Epidemiological studies on Japanese encephalitis virus

日本脳炎ウイルスの疫学的調査

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1. GENERAL INTRODUCTION

1.1. History of Japanese encephalitis virus

One report based on evolutional analysis suggests that Japanese encephalitis virus (JEV) was originated from its ancestral virus, evolved into the various genotypes (I-V) in the Indonesia-Malaysia region, and then spread across Asia (Mohammed *et al.*, 2011). Epidemics of encephalitis in humans and horses were first described in Japan in the 1870s, and one large endemic occurred in 1924. The pathogen was a filterable agent from human brain, but had not been identified or characterized (Miyake, 1964). The first JEV isolate, Nakayama strain, was isolated in 1935 from the brain of a fatal human case (Lewis *et al.*, 1947), and the other JEV was also recovered from brain of a sick horse in 1937 (Nakamura *et al.*, 1967). Also, as mosquito transmission of JEV was suspected during the early 1930s, isolation of JEV from *Culex* (*C.*) *tritaeniorhynchus* in Okayama Prefecture, Japan, was reported in 1938 (Mitamura *et al.*, 1938). Further studies identified that pigs and birds were the principle amplifiers and *C. tritaeniorhynchus* was responsible for transmission of JEV among these vertebrates and from amplifier to humans and horses (Buescher *et al.*, 1959). Subsequently, JEV has been found throughout Southeast Asia and Western Pacific regions, and the virus was most recently isolated in Papua New Guinea and the Torres Straits of Australia (Hanna *et al.*, 1996; Johansen *et al.*, 2000; Mackenzie *et al.*, 2001).

1.2. Virus properties

JEV is a member of genus *Flavivirus*, family *Flaviviridae*, and closely related to West Nile virus, St. Louis encephalitis virus, Murray Valley encephalitis virus, which all belong to Japanese encephalitis serocomplex (Mackenzie *et al.*, 2002).

Flavivirus is a single stranded positive sense RNA virus wrapped in a nucleocapsid and surrounded by a lipid envelope. The length of RNA genome is

approximately 11kb and encodes 3 structural proteins, capsid protein (C), precursor to the membrane protein (prM) and envelope protein (E), and 7 nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (Castle *et al.*, 1985, 1986; Rice *et al.*, 1985; Wengler *et al.*, 1985).

The prM protein is cleaved during maturation and only M protein is incorporated into the virion (Markoff, 1989). The pr protein is produced during maturation of virus particles within the secretory pathway, and is a small proteolytic fragment of the precursor prM protein (Stadler *et al.*, 1997). The pr protein can protect immature virions against premature fusion with the host membrane (Zhang *et al.*, 2003). Co-synthesis of the prM protein with the E protein is necessary for proper folding, membrane association, and assembly of the E protein (Konishi *et al.*, 1993).

The E protein is the major antigenic determinant on virus particle, plays an important role in fusion during virus entry (Wengler *et al.*, 1985), and associates with induction of protective neutralizing antibody response in hosts (Kimura-Kuroda *et al.*, 1988). The E protein is a head-to-tail homodimer with three structurally distinct domains, a central β-barrel (domain I, amino acids 1-51, 134-195, 284-296), an elongated dimerization region (domain II, amino acids 52-133, 196-283), and a *C*-terminal immunoglobulin-like module (domain III, amino acids 297-395) (Luca *et al.*, 2012; Nybakken *et al.*, 2006; Rey *et al.*, 1995). The domain I and domain III contain virus-specific epitopes, while domain II contains flavivirus cross-reactive epitopes (Mandl *et al.*, 1989).

Although the C protein is the lowest conserved region among flavivirus proteins, the structural properties, such as hydrophobicity profile, abundance of basic amino acid residues, and secondary and tertiary structures, are well conserved (Dokland *et al.*, 2004; Jones *et al.*, 2003; Ma *et al.*, 2004). Since the C protein has RNA-binding

ability by the basic amino acid clusters at its amino and carboxyl termini, it is believed that it binds to the genomic RNA to form a nucleocapsid (Khromykh *et al*., 1996).

The NS1 protein appears to be essential for virus viability, and contains two conserved *N*-glycosylation sites and 12 invariant cysteine residues (Lee *et al*., 1989; Mason, 1989). The NS1 protein exists briefly as a monomer and then undergoes dimerization within the host cells (Winkler *et al*., 1989). Also, the NS1 protein is released from infected cells as a hexamer, and it forms a lipoprotein particle with an open barrel protein shell and a prominent central channel rich in lipids (Flamand *et al*., 1999; Gutsche *et al*., 2011). Although it is suggested that the NS1 protein plays a role in viral RNA replication (Hall *et al*., 1999), a function of the NS1 protein have been still unclear.

The NS2A protein is a hydrophobic, multifunctional, and membrane-associated protein involved in RNA replication and virus assembly (Leung *et al*., 2008; Mackenzie *et al*., 1998). It is suggested that the NS2A protein inhibits the host antiviral interferon response (Liu *et al*., 2004, 2006).

The NS2B is also a small membrane-associated protein (Clum *et al*., 1997). The NS2B protein forms the complex with the NS3 protein, and plays a role as a cofactor of the NS3 protease activation (Arias *et al*., 1993; Falgout *et al*., 1991).

The NS3 protein is a large multifunctional protein, with several activities required for polyprotein processing and RNA replication. *N*-terminal region of the protein is the catalytic domain of the NS2B-NS3 serine protease complex, and *C*-terminal region contains RNA helicase and RNA-stimulated nucleoside triphosphatase activation domain (Chambers *et al*., 1990; Gorbalenya *et al*., 1989a, b; Li *et al*., 1999). Also, the NS3 protease with the NS2B cofactor induces caspase 3 activation and mitochondria-mediated apoptosis (Yang *et al*., 2009).

The NS4A and the NS4B are small hydrophobic proteins. The NS4A protein co-

localizes with RNA replication complex, and plays an important role in RNA replication (Mackenzie *et al.*, 1998). It is recently reported that the NS4A functions as a cofactor of the NS3 helicase activity (Shiryaev *et al.*, 2009), and protects host cells from death and enhances virus replication by inducing autophagy of host cells (McLean *et al.*, 2011). Both the NS4A and the NS4B associate with the negative modulation of the interferon response in host cells (Evans *et al.*, 2007; Lin *et al.*, 2008; Muñoz-Jordán *et al.*, 2005).

The NS5 protein is a large, highly conserved, and multifunctional protein with methyltransferase and RNA dependent RNA polymerase (Chu *et al.*, 1987). The methyltransferase domain on the *N*-terminal region of NS5 the protein is involved in modification of the 5' cap (Egloff *et al.*, 2002).

1.3. Epidemiology

As described above, JEV is widely endemic in the Southeast Asia and Western Pacific regions (Mackenzie *et al.*, 2001), with the annual incidence of the disease being approximately 50,000 cases and 10,000 deaths in humans (Erlanger *et al.*, 2009). Recently, the number of incidence has been stable or declined dramatically in Japan, South Korea, Taiwan, China, Nepal, Sri Lanka, and Thailand due to the effective vaccination program with regular surveillance. In contrast, the epidemic activity in countries such as, India, Cambodia, Bangladesh, Indonesia, Laos, Myanmar, and Pakistan, where vaccination program or regular surveillance are not in place, has been increased in recent years (Erlanger *et al.*, 2009).

In Japan, although there were several thousand JE patients during the 1950s, JE cases in human decreased dramatically in the 1980s due to the vaccination program started in 1967 (IDSC, 2003). In recent two decades, annual JE cases in human are less than ten. From 2003 to 2008, total of 33 JE cases were reported in Japan and all cases

occurred in the western part of Japan, especially in Kyushu and Shikoku districts (IDSC, 2009). As well as human, since 1986, there has been no report of equine JE in Japan due to the effective vaccination program and improvement of hygiene around horses. However, one unvaccinated horse died from JE in Tottori Prefecture in 2003, and horses around the diseased horse also showed significant increase of antibodies against JEV during this endemic period (Yamanaka *et al*., 2006).

Annual sero-survey in pigs as indicator of the JEV activity is performed by National Institute of Infectious Diseases in Japan by detecting HI antibodies against JEV. In recent years, the earliest detection of positive pigs has been around May in Okinawa and around July in the other parts of Japan. In 2008, JEV-positive pigs were detected in 34 out of 35 prefectures, and more than 50% of pigs were seropositive for JEV in 24 prefectures, mainly in western part of Japan (IDSC, 2009).

Therefore, although the number of the JE cases has been decreased in both human and horses, there is still a risk of disease by JEV infection in Japan, if the immunization against JEV is not enough.

1.4. Diseases and pathogenesis

The incubation period of JEV after infection to human is about 5 to 15 days. The illness begins with 2 to 4 days prodromal phase of headache, fever, chills, anorexia, nausea, vomiting, dizziness and drowsiness. These symptoms are followed by the appearance of nuchal rigidity, photophobia, altered states of consciousness, hyperexcitability, and various objective neurological signs (Dickerson *et al*., 1952).

Since there is no JEV-specific therapy, only supportive therapy is available. Although cerebral edema has been postulated to be an important factor in pathogenesis, control trial of high-dose corticosteroid therapy failed to show benefit in clinical outcome

(Hoke *et al.*, 1992). Anti-trypanosoma drug, suramin, showed a complete protection against experimental JEV infection in a mouse model (Swarup *et al.*, 2008). *In vitro* analysis using Vero cells showed that, a furanonaphthoquinone derivative, FNQ3, has some antiviral activity (Takegami *et al.*, 1998). However, safety and *in vivo* effectiveness of these drugs remains unknown (Burke *et al.*, 2001).

In horses, the incubation period is 8 to 10 days. Symptoms are varied in severity of JE. Some horses have a transient fever, anorexia and lethargy, which last only for 2 or 3 days and recover without any complication. Other horses develop encephalitis. In milder cases, the horse has a fluctuating fever and neurological signs that commonly include difficulty of swallowing, incoordination, transient neck rigidity, radial paralysis or impaired vision, and usually recover within a week. In severe cases, the horse has a high fever, aimless wandering, violent and demented behavior, occasional blindness, profuse sweating and muscle tremors, and these symptoms are often followed by collapse and death within 1 or 2 days. This severe case is uncommon that occurs in less than 5% of symptomatic horses (CFSPH, 2007; OIE, 2009)

In pigs, JEV is usually characterized as a causative agent of reproductive disease, such as the birth of stillborn or mummified fetuses (OIE, 2009), and it is rare for infected pigs to become encephalitis (Nakamura *et al.*, 1967). Most nonpregnant pigs are asymptomatic or experience only a transient febrile illness. Disturbances of spermatogenesis can cause temporal infertility in boars (CFSPH, 2007).

1.5. Diagnosis

In fatal cases, virus isolation and demonstration of viral antigen in brain tissue are feasible for a definitive diagnosis. Rapid diagnosis may be carried out by staining for antigen in mononuclear cells collected from cerebrospinal fluid (CSF) (Mathur *et al.*,

1990). From up to one third of patients on acute phase, virus may be isolated from their CSF, which is correlated with a poor prognosis. Serum antibody tests, such as the hemagglutination inhibition (HI), complement fixation (CF), and virus-neutralization (VN) tests, are also applicable for diagnosis of JEV infection. Cross-reactions with other flaviviruses, especially West Nile virus (WNV), may complicate the serologic diagnosis in place where various flaviviruses are endemic. Therefore, monoclonal epitope-blocking immunoassays may be used to identify JEV-specific antibody among patients in these areas (Burke *et al.*, 1982a, b).

1.6. Prevention and control

The most effective way of controlling JEV is the use of vaccine. Vaccination of horses with formalin-inactivated vaccines was the first successful application and afforded significant protection during an epizootic in Japan in 1948 (Hoshi *et al.*, 1948). The first inactivated vaccines for human was prepared in 1954, which was based on the formalin-inactivated strain Nakayama grown in adult mouse brain (Takaku *et al.*, 1968). In 1972, live attenuated vaccines for use in pig was also licensed in Japan, which was a critical method to interrupting transmission and amplification of JEV, resulting in a prevention of human infections with JEV (Yoshida *et al.*, 1981). After the successful vaccination program of JEV in Japan, the technology developed in Japan was transferred to other Asian countries. Vaccine manufacture was started in Taiwan, Korea, India, Thailand, Russia and Vietnam from 1960s to 1980s (Barrett *et al.*, 1997). In Peoples' Republic of China, following the successful development of inactivated JEV vaccine in other Asian countries, an attenuated JE vaccine, SA14-14-2 was developed in 1988 (Xin *et al.*, 1988). In Japan, due to the side effect, acute disseminated encephalomyelitis, of mouse brain-derived JE vaccine (Menge *et al.*, 2005; Plesner *et al.*, 1998), the strong

recommendation of JE vaccination was halted in 2005 (Kurane *et al*., 2005; Okabe *et al*., 2005). To dissolve the side-effect by mouse brain-derived JE vaccine, new Vero cell-derived inactivated JE vaccine (Beijing-01 strain) was developed and licensed, and the recommendation of JE vaccination was resumed in 2009 (IDSC, 2009).

Besides the vaccination of amplifiers or dead-end host, vector control is another way of prevention from JEV infection. As an environmental management for vector control, alternate wet and dry irrigation (AWDI) can substantially reduce vector breeding, along with saving water, increasing rice yields, and reducing methane emission (Keiser *et al*., 2005). However, an effective irrigation requires well-organized educational programs, sufficient water at specific times during the rice growing cycle, and an adequate infrastructure (Erlanger *et al*., 2009; Rajendran *et al*., 1995). As a chemical vector control, insecticides such as pyrethroids, organophosphates, and carbamates are used, which can break the transmission cycle of arbovirus in the short term. However, since JE vectors prefer irrigated rice fields that are often heavily exposed to pesticide selection pressure, rising levels of insecitide resistance have compromised the effectiveness of this vector control program (Erlanger *et al*., 2009; Karunaratne *et al*., 2000).

