ORIGINAL ARTICLE

SEROPREVALENCE OF JAPANESE ENCEPHALITIS VIRUS AMONG DOMESTIC ANIMALS AND RATS IN MINDANAO, PHILIPPINES

Emmanuel T. Baltazar, DVM, MS, PhD[1]*, Jose Alexander C. Abella, DVM, MS, PhD[1], Alan P. Dargantes, DVM, MTVSc, PhD[1], Luzviminda T. Simborio, DVM, MS, PhD[1], Reu Caesar James T. Bautista, DVM[1], Nikko Manuel A. Dinopol, Valeen Drex Bendette M. Ebora, DVM[1], Hiroshi Shimoda, DVM, PhD[2], Ryusei Kuwata, PhD[3], Eiichi Hondo DVM, PhD[4] and Ken Maeda, DVM, PhD[5]

[1]College of Veterinary Medicine, Central Mindanao University, University Town, Musuan, Bukidnon, 8714 Philippines; [2]Laboratory of Veterinary Microbiology, Joint Faculty of Veterinary Medicine, Yamaguchi University, 1677-1 Yoshida, Yamaguchi City 753-8515, Japan; [3]Faculty of Veterinary Medicine, Okayama University of Science, 1-3 Ikoinooka, Imabari City, Ehime Prefecture, 784-8555 Japan; [4]Laboratory of Animal Morphology, Nagoya University, Nagoya City, Japan; [5]Department of Veterinary Science, National Institute of Infectious Diseases, Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

ABSTRACT

The Japanese encephalitis virus (JEV), an arthropod-borne virus chiefly transmitted by mosquitoes, can infect humans, domestic, and wild animal species with usually subclinical manifestation in humans, and animals but can cause acute encephalitis in humans and horses, and reproductive illness in pigs. This serological survey confirmed JEV antibodies in various domestic animals and rats in Mindanao by indirect ELISA with an overall apparent seroprevalence of 12.6% (570/4,525 total samples). Animal species variation was observed, which can be attributed to the differences in behavior and raising management, and feeding behavior of mosquito vectors, and some are amplifying hosts of the virus. Regional variation was also scrutinized, which may be due to differences in vegetation, altitude, and water bodies influencing temperature and humidity that influence feeding behavior and reproduction of mosquito vectors. Results indicate that JEV has been circulating among domestic animals and rats, showing a potential human risk through mosquito bites. Results of this study may widen the understanding of the epidemiology of JEV in Mindanao.

Keywords: *Japanese encephalitis virus, seroprevalence, animals, Mindanao, Philippines*

Philipp. J. Vet. Med., 59(1): 46-52, 2022

INTRODUCTION

Mindanao and its smaller islands make up Southern Philippines. This extensive island group (120,800 km² of land area) has three types of climates (Types II, III, and IV), with types III and IV taking over majority of Mindanao and type I only observed in Surigao and the Dinagat Islands (DOST-PAGASA, 2010; PSA, 2005). It is inhabited by approximately 25.5 million people and has an estimated livestock population of 14.4 million heads (PSA, 2018).

Japanese encephalitis virus (JEV) is an arthropod-borne zoonotic virus of the genus *Flavivirus* under family *Flaviviridae.* The virus has only one serotype and at least five genotypes (Solomon *et al*., 2003). It is transmitted by mosquitoes mainly, *Culex tritaeniorhynchus,* although it may vary in different geographic locations (Mackenzie *et al*., 2004). *Culex tritaeniorhynchus* is known to lay eggs in irrigated rice paddies and in other pools of stagnant water (WHO, 2018). These mosquitoes are abundant in the Philippines, including Mindanao (Miyagi *et al*., 1985). Among several animals which are known as hosts of JEV, pigs are the most significant amplifier hosts because of high JEV titers during viremia in infected pigs (Ilkal *et al*., 1994). Ardeid birds are known to be the natural reservoir hosts of the virus and the infection is widely endemic among humans in Asia, including the Philippines

