

## Incidence of Japanese Encephalitis among Children is associated with the Presence of Pigs in Bali, Indonesia

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### ABSTRACT

Japanese encephalitis (JE) is an encephalitic arboviral disease transmitted by mosquitoes with pigs as one of its amplifying hosts. JE is still endemic in Bali and affects mostly children. People in Bali still rear pigs and live in close proximity to irrigated rice fields which is a perfect habitat for mosquitoes. However, few studies have examined the seroprevalence of JE virus (JEV) in pigs as a risk factor for JE in children in Bali together with pigpen location from houses and irrigated rice fields. Therefore, blood samples were collected from pigs in 5 regencies in Bali where JE cases were reported. Indirect enzyme-linked immunosorbent assays were performed on the sera to detect IgM and IgG antibodies against JEV. A total of 80 pig sera were assayed, 60% of which were positive for antibody against JEV. The seroprevalence of JEV-IgM and JEV-IgG was 20% and 40%, respectively. There were three risk factors that had significant associations ( $p < 0.05$ ) with JE in children such as JEV-IgG-positive pigs, pigpen location  $<10$  meters from the household, and distance of  $<500$  meters between irrigated rice field and pigpen with an IgG-positive pig. In conclusion, the presence of pigs still plays an important role in the JEV transmission cycle in Bali, as demonstrated by the high seroprevalence of JEV-IgG in pigs, and that pigs kept close to households and irrigated rice fields were risk factors for JE in Bali.

**Keywords:** Japanese Encephalitis, IgG, IgM, pig, children.

### INTRODUCTION

Japanese encephalitis (JE) is a zoonotic disease caused by Japanese encephalitis virus (JEV) infection which is transmitted by mosquitoes from pigs as the main amplifier host to human and other vertebrates. JEV is still a major cause of viral encephalitis in Asia and can cause severe brain damage in children. There are 24 JE endemic countries in Asia, including Indonesia, according to the WHO/UNICEF survey in 2011–2012. The number of JE cases have decreased drastically

recently, because some countries have implemented vaccination programs among children. In Indonesia, JE cases are still frequently noted since there is no implementation of vaccination programs. Moreover, JE cases are not well documented due to the lack of support for JE diagnosis in health facilities, and JE is still a neglected disease in Indonesia.<sup>1</sup>

The highest number of JE cases in Indonesia occurred in Bali, a small island located in the middle of the Indonesian archipelago. In 2006, Kari et al. reported that the JE incidence

rate was 8.2 per 100,000 children under 10 years, 10% of whom died and 37% had sequelae. It is believed that the actual number of JE cases in children is higher than the reported cases due to poor surveillance and lack of diagnosis.<sup>2</sup> The latest number of cases of JE infection from Bali Province Health Department during 2014–2015 were as many as 44 children diagnosed with JE among 452 viral encephalitis cases (unpublished). JE positive cases in children should be a major concern as the disease is zoonotic.<sup>3</sup>

Pig rearing is the main risk factor for JE transmission to humans and rearing pigs is common in Bali. The population could reach 860,117 pigs, whereas the total area in Bali is only 5.636 km<sup>2</sup>.<sup>4</sup> Pigs serve as amplifier hosts for JE because the virus replicates more efficiently in pigs than in other animals. In the viremia stage, a pig is the crucial reservoir for JE transmission to other vertebrates or humans in the presence of mosquitoes. The mosquito *Culex tritaeniorhynchus* is a competent JE vector and is found very abundantly throughout the year in the tropics. Irrigated rice fields are one of the main breeding sites for these mosquitoes.<sup>5</sup> Bali is potential for their breeding sites since it has 80,886 ha of irrigated rice fields.<sup>4</sup> There was a significant relationship between rice fields and *Cx. tritaeniorhynchus* density in the Republic of Korea and quantification of rice fields could be a parameter to predict the abundance of the mosquitoes.<sup>6</sup>

