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Seroepidemiology of Japanese Encephalitis Virus Infection in Bats and Pigs in West Kalimantan, Indonesia

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Japanese encephalitis virus is a significant cause of fatal encephalitis in humans, particularly affecting infants and young children. The virus is mosquito-vectored, and a wide range of vertebrate hosts has been implicated in its ecology. Infection of domestic pigs is epidemiologically linked to spread of the virus to humans. In this serological study evidence of Japanese encephalitis virus infection has been identified from a survey of 610 pigs from 6 districts in West Kalimantan Province, Indonesia (Pontianak, Landak, Sanggau, Sambas, Bengkayang and Singkawang). Some 84 *Pteropus vampyrus* bats from Pontianak district and 15 *Cynopterus brachyotis* bats from Singkawang district were studied. As much as 84% of pig sera and 12% of *P. vampyrus* sera reacted on Japanese encephalitis virus and flavivirus C-ELISA. None of the *C. brachyotis* showed evidence of infection. JEV infection appears to be endemic in pigs in Kalimantan and encephalitis cases due to JEV have been reported in humans. The presence of JE antibodies in *P. vampyrus* suggests that this species may act as a natural reservoir host of JE. While the latter does not mean that flying foxes are the source of infection for pigs, our finding of increased JE seroprevalence in pigs combined with increased incidence of human cases, clearly demonstrates the need for ongoing surveillance and further investigation of the epidemiology and ecology of this virus. This is the first report of apparent Japanese encephalitis virus infection in *P. vampyrus*, and adds to the list of zoonoses associated with this species. The findings indicate a high Japanese encephalitis virus prevalence in domestic pigs and identifies a significant risk of 'spill-over' human infection and disease.

Key words: Japanese encephalitis, antibodies, bats, pigs

The Japanese encephalitis virus (JEV) (family *Flaviviridae*, genus *Flavivirus*) is a significant cause of encephalitis in humans, especially in children under 10 (Tsai 2000). The virus has a primary enzootic cycle between mosquitoes and water birds. Infected pigs amplify the viral load, and thereby facilitate human infection through vectors such as *Culex tritaeniorhynchus* (Van Peenan *et al.* 1975; Ompusunggu *et al.* 2008).

A wide range of vertebrates has been implicated as hosts for JEV including ruminants, poultry, horses, rabbits, pigs, mice, monkeys, and bats (Zhang *et al.* 1990; Arunagiri *et al.* 1993; Sendow *et al.* 2000). Clinical disease has been reported only in pigs, horses, and humans. Infection in adult pigs is usually asymptomatic, although in pregnant pigs, JEV infection can cause reproductive failure such as abortion, stillbirth or foetal mummification (Jia *et al.* 2005; Dong *et al.* 2006). Clinical presentation in horses includes fever and evidence of mild to severe encephalitis (Ellis *et al.* 2000).

In Indonesia, JEV has been reported in humans in East Java, West Sumatera, East Nusa Tenggara, Bali, and West Papua (Spicer 1997; Kari *et al.* 2006; Ompusunggu *et al.* 2008). Van Peenan *et al.* (1975) also reported the isolation of JEV from *Culex* mosquitoes in West Java. Sendow *et al.* (2000) reported antibodies against JEV in pigs from a number of Indonesian provinces including West Kalimantan. Recent reports have identified bats as a natural host of a range of significant human and animal viral pathogens, including Nipah, Hendra, SARS, and lyssaviruses. Spill-over from bats has led to both major epidemics and sporadic cases of disease in humans and domestic animals (Calisher *et al.* 2003; Mackenzie and Field 2004). However, evidence of JEV infection has not previously been reported for bats in Indonesia.

This study was undertaken to investigate flavivirus, and particularly JEV, infection in pigs and bats in West Kalimantan, and provides baseline information on the detection of JE C-ELISA antibody reactions in pigs and bats. The evidence of JEV infection in *P. vampyrus* is the first report of infection of this species in Indonesia. The paper also discusses JE in West Kalimantan Province in relation to encephalitis in humans in 2007.

MATERIALS AND METHODS

Case Reports. Observed encephalitis case data was collected from pig farms and bat sellers in West Kalimantan.

Sample Collections. Bat sera were obtained by bleeding specimens of *P. vampyrus* sold for human consumption by local bat sellers in the Pontianak District. These bats are typically caught by local people using kites with fishing hooks attached to the kite-string. Sera were obtained from *Cynopterus brachyotis* bats sourced from a bird trapper in the Singkawang District. The age of bats ranged from juvenile to adult. Typically, 2.0 ml of blood was collected from the uropatagial vein. Sample details are presented. For pig sera,

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a non-random, convenience sample of pigs from multiple farms in the six West Kalimantan districts was bled. Blood was collected from a prominent ear vein, or from the cephalic vein. Data on age, sex, and location were recorded.