***FOR CORRESPONDENCE**:
(e-mail: etbaltazar@yahoo.com)

(WHO, 2006), causing meningitis and encephalitis with a significant number of mortality (Lopez *et al*., 2015). In animals, antibodies to JEV were first detected in the Philippines in 1943 among horses (Hammon *et al*., 1958). On the other hand, the first serologically confirmed case in humans was that in an American soldier stationed in the country in 1956 (Southam, 1956). Since then, human cases have also been reported in Mindanao (Southern Philippines) with mortalities in some years (DOH, 2019). Despite the importance of animals as potential sources of JEV in humans, limited epidemiological studies have been conducted in the Philippines (Lopez *et al*., 2015). In this study, indirect enzyme-linked immunosorbent assay (ELISA) was employed to detect the presence of JEV antibodies in the sera of various potential animal host species in Mindanao, Philippines. Specifically, this study compared the seroprevalence of JEV antibodies by species, i.e., pigs, dogs, cattle, buffaloes, goats, and rats, and by region. The results of this study will enhance the understanding of the epidemiology of JEV in Mindanao, Philippines.

MATERIALS AND METHODS

Sampling Areas

The serosurvey of domestic and wild animal species was conducted in all of the following regions of Mindanao: Zamboanga Peninsula (Region IX), Northern Mindanao (Region X), Davao (Region XI), SOCCSKSARGEN (Region XII), and Caraga (Region XIII), except BARMM (Bangsamoro Autonomous Region of Muslim Mindanao) due to issues on the accessibility of the area during the conduct of the study (Figure 1). Stratified random sampling was employed to identify the research areas with the region, province, town, and barangay as the strata (Table 1). At least 30% of the provinces in each region were included in the survey. From each of the identified provinces, 20% of its towns/cities were randomly selected, and 10% of the barangays in those chosen towns/cities were randomly selected. Randomization was done using random numbers generated in Microsoft Excel. Overall, eight provinces from five regions were sampled, with 86 barangays from 22 municipalities/cities.

Table 1. Sampling matrix of the provinces, towns/cities, and barangays surveyed in each region in Mindanao, Philippines.

Region	Province	Town/City	Barangays	Total
Region IX	Zamboanga del Sur	Aurora	Poblacion, Kauswagan, Lintugop, Lubid	4
		Mahayag	Poblacion Upper Mahayag, Boniao, Tuboran	3
		Molave	Madasigon Poblacion, Rizal, Dipolo	3
		Pagadian	Sto. Nino, Sta Maria, Tulangan, Lala, Kahayagan	5
		Ramon Magsaysay	Mabini, Poblacion, Esperanza	3
		San Miguel	Laperian, Dumalian	2
		Kibawe	Labuagon, Old Kibawe	2
		Maramag	Dologon, Panadtalan, Anahawon	3
Region X	Bukidnon	Pangantucan	Poblacion, Lantay, Adtuyon	3
		Valencia	Kahaponan, San Isidro, Concepcion, Laligan	4
	Camiguin	Catarman	Poblacion, Alga, Compol	2
Region XI	Davao del Sur	Davao City	Bankas Heights, Bago Oshiro, Tugbok Proper, Matina Aplaya, Malagos, Matina Pangi, Callawa, Indangan, Vicente Hizon Sr., Biao Joaquin, Tawan-Tawan, Taman, San Isidro, Lamanan, Tibungko, Alejandra Navarro, Dominga Malamba	18
		Digos City	Zone 1 Poblacion, Mahayahay, Soong	3
	Davao del Norte	Sto Tomas	New Katipunan, Talomo	2
		Tagum	Cuambugan, Pandapan	2
Region XII	South Cotabato	General Santos City	Mabuhay, Tambler, Ligaya	3
		Polomolok	Poblacion, Palkan	2
Region XIII	Agusan del Sur	Bayugan	Noli, Maygatasan, Fili	3
		Esperanza	Langag, New Gingoog, Duangan, Guadalupe, Hawilian	5
		Talacogon	Del Monte, Labnig	2
	Agusan del Norte	Butuan City	Baan KM3, Ampayon, Taguibo, San Mateo, Dumalagan, Kinamlutan, Pinamanculan, Pianing, Bonbon	9
		Buenavista	Rizal, Sangay, Malpoc	3
Total	**8**	**22**		**86**