1.7. Unknown facts

In Japan, there is a question how JEV can spend during winter seasons. Although pigs are known as major amplifiers of JEV in summer season during June and October, they are unlikely to be a host of JEV in winter seasons, because they are shipped before 6 months old. So far, there are several hypotheses how JEV is kept in winter seasons; carried from continents by migrating birds or mosquitoes, kept by wild animals, and kept by mosquitoes which pass the winter season. However, there is no clear answer to this question, yet. Furthermore, it is doubtful whether pig is only an amplifier in summer

seasons. In the case of West Nile virus (WNV), birds are thought to be main amplifiers. However, previous studies on WNV show that experimentally infected chipmunks develop high level of viremia enough to infect vector mosquitoes (Platt *et al*., 2007), and persistent shedding of virus in urine up to 8 months among experimentally infected golden hamsters (Tesh *et al*., 2005). These facts show the possibility of amplifier for JEV other than pigs. Therefore, it is important to clarify these questions to understand the transmission cycle of JEV, and to prevent and control JEV and other flaviviruses.

2. CHPATER 1

Dogs, but not cats, are good sentinels for

Japanese encephalitis virus infection

2.1. ABSTRACT

In this chapter, to assess the indirect risk of JEV infection to human, serosurvey of JEV in companion animals was performed. First, dogs and cats in Yamaguchi prefecture, in the western part of Japan, were examined, resulting that 17% (17 out of 100) of dogs and 1% (3 out of 292) of cats were seropositive for JEV. Furthermore, I examined seroprevalence of dogs from throughout Japan, resulting that 25% (164 out of 652) of dogs had VN antibodies against JEV. Dogs in northern part of Japan, Hokkaido (0%) and Tohoku (9%), showed significantly lower seroprevalence than other districts, while dogs in south-western part of Japan, Shikoku (61%) and Kyushu (47%) showed significantly higher seroprevalence than other districts. Interestingly, 8% (18 out of 222) of indoor-only family dogs and 21% (86 out 413) of dogs in urban/residential areas were seropositive, which indicate that the risk of JEV infection in human remain still high in Japan, especially in the south-western part. Dogs may be a good sentinel to assess the risk of JEV infection in residents, as they share their living spaces with human and are not vaccinated for JEV.

2.2. INTRODUCTION

As mentioned in GENERAL INTRODUCTION, annual serosurveys for antibodies to JEV in pigs, which are the main amplifiers of JEV, show high seropositivity in western part of Japan (Arai *et al.*, 2008). Although this data indicates that JEV remains endemic in Japan and humans still possess high risk of JEV infection, it is discussed whether serosurveys of pigs accurately reflect risk of infection to humans because pig farms are apart from human society, urban/residential areas. Therefore, another method for monitoring JEV infection risk to humans in urban/residential areas is necessary.

The serosurvey of cats and dogs were conducted for West Nile Virus (WNV) during epidemic infection in human in United States. The serum samples were screened by use of the plaques reduction VN test, and as the result, 26% of dogs and 9% of cats were seropositive in St Tammany Parish and the Slidell community, United States (Kile *et al.*, 2005). Also, the serologic survey of juvenile dogs for WNV was conducted in Houston. Seroconversion in the serum samples were detected 6 weeks before the first reported human case (Resnick *et al.*, 2008). These serologic surveys showed that dogs and cats may be useful sentinels for evidence of WNV, and since JEV is genetically close to WNV, dogs and cats may also be good sentinels for JEV.

In this chapter, I conducted serosurvey of JEV in dogs and cats, which share living space with humans in order to discuss the risk of JEV infection in urban/residential areas, and to assess whether companion animals could be useful sentinels for JEV.

2.3. MATERIALS AND METHODS

2.3.1. Cells

Vero cells (Vero 9013, JCRB number; JCRB9013), originated from African green monkey, were purchased from human science research resource bank (HSRRB, Japan), and cultured in Eagle's minimum essential medium (EMEM; GIBCO, U.S.A.) with 5% heat-inactivated fetal calf serum (FCS; Hyclone®, Canada), 1mM sodium pyruvate, 100U/ml of penicillin and 100μg/ml of streptomycin (GIBCO, U.S.A.) at 37°C in 5% CO_2. Mosquito-originated C6/36 cells (JCRB number; IFO 50010) were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, U.S.A.) with 10% heat-inactivated FCS, 100U/ml penicillin and 100μg/ml streptomycin at 28°C in 5% CO_2.

2.3.2. Virus

JEV/sw/Chiba/88/2002, which was kindly provided by Dr. Tomohiko Takasaki (National Institute of infectious Diseases, Japan), was isolated from peripheral blood mononuclear cells of a healthy pig in 2002, and was genetically classified into genotype I (Nerome *et al.*, 2007). The virus was propagated in C6/36 cells in DMEM with 2% FCS at 28°C and was stored at -80°C until use.

2.3.3. Serum samples

A total of 100 dog serum samples from 2006 to 2007 and 292 cat serum samples (215 samples from 1997 to 1999, 77 samples from 2004 to 2005) were collected from veterinary hospitals in Yamaguchi, and a total of 652 dog serum samples from November 2006 to October 2007 were collected from veterinary hospitals throughout Japan. All sera were inactivated by incubation at 56°C for 30 min and then stored at -20°C until use.

2.3.4. Plaque assay for titration of viruses

Viral infectivity was measured by plaque formation assay. Serially diluted viruses were inoculated onto Vero 9013 cells in a 6-well plates (Sumitomo Bakelite, Japan). After incubation for 90 min at 37°C in 5% CO_2, the cells were washed twice with EMEM and overlaid with 0.8% agarose (Seaplaque agarose, FMC BioProducts, U.S.A.) in EMEM containing 5% FCS. The plates were then incubated at 37°C in 5% CO_2 for 4 days. The cells were fixed with 5% buffered formaldehyde for 1 hour, and the agarose layers were removed. After staining with crystal violet, plaques were counted.

2.3.5. VN test

To determine the presence of VN antibody against JEV in sera, 80% plaque reduction VN test was carried out according to the previous report (Ohno *et al.*, 2009). For the first screening of JEV-positive sera, sera were diluted to 1:5 in EMEM containing 2% FCS. To determine VN titer, sera were serially two-fold diluted in EMEM containing 2% FCS. The diluted sera or medium alone were mixed with equal volumes of virus solution containing 100 PFU of JEV/sw/Chiba/88/2002 and then were incubated at 37°C for 90 min. After incubation, the mixtures were added to 6-well plates that were subconfluented with Vero 9013 cells and the plates were incubated at 37°C for 90 min, washed twice with EMEM and overlaid with 0.8% agarose in EMEM containing 5% FCS. The plates were then incubated at 37°C in 5% CO_2 for 4 days. The cells were fixed with 5% buffered formaldehyde for 1 hour, and the agarose layers were removed. After staining with crystal violet, plaques were counted. Sera that reduced number of plaques by more than 80% comparison with the mean number of plaques in control wells were considered as seropositive according to the previous report of WNV seroprevalence in cats and dogs (Kile *et al.*, 2005).

2.3.6. Statistical analysis

To analyze the results statistically, chi-square and Fisher's exact probability tests were performed. The significant level was $p < 0.05$.

2.4. RESULTS

2.4.1 JEV infection to companion animals in Yamaguchi, Japan

The seroprevalence of JEV in 100 dogs and 292 cats in veterinary hospitals in Yamaguchi, was examined by VN test. The result showed that 17% of dogs and 1% of cats were seropositive for JEV. The seropositivity in dogs was about 17-fold higher than that in cats. In addition, outdoor-only family dogs (38%) were about 4-fold more seropositive than indoor-only family dogs (10%) ($p < 0.05$). There were no significant difference between antibody prevalence in male (14%) and female (20%) dogs ($p > 0.05$) (Table 2-1).

2.4.2 JEV infection to dogs throughout Japan

Serum samples from 652 dogs collected from every prefecture in Japan between 2006 and 2007 were examined for seroprevalence of JEV. The results showed that 25% of dogs had VN antibodies against JEV. In Hokkaido and Tohoku districts, 0% and 9% of dogs, respectively, were seropositive. The seropositivities were significantly lower than in other districts ($p < 0.05$). In contrast, 61% and 47% of dogs in Shikoku and Kyushu districts, respectively, were seropositive. These levels were significantly higher than those in other districts with the other districts ($p < 0.05$). Seropositivity to JEV in Kanto (17%), Chubu (18%), Kinki (23%) and Chugoku (26%) districts did not show any significant difference ($p > 0.05$) (Table 2-2, Fig. 2-1). In addition, 45% of outdoor-only family dogs, and 8% of indoor-only family dogs were seropositive for JEV, thus confirming that outdoor-only family dogs were 5.5-fold more likely to be seropositive than indoor-only family dogs ($p < 0.05$). Especially, 75% and 77% of outdoor-only family dogs were seropositive in Shikoku and Kyushu districts, respectively. Regarding the areas of residence, 21% of dogs in urban/residential areas and 44% of dogs in rural area were

17

seropositive; the results for rural areas were significantly higher than urban/residential areas (Table 2-2). There was no significant correlation between ages of dogs and seopositivities to JEV (data not shown).

2.5. DISCUSSION

In Yamaguchi, seropositivity for JEV was significantly higher among dogs (17%) than cats (1%). From 1954 to 1955, similar results were reported: 55% of dogs and 10% of cats were seropositive for JEV (Nakamura *et al*., 1967). In addition, 26% of dogs and 9% of cats in Louisiana, and 5% of dogs and no cats in New York were seropositive for West Nile virus (WNV) (Kile *et al*., 2005; Resnick *et al*., 2008). As dogs appear to be more sensitive indicators of several flavivirus infections than cats, I conducted a serosurvey of dogs throughout Japan.

A previous reports on host feeding pattern of JEV and WNV vectors showed that the vectors, Culex spp, feed on various mammals, including dogs, cats, and humans and they tend to feed on dogs more than cats and humans (Mitchell *et al*., 1973; Molaei *et al*., 2007). These reports are consistent with our results that dogs showed higher seropositivity than cats and humans, and also indicate that some JEV vectors do accidentally feed on humans.

The nationwide serosurvey indicated that JEV prevalence is significantly lower in the Hokkaido and Tohoku districts and significantly higher in the Kyushu and Shikoku districts (Figure 2-1). The results of annual serosurveys in pigs have also shown that pigs in the western part of Japan tend to have higher seropositivity rates for JEV than those in the northern part. In addition, the number of human JE cases from 2005 to 2007 in Japan was 24, most of which occurred in the western part of Japan (IDSC, 2009). This is consistent with our data, suggesting that serosurveys in dogs accurately reflect JEV infection risk to humans in Japan.

In our study, 45% of outdoor-only family dogs were seropositive. A previous study on WNV showed that 69% of outdoor-only family dogs were seropositive during an epidemic of WNV infection in humans (Kile *et al*., 2005). A previous serosurvey of

JEV in Tokyo in Kanto district between 1954 and 1955 showed that 49% of stray dogs were seropositive (Nakamura, 1967). Both study results are similar to results in this study, indicating that the risk of JEV infection remains high in Japan, particularly in the western part of Japan. In addition, the confirmation of seropositivity among indoor-only family dogs (8%) indicates that JEV-infected mosquitoes may enter houses and that infants and elderly individuals, who tend to go outside less frequently, might be also at risk of JEV infection.

The seropositivity in rural areas (44%) was significantly higher than in urban/residential areas. This suggests that the existence of pig farms and rice paddies in rural areas is associated with environmental JEV levels. However, the relatively high seropositivity in urban/residential areas (21%), suggests that JEV infection risk in humans remains high even in these areas, where there are not so many pig farms and rice paddies. As pigs are kept apart from human society, serosurveys of pigs in urban/residential areas are limited. Therefore, dogs, which are found in all areas of Japan, may be better sentinels for JEV infection in these areas. However, it is not clear whether dogs become viremia after JEV infection, how long anti-JEV antibodies last and so on. Therefore, I conducted experimental infection of JEV against dogs in CHAPTER 2.

In conclusion, this study found that the risk of JEV infection remains high, even in urban/residential areas, when dogs were used as sentinels. Therefore, it is recommended that annual JEV surveillance should be carried out in pigs in Japan and that dogs should be surveyed every several years to assess the risk of JEV infection in humans in urban/residential areas.