The presence of pigs as a risk factor for JE has been studied elsewhere in the world; however, there is limited information about their role in Bali, Indonesia. Studies on the prevalence of antibody against JE among pigs was conducted in Bali in 2006 and 2008; however, there is no study assessing the seroprevalence of JE in pigs as a risk factor for JE in children in Bali.<sup>7,8</sup> JEV surveillance in pigs and its risk factors should be performed annually to understand the dynamics of JEV transmission in order to anticipate the emergence of JE.<sup>9</sup> Therefore, in this study, serological tests for immunoglobulin (Ig) M and IgG antibodies against JEV in pigs were conducted to investigate the prevalence of JE, to monitor its transmission in the areas where JE cases in human were reported, and to assess several risk factors contributing to JE in children.

## MATERIALS AND METHODS

### Ethical considerations

The study protocol was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Udayana University. All pig owners agreed to participate by signing the informed consent.

### Samples

Blood samples were from pigs collected during January–November 2015 from five regencies in Bali, namely, Buleleng, Negara, Tabanan, Badung, and Karangasem. The blood samples were collected based on three inclusion criteria. Firstly, the pigs had to be located within a radius of less than 500 meters from the houses of children who were JE cases and agreed to participate in the study. The distance of 500 meters is regarded as the effective flight range of the mosquito. Secondly, the pigs must have been reared prior to the children being confirmed as JE cases. The sera were separated by centrifugation, and stored at -20°C until they were processed further. Pigsty distance from the household and presence of an irrigated rice field nearby, as the risk factors, were also observed and recorded. The information about children with a positive JE diagnosis were obtained from the Ministry of Health, Bali Province (unpublished).

### Detection of anti-JEV IgM and IgG

Indirect enzyme-linked immunosorbent assay (ELISA) was performed to detect both IgM and IgG antibodies against JEV according to the protocol by Adi *et al.*<sup>8</sup> Ninety-six-well ELISA plates (Thermo Scientific™ Nunc™, USA) were coated with 100 µL JEV antigen diluted 1:50 in 0.1 M carbonate buffer, pH 9.6 at 4°C overnight. The plates were washed three times with ELISA washing buffer (0.01% Triton-X-100 in phosphate-buffered saline (PBS)). Thereafter, the reaction in each well was blocked with 200 µL of 3% skim milk in PBS, and incubated for one hour at 37°C. After being washed as described above, the pig sera were added to each well which was tested using the dilution 1:200 (1 µL serum : 200 µL 3% skim milk in PBS). The plates were washed three times and then reacted with 1:3000 diluted Rabbit anti-swine IgM-HRP (Rockland, USA) for IgM detection, or Goat anti-pig IgG-HRP (KPL, USA) for IgG detection, and kept at room temperature for one

hour. After the plates were washed three times as described above, 100  $\mu$ L substrate 1-Step™ Turbo TMB-ELISA (Thermo Fisher Scientific Inc., USA) was added and plates were incubated in the dark at room temperature for 10 min. Stop solution (50  $\mu$ L H<sub>2</sub>SO<sub>4</sub> 6 N) was added to each well and the optical density (OD) was read by an ELISA reader at 490 nm. The cut-off value was determined in each ELISA plate by calculating the mean of the negative control plus six times its standard deviation. Calculation of the covariance was required to determine the boundary between negative and positive results. The sera with optical density values that exceeded the cut-off value were considered as JEV-IgM or IgG positive.<sup>8</sup>

The chi-square test was conducted to analyze the association between risk factors of JEV-IgM-positive pig, JEV-IgG-positive pig, pigsty location from the house, distance between rice fields and pigsty, for the development of JE among children. The statistical significance level was set at  $p < 0.05$ .

