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Trials for Risk Assessment of Japanese Encephalitis Based on Serologic Surveys of Wild Animals

Mika Saito et al.*

*Department of Microbiology and Oncology, Graduate School of Medicine,
University of the Ryukyus, Okinawa
Japan*

1. Introduction

Japanese encephalitis (JE) is a severe and acute encephalitis with a high fatality rate, caused by Japanese encephalitis virus (JEV), a mosquito-borne flavivirus of the genus *Flavivirus*, family *Flaviviridae*. The JEV serogroup of flaviviruses includes West Nile virus, St. Louis encephalitis virus, and Murray Valley encephalitis virus which also cause encephalitis, though with some clinical variation (Mackenzie et al., 2007). JE is a major public health problem in the Asian region, accounting for more than 16,000 reported cases and 5,000 deaths annually. With the near eradication of poliomyelitis, JE is now the leading cause of childhood viral neurological infection and disability in Asia (Halstead and Jacobson, 2003). Approximately half of all survivors suffer from permanent neurological and/or mental impairments due to the invasion and destruction of cortical neurons and Purkinje cells by the virus (Johnson et al., 1985, Monath, 1986).

Historically, severe epidemics of JE had been reported during the summer season in Japan since the 19th Century and more than 1000 cases were reported annually in the 1960s. With the control of epidemics in Japan and Korea due to changes in agricultural and animal husbandry practices, and in part through vaccination, the number of JE cases markedly

* Takashi Ito², Yosuke Amano³, Junji Takara⁴, Katsushi Nakata⁵, Shoko Tamanaha⁵, Yuichi Osa⁶, Tomoo Yoshino⁷, Manabu Onuma⁸, Takashi Kuwana⁸, Go Ogura⁵, Masayuki Tadano¹, Daiji Endoh⁹ and Mitsuhiko Asakawa⁷

¹Department of Microbiology and Oncology, Graduate School of Medicine, University of the Ryukyus, Okinawa,

²Nago Zoological and Botanical Gardens, Okinawa,

³Nagamine Animal Clinic, Okinawa,

⁴Naha Veterinary Hospital, Okinawa,

⁵Laboratory of Subtropical Zoology, Faculty of Agriculture, University of the Ryukyus, Okinawa,

⁶Wildlife Section, Nature Conservation Department, Hokkaido Institute of Environmental Sciences, Hokkaido,

⁷Department of Pathobiology, School of Veterinary Medicine, Rakuno Gakuen University, Hokkaido,

⁸Biological Resource Laboratory, Laboratory for Intellectual Fundamentals for Environmental Studies, National Institute for Environmental Studies, Ibaraki,

⁹Department of Veterinary Radiology, School of Veterinary Medicine, Rakuno Gakuen University, Hokkaido
Japan

decreased, with less than 10 cases reported annually since the early 1990s (Arai et al., 2008). Meanwhile, JE emerged in Thailand, Vietnam and China in the 1980s. Although the incidence has since declined in these countries, recently the disease has emerged in new areas in Pakistan, and Oceania, particularly Papua New Guinea and northern Australia. The incidence of JE is increasing in countries like Bangladesh, Cambodia, India, Indonesia, Laos, Myanmar, North Korea, and Pakistan (Erlanger et al., 2009). Mackenzie et al (2007)