2.6. LEGEND FOR FIGURE

Figure 2-1. Geographical distribution of seropositivity for JEV among dogs from 2006 to 2007. Japan was divided into 9 districts: Okinawa, Kyushu, Shikoku, Chugoku, Kinki, Chubu, Kanto, Tohoku and Hokkaido. The numbers in parenthesis beside each pie graph indicate the number of examined samples. The size of each circle indicates the number of samples. Black and white parts of the pie charts show the proportion of seropositive and seronegative dogs, respectively.
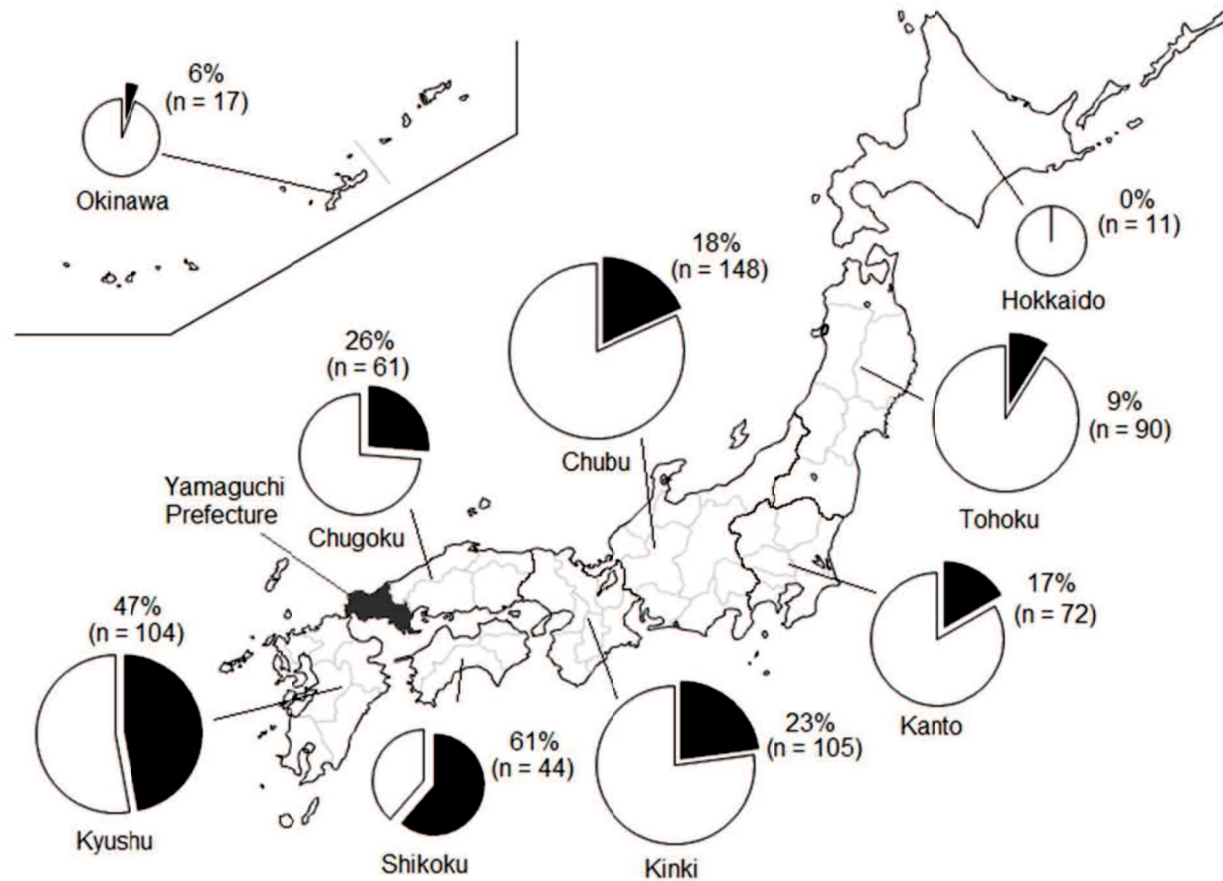
6%
(n = 17)

Okinawa

0%
(n = 11)

Hokkaido

18%
(n = 148)

Chubu

26%
(n = 61)

Yamaguchi
Prefecture

Chugoku

9%
(n = 90)

Tohoku

47%
(n = 104)

Kyushu

61%
(n = 44)

Shikoku

23%
(n = 105)

Kinki

17%
(n = 72)

Kanto

Figure 2-1 Geographical distribution of seropositivity for JEV among dogs from 2006 to 2007.

Table 2-1. Seroprevalence of JEV in dogs and cats in Yamaguchi

	Dogs				Cats
	Indoor	Outdoor	Both or unknown	Total	
Number of examined animals	58	21	21	100	292
Number of JEV-positive animals	6	8	3	17	3
% of positive animals	10	38	14	17	1

Table 2-2. Seroprevalence of JEV in dogs throughout Japan

	Where dog stays[a]			Area[a]			Total
	Indoor	Outdoor	Both or Unknown	Urban /Residential	Rural	Unknown	
Number of examined dogs	222	234	196	413	147	92	652
Number of JEV-positive dogs	18	105	41	86	65	13	164
% of positive dogs	8	45	21	21	44	14	25

[a] The following demographic information was recorded by questionnaire to owners: housing environment (indoor, outdoor, or both), and the location of the house at which the dog is kept (urban/residential or rural).

3. CHAPTER 2

Experimental infection to dogs with Japanese encephalitis virus

3.1. ABSTRACT

In this chapter, three dogs were experimentally infected with a high dose of JEV. Neither clinical sign nor abnormal result of blood examination except for C-reactive protein was observed in all dogs. VN titers rapidly increased at 1:1280 (No.1), 1:640 (No.2) and 1:320 (No.3), and were kept at the same level at least for 70 days after challenge. Virus isolation and RT-PCR from sera or peripheral blood mononuclear cells showed that any live virus or viral RNA was not detected in blood. In conclusion, dogs may be safe and useful sentinels to survey the human risk of JEV infection in urban areas, because any viremia and clinical sign are not developed in dogs after JEV infection.

3.2. INTRODUCTION

Since dogs, but not pigs, live together with their owners, seroprevalence of JEV in dogs indicate the human risk more strongly than that in pigs. In CHAPTER 1, serosurvey on JEV in Japan showed that 25% of dogs had VN antibodies against JEV. Furthermore, higher seropositivities were detected in the western part of Japan, Kyushu (47%) and Shikoku (61%) districts (CHAPTER 1; Shimoda *et al*., 2010). However, duration of anti-JEV antibodies and clinical symptoms after JEV infection in dogs still remain unknown. Furthermore, some canids were reported with encephalitis and myocarditis possibly associated with WNV, which belongs to Japanese encephalitis serocomplex (Cannon *et al*., 2006; Lichtensteiger *et al*., 2003; Read *et al*., 2005; Schwab *et al*., 2006).

In this chapter, dogs were experimentally infected with JEV to examine the clinical symptoms and the duration of anti-JEV antibodies in dogs.

3.3. MATERIALS AND METHODS

3.3.1. Animal experiments

Three female beagles (2 months old) without JEV antibody were purchased from NARC and were intraperitoneally and subcutaneously inoculated with 0.5ml of viral solution containing 5×10^6 plaque-forming units (PFU) of JaOH0566. JaOH0566 strain was isolated from JE patient in 1966 and was kindly provided by Dr. Ishikawa (Biken, Kagawa, Japan). Clinical signs were observed and body weight and temperature were measured every day. Blood samples were collected on day 0 to 14, 17, 21, 24, 28, 35, 42, 49, 56, 63, 70, and 77 postchallenge. The animal experiments were approved by Animal Research Ethics Board of Faculty of Agriculture, Yamaguchi University.

3.3.2. Sample treatments

Peripheral blood samples were transferred into untreated and EDTA-treated evacuated tubes (BD vacutainer; Becton, Dickson, and Compane, U.S.A.). Serum samples were separated from untreated whole blood by centrifugation at 3,500 rpm for 15min at 4°C and stocked at -80°C until use. Peripheral blood mononuclear cells (PBMC) were separated from 2ml of EDTA-treated whole blood mixed with 2ml of RPMI1640 medium (GIBCO, U.S.A.) by layering over 2ml of Lymphoprep^{TM} (Axis-Sheild, Norway) and centrifuging at $800 \times g$ for 45min at room temperature. Isolated PBMC was then washed with RPMI1640, resuspended in 1ml of Cellbanker (Nippon Zenyaku Kogyo, Japan) and stocked at -80°C until use. Blood smears were prepared from a drop of EDTA-treated blood, dried in air, and stained with Hemacolor (Merck, Germany). The slides were examined under light microscopy, and 300 white blood cells were counted. Each leukocyte was assigned to one of the following categories; lymphocytes, monocytes, segmented neutrophils, band neutrophils, eosinophils, basophils and other cells.

28

Complete blood count (CBC), excluding hematocrit (Ht) and packed cell volume (PCV) were measured using Sysmex KX-21 (Sysmex, Japan) and Ht and PCV were measured by centrifugation in hematocrit capillary. Creatine phosphokinase (CPK), creatinine (CRE), blood urea nitrogen (BUN), alanine aminotransferase (ALT), alkaline phosphatase (ALP), glutamate oxaloacetate transaminase (GOT), gamma-glutamyl transpeptidase (GGT) were measured using DRI-CHEM 7000V (Fujifilm, Japan). C-reactive protein (CRP) was measured by Laser CRP-2 (Arrows, Japan).

3.3.3. Cells

African green monkey-originated Vero9013 cells (JCRB number; JCRB9013) and mosquito-originated C6/36 cells (JCRB number; IFO 50010) were used in this study. The condition of culture media are the same as described in CHAPTER 1.

3.3.4. Viruses

Human-derived JaOH0566 (genotype III) and swine-derived JEV/sw/Chiba/88/2002 (genotype I) were used in this study. Human-derived JaOH0566 was isolated in 1966 from brain of JE patient (Shah *et al*., 2006). The propagation of these viruses were performed the same as described in CHAPTER 1.

3.3.5. Plaque assay for titration of viruses

Viral infectivity was measured by plaque formation assay described in CHAPTER 1.

3.3.6. VN test

The presence of VN antibody against JEV in sera was determined by 80% plaque

reduction VN test described in CHAPTER 1.

3.3.7. Virus isolation

Serially three-fold diluted PBMC or serum samples were incubated with Vero9013 or C6/36 cells in a 96-well plates at 37°C. Cytopathic effect (CPE) were monitored and if CPE was not found, the cells were passaged. After fifth passages, virus isolation was judged. Three days old suckling mice were intracerebrally inoculated with 20 μ l of mixed sera and the clinical sign was observed every day.

3.3.8. Detection of viral RNA

Total RNA was extracted from sera using QIAamp viral RNA mini kit (Qiagen, U.S.A.). Then, RT-PCR was carried out using one-step RT-PCR kit (Qiagen, U.S.A.) with primers, JEV1F (5'-GGA ACA GCA TGC AAA TCG AAG-3') and JEV2R (5'-ACC AGA AGG CCC AGC TGA AAA-3'). The RT-PCR products were analyzed by electrophoresis in 1.5% agarose gel. It was checked that this RT-PCR could detect 0.23 PFU of JEV in one reaction.

3.3.9. Statistical analysis

To analyze the results statistically, chi-square and Fisher's exact probability tests were performed. The significant level was $p < 0.05$.

3.4. RESULTS

3.4.1. Clinical signs and blood tests

While no clinical signs or increase in body temperature were observed during the observation period, transient decreases in body weight were observed on day 2 postinfection (dogs No. 1 and No. 2) and from day 1 to 3 postinfection (dog No.3) (Figure 3-1). Among the various blood parameters assayed, including a CBC and biochemical tests, only C-reactive protein (CRP; an index of inflammation) levels in sera were abnormally high. The CRP levels reached 2.35 mg/dl (dog No.1 - day 2), 5.6 mg/dl (dog No.2 – day 1) and 1.05 mg/dl (dog No.3 – day 2) before recovering to less than 1.0 mg/dl by 6 days after challenge (Figure 3-2).

3.4.2. VN titers

VN titers of sera collected from experimentally JEV-infected dogs against JaOH0566 were measured. The results showed that until 21 days after challenge in dogs No.1 and No.2, and 28 days in dog No.3, VN titers kept increasing before being maintained at similar levels for 70 days after challenge (Table 3-1). Interestingly, the sera (day 28 post infection) of all animals exhibited cross-VN activity to JEV/sw/Chiba/88/2002, which belongs to genotype I (data not shown).

3.4.3. Virus isolation and detection

To examine the presence of JEV in blood, sera and peripheral blood mononuclear cells collected on day 1 to 3, 7, 10, 14 and 17 postinfection were inoculated onto Vero9013 cells, and only sera were intracerebrally inoculated to suckling mice. Furthermore, sera collected on day 1 to 3 were examined by RT-PCR for detection of JEV RNA. As a result, neither the virus nor the viral genome was detected in blood by these methods (data not

shown).

3.5. DISCUSSION

Annual serological survey for JEV was performed among pigs in Japan, and showed high seropositivities, especially in western part of Japan (Arai *et al*., 2008). Furthermore, previous study about experimental JEV infection against pigs showed that VN titers of 1:20 to 1:80 were detected in all pigs on 14 days after challenge, and were kept at the detectable level until 47 days after challenge, with the exception of one pig (Williams *et al*., 2001). In our study, VN titers of every infected dog were kept at the same level at least for 70 days after challenge. These result suggested that dogs develop VN antibodies as well as pigs, and its duration period is long. In addition, the result of serological survey on JEV among dogs in CHAPTER 1 showed higher seropositivities in western part of Japan, which is consistent with the results of annual serological surveys among pigs.

In the previous study on WNV, although experimentally infected dogs did not show any clinical symptoms, viremia was detected in all infected dogs (Austgen *et al*., 2004). In addition, as described above, some canids were reported with encephalitis and myocarditis possibly associated with WNV (Cannon *et al*., 2006; Lichtensteiger *et al*., 2003; Read *et al*., 2005; Schwab *et al*., 2006). In contrast, no clinical symptom was observed and no viremia or JEV RNA was detected from blood samples of JEV infected dogs in this study. Furthermore, even during JEV was endemic in Japan, no JE cases in domestic animals, like dogs or cats, were reported (Nakamura *et al*., 1967). Although a previous report show that dogs develop encephalitis and viremia after experimental infection, they were all inoculated intracerebrally, which is not natural infection route of JEV (Hotta et al., 1964). These facts suggest that JEV infected dogs are unlikely to develop viremia via natural infection route, and a risk of human JEV infection from dogs is extremely low.

Previous study among dogs showed measurement of CRP can be a useful marker of inflammation and ideal cut-off value is 1.0mg/dl (Onishi *et al*., 2000). In this study, although clinical symptoms were not observed in any dogs, abnormal increase of CRP was observed in all dogs. It indicates that some inflammation had occurred in these dogs after inoculation of JEV, and suggests JEV infection might cause inflammation.

The ideal sentinels for serologic survey would be susceptible to infection, survive the infection, develop detectable antibodies, possess no risk of infection to handlers, and never develop a high enough level of viremia to infect vector mosquitoes (Resnick *et al*., 2008). The serological survey in CHAPTER 1 (Shimoda *et al*., 2010) and this study showed that dogs may be useful and safe sentinel to identify a risk of human infection, especially in cities and indoors.

3.6. LEGENDS FOR FIGURES

Figure 3-1. Body weight of JEV experimentally infected dogs. X-axis indicates the number of days after infection. Y-axis indicates the relative body weight of JEV infected dogs. The data were expressed as relative values, with Day 0 of each dogs being 100.