## RESULTS

Blood samples were collected from a total of 80 pigs from 43 households that met the inclusion criteria. The ELISA results showed that 60% of the pigs were positive for anti-JEV antibodies with 20% of them being positive for IgM and 40% for IgG (Table 1). The JEV-IgM in pigs was the highest in Badung with 6 (31.58%), followed by Negara 5 (25%), Karangasem 3 (18.75%), Tabanan 2 (13.33%), and Buleleng none. The JEV-IgG was also the highest in Badung at 10 (52.63%), Buleleng 5 (50%), Negara 8 (40%), Tabanan 5 (33.33%), and Karangasem 4 (25%).

The results of JE risk factors in children revealed that JEV-IgG-positive pigs were significantly associated with JE in children ( $p=0.01$ ). Pigsty location <10 meters from the household showed a significant association with JE in children ( $p=0.036$ ). Similarly, the distance between an irrigated rice field and pigsty <500 meters with an IgG-positive pig became a risk factor for JE in children ( $p=0.02$ ). Two other variables, JEV-IgM-positive pigs and distance between irrigated rice field and pigsty >500 meters, had no significant association with JE in children (Table 2).

## DISCUSSION

Seroprevalence of JEV in pigs in Bali and risk factors contributing to the JE cases in children were demonstrated in this study. Seroprevalence of anti-JEV antibody in pigs among regencies might not reflect the actual conditions; it could possibly be higher since the majority of pigs were sold by their owners as soon as JE cases in children around their houses became known. JEV seroprevalences found in this study were similar to those reported previously. Adi *et al.*, reported that the seroprevalence of JEV-IgG in pigs in Bali reached 32.2% in 2006 and 49% in 2008.<sup>9</sup> This proves that JEV is still actively circulating in Bali since it was first reported in 1960.<sup>9</sup> In other JEV-endemic countries in Asia, JEV seroprevalence in pigs were varied, ranging from 4.5%–70% in Japan, 60%–99% in Can Tho, Vietnam, and 59.8%–90.2% in Laos.<sup>10-13</sup>

JEV-IgG positivity in pigs was one of the significant JE risk factors in this study. The presence of anti-JEV antibodies, both IgM and IgG, among

**Table 1: Pig Seroprevalences of IgM and IgG against JEV in five regencies in Bali**

District	No. of pigs	JEV-IgM	JEV-IgG	Total
Buleleng	10	0	5 (50%)	5(50%)
Karangasem	16	3 (18.75%)	4 (25%)	7(43.75%)
Badung	19	6 (31.58%)	10 (52.63%)	16(84%)
Tabanan	15	2 (13.33%)	5 (33.33%)	7(46.67)
Negara	20	5 (25%)	8 (40%)	13(65%)
Total	80	16(20%)	32(40%)	48(60%)

**Table 2: Risk factors associated with JE among children in Bali Error! Not a valid link**

Risk Factors	JE Patient		RR (CI 95%)	p
	Positive	Negative		
JEV-IgM pig			1.164 (1.054 - 1.285)	0.119
	Positive	0		
	Negative	9		
JEV-IgG pig			15.667 (1.85-132.655)	0.002
	Positive	8		
	Negative	1		
Pigsty location from house			7.351 (0.873-61.883)	0.036
	<10 m	8		
	>10 m	1		
Pigsty location from rice field<500meter			0.714 (0.0565-0.903)	0.002
	IgG pig positive	8		
	IgG pig negative	0		
>=500 meter			1.056 (0.949-1.1174)	0.826
	IgG pig positive	0		
	IgG pig negative	1		

pigs showed that they had been infected with JEV at particular time periods. In pigs, IgM antibody appears in the serum during the acute phase after 2–3 days of infection and is still detectable for an average of three weeks.<sup>14</sup> Some pigs with IgM antibodies appeared to be infected very recently with JEV, which might indicate that the virus might have still been circulating among the pig population in the location at the time of blood sample collection. IgG can reflect the history of JEV infection in pigs. IgG antibodies appear one week post-infection, and remain detectable for some time longer than IgM.<sup>15</sup>

Blood samples from the pigs were drawn at least over a month since the children were diagnosed as JE positive. Thus, if JEV-IgG was already found in the blood, it means that the pigs were infected with JEV about the same time that the children got infected. In Bali, it takes time to diagnose a child who suffers from JEV by serologic ELISA because this examination is not widely available in health facilities. The sera must be sent to the central laboratory in the central province, Denpasar, for analysis by ELISA, and it generally takes more than a month to obtain the result due to facility and cost limitations. This might explain why IgG played a significant role

instead of IgM. As mentioned, IgG persists in blood for a longer time than IgM.

