THE FIRST REPORT ON HUMAN CASES SEROLOGICALLY DIAGNOSED AS JAPANESE ENCEPHALITIS IN INDONESIA

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Abstract. Although Japanese encephalitis (JE) virus was isolated from mosquitos in 1974, human JE cases have never been reported in Indonesia in spite of the prevalence of anti-JE antibodies among human and pig populations as well as abundant JE vector mosquitos. In this report, we describe serological diagnosis of JE cases in Bali, Indonesia, using IgM-capture ELISA both on serum and cerebrospinal fluid (CSF) of the patients. In the first series of our investigation (Series 1), we examined serum specimens from 12 patients with clinical diagnosis of viral encephalitis, meningitis or dengue hemorrhagic fever (DHF), and found 2 possible JE cases. In the next series (Series 2), we examined both serum and CSF from encephalitis patients and gave laboratory diagnosis of JE. One of them was suspected to have concomitant or recent infection with dengue virus, probably type 3. These results strongly indicated that JE has been prevalent in Bali, Indonesia.

INTRODUCTION

Japanese encephalitis (JE) has been a serious health problem in many parts of East, Southeast to South Asia (Thongcharoen, 1989; Sabchareon and Yoksan, 1998). While, relatively small number of patients have been reported in tropical Asia like Malaysia and the Philippines (Sinniah, 1989; Barzaga, 1989; Igarashi, 1998). Although isolation of JE virus from mosquitos was reported in Indonesia (van Peenen *et al*, 1974) and anti-JE antibodies among human and animals were prevalent (Hotta *et al*, 1970; Kanamitsu *et al*, 1979), no single human JE case has been confirmed either serologically or virologically in Indonesia, in spite of abundant vector mosquitos and amplifier vertebrates which can support transmission of the disease (Wuryadi and Suroso 1989). Two reports have been published on travelers who were diagnosed as JE based on their symptoms and serological findings after returning from Bali. The endemicity or sporadic outbreaks of JE in Bali have been conjectured by MacDonald *et al* (1989) and Wittesjo *et al* (1995).

We had an opportunity to carry out serological diagnosis on patients showing symptoms of acute encephalitis, meningitis, or dengue hemorrhagic fever (DHF), and found the possibility of some JE cases in Bali (Series 1). This finding stimulated us to confirm the presence of JE cases in another investigation (Series 2), by virus isolation and IgM-ELISA on sera and cerebrospinal fluid (CSF) of the patients showing acute encephalitis, and describe the results in this paper.

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MATERIALS AND METHODS

Patients in series 1

Six patients (No. 1 - 6) clinically diagnosed as viral encephalitis or meningitis, and another 6 (No. 7 - 12) as DHF, were selected for serological and virological examinations with informed consent (Table 1). They visited Udayana University Hospital between January 1996 to January 1997. Their blood specimens were collected on the day of admission as acute specimens and on the 8th hospital day as convalescent specimens, respectively.

Patients series 2

Five patients (Cases 1-5 in Tables 2 and 3, and Fig 1) with presumptive diagnosis of viral encepha-

Table 1

Serological findings of Series 1.

Patient No.	Period of specimen collection	Clinical diagnosis	Sro-dianosis	Total Ig-ELISA to JE (1:1,000)	JgM-captue ELISA (P/N ratio)				
					Anti - JE	- Dl	- D2	- D3	- D4
1	A	ECP	JE	8,200	1.411	0.953	1.149	1.259	1.181
	C			10,500	5.198	1.021	1.496	1.149	1.175
2	A	ECP	N	4,000	1.18	0.862	0.929	1.242	1.251
	C			9,000	1.297	1.22	1.306	1.555	1.465
3	A	MNG	N	17,000	1.35	1.075	1.845	1.266	1.243
	C			28,000	1.149	1.276	2.024	1.376	1.319
4	A	ECP	JE	16,200	5.018	1.108	1.265	1.175	1.241
	C			10,500	4.774	0.869	0.974	1.1	1.228
5	A	ECP	N	13,000	1.389	1.152	1.261	1.262	1.243
	C			27,000	1.541	1.276	1.33	1.395	1.383
6	A	ECP	D	42,000	1.986	1.63	3.738	1.496	1.626
	C			52,000	1.802	1.738	3.187	1.686	1.693
7	A	DHF	D	36,000	1.637	2.487	5.205	1.779	1.523
	C			33,000	1.826	3.11	5.886	2.172	1.939
8	A	DHF	D	23,000	1.828	2.311	4.539	1.838	2.234
	C			24,000	1.653	2.007	3.903	1.747	2.152
9	A	DHF	D(JE)*	4,300	1.623	2.241	4.683	1.906	1.681
	C			16,500	2.631	3.047	5.866	2.194	2.184
10	A	DHF	D	18,000	2.082	2.286	4.403	1.616	1.52
	C			22,500	1.22	1.407	2.136	1.316	1.363
11	A	DHF	D(JE)*	10,000	1.29	1.473	2.069	1.572	1.354
	C			19,500	1.485	1.609	2.504	1.605	1.33
12	A	DHF	D	22,000	1.551	1.593	2.522	1.483	1.404
	C			12,000	1.208	0.913	0.914	1.24	1.219

Patient No. is used only for the patients in Series 1.