Figure 3-2. The increase of CRP level in serum of experimentally JEV infected dogs. X-axis indicates the number of days after infection. Y-axis indicates the quantity of CRP in serum samples from JEV infected dogs.
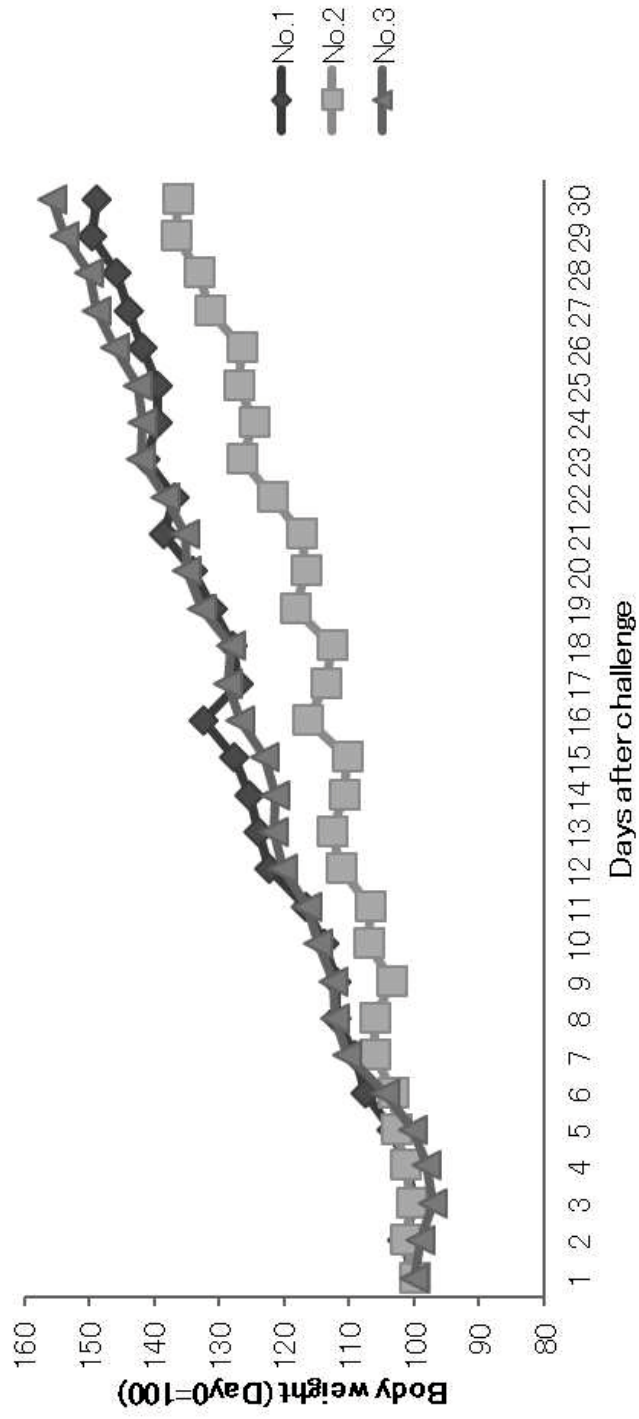
Figure 3-1. Body weight of JEV experimentally infected dogs
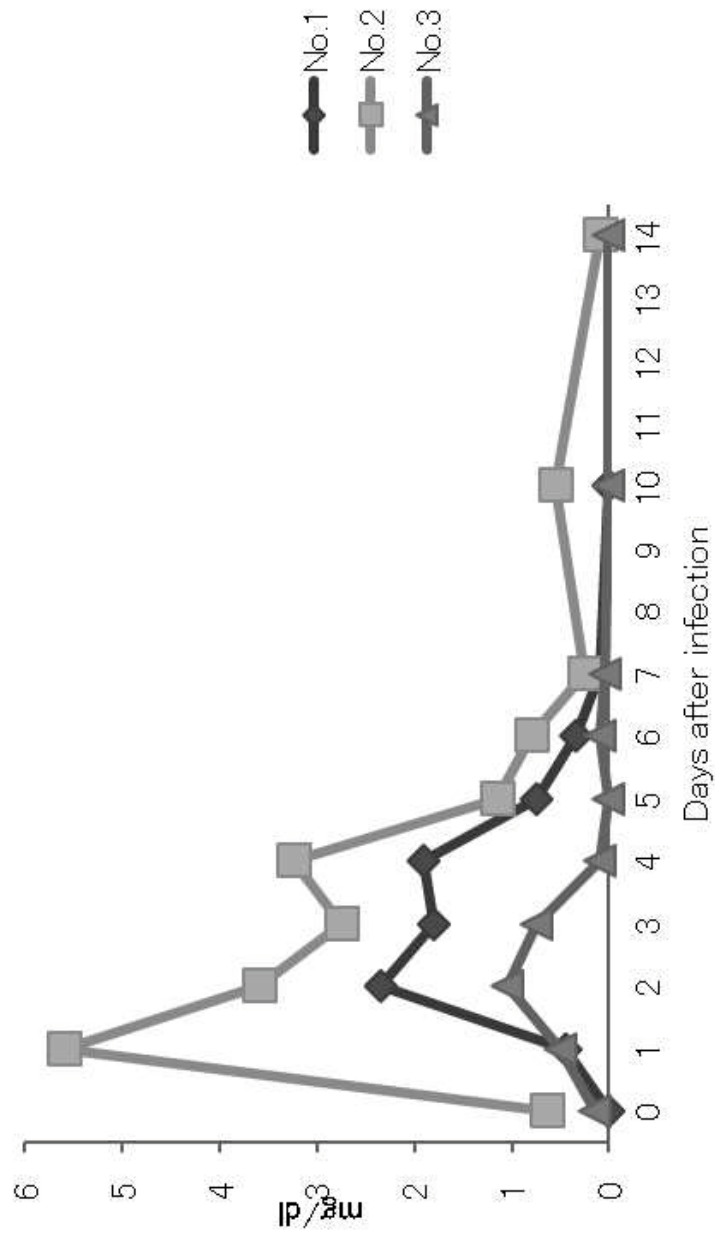
Figure 3-2. The increase of CRP level in serum of experimentally JEV infected dogs

Table 3-1. VN titers in JEV-infected dogs

Dogs	Days after challenge								
	0	7	14	21	28	35	42	56	70
No.1	<1:10	1:160	1:160	1:1280	1:640	1:1280	1:640	1:640	1:640
No.2	<1:10	1:320	1:320	1:640	1:640	1:640	1:640	1:320	1:320
No.3	<1:10	1:80	1:40	1:160	1:320	1:320	1:160	1:160	1:80

4. CHAPTER 3

Development and application of an indirect enzyme-linked immunosorbent assay

for serological survey of Japanese encephalitis virus infection in dogs

4.1. ABSTRACT

Although I described in the previous chapter that dogs are good sentinels to assess the risk of JEV infection in humans, a VN test has been the only method available for measuring JEV antibody levels in dogs. In this chapter, an indirect enzyme-linked immunosorbent assay (ELISA) for a serological survey of JEV infection in dogs, using purified viral particles as an antigen, was developed. In dogs inoculated experimentally with JEV, ELISA detected anti-JEV IgM at 3 days after infection, with IgM levels peaking at 7 days after infection. Anti-JEV IgG were detected at 14 days after infection and peaked on 21-28 days after infection. VN titers did likely correlate with the sum of anti-JEV IgM and IgG measured by the ELISA. To test the utility of the new assay, the seroprevalence of JEV infection among 102 dogs in Kyushu, Japan, was examined by both IgG ELISA and VN test. The correlation coefficient between IgG ELISA and VN test was 0.813 ($p<0.001$); comparison of IgG ELISA and VN test revealed a sensitivity and specificity of 82% and 98%, respectively. IgG ELISA was used to survey dogs in Bangkok, Thailand, with the results showing that 51% of these dogs were seropositive for JEV. These data suggest that even in the capital city of Thailand, the risk of infection with flaviviruses, including JEV, remains high. This indirect IgG ELISA for dogs is a useful and simple method to assess the risk of JEV infection to humans.

4.2. INTRODUCTION

In Thailand, 1,500 to 2,500 JE cases had been reported annually throughout the 1970s and the 1980s (Olsen *et al.*, 2010). In contrast, between 2005 and 2010, the annual number of encephalitis cases in Thailand ranged from 322 to 431, including 36 to 78 confirmed JE cases per year (Thailand Ministry of Public Health). This dramatic reduction in the number of JE cases over the past 20 years has been the result of an effective human vaccination program by the Thailand Ministry of Public Health. However, a recent (2009) serological survey showed that 39% of pigs are seropositive for JEV in Thailand; the seroprevalence of JEV was especially high (67%) in pigs in Chiang Mai province, which includes the second biggest city of Thailand (Prompiram *et al.*, 2011). In addition, the number of JE cases in Thailand still exceeds those in other vaccine-controlled countries, such as Japan and Korea (Erlanger *et al.*, 2009). However, recent human risk of JEV infection in the capital city, Bangkok, remains unknown.

Serological tests such as VN test and hemagglutination inhibition (HI) have been the primary assays used to detect JEV-specific antibodies in serum (Anderson *et al.*, 2011, Chiou *et al.*, 2007, Mall *et al.*, 1995, Ting *et al.*, 2004, Watanabe *et al.*, 2008). The HI requires a large volume of serum, while the VN test requires a special facility (e.g., biosafety level-2 or -3) and a high level of technical skill. In contrast, enzyme-linked immunosorbent assay (ELISA) does not require the use of live JEV, and the procedure is simple and requires only a small amount of sample. Other laboratories have reported the use of indirect IgG ELISA for JEV serological surveys among pigs and bats (Cui *et al.*, 2008, Hamano *et al.*, 2007, Nidaira *et al.*, 2007, Xinglin *et al.*, 2005, Yang *et al.*, 2006).

In the previous chapters, I demonstrated the utility of dogs as sentinels for JEV infection to human. Therefore, in this chapter, an indirect ELISA to detect JEV antibodies in dogs was developed using sera of dogs infected experimentally and domestic dogs in

41

Kyushu and Hokkaido, Japan. The test was then applied for a serological survey of dogs

in Thailand to assess the risk of human infection with flaviviruses including JEV.

4.3. MATERIALS AND METHODS

4.3.1. Cells

African green monkey-originated Vero9013 cells (JCRB number; JCRB9013) and mosquito-originated C6/36 cells (JCRB number; IFO 50010) were used in this study. The condition of culture media are the same as described in CHAPTER 1.

4.3.2. Virus

Swine-derived JEV/sw/Chiba/88/2002 (genotype I) were used in this study. The propagation of this virus was performed as described in CHAPTER 1.

4.3.3. Dog serum samples

Sera of dogs infected experimentally with JEV used in this study were sequentially collected and stored at -80 °C in CHAPTER 2 (Shimoda *et al*., 2011).

For the serological survey, a total of 183 family dog sera were collected from domestic dogs in Kyushu and Hokkaido districts, Japan, and Bangkok, Thailand. These samples were collected from veterinary hospitals in Japan, or by visiting the owners of the examined dogs in Thailand. All owners were informed of the research objectives before sampling and data collection. To classify the dogs examined in this study, the owners also were asked to provide data about the sex, age, breed, and housing of their dogs. Note that all sera used for the VN test were inactivated by incubation at 56 °C for 30 min to prevent the inactivation of JEV by complement in serum samples, and then stored at -20 °C until use.

4.3.4. VN test

The presence of VN antibody against JEV in sera was determined by 80% plaque

reduction VN test described in CHAPTER 1.

4.3.5. ELISA

For ELISA, inactivated JEV originating from the Beijing 01 strain was selected as the antigen. This strain had been propagated in Vero cells, inactivated with formaldehyde, and purified by ultracentrifugation, and the product was intended for use as a vaccine antigen in humans. The inactivated JEV was diluted to 5 μg/ml with adsorption buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6), and distributed at 100 μl per well into 96-well microplates (Maxisorp; Nunc, Roskilde, Denmark). Control wells received an equivalent volume of adsorption buffer without antigen. After incubation at 37 °C for 2 hrs, plates were placed at 4 °C overnight. The wells were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween20 (PBS-T) and then incubated with 100 μl per well of 0.1% bovine serum albumin (BSA) (Fraction V; Sigma, St. Louis, MO, U.S.A.) in PBS at 37 °C for 30 min. Test sera were diluted with PBS-T containing 10% FCS. Wells were washed three times with PBS-T, and then diluted sera were added to duplicate wells, and plates were incubated at 37 °C for 30 min. Next, the wells were washed three times with PBS-T and incubated with 100 μl per well of diluted peroxidase-conjugated sheep anti-dog IgG or goat anti-dog IgM antibody (Bethyl Laboratories, Montgomery, TX, U.S.A.) at 37 °C for 30 min. Following washing three times with PBS-T, 100 μl of horseradish peroxidase substrate kit (Bio-Rad, Hercules, CA, U.S.A.) was added to each well. After incubation at room temperature for 30 min, the enzymatic reaction was stopped by adding 100 μl of 2% oxalic acid to each well. The absorbance was measured by a spectrophotometer (Bio-Rad) with a 405-nm filter. All results were corrected by the value of the non-antigen control.

4.3.6. Optimization of concentration of ELISA antigen

The optimal concentration of the antigen was determined by cross-titration using the serially diluted antigen and the serially diluted serum from one mouse infected with JEV as the primary antibody. Based on the results of this preliminary study, an optimal antigen concentration of 5 µg/ml was selected (data not shown).

4.3.7. Statistical analysis

Chi-square test was performed to assess statistically the seroprevalence of JEV among dogs using IgG ELISA, and Pearson's correlation coefficient was calculated to determine the correlation between VN test and IgG ELISA. p values of < 0.05 were considered statistically significant.

4.4. RESULTS

4.4.1. Optimization of dilution of dog sera

The optimal serum dilution was determined using sera collected from three dogs infected experimentally with JEV. IgG ELISA was carried out using sera diluted to 1:100 and 1:500. The observed increases of JEV antibody after JEV challenge were consistent with the pattern seen by the VN test. Using 1:100 diluted serum, the OD value of IgG ELISA reached over 3.500 (the limit of detection) after day 14 in dogs No.1 and No.2 (data not shown). On the other hand, absorbance exceeded 3.500 only after day 21 in dog No.1 and on day 28 in dog No.2 using 1:500 diluted sera (Figure 4-1). In addition, in dog No.3, the highest absorbance in 1:500 diluted sera was 2.270 on day 28. Therefore, to avoid false-positives and to examine the correlation between the absorbance of IgG ELISA and VN titers, a dilution of dog sera of 1:500 was selected for subsequent phases of this study.