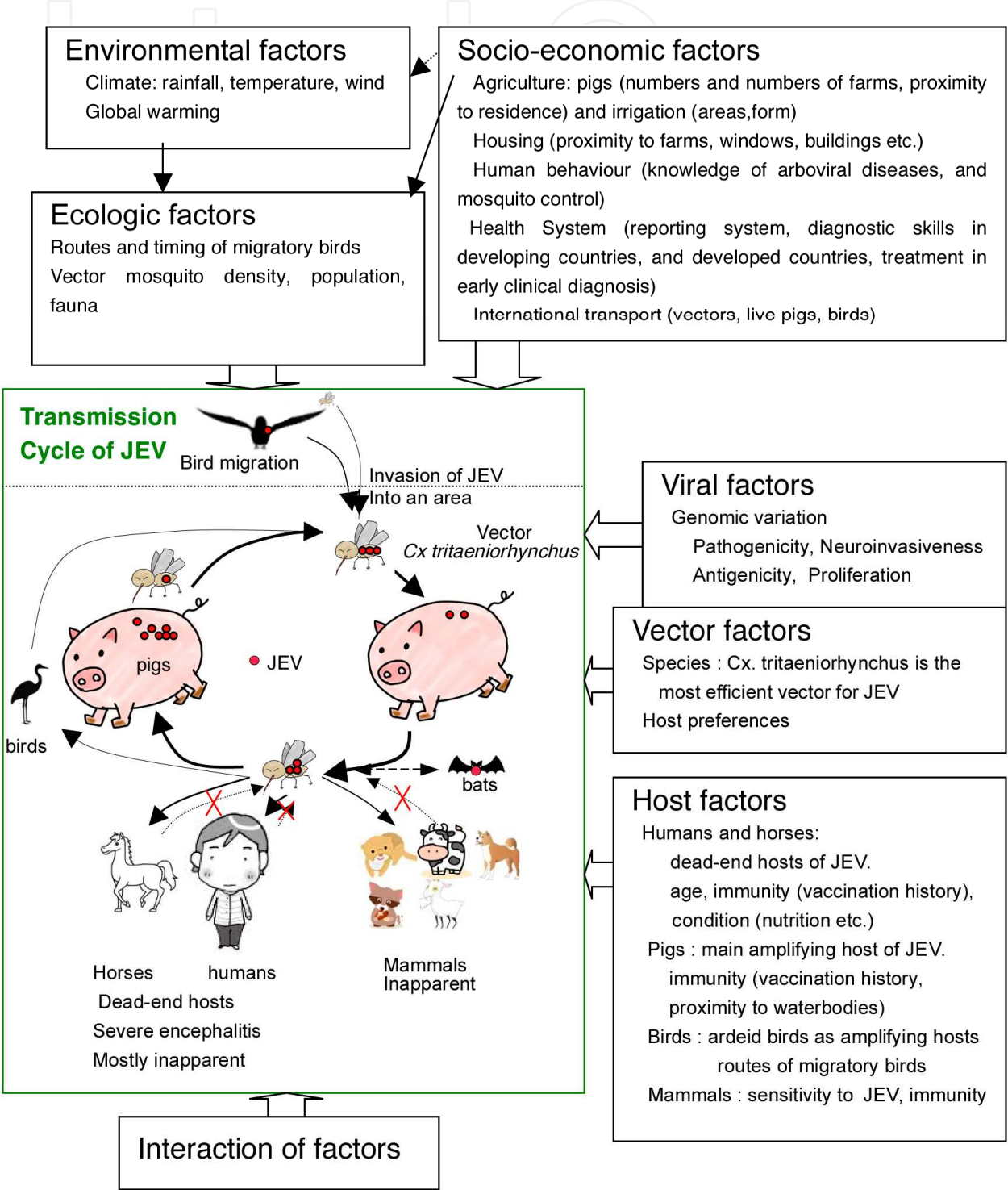


Fig. 1. Transmission cycle of JEV and Risk factors for JE

attributed this phenomenon to the propensity of JEV to spread and colonize new areas. Genetically, five genotypes (genotype 1, 2, 3, 4 and 5) of JEV had been distributed with geographic segregation (Chen et al. 1992, 1994, Uchil and Satchidanandam 2001). The distribution pattern has changed drastically in the last few decades, with a major shift from genotype 3 to genotype 1 in the 1990s in Japan, as well as Vietnam, China, and Korea (Ma et al., 2003, Chung et al. 1996, Nga et al. 2004.) This indicated that an exotic JEV was introduced into these countries and established a transmission cycle.

JEV is maintained in nature by transmission cycles involving *Culex* (*Cx.*) *sp.* mosquitoes and amplifying hosts. *Cx. tritaeniorhynchus* is the most efficient vector, and pigs and certain species of birds, particularly *ardeid* birds, are the most effective amplifiers. Humans and horses are incidental, known as dead-end hosts, and most infected mammals do not develop sufficient levels of viremia to infect mosquitoes. As JEV has a wide range of hosts, environmental changes such as global warming, and economic development including irrigation and animal husbandry practices influence its distribution and occurrence.

At present, no specific treatment for JE is available, and the efficacy of ongoing vaccines against various genotypes found in Asia is also controversial. Therefore, surveillance and risk assessment using an interdisciplinary approach are required for the control of JE, and also other flaviviral infections.

Risks of JE infection closely relate to the transmission cycles of JEV, and many factors involved there in. Factors worth considering when assessing risks are described briefly in Figure 1. Risks of JE occurrence in humans relate to both host and viral factors. The ratio of apparent to inapparent JE infections in humans is estimated at 1:200 to 1:1000. The frequency of exposure through infected mosquitoes becomes high in areas where JE is epidemic. Migratory birds, wind-blown vectors, and the transport of vectors and animals would be determinants of the invasion by JEV of new areas.

