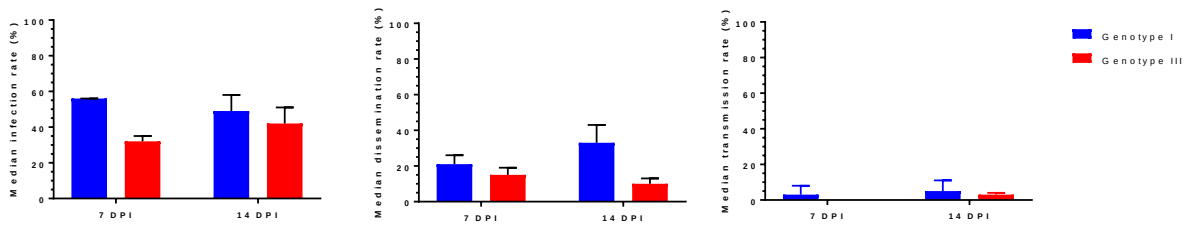


Figure 21 Median rates of infection, dissemination and transmission for JEV GI and GIII

5.4 Discussion

Like many viruses, the ability of JEV to successfully replicate in hosts determines its relative fitness (Domingo and Holland, 1997). We observed growth of virus *in vitro* and *in vivo* with all six strains of the JEV, belonging to either GI or GIII. All six strains of JEV reached peak titer, approximately 8-9 $\log_{10}$PFU/ml on 2 dpi in Vero cell with MOI of 0.01. When freshly-grown viruses were inoculated into ducklings, their body weight or temperature were not affected by the infection. But viruses were detected in serum obtained from ducklings on 1dpi to 3 dpi. There was no significant difference between the peak viremia titer among the six viruses tested ($p>0.05$). Similarly, no significant difference was observed when the means titers of JEV GI and JEV GIII were compared. We observed that the ducklings were seroconverted on 7 dpi and relatively higher antibody titer against JEV strains was developed on 21 dpi. JEV was also detected in oral and cloacal swabs obtained from ducklings inoculated with JEV GI and GIII viruses, as has also been reported by Ricklin et al., (2016) in pigs with the detection of genetic materials of JEV. But no infectious virus was detected in uninfected ducklings kept in MAR864 and Sagiyama strains group. This suggests that JEV is unlikely to be transmitted through the oral route in ducks. Both genotype viruses were found to be shed through oral and cloacal route, but little is known about significance of such shedding in the transmission of JEV. Shedding of JEV

was also recorded in nasal cavity washings in monkey when experimentally infected (Taniguchi et al., 1936). Overall, we were not able to demonstrate a difference in fitness between viruses from genotype I versus III based on replication in ducks, suggesting that differences in the ability to replicate in avian hosts is not the basis of genotype displacement.

Culex quinquefasciatus mosquitoes are known to be competent vectors for JEV in several areas of Asia (Mackenzie et al., 2004; Nitatpattana et al., 2005). With six strains of JEV belonging to either GI or GIII, showed *Culex quinquefasciatus* as competent vector for both genotypes of JEV. Since JEV infection in Vero cells inoculated with 7 dpi saliva content of mosquitoes, blood-fed with JEV GIII viruses was not detected, we concluded that JEV GIII has longer extrinsic incubation period than JEV GI in *Culex quinquefasciatus.* A shorter extrinsic incubation period of WNV 02 genotype strains of West Nile virus was reported to be the cause of WNV NY99 genotype displacement (Ebel et al., 2004; Moudy et al., 2007). Genotype displacement of WNV in the US and clade replacement of DENV in Nicaragua (Quiner et al., 2014) demonstrated that differences in replicative efficiency and transmission potential among circulating virus genotypes or clades, which is also applicable to JEV genotype displacement.