4.4.2. Determination of cut-off value

For any serological surveys, determination of the cut-off value is important. To determine the cut-off value, IgG ELISA was performed using 5 µg/ml of the JEV antigen and 1:500 diluted dog sera as the primary antibody. Eleven dog sera collected in Hokkaido, Japan, where there was no evidence of the prevalence of JEV in dogs (Shimoda *et al.*, 2010), were examined using IgG ELISA. The absorbance values obtained from these 11 specimens ranged from 0.013 to 0.200. The mean IgG ELISA value was 0.052 with a standard deviation (S.D.) of 0.053 (data not shown). The mean value plus 3 S.D. (a value of 0.211) was selected as the cut-off value between positive and negative results in this study.

4.4.3. Comparison of antibody response in dogs infected experimentally

In dogs infected experimentally with JEV, anti-JEV IgM was detected on day 3, peaked on day 7, and then gradually decreased. On the other hand, anti-JEV IgG increased on day 7 and peaked on day 21-28. For comparison, VN titers reached the first peak on day 7, and then started to increase again on day 14. Therefore, it is inferred that the titer detected by VN test corresponds to the sum of IgG and IgM concentrations (Figure 4-1).

4.4.4. Specificity and sensitivity of IgG ELISA

To investigate the specificity and sensitivity of IgG ELISA, 102 serum samples collected from dogs in Kyushu, Japan, were examined by both IgG ELISA and VN test. Of 49 serum samples judged positive by VN test, 40 and nine were positive and negative in the IgG ELISA, respectively; of 53 serum samples judged negative by VN test, only one was positive in the IgG ELISA (Table 4-1, Figure 4-2). Thus, in comparison with the results in VN test, the sensitivity and specificity of IgG ELISA were 82% and 98%, respectively. The correlation coefficient between IgG ELISA and VN test was 0.813 ($n=102$, $p<0.001$). These results indicated that the result of IgG ELISA showed significant correlation with that of VN test.

4.4.5. Seroprevalence of flaviviruses, including JEV, among dogs in Thailand

To survey JEV infection among dogs in Bangkok, Thailand, 70 serum samples were examined by IgG ELISA, revealing that 36 out of 70 dogs (51%) were seropositive for JEV. The seroprevalence among older dogs (>3 yrs) tended to be higher than that among young dogs (3 yrs and under), but no significant difference was observed. In addition, 29 out of 44 (66%) dogs which kept outside (group A) were seropositive for JEV; in contrast, all dogs which are kept inside (group B) were seronegative (Table 4-2).

47

The serum samples from dogs in Thailand were also screened using IgM ELISA, but the resulting absorbances were less than 0.3 (data not shown).

4.5. DISCUSSION

Previous studies in CHAPTER 1 and CHAPTER 2 showed that dogs are good sentinels to assess the risk of human infection with JEV, given that dogs share their life space with humans, are not vaccinated against JEV, do not display symptoms when infected, do not serve as amplifiers for JEV, and maintain VN titers for long periods after infection (Shimoda *et al*., 2010, 2011). These previous studies indicated that the VN test was a suitable method for serological surveys of JEV infection in dogs, but some sera, especially sera from wild animals, nonspecifically inhibited JEV infection in the VN test. Therefore, although surveys in humans typically employed a 50% plaque reduction assay, serological surveys in wild and companion animals were performed using an 80% plaque reduction assay (Ohno *et al*., 2009, Shimoda *et al*., 2010). In addition, the VN test is time-consuming and requires a special facility. Therefore, other methods for the detection of JEV antibodies are desirable. In this study, indirect ELISA was developed to detect immunoglobulin for JEV in dogs, and the assay was applied in a serological survey on JEV infection to assess the risk of JEV infection in humans.

A preliminary ELISA study (using sera from dogs infected experimentally with JEV) tested the use of an unpurified extract from Vero cells infected with JEV/sw/Chiba/88/2002 as the ELISA antigen. This extract yielded lower absorbances than those seen with inactivated JEV antigen (data not shown). Therefore, inactivated JEV was selected as the ELISA antigen for the present study. This inactivated JEV antigen corresponds to the JEV vaccine that has been used in Japan since 2009, and is derived from the Beijing 01 strain (JEV genotype III) that has been propagated in Vero cells, inactivated with formaldehyde, and purified by ultracentrifugation.

Thus, in the present study, different viruses, Beijing 01 and JEV/sw/Chiba/88/2002, were used in ELISA and VN test, respectively. To avoid non-

49

specific reaction in ELISA, the vaccine for human use originated from Beijing 01 was selected, because the vaccine is, to our knowledge, the most purified JEV reagents available. For VN test, JEV/sw/Chiba/88/2002 routinely used in our laboratory (Ohno *et al*., 2009; Shimoda *et al*., 2010, 2011) was selected, because the plaques formed by the virus are large and clear. VN test using this strain could detect JEV antibodies in various animal species, including dogs, raccoons, wild boars, and raccoon dogs (Ohno *et al*., 2009; Shimoda *et al*., *2010*). In addition, some reports described that antibodies to JEV are cross-reactive among various JEV strains by VN test (Kitano, 1989; Liu *et al*., 2011). Therefore, it seems that our ELISA and VN test can detect a broad range of JEV antibody.

Sensitivity and specificity of IgG ELISA, as well as correlation with VN test, were determined using domestic dogs in Kyushu, Japan, where there is no evidence of the prevalence of other flaviviruses. Absorbances in the IgG ELISA significantly correlated with VN titers (0.813; n=102, $p<0.001$), suggesting that the IgG ELISA is suitable for detection of JEV antibodies in dogs. Sensitivity (82%) of IgG ELISA in this study was not so high, as nine dogs that were seropositive by VN test were seronegative by IgG ELISA. Since sensitivity may have been decreased due to the use of highly diluted sera (1:500), these nine serum samples with inconsistent results were examined at higher serum concentrations (1:10, 1:20, 1:40, and 1:80). The results showed that significant increases of IgG ELISA values were obtained in 5 out of 9 samples (data not shown), suggesting these 5 dogs might be seropositive for JEV. In development of this IgG ELISA, the specificity was the first priority for avoiding false-positives. Therefore, 1:500 dilutions of sera were used as the primary antibody, providing reduced background (compared to 1:100 dilutions) and increased specificity (Fig.1 and 2).

In Thailand, only a few surveys for JEV infection have been reported recently (Olsen *et al*., 2010, Prompiram *et al*., 2011). In particular, the risk of human infection

with JEV in the capital city, Bangkok, remains unknown. In this study, a serological survey of JEV among dogs in Bangkok showed that 66% of dogs belonging to group A possessed antibodies to JEV, while JEV antibodies were not detected in any exclusively dogs belonging to group B. In Japan, the seroporevalence of dogs belonging to group A (45%) was 5.5-fold higher than that of dogs belonging to group B (Shimoda *et al*., 2010). These data suggest that the risk of human infection with JEV in Bangkok may be similar to or even higher than that in Japan.

Some inconsistent results between IgG ELISA and VN test also were observed in dogs in Thailand, as one of the putatively seronegative dogs by VN test yielded a high absorbance (of 2.084) by IgG ELISA (data not shown). It is hypothesized that this dog was infected with another flavivirus, such as dengue virus (DENV), which is endemic in Thailand (Hemungkorn *et al*., 2007). Upon testing by indirect IgG ELISA (using DENGUE IgG INDIRECT ELISA (Panbio, Queensland, Australia) only as the DENV antigen), this serum sample displayed high ELISA titer against DENV (data not shown). Thus, this dog may have been infected with DENV.

ELISA of sera from dogs infected experimentally with JEV showed that anti-JEV IgM appeared earlier than IgG and then rapidly disappeared (Fig.1), which is consistent with the results of a previous study on experimental infection of JEV against pigs (Ohkubo *et al*., 1984). These data suggest that the detection of anti-JEV IgM in this assay indicates recent infection with JEV. In the survey of Thai dogs, no dog possessed significant levels of anti-JEV IgM, suggesting that infection with JEV had occurred more than 2-3 weeks before sampling. However, the values of the indirect ELISA for detection of anti-JEV IgM are not quantitative, since the binding strength of IgM is weaker than that of IgG, and the absorbance of anti-JEV IgM also may depend on the amount of anti-JEV IgG. The development of an IgM-capture ELISA, which has been used previously

in serological surveys of humans and pigs (Hamano *et al*., 2007, Jacobson *et al*., 2007), might be required for the assessment of recent JEV infection.

In conclusion, an indirect ELISA was described for the detection of JEV antibodies in dogs; this assay allowed us to examine a large number of samples at once and to assess the levels of anti-JEV immunoglobulins in canine specimens quantitatively. This method is expected to facilitate the assessment of the human risk of JEV infection.

4.6. LEGENDS FOR FIGURES

Figure 4-1. Enzyme-linked immunosorbent assay (ELISA) values using 1:500 diluted sera and VN titers in dogs infected experimentally with Japanese encephalitis virus (JEV). Absorbances in IgG (closed circle) and IgM (closed triangle) ELISA are compared with VN titers (open diamond) (Shimoda *et al*., 2011).

Figure 4-2. Comparison of IgG ELISA values using 1:500 diluted sera and VN titers among dogs in Kyushu, Japan. Absorbances in IgG ELISA and VN titers are plotted. Closed circles, positive by both IgG ELISA and VN test; closed squares, negative by both IgG ELISA and VN test; open circles, positive only by VN test; open squares, positive only by IgG ELISA. The cut-off value for IgG ELISA was set at 0.211 (see main text).
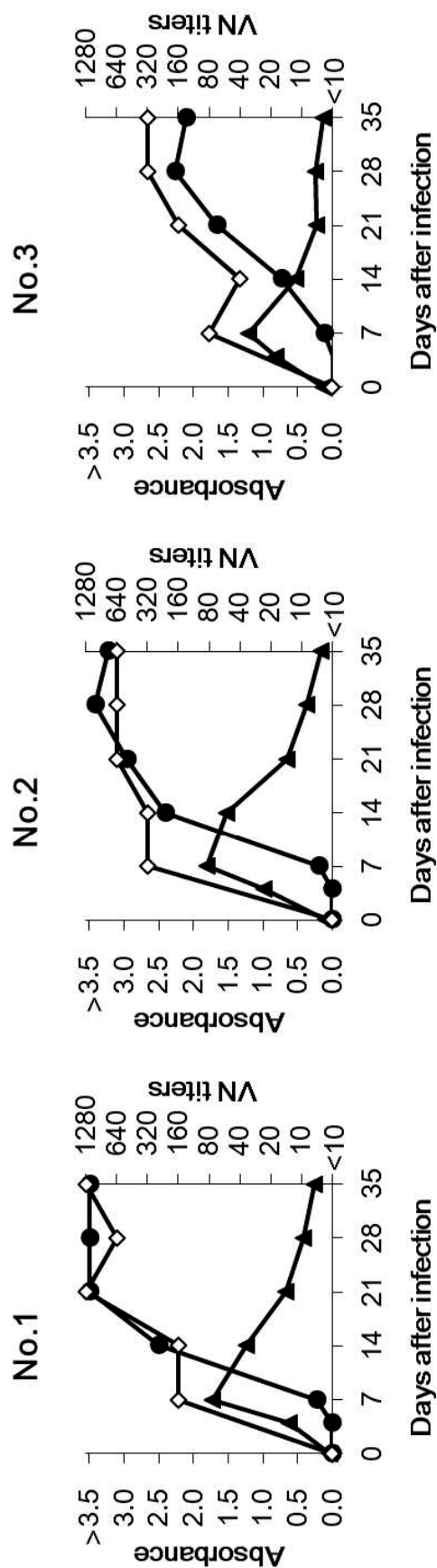
Figure 4-1. Enzyme-linked immunosorbent assay (ELISA) values using 1:500 diluted sera and VN titers in dogs infected experimentally with Japanese encephalitis virus (JEV).
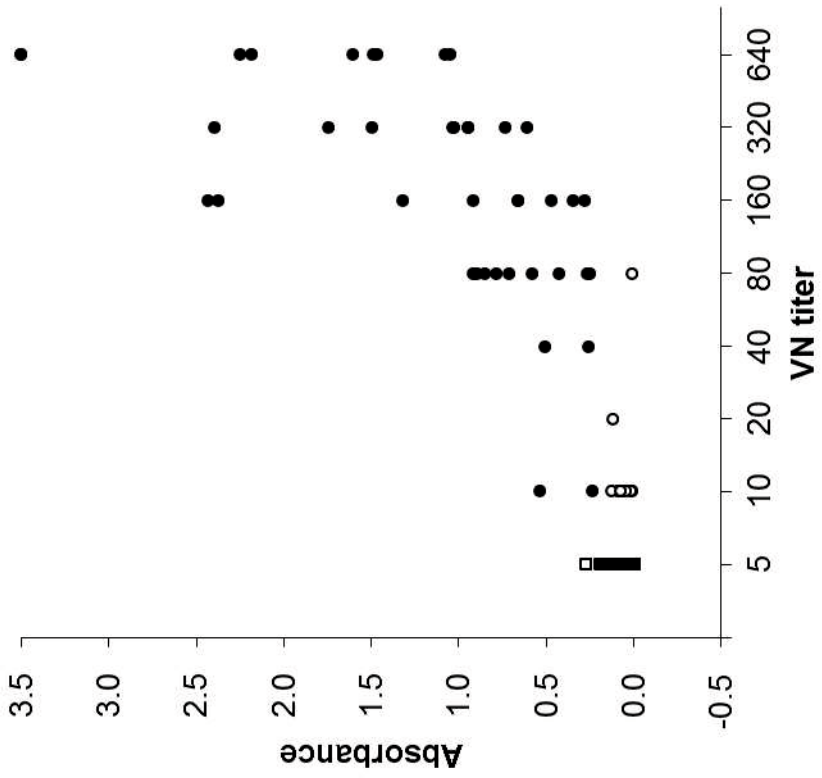
Figure 4-2. Comparison of IgG ELISA values using 1:500 diluted sera and VN titers among dogs in Kyushu, Japan.