A: acute, C: convalescent. Clinical diag; clinical diagnosis.

ECP; viral encephalitis, MNG: meningitis, DHF: dengue hemorrhagic fever, JE: Japanese encephalitis, N: not diagnosed, D1~4: dengue type 1~4. *JE is the second candidate.

litis, visiting Udayana University Hospital between April 2 to May 9, 1997, were investigated under suspicion of JE. The 3rd to the 5th cases lived in Denpasar, which is an urban area with small rice fields and small pig farms; the 1st case lived in a village 59 km from Denpasar, and the 2nd case in a village 18 - 20 km from Denpasar, respectively (Fig 1). These 2 villages were surrounded by rice fields and pig farms, although the fields were not irrigated but dry at the time of their disease. Patients were admitted to Udayana University Hospital gave informed consent, and were examined by clinical symptoms as well as cytokine levels in blood. Their blood and CSF specimens were collected on the day of admission, the 8th hospital day, and on the day of discharge. Serological investigations were carried out on serum and CSF specimens as described below.

Indirect micro ELISA to measure total immunoglobulin (Ig) titer to JE

This test was performed according to Igarashi *et al* (1981a), and Bundo *et al* (1982). The test serum was diluted 1:100 and 1: 1,000 in PBS-Tween, and distributed into wells in 96 well ELISA plates which had previously been coated with formalin-inactivated, purified JE vaccine concentrate as assay antigen. The step was followed by reaction with horseradish peroxidase (HRPO)-conjugated anti-human Ig, and the enzyme reaction with substrate solution containing o-phenylene diamine and hydrogen peroxide. The reaction was stopped by sulfuric acid and the OD value at 492 nm was recorded by a microplate ELISA reader. The OD obtained by each test specimen was compared with those developed by serial dilution of a standard positive serum in order to calculate the ELISA titers of the test sera (Morita *et al*, 1982).

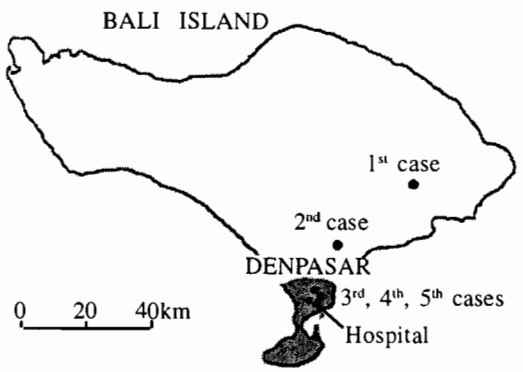

Fig 1–Map of Bali island, Indonesia showing residence of 5 cases in Series 2.

IgM-capture ELISA

This test was done according to Bundo and Igarashi (1985) and Igarashi and Antonio (1997). The 96-well ELISA plate was coated with anti-human IgM (m chain specific) goat IgG (Cappel, USA) at 4°C overnight, followed by blocking with Blockace (Yukijirushi, Japan). After washing, the wells in the plates were reacted with the test specimens as well as standard positive and negative sera at 1:100 dilution in PBS-Tween. After the reaction and washing, the assay antigen prepared as infected C6/36 cell culture fluid and diluted to 25 ELISA units was added into the wells. After the incubation, the plate was washed and successively reacted with HRPO-conjugated anti-flavivirus IgG, which was prepared from high titered DHF patients' sera, followed by washing and then substrate solution. The HRPO reaction was stopped and OD was recorded as in the case of indirect ELISA. The P/N ratio was calculated by dividing the ELISA-OD of the specimen by that of the standard negative serum. The specimen with P/N ratio equal to or greater than 2.0 was considered as positive.

Clinical record, cytokine levels, and virus isolation

The mental status score and pediatric coma scale concerning encephalitis symptoms are explained in the legend of Table 2, and abbreviated as M/C in abnormal and (M/C) in normal. TNFα and IL-6 levels were measured by commercial ELISA kits and presented as picogram/ml. Virus isolation from serum and CSF was carried out by inoculation to C6/36 mosquito cell line (Igarashi *et al*, 1981b).