In this study, a serologic survey of domestic and migratory wild mammals and wild birds was conducted in Hokkaido and Okinawa, Japan, in order to assess risks for JE in humans. The necessity of assessing risks based on serosurveys of wild animals is discussed.

2. Trials for risk assessment of Japanese encephalitis – a serosurvey of wild animals in Japan

2.1 Study design

2.1.1 Study areas

A seroepidemiologic study of wild animals was conducted in Hokkaido, northern Japan, about 850 km from Tokyo, and on Okinawa Island; a small subtropical island, about 2,000 km southwest of Tokyo (Figure 2).

2.1.2 Collection of serum samples from wild mammals

Alien mammals such as the raccoon *Procyon lotor* in Hokkaido and Honshu, and the mongoose *Herpestes auropunctatus* on Okinawa island threaten ecological destruction as predators of endangered species, and government projects to control their population were conducted with the permission of the Ministry of the Environment, Japan. Sera were obtained from 45 raccoons captured in central Hokkaido in 2005, and Mongooses captured in Okinawa between 2001 and 2005 (Saito et al, 2009a). Two serum samples were obtained

from injured flying foxes, *Pteropus dasymallus inopinatus* Kuroda, rescued on Okinawa in 2007 and 2008. The isolation of viruses from all serum samples was attempted without success.

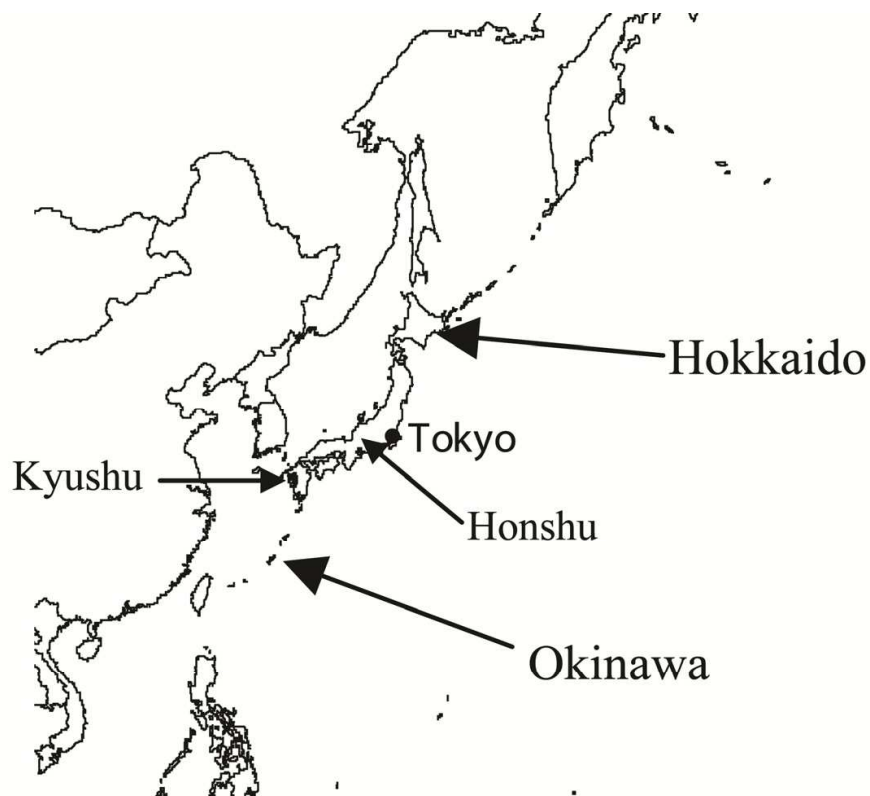


Fig. 2. Location of study areas

2.1.3 Collection of serum samples from wild birds

A total of 50 wild ducks were captured in eastern Hokkaido in 2008, with permission from the Ministry of the Environment, Japan. Eight Spot-billed Ducks (*Anas poecilorhyncha*), 17 Mallards (*A. platyhynchos*), 1 Eurasian Wigeon (*A. penelope*), and 24 Northern Pintails (*A. acuta*) were subjected to a serosurvey. Spot-billed Ducks are recognized as migrant breeders, Mallards as residents breeders or wondering birds, and Eurasian Wigeons and Northern Pintails as winter visitors in Hokkaido.