Table 4-1. Comparison of the results between VN test and IgG-ELISA among dogs in Kyushu, Japan

IgG-ELISA	VN test		Total
	Positive	Negative	
Positive	40	1	41
Negative	9	52	61
Total	49	53	102

Table 4-2. Seroprevalence of JEV by IgG-ELISA among dog population in Bangkok, Thailand

	Housing			Age (year)			Total
	Indoor	Outdoor	Both	0-3	4-6	7-	
No. of examined dogs	9	44	17	13	33	24	70
No. of JEV-positive dogs	0	29	7	5	17	14	36
% of JEV-positive dogs	0	66	41	38	52	58	51

5. CHAPTER 4

Seroprevalence of Japanese encephalitis virus infection

in Japanese macaques (*Macaca fuscata*) in Japan

5.1. ABSTRACT

In this chapter, a serosurvey of JEV in Japanese macaques (*Macaca fuscata*) reared in Aichi Prefecture was performed using purified JEV as an antigen for ELISA. The results showed that 146 of 332 monkeys (44.0 %) were seropositive for JEV. In addition, 35 out of 131 monkeys (26.7%) born in the facility were seropositive and the annual infection rate in the facility was estimated as 13%. Our results provide the evidence of frequent exposure of JEV to Japanese macaques, suggesting that there is a risk of JEV transmission to humans by mosquitoes.

5.2. INTRODUCTION

Although only a few human JE cases have occurred in Japan, an annual serological survey of JEV in pigs showed that it was endemic especially in the western part of Japan (Arai *et al*., 2008). In addition, various wild and domestic mammals, including wild boars, raccoons, raccoon dogs and dogs were seropositive for JEV (Hamano *et al*., 2007; Nidaira *et al*., 2007; Ohno *et al*., 2009; Shimoda *et al*., 2010). Therefore, there is still a high risk of JEV infection in various mammals in Japan, especially in the western part.

The seroprevalence of JEV among various species of monkeys has been examined previously. Cynomolgus monkeys (*Macaca fascicularis*), Japanese macaques (*Macaca fuscata*), green monkeys (*Chlorocebus sabaeus*) and pig-tailed macaques (*Macaca nemestrina*) were seropositive for JEV in several Asian countries (Inoue et al., 2003; Nakgoi *et al*., 2013; Yamane 1974; Yuwono *et al*., 1984). In contrast, there was no evidence of JEV infection in toque macaques (*Macaca sinica*) in Sri Lanka where JEV is endemic (Peiris *et al*., 1993). Although there are reports of JEV infection in various monkey species in Asia, the recent prevalence in Japan remains unknown. Therefore, an assessment of the prevalence of JEV in monkeys in Japan will provide information about the potential risk of transmission of JEV infection to humans.

In this chapter, I performed a serological survey of Japanese macaques using ELISA and assessed the current risk of JEV infection to monkeys in Japan.

5.3. MATERIALS AND METHODS

5.3.1. Cells

African green monkey-originated Vero9013 cells (JCRB number; JCRB9013) and mosquito-originated C6/36 cells (JCRB number; IFO 50010) were used in this study. The condition of culture media are the same as described in CHAPTER 1.

5.3.2. Virus

Swine-derived JEV/sw/Chiba/88/2002 (genotype I) were used in this study. The propagation of this virus was performed the same as described in CHAPTER 1.

5.3.3. Monkey serum samples

A total of 332 serum samples were collected from Japanese macaques (*Macaca fuscata*) housed at the Primate Research Institute (PRI), Kyoto University in Aichi Prefecture, Japan. Among them, 131 monkeys were born at PRI. All monkeys examined in this study were bled in the environment where they have chance to be bitten by mosquitoes frequently. These experiments were carried out according to rules of Kyoto University and guidelines for experimental animal welfare. Bleeding was performed under ketamine hydrochloride anesthesia. All sera used for ELISA were stored at -20°C until use.

5.3.4. VN test

The presence of VN antibody against JEV in sera was determined by 80% plaque reduction VN test described in CHAPTER 1.

5.3.5. ELISA

To determine the seroprevalence of JEV among monkeys, indirect ELISA using inactivated JEV, Beijing 01 strain, was carried out as described in CHAPTER 3. Peroxidase Conjugated Purified Recomb® Protein A/G (Thermo Fischer Scientific, Rockford, IL, U.S.A.) was used instead of a second antibody.

5.3.6. Statistical analysis

To analyze the results statistically, chi-square test was performed. p values of < 0.05 were considered statistically significant.

5.4. RESULTS

5.4.1. Determination of cut-off value

To determine the cut-off value, samples from 18 monkeys that were born after the last summer and had not experienced summer time yet and their mothers confirmed as JEV-negative by VN test, were examined by ELISA. The absorbance values obtained for these specimens ranged from 0.015 to 0.439, and the mean absorbance value was 0.123 with a standard deviation (S.D.) of 0.107. The mean value plus $3 \times$ S.D. (a value of 0.444) was selected as the cut-off value between positive and negative results in this study. Only one monkey whose mother was JEV-positive by VN test became JEV-positive in ELISA (Fig. 5-1) Since this baby monkey was three months old and had never spend the summer, the detected antibody must be originated from maternal antibody against JEV from its mother.

5.4.2. Seroprevalence of JEV among monkeys in Japan

To survey JEV infection in monkeys in Japan, 332 serum samples were examined by ELISA. The results revealed that 146 out of 332 monkeys (44%) were seropositive for JEV. The seroprevalence among older monkeys (over 7 years old, 68%) was significantly higher than that among younger monkeys (0-3 years old, 12%) ($p<0.05$). There were no significant difference in seroprevalence between males (43%) and females (44%) (Table 5-1).

5.4.3. JEV infection to monkeys born and raised only in Aichi Prefecture, Japan

To determine the annual seroprevalence among monkeys in Japan, I focused on monkeys born and raised in the same facility in Aichi Prefecture. The results showed that 35 out of 131 monkeys (27%) were seropositive for JEV (Table 5-2). The seroprevalence

of JEV increased with age in these monkeys, which was consistent with results from other monkeys in Japan. The annual seroprevalence was calculated according to the assumption that JEV antibodies are maintained in Japanese macaques throughout their life. As a result, approximately 13% of monkeys were infected annually with JEV in Aichi Prefecture (Table 5-2).

5.5. DISCUSSION

The ELISA used in this study was established previously for surveillance of JEV among dogs in Thailand (CHAPTER 3; Shimoda *et al*., 2013). I adapted the method for surveillance of monkeys using protein A/G instead of a secondary antibody, and the results of ELISA were correlated with those of VN tests (data not shown). Therefore, this method can be used for surveillance in monkeys as well as dogs. Furthermore, protein A/G is reported to have a broad binding ability for various mammalian immunoglobulins including monkeys, deer, wild boar and raccoon dogs (Inoshima *et al*., 1999) which may mean it can be used for surveillance of JEV in many species of wild mammals.

Serosurveys have indicated that the risk of JEV infection is higher in the western part of Japan than in eastern or northern parts (Arai *et al*., 2008; Shimoda *et al*., 2010). In this study, 27% of monkeys in Aichi Prefecture, which is located in the middle part of Japan, were seropositive for JEV. Therefore, there may be a higher risk of JEV infection in monkeys in western parts of Japan, such as Kyushu and Shikoku districts. One old serological study (before 1980s) on JEV prevalence in 34 Japanese macaques bred in Aichi Prefecture indicated that 29.7% of them were seropositive (Yuwono *et al*., 1984). In this study, 27% of Japanese macaques born in the same facility were seropositive for JEV. Therefore, the prevalence of JEV among monkeys appears to have been maintained since the 1980s. In addition, many macaques seropositive for JEV will be protected from the diseases by JEV, but not from JEV infection, because humans vaccinated with inactivated JEV vaccine developed NS1 antibody by natural infection of JEV without any severe symptoms (Konishi et al. 2002, Matsunaga et al. 2008).

To estimate the lowest annual seroprevalence of JEV, it was assumed that the antibody against JEV is maintained in Japanese macaques throughout their life. However, the duration of human antibody to JEV NS1 was estimated as 4.2 years (Konishi and Kitai,

2009). Another study on JEV vaccination indicated that 18%, 47%, 82%, and 100% of human were estimated to become virus neutralization antibody negative at 5, 10, 15, and 20 years after the second series of routine vaccination, respectively (Abe et al. 2007). In experimentally JEV-infected dogs, virus-neutralization titers kept increasing until 21-28 days after infection, and slightly decreased at 70 days after infection (Shimoda et al. 2011). These reports indicated that JEV antibody are maintained for a long time, but not throughout their life. Therefore, the annual seroprevalence of JEV infection must be much higher, over 13%.

In this study, the seroprevalence of JEV increased with the age of the monkeys. This result seems to be correlated with the period of exposure to JEV-infected mosquitoes. Although there is no available data about feeding pattern of *Culex tritaeniorhynchus*, main vector of JEV, against humans and non-human primates, some other mosquito species, such as *Aedes* and *Haemagogus* species, show a similar feeding pattern between humans and non-human primates (Marassa et al. 2009). Therefore, humans like Japanese macaques will be bitten by JEV-infected mosquitoes around this facility. However, since main amplifiers of JEV are pigs and birds, Japanese macaques must not be reservoir for transmission of JEV to human.

In conclusion, since many Japanese macaques developed and maintained antibodies to JEV, there is a risk of JEV transmission to humans by JEV-carrying mosquitoes. In addition, since the prevalence of JEV among monkeys this facility has not changed over the last 30 years, continuous surveillance for JEV infection and vaccination of JEV in Japan is required.

LEGEND FOR FIGURE

Figure 5-1. ELISA values to JEV in infant Japanese macaques and their mother. The lanes "VN (+)" and "VN (-)" represent positive and negative for JEV neutralizing antibodies in mother monkeys, respectively. Infant monkeys that were born after the last summer and their mothers were negative for JEV by VN test were assumed to be JEV negative for determination of the cut-off value.
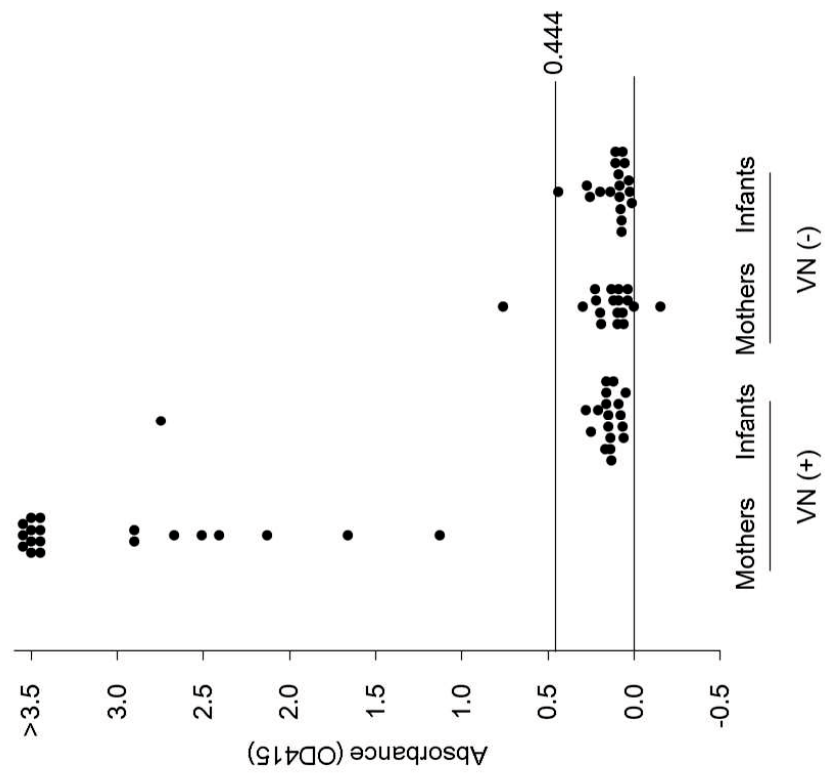
Figure 5-1. ELISA values to JEV in infant Japanese macaques and their mother.

Table 5-1. Seroprevalence of JEV in Japanese macaques that were gathered from throughout Japan and raised in Aichi Prefecture, Japan.

	Sex		Age			Total
	Male	Female	0-3	4-7	8-	
No. of examined monkeys	111	221	105	73	154	332
No. of positive monkeys	48	98	13	28	105	146
% of positive monkeys	43	44	12	38	68	44

Table 5-2. Seroprevalence of JEV in Japanese macaques born and raised in the same facility in Aichi Prefecture, Japan.

	Sex		Age			Total
	Male	Female	0-3	4-7	8-	
No. of examined monkeys	63	68	93	18	20	131
No. of positive monkeys	17	18	13	8	14	35
% of positive monkeys	27	26	1	44	70	27
% of annual positive rate[a]	15	12	-	-	-	13

a: Annual positive rate was calculated according to assumption that the antibody against JEV is maintained in Japanese macaques throughout their life.

6. CHAPTER 5

Production and characterization of monoclonal antibodies

to Japanese encephalitis virus

6.1. ABSTRACT

In this chapter, eighteen monoclonal antibodies (MAbs) to recent Japanese encephalitis virus (JEV) genotype I were produced and characterized by VN test, western blot analysis, indirect immunofluorescence assay and enzyme-linked immunosorbent assay. All MAbs recognized only envelope (E) protein or conformational epitope of E and precursor membrane (prM) proteins. Two MAbs (7E5 and 3-3H8) showed VN activity to JEV/sw/Chiba/88/2002 and JaOH0566, and the escape mutants were produced by addition of the MAbs to culture medium. All escape mutants possessed a change of glutamine to histidine at the position of 52 of E protein, suggesting that these neutralizing MAbs recognize the domain I-II hinge region of E protein. Five MAbs recognized all examined flaviviruses, two were specific to JEV and one was specific to JEV serocomplex. These MAbs may be useful for differentiation and diagnosis of flaviviruses.