C-ELISA Test. Serological testing was conducted using a Competitive ELISA (C–ELISA) adapted from Lunt (1998) and Pant *et al.* (2006). Inactivated JEV antigen and monoclonal antibody (MAB) 989 was provided by the Australian Animal Health Laboratory (AAHL), Geelong, Australia. JEV antigen at an optimal dilution (1:200) in carbonate buffer (pH 9.0) was added (50 µl per well) to flat bottom polystyrene microtiter plates. Plates were incubated at 37°C for 1 h and used immediately, or kept at 4°C for use next-day. Plates were then triple-washed in Phosphate buffered-saline and Tween 20 (PBS-Tween 20). Test sera, positive control sera, and negative control sera were diluted 1:10 in buffer solution and added to the plates (50µl per well). Plates were incubated at room temperature for one hour with agitation and then again triple-washed with PBS-Tween 20. JE MAB 989 at optimum dilution (1:3 000) was added to all wells (50 µl per well) except for the MAB control well. Plates were then incubated for a further 30 min at room temperature with agitation and then triple-washed with PBS-Tween 20. Anti-mouse horseradish peroxidase conjugate at optimum dilution (1:2 000) was then added to all wells (50 µl per well) and the plates were incubated for a further 30 min at room temperature with agitation. After a final wash with PBS-Tween 20, TMB substrate was added (50 µl per well) to all plates, which were then incubated for a further 10 to 20 min at room temperature with agitation. The reaction was stopped with 50 µl per well of stopping solution (1.0 M H_2SO_4). Plates were then read in a *Beckman Coulter* ELISA reader using a 450 nm filter. Negative sera produced a blue colour while JE positive sera produced reduced or no colour.

Percentage inhibition was calculated by the following formula:

$$\% \text{ inhibition} = \frac{100 - (100 \times \text{OD test serum } (A_{450nm})}{\text{OD mean negative control serum } (A_{450nm})};$$

OD: optical density

Test serum was judged as positive if 50% inhibition or above was obtained.

RESULTS

Bat Sera. A total of 99 bat sera was tested for antibodies against JEV, being 84 *P. vampyrus* from Pontianak District and 15 *C. brachyotis* from Singkawang District. Ten *P. vampyrus* (12%) gave a positive ELISA test while none of the *C. brachyotis* was positive (Table 1).

Pig Sera. Pig sera were also collected from 6 districts in West Kalimantan Province, including Pontianak, Landak, Sanggau, Sambas, Bengkayang, and Singkawang Districts (Fig 1). The serological results show that antibodies against JEV were detected in 514/610 (84%) of the pigs tested in West Kalimantan, with prevalence varying from 66% to 99%. The lowest prevalence was detected in Pontianak District and the highest prevalence in Singkawang district (Table 1). JEV reactors were found in pigs of all age classes, with prevalence increasing with age overall (Table 2). There was no significant difference between antibody prevalence rates for male and female pigs overall (Table 2).

Field Observations. Encephalitis cases were not observed during the field observation among pig farmers and bat sellers. However, encephalitis cases in humans had been reported by the Health Department surveillance in 2006.

DISCUSSION

The reactivity in *P. vampyrus* (Table 1) adds to the list of emerging diseases linked to fruit bats and flying foxes. The absence of reactivity in *C. brachyotis* could plausibly reflect a lack of exposure, a lack of susceptibility, or the limited sample size used here.

There have been previous reports implicating bats as possible hosts of JEV. In 1974, flaviviruses distinct from JEV were isolated from bats in Japan (Miura and Kitaoka 1977). The same report noted that 1-10 % of bats had antibody to JEV. Calisher *et al.* (2003) reported that some bat species (*Hipposideros armiger terasensis* and *Miniopterus schreibersii*) had been identified as a natural reservoir host for JEV. Mackenzie *et al.* (2004) noted that bats could be experimentally infected with JEV. In Indonesia, Winoto *et al.* (1995) reported JEV antibodies in 24/157 (15%) of unidentified bats caught in Sintang, West Kalimantan Province.

The results of this study indicate that *P. vampyrus* in West Kalimantan have been exposed to infection with JEV or a related flavivirus. This is the first report implicating JE infection in *P. vampyrus* in Indonesia. The results also identify JEV as one of a growing list of emerging diseases associated with fruit bats. Further work is needed to identify the role bats' play in the ecology and epidemiology of JEV infection. Bats may be an effective reservoir of JEV, or alternatively, be inconsequential adventitious dead-end hosts. However, isolation of the virus or the detection of JEV nucleic acid is needed to confirm infection specifically with JEV, rather than a related flavivirus that has elicited an antibody cross reaction.