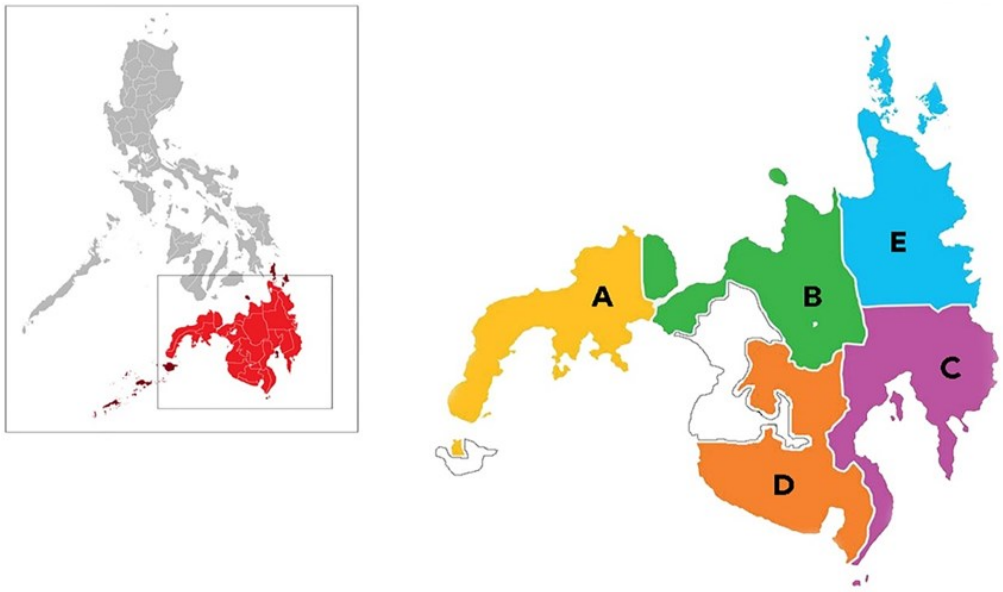

Figure 1. Map of Mindanao, Philippines showing the sampled regions: Region IX/Zamboanga Peninsula (A), Region X/Northern Mindanao (B), Region XI/Davao Region (C), Region XII/ SOCCSKSARGEN (D) and Region XIII/Caraga (E).

Sample Size

The minimum sample size of 139 for each domestic animal species (cattle, buffalo, goats, dogs, pigs) and rats in each region was computed using Epitools Epidemiological Calculators (Sergeant, 2018) based on the following assumptions: (a) 10% disease prevalence; (b) 95% confidence level; (c) 5% precision; and, (d) an infinite population for a particular animal species. The sample size for each animal species per region was increased to 144 to increase reliability. A total of 4,525 animals (domestic animals and rodents) were sampled in Mindanao.

Sampling

This study was conducted with Central Mindanao University bioethics (IERC Control Number: 0131-2019) and Institutional Animal Care and Use Committee (Protocol Number: 2018-43B) clearances, and gratuitous permits (R-10 2017-01, R9-02-2018) of the Department of Environment and Natural Resources. Furthermore, written consent from each respondent/animal owner was secured before the survey and interview.

Blood samples were collected following standard protocols. The animals were physically restrained and blood samples were collected at the following sites: jugular/tail vein for cattle and buffaloes, jugular vein for goats, cephalic vein for dogs, anterior vena cava/or its surrounding vessels for pigs, and heart for rats. Blood samples were obtained using sterile vacutainers/hypodermic syringes. Proper disinfection and antisepsis were observed throughout the blood collection.