6.2. INTRODUCTION

Many methods have been used for serological survey of JEV in various animals, such as VN test, hemagglutination inhibition (HI) assay and enzyme-linked immunosorbent assay (ELISA). Among them, VN test is currently "gold standard" for the serological differentiation of flavivirus infections (Maeda *et al.*, 2013). However, VN test requires a special facility, high level of technical skill, and a large volume of serum samples. In contrast, ELISA does not require the use of live virus and the procedure is simple and requires only a small amount of serum samples. I and other laboratories have reported the usability of indirect IgG ELISA for serological survey of JEV (Cui *et al.*, 2008; Hamano *et al.*, 2007; Niwa *et al.*, 1991; Shimoda *et al.*, 2010; Xinglin *et al.*, 2005; Yang *et al.*, 2006). Although indirect ELISA allows us to examine a large number of samples at once, the cross-reactivity of the antibody with other flaviviruses obstructs us to perform the JEV-specific diagnosis. Dengue virus (DENV) and JEV have overlapped geographic distributions in several countries in Southeast Asia (Mackenzie *et al.*, 2004), and also in Japan, both JEV and tick-borne encephalitis virus (TBEV) have spread. Therefore, diagnostic tools to differentiate JEV from other flaviviruses are required.

In Asia including Japan, a main genotype of JEV has changed from genotype III to I since 1990's (Chen *et al.*, 2011; Ma *et al.*, 2003; Nga *et al.*, 2004; Pan *et al.*, 2011). Although there is a report that antigenicity between genotypes I and III is different (Saito *et al.*, 2007), the difference of antigenicity among JEV genotypes has been obscure.

In this study, monoclonal antibodies to recent JEV genotype I were produced and the specificities were compared among various flaviviruses.

6.3. MATERIALS AND METHODS

6.3.1. Cells

African green monkey-originated Vero cells (JCRB number; JCRB9013) and mosquito-originated C6/36 cells (JCRB number; IFO 50010) were used in this study. The condition of culture media are the same as described in CHAPTER 1. The P3U1 cells derived from mice myeloma cells were maintained in RPMI1640 (GIBCO) supplemented with 10% heat-inactivated FCS, 100 units/ml penicillin, 100 μ g/ml streptomycin and 55 μ M of 2-mercaptoethanol (GIBCO) at 37°C under 5% CO_2.

6.3.2. Viruses

Human-derived JaOH0566 (genotype III) and swine-derived JEV/sw/Chiba/88/2002 (genotype I) were used in this study. The propagation of these viruses were performed the same as described in CHAPTER 1.

6.3.3. Production of MAbs to JEV

BALB/c mice (Five weeks old, male) were intraperitoneally immunized with JEV/sw/Chiba/88/2002 3 or 4 times at intervals of three weeks. The mice were euthanized 3 to 7 days after the final immunization and the splenocytes were fused with P3U1 myeloma cells by using 50% polyethylene glycol solution (Hybri-Max^{TM}; Sigma-Aldrich, St. Louis, Mo, U.S.A.). The hybridoma cells were diluted in selection media, GIT media (Wako, Osaka, Japan) containing 2% hypoxanthine-aminopterin-thymidine (HAT) supplement (GIBCO), 10% BM-Condimed H1 Hybridoma Cloning Supplement (Roche Diagnostics, Mannheim, Germany) and 10% FCS, and incubated for 7 to 10 days at 37°C and 5% CO_2. The screening of hybridomas was performed by either VN test or indirect

immunofluorescence assay (IFA) using JEV/sw/Chiba/88/2002 as described below. Hybridomas producing MAbs specific for JEV were selected and subsequently cloned by the limiting dilution method. The cloned hybridomas were intraperitoneally injected into pristane-treated BALB/c mice (Five weeks old, male) to produce ascites. The immunoglobulin subclass of MAbs was determined using an IsoStrip Mouse Monoclonal Antibody Isotyping Kit (Roche Applied Science) according to the manufacturer's instructions.

6.3.4. VN test

To determine the VN activity of MAbs, VN test was carried out as described in CHAPTER 1.

6.3.5. ELISA

ELISA was carried out using inactivated JEV, Beijing 01 strain, as described in CHAPTER 3. Peroxidase-conjugated goat anti-mouse IgG+A+M (Cappel Laboratories, Cochranville, PA, U.S.A.) was used as a second antibody.

6.3.6. Western blot analysis

Vero cells were infected with JEV/sw/Chiba/88/2002, and then incubated at 37°C and 5% CO_2 until cytopathic effect (CPE) was observed. The cells were removed from dishes with 0.02% EDTA in PBS. After centrifugation at 200 $\times$ g for 5 min at 4°C, the supernatant was removed and the cells were suspended in PBS. Then, suspended cells were mixed with equal volume of 2$\times$ concentrated sample buffer (6.25 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 20% glycerol, 0.001% bromophenol blue).

These samples were boiled for 3 min, placed on ice for 3 min and centrifuged at 13,000 × *g* for 3 min at room temperature. Then, the cell lysates were electrophoresed on 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane (Immobilon-P; Millipore, Billerica, MA, U.S.A.). After blocking with Tris-buffered saline (TBS) containing 3% gelatin (EIA Grade Reagent Gelatin; Bio-Rad) for 45 min at 37°C, the membrane was washed three times with TBS containing 0.05% Tween 20 (T-TBS). After incubation with diluted MAbs for 45 min at 37°C, the membrane was washed three times with T-TBS. Then, the membrane was reacted with peroxidase-conjugated goat anti-mouse IgG+A+M (Cappel Laboratories) for 45 min at 37°C. After washing the membrane with T-TBS and TBS three times each, the specific bands were visualized by 3,3'-diaminobenzidine tetrahydrochloride (DAB; Wako)

6.3.7. Construction of plasmids and transfection to cells

Viral RNA was extracted from virus-infected Vero cells using QIAGEN RNeasy Mini Kit (QIAGEN, Germantown, MD, U.S.A.) according to the manufacturer's instructions. First strand cDNA was transcribed by RNA LA PCR™ Kit (AMV) Ver.1.1 (TAKARA, Shiga, Japan) using random 9 mer at 30°C for 10 min, 42°C for 30 min, 70°C for 15 min and 4°C for 5 min. The cDNA was amplified using primer pairs, EcoprMF (5'-ACA GAA TTC ACC ATG GGA GGA AAT GAA AGC TCG-3') and BglMR (5'-ACA AGA TCT TCA ACT GTA AGC CGG AGC GAC C-3') for prM, EcoprMF and BglER (5'-ACA AGA TCT TCA GGC ATG CAC ATT GGT CGC-3') for prME, and EcoEF (5'-ACA GAA TTC ACC ATG CAA CGT GTG GTG TTT ACT ATT C-3') and BglER for E. Amplified cDNAs were treated with restriction enzymes *Eco*RI and *Bgl*II, and then cloned in the *Eco*RI-*Bgl*II site of pCAGGS plasmid (Niwa *et al*., 1991). The resultants

were named as pCAG-prM, pCAG-E and pCAG-prME, respectively. Then, 293T cells were transfected with expression plasmids using Lipofectamine™ LTX and Plus™ Regent (Invitrogen, Carlsbad, CA, U.S.A.) and analyzed by IFA.

6.3.8. IFA

Subconfluent monolayers of Vero cells were infected with viruses, including JEV genotype I (JEV/sw/Chiba/88/2002, JEV/eq/Tottori/2003, Jakagawa2000), JEV genotype III (JaOH0566, JaGAr-01), JEV genotype V (Muar), Murray Valley encephalitis virus (MVEV), WNV (NY99), DENV serotype 1 (Hawaii), serotype 2 (NC), serotype 3 (H87), serotype 4 (H241), yellow fever virus (YFV) (17D), and TBEV (Sofin). After incubation at 37°C and 5% CO_2 until CPE was observed, infected cells were removed from dishes with 0.02% EDTA in PBS. After centrifugation at 200 $\times$ g for 5 min at 4°C, the supernatant was removed and the cells were suspended in PBS. The cells were placed on micro slide glass (Matsunami, Osaka, Japan), air-dried, and fixed with cold acetone for 10 min at -20°C. 293T cells transfected with expression plasmids were also treated the same as described above. Then, the cells were incubated with MAbs at 37°C for 30 min. After washing the cells three times with PBS, fluorescein-conjugated goat anti-mouse IgM+IgG+IgA (Southern Biotech, Birmingham, AL, U.S.A.) was added and the cells were incubated for 30 min. The cells were washed three times with PBS and preserved with mounting fluid, 90% glycerol in 0.05M sodium carbonate and 0.05M sodium hydrogen carbonate. Cells were examined using a Nikon Optiphot-2 EFD-3 Fluorescence phase contrast Microscope (Nikon Corporation, Tokyo, Japan).

6.3.9. Selection of neutralization-resistant escape mutant

Neutralization-resistant escape mutants were selected by harvesting JEV in Vero cells in the presence of neutralizing MAbs (7E5, 3-3H8). Briefly, Vero cells were infected with JEV/sw/Chiba/88/2002 or JaOH0566 at a multiplicity of infection of 0.01. After incubation at 37 °C for 90 min, cells were washed twice and EMEM containing 2% FCS and 1 % ascites of MAbs was added. The supernatant was collected when CPE was observed. After repeating these steps three times, the supernatant was cloned by plaque cloning and the obtained mutants were confirmed by IFA. The escape mutants were named as 7E5 mar Chiba, 7E5 mar JaOH0566 and 3-3H8 mar Chiba.

6.3.10. Sequence analysis

Viral RNA was extracted from virus-infected Vero cells using QIAGEN RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. First strand cDNA was transcribed by RNA LA PCR^{TM} Kit (AMV) Ver.1.1 (TAKARA) using random 9 mer at 30°C for 10 min, 42°C for 30 min, 70°C for 15 min and 4°C for 5 min. To analyze the nucleotide sequences of JEV structural proteins (C, prM, E), cDNA was amplified by PCR using a primer pair J8 (nt 1-18) and J6 (nt 2845-3865) (Yun et al., 2003) at 94°C for 2 min, followed by 30 cycle of denaturation at 94°C for 30 sec, annealing at 65°C 30 sec, and extension at 72°C for 4 min. PCR products were electrophoresed on 0.8% agarose gel and an approximately 4 kbp fragment was purified with the QIAGEX-Ⅱ Gel Extraction Kit (QIAGEN) and analyzed by direct sequencing with the BigDye Terminator Cycle Sequencing Kit ver3.1 (Applied Biosystems, Austin, TX, U.S.A.) according to the manufacturer's instructions.

6.4. RESULTS

6.4.1. Production and characterization of MAbs against JEV

Total of 18 hybridomas were finally isolated and cloned. The heavy chain subclasses of MAbs were determined as described in Table 6-1, and their light chains were all kappa isotype.

To determine the protein recognized by MAbs, expression plasmids, pCAG-prM, pCAG-E, pCAG-prME and a mixture of pCAG-prM and pCAG-E were transfected to 293T cells and IFA was carried out. All MAbs recognized prME protein and co-expressed prM and E. Among them, seven recognized E protein alone and none recognized prM protein alone (Table 6-1). Three MAbs (3-1A5, 3-2D6, 3-3B10) recognized a molecular mass of 50 kDa by western blot analysis under non-reducing condition (Table 6-1).

To determine the binding ability to purified vaccine antigen, Beijing 01 (genotype III), ELISA was performed. Six MAbs (3-1A1, 3-1A5, 3-2A1, 3-2D6, 3-4B3 and 3-3B10) showed high reactivity with purified JEV antigen (Absorbance > 3.5), while three MAbs (1-1A7, 3-1C2, 4-3E8) showed no or less reactivity (Absorbance < 1.0) (Table 6-1).

6.4.2. Cross-reactivity of MAbs to various flaviviruses

To determine the cross-reactivity of the MAbs with various flaviviruses including genotypes I, III and V of JEV, MVEV, WNV, serotypes 1, 2, 3 and 4 of DENV, YFV, and TBEV, each MAb was examined by IFA using Vero cells infected with these viruses. As the result, five MAbs (3-1A1, 3-1A5, 3-2A1, 3-2D6, 3-4B3) cross-reacted with all flaviviruses examined in this study and two MAbs (3-1A2, 4-4A8) specifically recognized all genotypes of JEV (Table 6-2). One MAb (3-1D7) recognized JEV, MVEV,

and WNV, suggesting that 3-1D7 is specific for JEV serocomplex (Table 6-2).

6.4.3. Analysis of escape mutants

To identify the epitope recognized by MAbs with VN activity, these escape mutants, 7E5 mar Chiba, 3-3H8 mar Chiba and 7E5 mar JaOH0566, were selected by harvesting JEV in the presence of MAb 7E5 or 3-3H8. Viral genome encoding C, prM and E was compared between parent strains and escape mutants. As the result, only one amino acid change was identified in the E proteins of 7E5 mar Chiba and 3-3H8 mar Chiba at E52 (Gln→His) and two amino acid changes were identified on the E protein of 7E5 mar JaOH0566 at E52 (Gln→His) and E276 (Ser→Arg) (Table 6-3). Based on these sequence analysis of escape mutants, MAbs 7E5 and 3-3H8 recognized the domain I-II hinge region of E protein, which has been previously described as an important neutralizing epitope for JEV (Hasegawa *et al*., 1992; Kobayashi *et al*., 1985; Luca *et al*., 2012; Morita *et al*., 2001).

6.5. DISCUSSION

In this study, 18 MAbs to a recent field isolate belonging to genotype I were produced and characterized. Among them, two MAbs (3-1A2, 4-4A8) were specific to JEV, and one MAb (3-1D7) was to JEV serocomplex. In contrast, five MAbs (3-1A1, 3-1A5, 3-2A1, 3-2D6, 3-4B3) were cross-reactive among all flaviviruses, including MVEV, WNV, DENV, YFV and TBEV. These JEV- or JEV serocomplex-specific MAbs and cross-reactive MAbs may be useful for the detection of flaviviruses and may be applicable for the differentiation of JEV infection from other flavivirus infection by immunohistochemistry and competitive ELISA using these MAbs.

I showed that MAbs 7E5 and 3-3H8 recognized the same epitope located on the domain I-II hinge region of E protein. Specifically, sequence analysis of 7E5 and 3-3H8 escape mutants showed a common amino acid change at E52 (Gln→His). This residue has been previously described as an important epitope recognized by MAbs against JEV with strong neutralizing activity (Hasegawa *et al*., 1992; Kobayashi *et al*., 1985; Luca *et al*., 2012; Morita *et al*., 2001), confirming that the domain I-II hinge region of E protein is a major target of VN antibody.