A total of 72 injured birds, mostly from traffic-related accidents, rescued in animal hospital and zoos as wildlife relief facilities on Okinawa from 2007 to 2009, were subjected to a serosurvey. The order, family, and species of the birds are listed in Table 1. Eleven orders, 17 families, and 28 species were collected. Roughly, the birds were classified seasonally, seven individuals (one species) as migrant (summer) breeders, 29 (13 species) as winter visitors, 34 (12 species) as resident breeders, and 2 (2 species) as wondering breeders in Okinawa. Brains, livers, hearts, and spleens were sampled from dead bodies. The isolation of viruses from all samples was attempted without success.

2.1.4 Positive and negative control sera for birds

To conduct the serologic survey of wild birds, negative and positive control sera were used. As maternal antibody levels declined 2 weeks after hatching in chickens, and 3 weeks after

hatching in ducks, sera from 10 chicks reared for 2 weeks after hatching in a cage without mosquito bites, and 70 Aigamo juvenile ducks, a cross between wild ducks (*A. platyrhynchos*) and domestic ducks (*A. platyrhynchos* var. *domesticus*), reared for 50 days after hatching in a room until early May, not the breeding season for mosquitoes, 2008, in Hokkaido, were used as negative control. Positive sera for JE were produced in chickens by immunization with an inactivated JE vaccine for animals (Kyoto Biken, Japan) three times weekly, starting from 2 weeks of age, and bleeding the animals 2 weeks after the last immunization. Serum samples from 7 Aigamo ducks infected by West Nile virus (NY-99 strain) were provided and used as reference sera for flavivirus infection (Shirafuji et al., 2009).

Order	Family	species	English name	n (+)*	seasonal
Anseriformes	Anatidae	<i>Aythya fuligula</i>	Tufted duck	1 (1)	winter
		<i>Anas poecilorhyncha</i>	Spotbilled duck	1 (0)	residents
Caraciformes	Alcedinidae	<i>Halcyon coromanda</i>	Ruddy Kingfisher	7 (0)	summer
Pelecaniformes	Ardeidae	<i>Bubulcus ibis</i>	Cattle Egret	2 (2)	winter
		<i>Egretta alba</i>	Great Egret	2 (1)	winter
		<i>Egretta intermedia</i>	Intermediate Egret	3 (0)	winter
		<i>Ardea cinerea</i>	Grey heron	4 (1)	winter
		<i>Egretta sacra</i>	Pacific reef egret	1 (0)	residents
Accipitriformes	Accipitridae	<i>Butastur indicus</i>	Grey-faced buzzard	7 (0)	winter
		<i>Accipiter gularis</i>	Japanese sparrow hawk	1 (1)	residents
	Pandionidae	<i>Pandion haliaetus</i>	Osprey	1 (0)	winter
Falconiformes	Falconidae	<i>Falco peregrinus</i>	Peregrine Falcon	1 (0)	winter
Procellariiformes	Procellariidae	<i>Puffinus pacificus</i>	Wedge-tailed shearwater	1 (0)	wondering
Columbiformes	Columbidae	<i>Streptopelia orientalis</i>	Eastern turtle dove	5 (2)	residents
		<i>Sphenurus formosae</i>	Whistling Green-pigeon	5 (0)	residents
		<i>Columba livia</i>	Rock dove	1 (0)	residents
Passeriformes	Turdidae	<i>Turdus pallidus</i>	Pale Thrush	3 (0)	winter
		<i>Monticola solitarius</i>	Blue Rock Thrush	1 (0)	residents
	Hirundinidae	<i>Hirundo tahitica</i>	Pacific swallow	1 (0)	residents
	Corvidae	<i>Corvus macrorhynchos</i>	Jungle Crow	3 (0)	residents
		<i>Ninox scutulata</i>	Brown hawk owl	11 (2)	residents
Stringiformes	Stringidae	<i>Otus lempiji</i>	Sunda Scops-owl	3 (0)	residents
		<i>Larus crassirostris</i>	Black-tailed gull	2 (0)	winter

Order	Family	species	English name	n (+)*	seasonal
	Charadriidae	<i>Vanellus vanellus</i>	Northern lapwing	1 (0)	winter
	Scolopacidae	<i>Numenius phaeopus</i>	Whimbrel	1 (0)	winter
	Stercoradriidae	<i>Stercorarius longicaudus</i>	Long-tailed jaeger	1 (0)	wondering
Gruiformes	Rallidae	<i>Gallinula chloropus</i>	Common Moorhen	1 (0)	residents
		<i>Felica atra</i>	Coot	1 (0)	winter
Total (number of positive)				72 (10)	