During field sampling and before centrifugation, freshly collected blood samples in vacutainer tubes were allowed to stand in a slanting position inside buckets with ice packs. Then, centrifugation was done for 15 minutes at 3,000 rpm. The extracted sera were placed in individual microcentrifuge tubes and stored in a portable freezer at -20°C during transport, and at -80°C in the laboratory freezer until further use.

ELISA

ELISA for surveillance of JEV among various animals was performed using protein A/G as detecting protein according to previous report (Shimoda *et al.,* 2014 & 2019). Briefly, JEV or mock-infected BHK-21 cells were lysed with RIPA buffer [25 mMTris-HCl (pH 7.6), 150 mM sodium chloride, 1% sodium dodecyl sulfate, 1% sodium deoxycholate, and 1% Triton X-100], and was used as antigen for ELISA (Suzuki *et al*., 2015). The antigen concentration was adjusted to 5 µg/mL with an adsorption buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6). Diluted antigen was added to 96-well microplates at 100 µL/well (Maxisorp; Nunc, Roskilde, Denmark). After incubation at 37°C for two hours, plates were placed at 4°C until use. Non-adsorbed antigens were discarded. One percent (1%) Block Ace (Dainippon Pharmaceutical, Osaka, Japan) in Phosphate Buffered Saline then was added at 200 µL/well. The plates were then incubated at 37°C for 30 minutes. After washing the wells three times with PBS containing 0.05% Tween 20 (PBS-T), diluted serum (1:100 in PBS-T containing

0.4% Block Ace) was added at 100 µL/well to duplicate wells, and the plates were incubated at 37°C for 30 minutes. Then, the wells were washed three times with PBS-T before the addition of 100 µL/well of Peroxidase Conjugated Purified Recomb® Protein A/G (Thermo Fisher Scientific, Waltham, MA, USA) diluted with PBS-T containing 0.4% Block Ace, and plates were incubated at 37°C for 30 minutes. Following three washes with PBS-T, 100 µL of horseradish peroxidase substrate (Bio-Rad, Hercules, CA, USA) was added to each well. Then, after incubation at room temperature for 30 minutes, the enzymatic reaction was stopped by adding 100 µL of 2% oxalic acid to each well. The optical density (OD) was measured in a spectrophotometer (ELx808, BioTek Instruments, Winooski, Vermont, USA) using a 405-nm filter. The resulting OD values were corrected by subtracting the average OD values of antigen wells with the average OD values of mock-infected cells. For this ELISA, the OD values of ≥0.5 were considered seropositive and OD values of <0.5 were tentatively considered seronegative.

Statistical Analysis

The apparent Seroprevalence of JEV was computed using a published formula (Thrusfield, 2007). Prevalence was computed by dividing the number of positive samples by the total number of tested animals and was expressed as a percentage.The odds ratio (OR) and its 95% confidence interval (95% CI) were computed. Fisher's exact test was also used to determine the significant differences on the prevalence estimates among and between animal species and regions included in the study. In all statistical tests, a significance level of $p<0.05$ was used.

RESULTS AND DISCUSSION

Seroprevalence of Japanese encephalitis virus among domestic animals and rats in Mindanao

Overall, 13% (570/4525; 95% Confidence Interval, CI: 11.7-13.6) of the pigs, goats, cattle, buffaloes, dogs, and rats sampled in Mindanao (Table 2) harbored antibodies against Japanese encephalitis virus using indirect ELISA. Pigs had the highest JEV seroprevalence at 27.7% (CI: 24.6-31.0), followed by goats (13.4%, CI: 11.1-16.0), cattle (12.3%, CI: 10.2-14.9), buffaloes (10.5%, CI: 8.5-12.9), dogs (8.8%, CI: 7.0-11.0), and rats (2.4%, CI: 1.5-3.7).