Previous attempts to explain JEV genotype displacement have often ignored the role of ecologically relevant avian hosts and mosquito vectors. A shorter extrinsic incubation period for GI could give it an evolutionary advantage through a faster lifecycle and quicker spread through susceptible populations of hosts. Characterizing the replicative efficiency of JEV genotypes I and III in Indian runner ducks showed no difference between genotypes, coupled with mosquito experiment showing a shorter extrinsic incubation period in *Culex quinquefasciatus* mosquitoes, lends support to this explanation for GI displacement. Genetic variation between genotypes has

been thought to have played a major role in viral fitness and hence the ability of JEV GI to displace JEV GIII in Asia and JEV GII in Australia (Schuh et al., 2014; Sarkar et al., 2012a, Pyke et al., 2001; Schuh et al., 2014; Han et al., 2015; Ma et al., 2003; Mohammed et al., 2011 Nga et al., 2004). In contrast, our study suggests a difference in extrinsic incubation period in mosquitoes as a responsible factor in genotype displacement. The broader impact of the proposed study is that exploration of host-virus interaction by the utilization of ecologically relevant hosts and vectors will elucidate the mechanistic basis behind genotype emergence and displacement. Viral and host ecology are key components of disease emergence and spread, and this finding can extend into an exploration of other zoonotic viruses that co-circulate, co-evolve and show displacement events.

CHAPTER 6: CONCLUDING REMARKS

Japanese encephalitis (JE) is an important public health threat in Nepal and other regions of Asia, and is a potential emerging disease in many areas of the world. The ecology of Japanese encephalitis virus (JEV) transmission as a mosquito-borne pathogen is reflected in the high vulnerability of people living in rural areas in association with livestock hosts and rice paddy farming. Despite its importance, surprisingly little research has been conducted in Nepal to understand factors important in virus transmission and to characterize the fundamental ecology of the virus. The studies reported in this dissertation had multiple objectives, all targeting an enhanced understanding of JEV in Nepal and more generally in other endemic regions.

First, I conducted a seroprevalence study in Rupandehi district of Nepal to estimate the level of exposure to JEV among important domestic livestock reservoir hosts. I detected anti-JEV antibodies in pigs, ducks, and chickens of Nepal, and the estimated seroprevalence was 14.7% in pigs, 11.8% in ducks, and 6.7% in chickens. In conjunction with the seroprevalence study, I trapped and identified mosquitoes at eight sites in the district and was able to confirm a high abundance of vectors known to transmit JEV, again supporting the contention that the environment was highly conducive to virus transmission. These results indicate that people living in the Rupandehi district are at substantial risk of contracting JEV at a household level and that the virus transmission was high in the recent past. Finally, I conducted a knowledge, attitudes and practices survey among livestock owners in Rupandehi district to evaluate sociological factors that could influence JEV transmission. I found that a large majority of people engaged in agriculture in Nepal cannot afford the cost of vaccination of their families and certainly not their livestock. Despite the importance of JE, the survey results indicated that

people living in close contact with livestock, especially those domestic animals serving as potential reservoir hosts of JEV, are often unaware of the disease risk from their livestock. Future research and development should focus on the production of a safe and efficacious vaccine for humans and pigs that is affordable and requires a single-dose for long-lasting immunity.

Second, I evaluated two hypotheses to explain the phenomenon of JEV genotype displacement. I speculated that viruses belonging to genotype I versus genotype III have different relative fitness in either avian hosts or mosquitoes. I inoculated groups of ducklings with three low-passage viruses from each genotype and measured magnitude and duration of viremia. All of the virus-inoculated ducklings developed viremia, but differences in peak virus titer were not observed between the viruses representing the two genotypes. As expected, none of the infected ducklings manifest overt clinical disease. To evaluate vector competence, I fed *Culex quinquefasciatus* mosquitoes on blood meals containing the same six viruses evaluated in ducklings, and measured rates of infection, dissemination and transmission. Each of the viruses was able to infect and disseminate in the mosquitoes. The most striking difference observed in the mosquitoes was a shorter extrinsic incubation period for viruses bellowing to genotype I. This could at least partially explain the ability of genotype I viruses to displace those from genotype III in many parts of Asia.

Currently JEV is the leading cause of vaccine-preventable encephalitis in Asia. People from low and middle income countries like Nepal, where environmental and socioecological factors are favoring the existence of JEV transmission, could face large epidemics of JEV if human vaccination is not included in national immunization plans or is discontinued. The

genotype displacement events of other flaviviruses have often involved abrupt shifts in disease incidence, severity and have several potential impacts on international public health efforts to control this disease. This includes the possibility that currently available vaccines are now antigenically mismatched. My study substantially lends empirical support to the potential importance of variation in EIP as an explanation for genotype displacement in JEV, and will allow theoreticians to formulate a mathematical model to explore the impact of this variation at a population level.

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