* n=number of rescued birds. Values in parenthesis are number of JEV antibody-positive birds

Table 1. The list of injured birds rescued in Okinawa and the number of birds positive for JEV antibodies

	Chicken Negative Control (n=7)	Chicken JE vaccinated (n=4)	Aigamo duck Negative Control (n=70)	Aigamo duck WNV infected (n=7)
FRNT ₅₀	0 %	100 %	55.7 %	100 %
FRNT ₉₀	0%	100 %	1.4 %	85.7 %

Table 2. Neutralization antibody titers against JEV Nakayama with different stringencies

2.1.5 Serologic survey

Fifty percent focus reduction neutralization tests (FRNT₅₀) were conducted to measure neutralizing antibody titers against JEV strains, Nakayama (prototype strain) and Beijing-1 (laboratory strain), for mammals as described previously (Saito et al., 2009a). Ninety percent focus reduction neutralization tests (FRNT₉₀) were employed to measure neutralizing antibody titers against the Nakayama strain for wild birds. The testing was done on BHK-21 cells in 96-well microplates with an immuno-staining method as described previously (Saito et al., 2007).

2.2 Results

2.2.1 Serosurvey of wild mammals

A single sample from 45 raccoons (2.2 %) in Hokkaido had antibody titers of 70 and 40 against the Nakayama and Beijing-1 strains, respectively.

Previously obtained serologic data, showing that 35.4 % (85/240) of mongooses had antibodies for JEV (Saito et al., 2009a), was re-analyzed by GIS on different scales (Figure 4). The analysis on a small scale showed barns, bodies of water and rive paddies to be near the capture points of mongooses with high titers of JE antibody.

Both of the flying foxes captured in Okinawa had antibodies against Nakayama, with titers of 17 and 14, respectively.

2.2.2 Serosurvey of wild birds

To evaluate the reliability of testing, both FRNT₅₀ and FRNT₉₀ at different stringencies, were conducted on negative and positive control (JE immunized and WNV infected) samples of chickens and Aigamo ducks (Table 2) (Buckley et al, 2003). Non-specific reactions were observed at a high rate (55.7 %) among negative control ducks, by FRNT₅₀. Even by FRNT₉₀, a single duck showed a non-specific reaction, although the titer was low as 10. Serologic tests using Negative controls of chickens and JE-vaccinated chickens clearly showed all samples to be negative and all to be positive by FRNT₅₀ and FRNT₉₀. WNV-infected ducks as reference showed positive for JE antibody by both stringencies of test, except a single sample by FRNT₉₀. Given the results, FRNT₉₀ against JEV Nakayama was employed for the seroepidemiologic survey of wild birds.

Among 50 serum samples from 4 species of ducks; spot-billed ducks, mallards, northern pintails and Eurasian wigeons, in Hokkaido, only a single sample from spot-billed ducks (2%) recognized as migrant breeders had JE antibodies (Figure 3).