A high level of seropositive pigs was detected by the JE C-ELISA. Table 1 shows that antibodies against JEV were

Table 1 Japanese encepfralitis virus antibody detection by ELISA in pigs and bats from West Kalimantan

Sampling location (District)	Pigs			*Pteropus vampyrus*			*Cynopterus brachyotis*		
	Sample size	Number	Positive (%)	Sample size	Number	Positive (%)	Sample size	Number	Positive (%)
Pontianak	86	57	66	84	10	12	NA		
Landak	64	56	88	NA	NA		NA		
Sanggau	74	60	81	NA	NA		NA		
Singkawang	162	161	99	NA	NA		15	0	0
Sambas	100	79	79	NA	NA		NA		
Bengkayang	124	101	81	NA	NA		NA		
Total	610	514	84	84	10	12	15	0	0

NA: not available

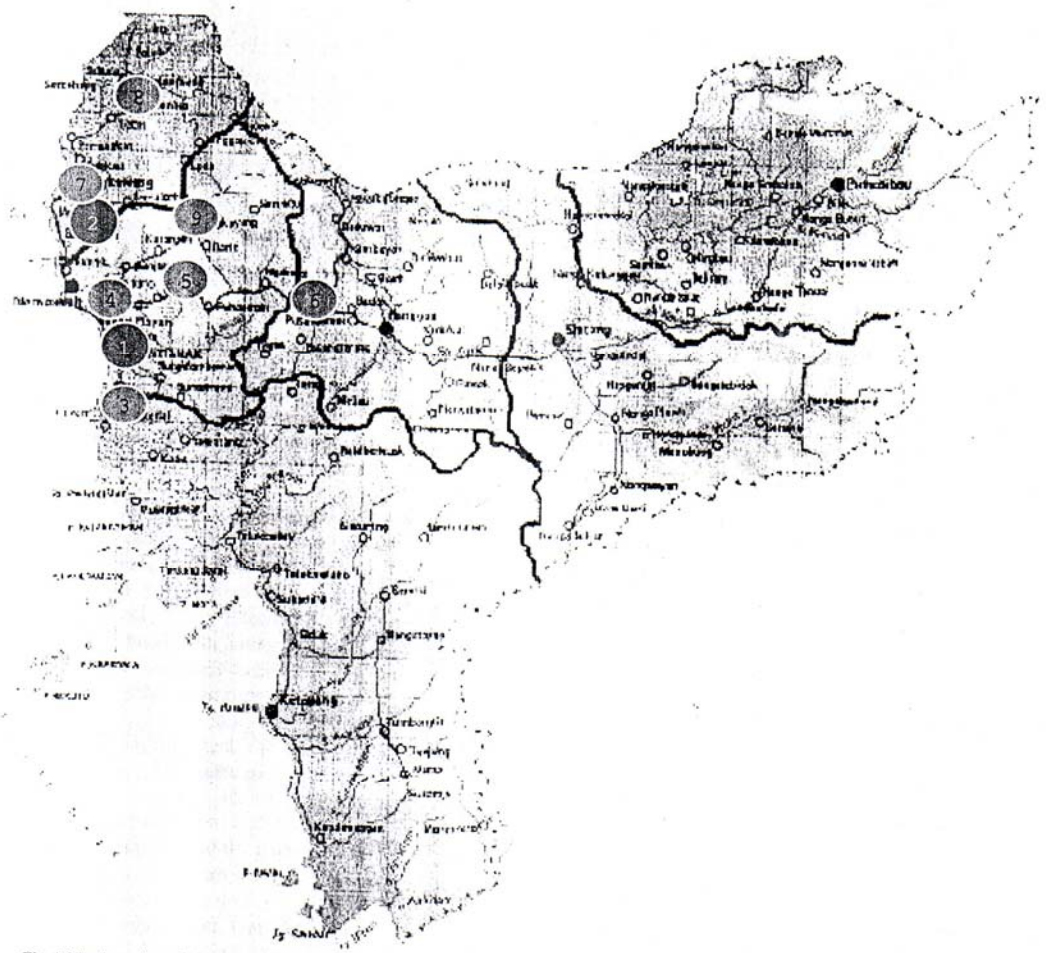

Fig 1 The location of pig and bat sample collection sites in West Kalimantan Province. The distribution location of bats sample collection sites in West Kalimantan Province: 1, *Pteropus vampyrus*, Pontianak District; 2, *Cynopterus brachyotis*, Singkawang District, and the distribution location of pigs sample collection sites in West Kalimantan Province: 3,4, Pontianak District; 5, Landak District; 6, Sanggau District; 7, Singkawang City; 8, Sambas District, 9, Bengkayang District.

detected in 84% pigs tested in West Kalimantan. Most farms were located in rural areas, and some of those farms were located near elements that may be implicated in vector or virus maintenance, such as poultry, fish ponds and wild bird nesting areas. JEV reactors were found in pigs of all age classes, with prevalence increasing with age overall. There was no significant difference between antibody prevalence rates for male and female pigs.