Badung has the highest seroprevalence, whereas Buleleng has the lowest among regencies. There are many factors that influenced differences in seroprevalence between regencies in this study. One of the factors is the weather condition at the time of sampling. Mostly, samples were taken at the end of the rainy season between January and March, except those from the regency of Buleleng that were taken during the prolonged dry season between June and October. Normally, the mosquito population declines in the dry season which decreases the JE transmission between pigs.

Pigsties locations less than 10 meters from households and the presence of JEV-IgG-positive pigs less than 500 meters from irrigated rice fields were also found to be risk factors for JE in children in this study. These results are consistent with a previous study by Liu *et al.* (2010) in Bali. In that study, they found that the ownership of pigs and rice cultivation were associated with the risk of JE in children.<sup>16</sup> The same results were also found in other Asian countries such as Thailand, Nepal, China,

Vietnam, and Laos.<sup>13,17-19</sup> Furthermore, a study in Laos in 2008 found that the JEV seroprevalence in humans who lived near irrigated rice fields doubled from 19.4% to 42.4%.<sup>13</sup>

The majority of Balinese are still rearing pigs near their households. As JEV is not transmitted directly from pigs to humans but through mosquitoes of the genus *Culex*, especially by *Cx. tritaeniorhynchus*, the presence of mosquitoes is essential for transmission of JEV from animals to humans. These mosquitoes can be zoophilic (sucking animal blood) and anthropophilic (sucking human blood).<sup>20</sup> The pigsties are usually located immediately behind the house with a distance of less than 10 meters for practical reasons and security. Moreover, due to the rapid population growth, many houses are built in the area nearby rice fields. This means that the pigsties were also close to the rice fields. The presence of pigs and irrigated rice fields around human habitation provides a suitable condition for mosquitoes to transmit JEV from pigs to human.<sup>6</sup> Some studies had reported that the average flight range of *Culex* mosquitoes, such as *Cx. Annulirostris*, was around 4.4 km while the mosquito species *Cx. quinquefasciatus*, *Cx. tarsalis*, and *Cx. stimagtosoma* have an effective flight range as far as 1 mile (1.6 km).<sup>21, 22</sup> According to Solomon, the preferable location of pigsties from human habitats should be more than 5 km.<sup>5</sup>

Implementation of JE vaccination for children is essential to eliminate JE; however, countries like Indonesia that do not have vaccination

programs, a biological vector control management together with an alternate wet and dry irrigation system have the potential to reduce the level of transmission and JE burden.<sup>23</sup> Moving pigsties at least 5 km away from human habitation was not effective to reduce JE-infected mosquitoes because there are other amplifier hosts for JEV, like wading birds, and moving pigsties at least 5 km away from human habitation is certainly not suitable for a small island like Bali. Vaccination of pigs is another strategy for controlling JE transmission but the effectiveness of the vaccine will decline in young pigs because maternal antibodies normally still persists for 3–6 months, and it is also costly for routine vaccination of newborn pigs.<sup>17, 19, 24</sup> Further studies are required to establish effective methods for preventing pigs from being infected with JEV in the absence of vaccination in countries like Indonesia. Studies to determine the viral titers in pigs to assess their ability to act as an amplifying host are also warranted.

## CONCLUSIONS

Pigs play a major role in JEV transmission in Bali, JEV-IgG-positive pigs that are kept close to households and irrigated rice fields are risk factors for JE.

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