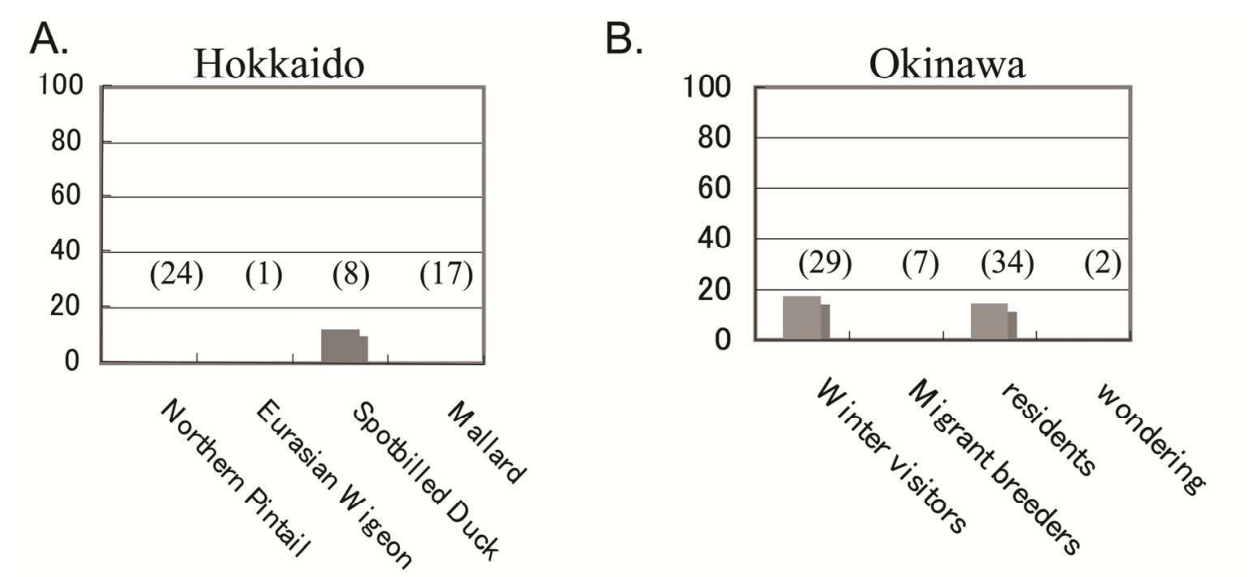


Fig. 3. Antibody-positive rates of ducks captured in Hokkaido (A) and injured birds rescued in Okinawa (B) against the Nakayama strain. Northern Pintails and Eurasian Wigeons are recognized as winter visitors, Spotbilled Ducks as migrant breeders, and Mallards as residents, and partly wonderers. Values in parentheses show numbers of bird species or seasonal groups

A total of 10 (14.2%) sera of wild birds in Okinawa had JE antibodies. Five (2 Cattle Egrets, 1 Grey heron, 1 Great Egret, and 1 Tufted duck) were from 29 winter visitors and 5 (2 Brown hawk owls, 2 Eastern turtle doves, and 1 Japanese sparrow hawk) from 34 resident breeders. Four of the five positive sera in winter visitors were from *ardeid* birds. None of the migrant breeders had antibodies (Table 1 and Figure 3).

3. Discussion

A seroepidemiologic study was conducted on wild mammals and wild birds in Hokkaido and Okinawa, Japan, and risks for JEV infection and JEV invasion were evaluated by

knowing local transmission activity and the seroprevalence of migratory species, respectively. JEV activity in Hokkaido is known to be low, as demonstrated by serosurveys of pigs (Infectious Disease Surveillance Center), although focal outbreaks occurred in pigs in the 1980s (Takashima et al. 1988). In Okinawa, high JEV transmission rates have been demonstrated by serosurveys of pigs indicating the longest JEV active season in Japan, and the virus has been isolated even in winter (Ura, 1976, Tadano et al. 1994, Infectious Disease Surveillance Center). About 100 JE cases were reported with fluctuations until the mid 1960s. The last reported case was in 1998. Seroconversion in pigs was used for JE surveillance for many years, nationwide in Japan, and thus is generally accepted as reflecting the JEV transmission rate in an area.

We reported a serosurvey of mongooses in Okinawa, valuable for understanding the local transmission activity of JEV in nature. By using mongooses under control projects, it is possible to analyze capture points and antibody titers, enabling estimates of high transmission areas on a small scale (Figure 4A) (Saito et al., 2009a). The environmental condition at the capture point for animals with high antibody titers were suitable for the transmission of JEV, i.e. rice fields and bodies of water for the breeding of vector mosquitoes and barns for pigs as an amplifier of JEV (Figure 4B). However, mongooses mostly inhabit only the Okinawa and Amami islands in Japan. By contrast, raccoons, another alien species is distributed over a wide range in Japan, though not in Okinawa. In this study, a serosurvey of raccoons was conducted in Hokkaido, showing low positive rates with only a single sample having JE antibodies, thus implying low transmission of JEV there. Ohno et al (2009) also conducted a comparative serosurvey of raccoons in Hokkaido and in the Kinki area of western Japan, showing a high prevalence of JEV in western and low prevalence in northern areas. Thus, the animal appears to be a good sentinel for JEV infection. In this study, the suitability of risk assessments with serosurveys of alien animals under control projects, together with fundamental data analysis is indicated.

We also conducted a serosurvey of migratory species. It was reported that flying foxes have a potential role in the transmission cycle and dispersal of JEV (van den Hurk AF et al, 2009). Antibodies against JEV with low titers were observed in both flying foxes caught in Okinawa, a species that can fly short distances within the island or to nearby islands. Flying foxes might play a role in the dispersal of JEV on the island.