The assumption that JE C-ELISA reactions is evidence of JEV infection requires careful interpretation. It is possible that antibody reacts against closely related flaviviruses. In particular Murray Valley Encephalitis (MVE) virus and to a lesser extent, Kunjin, virus which can cross-react with JE antigen to yield positive reactions (Williams *et al.* 2001). Less closely related flaviviruses are unlikely to induce clear cross-reactive antibodies. MVE virus has not been reported in humans or animals in Indonesia. Sendow (unpublished data) showed that none of 384 pig sera from North Sumatera and Riau tested positive for MVE antibodies by the Serum-Neutralization test. Therefore it is unlikely that exposure to MVE virus can be attributed as the source of JE C-ELISA reactivity in this and other Indonesian studies. Further studies will be needed to also exclude the less likely Kunjin virus.

Sendow *et al.* (2000) reported antibodies against JEV in 2% of West Kalimantan pigs surveyed in 1995. Use of the haemagglutination inhibition (HI) test reported a higher level of 57% in pigs sampled in Sintang, West Kalimantan Province (Winoto *et al.* 1995). The difference between these studies may indicate fluctuation in prevalence both between, and within, populations sampled at different locations. Alternatively, limited sample size and/or the testing of a particular age cohort (such as young piglets) could skew results. Nonetheless, the findings of the current study suggest an increased prevalence of JEV infection in pigs of West Kalimantan province.

Table 2 Japanese encephalitis virus antibody detection by ELISA in pigs from West Kalimantan according to age and sex

Sampling location	Positive according to age (%)					Positive according to sex (%)			
	</=2 months	>2-3 months	>3-6 months	>6-12 months	>1 year	Male		Female	
Pontianak District	0/9 (0%)	18/36 (50%)	5/5 (100%)	18/19 (95%)	16/17 (94%)	3/9 (33%)	CI 7-70	53/77 (70%)	CI 59-80%
Singkawang District	NA	NA	69/69 (100%)	34/35 (97%)	58/58 (100%)	NA	NA	NA	NA
Sambas District	19/22 (86%)	38/42 (90%)	13/21 (62%)	9/14 (64%)	NA	7/8 (88%)	CI 47-99	72/92 (78%)	CI 68-86%
Bengkayang District	3/3 (100%)	45/49 (92%)	50/69 (72%)	2/2 (100%)	1/1 (100%)	20/30 (67%)	CI 47-83	81/94 (86%)	CI 78-92%
Total	22/34 (65%)	101/127 (80%)	137/164 (84%)	63/70 (90%)	75/76 (99%)	30/47 (64%)	CI 49-77	206/263 (78%)	CI 73-83%

NA: not available, CI: confident limit (%).

JEV remains a significant (and in certain regions an increasing) cause of human encephalitis in Indonesia. Kari *et al.* (2006) showed that 4 of 86 Balinese children presenting with primary clinical signs of fever, convulsion, and loss of consciousness, were JE-positive by ELISA. The same study reported that 70% of a sample of 400 Balinese pigs tested positive for antibodies to JEV. Increased vector abundance associated with breeding sites provided by year-round rice cropping in Indonesia has been identified as factor likely to promote an increase in JEV infection (Kari *et al.* 2006).

An increased incidence of infection in pigs in Indonesia should concern public health authorities, given the role of pigs in the transmission of JEV to humans. The detection by the Indonesian Health Department of cases of encephalitis and antibodies against JEV in human populations of West Kalimantan in 2006 is consistent with 'spill-over' of the virus population following high rates of infection in pigs. Early warning of such threats could be usefully signalled by surveillance of pig populations and this highlights the value of a 'one-health' approach to the management of zoonotic diseases. However, resources for such cross-disciplinary investigations continue to be limited in Indonesia.

Our findings demonstrate that JEV infection of pigs commonly occurs in West Kalimantan and is plausibly linked to an increased incidence of human JE cases. JEV can infect a range of avian and mammalian species, including *P. vampyrus* as illustrated in our study. These hosts can be deemed significant for human health only by defining linkages to disease outbreaks. Such evidence is available for pigs in West Kalimantan. Plausible approaches to reducing the incidence of human disease include removing pig farms from proximity to human housing. Even if this did not fully eliminate JEV risk (van den Hurk *et al.* 2008), increasing the level of farm sanitation and reducing the availability of mosquito breeding sites is advisable. It is appropriate to reiterate here that human JE cases result from the bites of an infected mosquito, not from consumption of pork (or bat meat). The forecasting of imminent outbreaks of this human disease can be better achieved by combining the ongoing and structured surveillance for JEV activity with the identification of other risk factors.

Further study on genotyping and isolation must be conducted in both species to better define the hazard for human health.

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