RESULTS

Series 1

All 6 DHF patients (No. 7 - 12) were serologically diagnosed as dengue virus infection by the IgM-ELISA, because their P/N ratios were over 2.0 using dengue, especially type 2 antigen. Their convalescent sera on the 8th day of hospitalization possessed high titers of total Ig-ELISA to JE antigen (1:12,000 to 1:33,000).

On the other hand, among 6 patients of viral encephalitis or meningitis (No. 1 - 6), 2 cases (No.1 and 4) possessed significant P/N ratio (over 2.0) of anti-JE IgM-ELISA as shown in Gothic. Their total Ig-ELISA titer to JE antigen on the 8th hospital day was 1:10,500. These data indicated that these 2 patients suffered from JE, and JE virus infection has been prevalent in Bali.

Table 2
Clinical explanation of the five cases in Series 2. Mental status score (on numerator) and pediatric coma scale (on denominator) by Simpson and Reilly, neurological symptoms and sequelae.

	1st case	2nd case	3rd case	4th case	5th case
Age(year,month), sex	5 and 6/f	0 and 6/f	1 and 9/m	4 and 1/f	3 and 6/f
Admission day of illness	4th day	4th day	4th day	3rd day	4th day
Highest fever	37.2°C	37°C	39.2°C	38.5°C	38°C
Mental and coma, score M/C	2/10 1/14)*	3/6(1/10)	3/4 (1/12)	3/7 (1/13)	3/7 1/13
Abnormal M/C was seen till the hospital day	3rd day	6th day	37th day	12th day	6th day
Neurological-symptoms	(+)	(+)	(+)	(+)	(+)
Hospital day	till 4th day	till 20th day	till 37th day	till 4th day	till 2nd day
Sequela	(-)	(±) Babinski reflex	(+) monoplasia	(-)	(-)

Mental status score [M] Alert: 1, Lethargic: 2, Obtunded: 3, Comatose: 4

Pediatric coma scale [C]

Eyes open		Best verbal response		Best motor response		Normal aggregate score [C]	
Spontaneously	4	Oriented	5	Obeys commands	5	Birth to 6 months	9
To speech	3	Words	4	Localizes pain	4	6 to 12 months	11
To pain	2	Vocal sounds	3	Flexion to pain	3	1 to 2 years	12
Not at all	1	Cries	2	Extension to pain	2	2 to 5 years	13
		None	1	None	1	Over 5 years	14

*Legend of the fractional numbers and etc in Table 2.
Figures on numerator: The worst one of mental status scores duling hospital care.
Figures on denomintor: The worst one of aggregate scores which is composed of scores of the eyes open, the best verbal response and best motor response) during hospital care.
Figures in parenthesis: Normal mental status score / normal aggregate score at the said age.
Ordinal day numbers: Until the said hospital day, M/C score's were abnormal.

Series 2: Clinical features of the patients

Encouraged by the results in Series 1, we further investigated 5 more presumptive JE cases, focusing on the IgM-ELISA of CSF. Clinical explanations of these 5 cases as to mental and coma scores, neurological symptoms and sequelae are shown in Table 2 (the 1st to the 5th cases) and described below:

The 1st case: A 5 year and 6 months old female, admitted on the 4th day of illness, with the highest body temperature at 37.2°C. Her fever persisted for 5 days including pre-admission period. Her worst mental status score was 2, and the worst coma scale was 10, *ie* 2/10 against normal score (1/14). These abnormal scores and scales continued until the 3rd hospital day. Her neurological symptoms including slightly impaired movements of both arms and legs were observed until the 4th hospital day. Ventilation support was used for one day. The CSF on admission showed mononuclear cells at 9/mm^3, protein level at 35.2 mg/dl, and glucose level at 94 mg/dl, respectively.

The 2nd case: The body temperature about 37.0°C persisted for 5 days. Before admission, she suffered from seizures. M/C were 3/6 against (1/10) of normal. Abnormal scores continued until the 6th hospital day. Babinski reflex was observed until the 20th hospital day of her discharge. Movement of arms and legs was slightly impaired: until the 4th hospital day on the right side, and the 8th day on the left. The CSF on admission showed cell count of 168/mm^3, with 82.2% mononuclear and 18.0% polynuclear cells, respectively, and protein level of 39.2 mg/ml and glucose level of 67 mg/dl. She suffered from marginal sequelae.

The 3rd case: Seizures were observed before and

Table 3
IgM-capture ELISA to JE and dengue type 1-4 in Series 2.