Interestingly, in this serosurvey of wild birds, all of the positive ducks collected in Hokkaido, 2008 were Spotbilled ducks, a migrant breeder in Hokkaido, and seemed to have been infected in the south (Saito et al, 2009b). Results from our studies of raccoons and wild ducks showed low transmission rates of JEV in Hokkaido, consistent with those for pigs and other species.

The yearly sero-conversion of JEV in pigs starts in Okinawa, and moves to the north. This phenomenon may be caused by the migration or hibernation of JEV. The migratory flyways of migrant breeders such as the Ruddy Kingfisher correlate with this movement, from south, Australia, to north, Siberia, through Okinawa, and mainland Japan. However, no migrant breeder tested negative for the JEV antibody in this study. Nabeshima et al (2009) described that JEV is frequently introduced into Japan from Southeast Asia and continental east Asia, based on a molecular epidemiologic study and chronological order. Okinawan genotype 1 strains are closely related to strains from other parts of Japan, Korea and China, but different from Australian strains (Saito et al., 2007). Recently genotype 1 with the same subcluster as Kyushu and Shanghai appeared in Taiwan, in 2007, approximately 10 years after their first

appearance in China, Korea, and Japan (Huang et al, 2010). Thus, the direction of JEV's introduction seemed to be from north to south. Five winter visitors with antibodies were observed, 2 Cattle Egrets, 1 Grey Heron, 1 Great Egret and 1 Tufted duck. They are thought to have been infected in the north, not in Okinawa, because of seasonal characteristics. *Ardeid* birds, Egrets and Herons are known amplifiers of JEV and involved in JEV invasion (Buescher EL et al, 1959). From the results of this study, together with molecular epidemiologic studies, JEV is suggested to have been introduced into Okinawa from Honshu, and Kyushu, possibly by a winter visitor such as *ardeid* birds. The flyways of most *ardeid* birds remain unknown. Their elucidation and continuous epidemiologic and virologic studies will provide important information for evaluating risks of JEV invasion. Migratory birds are one of the determinants with which to assess invasive viruses like not only JEV, but also flaviviruses with a similar epidemiology.