All MAbs in this study recognized prME protein, and among them seven MAbs recognized E protein alone and none did prM protein alone. The previous study indicates that the prM protein assists in the proper folding of E protein (Konishi *et al*., 1993). Therefore, the other eleven MAbs may recognize the conformational epitope in the presence of prM protein.

In conclusion, eighteen MAbs to JEV possessed different reactivity with flaviviruses. As several flaviviruses have been endemic in some regions, these MAbs should be the useful tools to detect flavivirus infection and to differentiate JEV infection

from the other flavivirus infections.

Table 6-1. Characterization of MAbs

| MAbs | Isotype | IFA[a] | | | | ELISA[a] (G III) | Western blot[a] (G I) | VN test | |
		prM	E	prME	prM+E			Chiba (G I)	JaOH0566 (G III)
3-1A1	IgG2a	-	+	+	+	>3.50	-	<1:100	NT[b]
3-1A5	IgG2a	-	+	+	+	>3.50	+	<1:100	NT
3-2A1	IgG2a	-	+	+	+	>3.50	-	<1:100	NT
3-2D6	IgG2a	-	+	+	+	>3.50	+	<1:100	NT
3-4B3	IgG2a	-	+	+	+	>3.50	-	<1:100	NT
3-3B10	IgG2b	-	+	+	+	>3.50	+	<1:100	NT
1-1A7	IgM	-	-	+	+	0.05	-	<1:100	NT
3-1D7	IgG3	-	-	+	+	2.40	-	<1:100	NT
3-3D1	IgM	-	-	+	+	2.05	-	<1:100	NT
4-4E9	IgG2a	-	-	+	+	1.82	-	<1:100	NT
3-2D1	IgM	-	-	+	+	2.35	-	<1:100	NT
3-1A2	IgG2a	-	-	+	+	2.38	-	<1:100	NT
4-4A8	IgM	-	-	+	+	3.30	-	<1:100	NT
3-1C2	IgG2a	-	-	+	+	0.09	-	<1:100	NT
3-1B5	IgG2a	-	-	+	+	2.53	-	<1:100	NT
7E5	IgM	-	-	+	+	1.53	-	1:102400	1:102400
3-3H8	IgM	-	-	+	+	1.65	-	1:102400	1:204800
4-3E8	IgG2a	-	+	+	+	0.28	-	<1:100	NT

a 100-fold diluted mouse ascites was used for IFA, ELISA and western blot analysis.

b NT: Not tested

Table 2. Cross-reactivity of mAbs against various flaviviruses

MAbs	JEV GI			JEV GIII		JEV GV	MVEV	WNV	DENV				YFV	TBEV
	Chiba	Tottori	Kagawa	JaOH	JaGAr				1	2	3	4		
3-1A1	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3-1A5	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3-2A1	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3-2D6	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3-4B3	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3-3B10	+	+	+	+	+	+	+	+	+	+	+	+	+	-
1-1A7	+	+	+	+	+	+	-	+	+	+	+	+	-	-
3-1D7	+	+	+	+	+	+	+	+	-	-	-	-	-	-
3-3D1	+	+	+	+	+	+	-	+	-	-	-	-	-	-
4-4E9	+	+	+	+	+	-	+	+	-	-	-	-	-	-
3-2D1	+	+	+	+	+	+	+	-	-	-	-	-	-	-
3-1A2	+	+	+	+	+	+	-	-	-	-	-	-	-	-
4-4A8	+	+	+	+	+	+	-	-	-	-	-	-	-	-
3-1C2	+	+	+	-	+	+	-	-	-	-	-	-	-	-
3-1B5	+	+	+	+	+	-	-	-	-	-	-	-	-	-
7E5	+	+	-	+	-	-	-	-	-	-	-	-	-	-
3-3H8	+	+	-	+	-	-	-	-	-	-	-	-	-	-
4-3E8	+	+	-	+	-	-	-	-	-	-	-	-	-	-

a: IFA was performed using 100-fold diluted mouse ascites.

Table 3. Amino acid changes in neutralization-resistant escape mutants

Viruses	E52 (Domain II)	E276 (Domain II)
Parents		
Chiba	Q	S
JaOH0566	Q	S
Escape mutants		
7E5 mar Chiba	H	S
3-3H8 mar Chiba	H	S
7E5 mar JaOH0566	H	R

7. GENERAL CONCLUSION

In this dissertation, to understand the present situation of JEV, author examined the seroprevalence of JEV among dogs, cats and monkeys in Japan and dogs in Thailand using classical and our established novel method for detecting antibodies against JEV. Also, for further development of novel, simple, and specific method to detect both antibody and antigen of JEV, MAbs were produced.

The ideal sentinels for serological survey would be susceptible to infection, survive from the infection, develop detectable antibodies, pose no risk of infection to handlers, and never develop a high level of viremia enough to infect vector mosquitoes (Resnick *et al*., 2008). The study in CHAPTER 1 found the risk of JEV infection remains high, even in urban/residential areas, by using dogs as sentinels, and in CHAPTER 2, it was confirmed that dogs fulfil the requirements as a good sentinel for serosurvey of JEV infection. Therefore, it is recommended that annual JEV surveillance should be carried out in pigs in Japan and that dogs should be surveyed every several years to assess the risk of JEV infection in humans in urban/residential areas.

In CHAPTER 3, a novel method for serosurvey of JEV was developed. An indirect ELISA using JEV antigen was established and applied for the detection of JEV antibodies in dogs; this assay allowed us to examine a large number of samples at once and to assess the levels of anti-JEV immunoglobulins in canine specimens quantitatively. This simple method is expected to facilitate the assessment of the human risk of JEV infection.

In CHAPTER 4, seroprevalence among non-human primates in Japan was described, and revealed that many Japanese macaques possessed antibody against JEV.

Therefore, there may be a risk of JEV transmission to humans by JEV-carrying mosquitoes. In addition, since the prevalence of JEV among monkeys in Japan had not changed over 30 years, we have to keep giving an attention to JEV infection in Japan.

In CHAPTER 5, eighteen MAbs to JEV were produced and each possessed different reactivity with flaviviruses. As several flaviviruses have been endemic in some regions, these MAbs must be the useful tools to detect flavivirus infection and to differentiate JEV infection from the other flavivirus infection.

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10. BIOGRAPHY

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11.1. Original Papers

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Epidemiological studies on Japanese encephalitis virus
日本脳炎ウイルスの疫学的調査

　　日本脳炎ウイルス（JEV）はフラビウイルス科フラビウイルス属に属するウイルスで、国内では第 4 類感染症に指定されている。JEV はブタを増幅動物とし、カを媒介し、ヒトやウマに重篤な脳炎症状を引き起こす。世界中で年間約 50000 名の患者、約 10000 名の死者が報告されているが、国内ではワクチン接種の効果、養豚場の隔離、媒介蚊の減少などにより近年では年間 10 名以下に抑えられている。しかし、ブタにおける毎年の調査では西日本を中心に依然として高い抗体陽性率が認められ、いまだに JEV は国内で蔓延していると考えられる。

　　本研究は JEV の生態を解析することを目的とし、全 5 章より構成される。

第1章　　伴侶動物における JEV に対する抗体保有状況の調査

　　毎年 JEV 抗体調査が実施されている豚は人の生活空間から隔離されているため、人への感染リスクを示しているかは明らかではない。そこで、本章では人と生活空間を共有する伴侶動物において JEV の抗体保有状況の調査を中和試験にて実施した。

　　山口県内のイヌやネコの調査では、100 頭中 17 頭（17%）のイヌ、292 頭中 3 頭（3%）のネコが JEV 抗体陽性となった。この結果より、イヌの方が陽性率が高く、感受性が高いと考えられたため全国調査をイヌで実施することとした。

　　全国のイヌ 652 頭の血清を用いて実施した結果、164 頭（25%）のイヌが陽性と判定された。地方別にみると、北海道や東北地方ではそれぞれ 0% と 9% と、他地域と比較して有意に低い陽性率が示された。一方で四国、九州地方ではそれぞれ 61% と 47% と、他地域と比較して有意に高い陽性率が示された。また、都市部のイヌや（21%）や室内飼育犬（8%）でも陽性個体が多く認められた。

　　以上より、西日本を中心として未だに JEV が蔓延していることが本章で明らかとなった。室内飼育犬でも陽性が認められたことから、あまり外出しないお年寄りや乳児にも感染のリスクがあると考えられる。犬における調査では豚では調査することのできない、室内や都市部における JEV の感染リスクを評価することが可能であり、調査対象として有用であると考えられる。

第2章　　イヌにおける JEV の感染実験

　　本章では JEV の蔓延状況の調査におけるイヌの安全性および有用性の評価、またイヌに

おける JE 発症の有無を調べるためにイヌに対して JEV の感染実験を実施した。

　ビーグル（2 か月齢、メス）3 頭に対して JaOH0566（遺伝子型 III、1x10^{7}PFU）を静脈内および皮下接種後、毎日体重と体温の測定と臨床症状の観察を行った。採取した血液を用いて血液生化学検査、全血球算定、白血球数の算定、中和試験、ウイルス分離・検出を行った。

　その結果、実験感染犬では発熱などの臨床症状は認められなかった。血液検査では炎症マーカーである C-反応性蛋白 のみ上昇が認められたが、その他の血液検査項目では異常が認められなかった。中和抗体価は 7 日目には上昇し、21-28 日目で最も高い値となり、実験終了の 70 日目まで持続していた。血液からウイルスは分離・検出されなかった。

　今回の結果より、イヌは JEV 感染後、ウイルス血症を起こさず不顕性感染し、中和抗体が長期間持続することが示された。イヌはヒトへの JEV 感染のリスクを調査する際に安全かつ有用な対象であると考えられる。

　第 3 章　簡便で有用な新たな JEV 抗体診断系の確立

　前章までに日本脳炎ウイルスの蔓延状況を調査する上でイヌが有用であることを申請者らは示した。しかし、これまで実施してきた中和試験は手技が煩雑、生のウイルスを使用、多量の血清を使用するなど多くの欠点がある。そこで、本研究では簡便で有用な抗体調査手法とし ELISA 法による新たな診断系の確立を試みた

　ワクチン用不活化 JEV 抗原（Beijing01）を抗原として ELISA 法を確立した。実験感染犬の血清を用いて条件検討（血清 500 倍希釈）を行い、カットオフ値（OD>0.211）を設定するために JEV の流行がない北海道のイヌの血清を用いた。九州地方のイヌで調査を実施し、中和抗体価と値を比較したところ、有意な相関性（相関係数：0.813［$p < 0.001$]）が認められた。また、ELISA の感度は 82%、特異性は 98% であった。

　確立した ELISA の系を用いて、タイの首都であるバンコクのイヌについて抗体の保有状況の調査を実施した。調査した全 70 頭のうち 30 頭（43%）で JEV 抗体陽性と判定された。

　以上のことより、本章では簡便で有用な新たな抗体診断系の確立に成功し、結果は中和試験の結果と相関していた。タイのバンコクで約半数のイヌが JEV 抗体を保有しており、タイでは都市部でも JEV にヒトが感染するリスクが高いということが明らかとなった。本章で確立したイヌにおける JEV 抗体検出系はヒトへの JEV 感染のリスクを調査する上で有用であると考えられた。

　第 4 章　サルにおける JEV 抗体保有状況の調査

　本章では第 3 章で確立した ELISA 系を用いて、ヒトと近縁な霊長類であるニホンザルにおいて抗体の保有状況を調査した。二次抗体には様々な動物の抗体に結合する HRP 標識 Protein A/G を用いた。

　カットオフ値（0.444）は中和抗体陰性の母親から産まれた 0 歳齢のサル 18 頭から算出した。そして、愛知県の霊長類研究所で飼育されているニホンザル 332 頭を調査した結果、146 頭（44%）で陽性が認められた。研究所で産まれた個体に限定すると、131 頭中 35 頭（27%）で陽性となり、年間感染率を算出したところ、13% となった。

　以上のことから、本章では多くのニホンザルが JEV に感染していることが明らかとなり、

ヒトへの感染リスクが十分存在することが示された。

　　第 5 章　　JEV に対する単クローナル抗体の作製とその性状解析

　　本章では日本脳炎ウイルスの遺伝子型 1 の株に対する単クローナル抗体を作製し、その性状解析を行った。

　　JEV/sw/Chiba/88/2002（遺伝子型 I）をマウスに免疫し、その脾細胞よりハイブリドーマを作製した。ハイブリドーマのスクリーニングには中和試験もしくは間接蛍光抗体法（IFA）を用いた。スクリーニングの結果、18 個のハイブリドーマで単クローナル抗体の産生が認められた。

　　ハイブリドーマをマウスに接種することで得られた腹水またはハイブリドーマの培養上清を用いて、単クローナル抗体の性状解析を実施した。IFA により、すべての抗体が日本脳炎ウイルスの構造蛋白である E 蛋白または prM 蛋白を認識していることが明らかとなった。また、18 個中 2 個の抗体が中和活性を有していた。その抗体に対する中和回避変異体を作製し、エピトープの同定を試みたところ、2 個の抗体とも共通の部位（E 蛋白の 52 番目）に変異が認められた。

　　様々なフラビウイルスに対する抗体の反応性を IFA にて評価した。その結果、5 個の抗体が調べたすべてのフラビウイルスに対して反応性を示した。一方で 2 個の抗体が日本脳炎ウイルスに、1 個が日本脳炎ウイルス血清群に対して特異的な反応性を示した。

　　本章で作製に成功したこれらの単クローナル抗体を用いることで、特異的、簡便かつ高感度の疫学調査手法の開発が可能となると考えられる。

本研究ではネコ、イヌ、サルにおける JEV 感染状況を調査することで、日本国内では依然として JEV が蔓延していることが明らかとなった。また、タイにおいては都市部でバンコクで高い陽性率が示されたことから、ヒトへの JEV 感染リスクは高いと考えられる。今回、確立した ELISA 系は簡便で有用であり、HRP 標識 Protein A/G を二次抗体として用いることで様々な野生動物に対して適用可能であると考えられる。さらには、作製した単クローナル抗体は JEV 特異的あるいはフラビウイルス共通の抗原・抗体検出系が開発に有用であると考えられ、今後の発展が期待される。