The prevalence of JEV in pigs (27.7%) was highest ($p<0.05$) among all tested animals in Mindanao. A previous study isolated JEV belonging to GIII from Mindanao (Kuwata *et al.*, 2020). Results of the current study mean that pigs in the sampled areas were most likely to harbor JEV antibodies than those other tested animals (OR (95% CI): 15.9 (9.6, 26.4)). This can be attributed to the life cycle of the Japanese encephalitis virus in which pigs are considered to be the most significant amplifying hosts in the

Table 2. Seroprevalence of JEV antibodies in Mindanao, Philippines by animal species.

Animal Species	n Tested	n Positive	% Apparent Prevalence (95% CI)	Comparison	Odds Ratio (95% CI)
a. Pigs	762	211	27.7 (24.6-31.0)	a vs. b* a vs. c* a vs. d*	15.9 (9.6-26.4)
b. Goats	762	102	13.4 (11.1-16.0)	b vs. c^{ns} b vs. d^{ns} b vs. e*	6.4 (3.8-10.9)
c. Cattle	754	93	12.3 (10.2-14.9)	c vs. d^{ns} c vs. e^{ns} c vs. f*	5.9 (3.5-9.9)
d. Buffaloes	760	80	10.5 (8.5-12.9)	d vs. e^{ns} d vs. f*	4.9 (2.9-8.3)
e. Dogs	763	67	8.8 (7.0-11.0)	a vs. e* e vs. f*	4.0 (2.3-6.9)
f. Rats	724	17	2.4 (1.5-3.7)	a vs. f* b vs. f*	1.0 (0.5, 2.0)
TOTAL	**4,525**	**570**	**13 (11.7-13.6)**		

N- Number of samples; CI- Confidence Interval; ns- Not significant; *- Significant at 5%

supply of virus in the blood for infection of feeding mosquitoes (Ilkal *et al*., 1994). Also, pigs develop high-prolonged viremias compared to other animals, and, therefore, they may serve as a bridge for the infected vector mosquitoes to initiate epizootics and epidemics among susceptible humans (Endy & Nisalak, 2002). Results of this study indicate that JEV-seropositive pigs could pose a threat to human health, especially that most of those tested were "smallhold pigs" which constitute about 64% of pigs in the Philippines (PSA, 2018). As such, they are raised close to the human dwellings, thereby enhancing the likelihood of human transmission.

Interestingly, next to pigs, JEV seroprevalence estimates in goats (13.4%), cattle (12.3%) and buffaloes (10.5%) are similar. This could be accounted to the host preference of Culex tritaeniorhynchus, the primary JEV vector, which predominantly feeds on cattle, goats, buffaloes, and humans (Arunachalam et al., 2005). Moreover, it was observed in this study that most cattle, goats, and buffaloes are commonly tethered or pastured near swampy fields, rice paddies, or stagnant water sources where the vector mosquitoes may favorably breed and reproduce, thus, increasing the risk of these animals in getting infected with JEV. Dogs (8.8%) and rats (2.4%) among the animals tested, have the lowest JEV seroprevalence, which could also be accounted to the mosquito vector preference for larger and less active mammalian hosts (Mwandawiro et al., 1999). Likewise, rats with only 17 infections of JEV are the least likely to be infected with JEV than other tested animals (OR (95% CI): 1.0 (0.5, 20.0)).

Seroprevalence of Japanese encephalitis virus among domestic animals and rats by region

Results show that JEV seropositive animals were widely distributed in the five regions of Mindanao surveyed (Table 3). The JEV seroprevalence differs from region to region which may be due to variations in geographical position including vegetation, altitude, and water bodies (Shimoda *et al.,* 2013). Thus, influencing the temperature and humidity, in which changes of these environmental factors can directly affect the feeding behavior and reproduction of the mosquito vectors accordingly (Pearce *et al*., 2018).

Nevertheless, the highest number of JEV seropositive animals in Mindanao is in Region XIII (Caraga; 23.5%), implying that the animals sampled in this region are most likely to be positive with JEV infection than animals in other regions in Mindanao (OR (95% CI): 8.9 (6.1-13.1)). In contrast, animals sampled in Region IX are the

Table 3. Seroprevalence of JEV antibodies in Mindanao, Philippines by region.