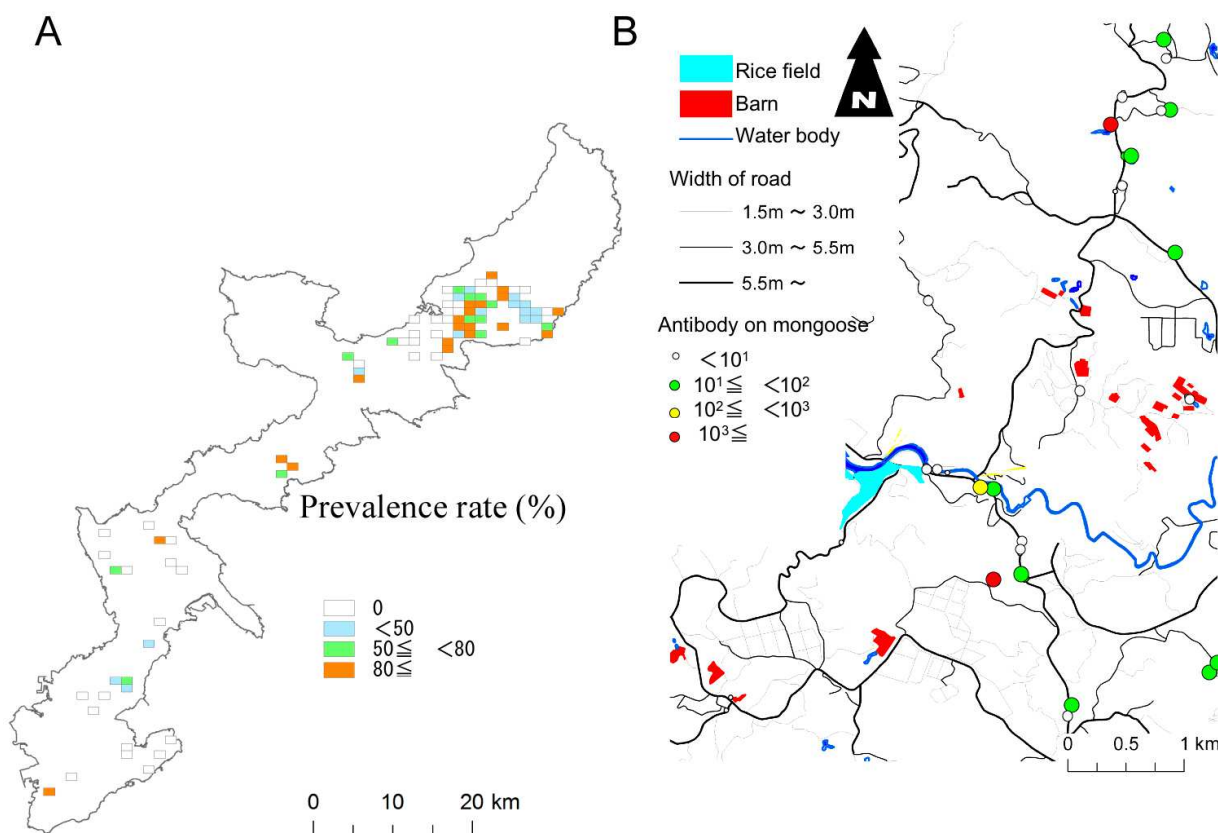


Fig. 4. Map of Okinawa Island used for the GIS analysis using mesh with prevalence rates of JE antibody in mongooses (ref. Saito et al, 2009a) (A). Map of high prevalence areas with the location of farms (B). Dots show capture points and the JE antibody titer

JEV antibodies were detected in 14.3 % (5/35) of resident breeders in Okinawa between 2007 and 2009, a relatively low rate. This however may be due to the inclusion of data from a variety of species and sizes, differing in sensitivity and mosquito preference. In Okinawa, serosurveys of pigs have showed that seroprevalence has gradually decreased since the mid 2000s and was less than 20 % in 2009 and 2010, which indicated that low transmission activity (Infectious Disease Surveillance Center) might correlate with seroprevalence in resident breeders and mongooses.

The Ministry of Health, Labour and Welfare in Japan discontinued the recommendation for JE vaccinations due to adverse events for 5 years from 2005. During this period, no epidemic of JE occurred in Japan, and no cases were reported in Okinawa either. Risk assessments for JE should be strengthened to evaluate the quick and drastic changes in natural transmission that might be occurring in Okinawa with an interdisciplinary approach, including studies of mosquitoes, serosurveys of people, domestic and wild animals, and vaccine evaluation. A reconsideration of JE control is required in step with recent changes.

4. Conclusion

The distribution and occurrence of JE are changing due to environmental influences. Therefore, risk assessment and surveillance are important for controlling JE. Serosurveys of pigs as amplifiers are the predominant means of assessing risks of JEV infection, and also sustainable. Wild mammals such as raccoons and mongooses are valuable sentinels for assessing natural transmission, and strengthen the notion of surveillance using pigs, while control programs for alien mammals provide the opportunity for a detailed geographic analysis of JEV transmission. Virologic and serologic surveys of wild birds, particularly *ardeid* birds as possible amplifiers, and bats should be sustained to assess risks of JEV invasion. Furthermore, they may reveal the mechanisms by which the distribution of JEV is expanding.

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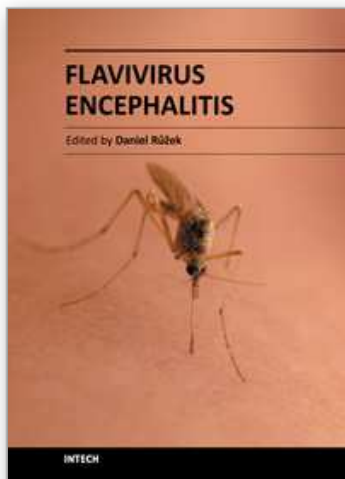
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Encephalitis is an inflammation of the brain tissue associated with clinical evidence of brain dysfunction. The disease is of high public health importance worldwide due to its high morbidity and mortality. Flaviviruses, such as tick-borne encephalitis virus, Japanese encephalitis virus, Murray Valley encephalitis virus, or St. Louis encephalitis virus, represent important causative agents of encephalitis in humans in various parts of the world. The book *Flavivirus Encephalitis* provides the most recent information about selected aspects associated with encephalitic flaviviruses. The book contains chapters that cover a wide spectrum of subjects including flavivirus biology, virus-host interactions, role of vectors in disease epidemiology, neurological dengue, and West Nile encephalitis. Special attention is paid to tick-borne encephalitis and Japanese encephalitis viruses. The book uniquely combines up-to-date reviews with cutting-edge original research data, and provides a condensed source of information for clinicians, virologists, pathologists, immunologists, as well as for students of medicine or life sciences.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
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InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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