Region	n Tested	n Positive	% Prevalence (95% CI)	Comparison	Odds Ratio (95% CI)
a. Region XIII *Caraga Region*	924	217	23.5 (20.9-26.3)	a vs. b* a vs. c*	8.9 (6.1-13.1)
b. Region X *Northern Mindanao*	841	159	19.0 (16.4-21.7)	b vs. c* b vs. d*	6.8 (4.6-10.0)
c. Region XI *Davao Region*	900	85	9.4 (7.7-11.5)	c vs. d^{ns} c vs. e*	3.0 (2.0-4.6)
d. Region XII *SOCCSKSARGEN*	900	77	8.6 (6.9-10.6)	a vs. d* d vs. e*	2.7 (1.8-4.1)
e. Region IX *Zamboanga Peninsula*	960	32	3.3 (2.4-4.7)	a vs. e* b vs. e*	1.0 (0.61-1.65)
TOTAL	**4,525**	**570**	**13 (11.7-13.6)**		

n- Number of samples; CI- Confidence Interval; a- Region XIII; b- Region X; c- Region XI; d- Region XII; e- Region IX; ns- Not significant; *- Significant at 5%

least likely to be infected with JEV (OR (95% CI): 1.0 (0.61-1.65)). This may be attributed to the fact that most randomly selected sampling sites in Region XIII have or are near rivers, lakes, swamps, marshes, and rice paddies. In addition, there are two large bodies of freshwater, i.e., Lake Mainit and Agusan Marsh, where breeding and reproduction for the mosquito vectors are suitable which likely causes the highest exposure of JEV to the animals among all regions studied. This area is followed by Region X (Northern Mindanao; 19.0%) where sampling sites have similar geographical description, i.e., with lakes, rivers, and rice paddies as in Region XIII, but have a higher average altitude of 246 meters above Mean Sea Level (m MSL) compared to 137 m MSL of Region XIII (PhilSurv, 2019) that directly influences the preference to mean temperature and humidity of the mosquito vectors for their breeding and reproduction (Pearce *et al*., 2018). The population of *Aedes and Culex* mosquitoes is strongly influenced by altitude, wherein their populations significantly decline with increasing altitude (Asigau *et al*., 2017). Thus, a reduced mosquito vector population could lessen the exposure of animals to JEV, resulting to a lower prevalence in the region. The blood collections in Regions XI (Davao Region; 9.4%), XII (SOCCSKSARGEN; 8.6%) and IX (Zamboanga Peninsula; 3.3%) were conducted during the hot, dry season, that may dry up most of the breeding sites for the mosquito vectors. Also, the sampling sites were primarily located on or near coconut plantations, pineapple plantations, and some areas were urbanized through community houses and buildings which might contribute to lower prevalence estimates in these regions because of the affected mosquito vector population.

This study serologically confirmed the presence of antibodies against JEV among various domestic animals and rats in Mindanao by indirect ELISA. Animal species variation on seroprevalence estimate may be due to differences in their behavior and raising management, feeding behavior of mosquito vectors, and some animals are amplifying hosts of the virus. Likewise, there is a regional variation which may be due to varied vegetation, altitude, and water bodies influencing the temperature and humidity which, in turn, influence the feeding behavior and reproduction of the mosquito vectors.

It is recommended that measures such as mosquito vector control, management of the environment, changes in agricultural practices, mitigation of public health risk, and exposure to good hygiene, education, and awareness be undertaken. Moreover, Local Government Units (LGUs) and concerned government offices shall conduct annual JEV surveillance in both humans and animals and formulate related ordinances that may help reduce the risk of JEV infections.

STATEMENT OF COMPETING INTEREST

The authors have no competing interests to declare.

AUTHOR'S CONTRIBUTION

All the authors contributed in full or in part in the conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, visualization, and in writing, reviewing, and editing of the paper.

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