Case and samples	IgM-capture ELISA to JEV and dengue virus type 1-4											
	Sera						CSF					
	anti-Dl	D2	D3	D4	anti-JE	Sero-diagnosis	anti-Dl	D2	D3	D4	anti-JE	CSF diagnosis
1st case												
Sample-1	0.763	1.733	1.769	0.891	15.84		1.731	1.79	2.64	1.66	25.33	
-2	0.797	1.903	1.811	0.948	23.25	JE	1.209	1.297	1.709	1.18	1.697	JE
-3	0.834	1.665	1.741	0.93	23.83		1.423	1.328	1.889	1.145	31.32	
2nd case												
Sample-1	0.716	1.494	2.027	0.906	22.93		1.949	1.751	2.672	1.541	16.16	
-2	0.69	1.414	2.23	0.886	21.81	JE	1.681	1.664	2.493	1.379	28.19	JE
-3	0.703	1.273	1.691	0.875	24.71		1.286	1.232	1.682	1.379	31.7	
3rd case												
Sample-1	0.68	1.215	1.589	0.91	4.587		2.134	1.851	2.456	1.883	5.932	
-2	0.76	1.375	1.682	0.961	19.39	JE	1.815	1.763	2.291	2.132	27.45	JE
-3	0.884	1.476	1.921	1.079	21.12		2	1.755	2.186	1.928	27.01	
4th case												
Sample-1	0.696	1.607	2.147	1.079	17.22		2.07	2.064	2.51	1.822	31.87	
-2	0.887	1.418	1.844	1.055	22.62	JE	1.893	1.9	2.288	1.899	34.62	JE
-3	0.86	1.215	1.87	1.092	19.18		1.679	1.858	2.197	1.517	33.98	
5th case												
Sample-1	0.954	1.646	2.271	1.281	1.339		1.955	1.984	2.437	1.702	4.563	
-2	0.921	1.602	2.262	1.196	1.247	D	2.013	2.232	2.576	1.697	4.135	JE
-3	1.048	2.866	2.866	1.334	1.456		1.986	1.919	2.583	1.612	7.747	

All values are shown by P/N ratio.
Sample No. 1 is admission sample. Sample No. 2 is that on the 8th hospital day. Sample No. 3 is that on the discharge day.
CSF diagnosis : diagnosis with CSF-P/N ratio.
JE : Japaneses encephalitis, D ; Dengue fever.

after admission. The fever persisted for 13 days with maximum at 39.2°C. M/C scores were 3/4 against the normal (1/12) from the 14th to the 37th hospital day of discharge. Ventilation support was used for 5 days. Movement of extremities was slightly to markedly impaired during 37 days of hospitalization, but after the 24th hospital day, movement of her right arm and leg and left leg showed recovery. Movement of the left arm remained impaired until the day of discharge, leaving slight monoplegia as sequela. The CSF on admission showed a cell count of 75/mm^3; with 85% mononuclear and 15 % polynuclear cells, respectively, and protein level of 23.2 mg/dl, and glucose level of 57 mg/dl. This case was the most severe one among the presented 5 cases.

The 4th case: Before and after hospitalization she suffered from seizures, and fever persisted for 11 days with maximum at 38.5°C. As for neurological symptoms, Babinski reflex was observed until the 4th hospital day. Movement of the 4 extremities was slightly impaired until the 4th hospital day, but thereafter returned to normal. Therefore, no sequelae remained on the 19th day of discharge. Ventilation support was used for 3 days. The CSF on admission showed a cell count of 50/mm^3, with 50% mononuclear and 50% polynuclear cells, respectively, and protein level at 18 mg/dl and glucose level at 77 mg/dl.

The 5th case: Seizures were observed before and after admission till the 1st hospital day. Fever persisted for 6 days, and movement of extremities was slightly impaired until the 2nd hospital day. Sequelae were negative. The CSF on admission showed cell count of 29/mm^3, with 75% mononuclear and 25% polynuclear cells, respectively, and protein level of 27.2 mg/dl and glucose level of 136 mg/dl.

Isolations of JE virus were attempted by inoculating each specimen to a different culture of C6/36 cells, and all specimens were found to be negative. Bacterial cultures of all CSF specimens of Series 2 were negative. As for treatment, all cases were given dexamethasone, and ampicillin for secondary infection and luminal for neuropsychiatric symptoms.

Series 2: Serodiagnosis

Results of IgM-capture ELISA using JE and dengue type 1-4 virus antigens on the 5 patient's sera and CSF in Series 2 are shown in Table 3, and described below.

The 1st case showed markedly high PIN ratio of anti-JE IgM ELISA in both sera and CSF except for the CSF collected on the 8th hospital day. Even on admission day (acute phase), the P/N ratios of both serum and CSF were very high. The data were sufficient for diagnosis of JE.

The 2nd and the 4th cases showed high P/N ratio of anti-JE IgM ELISA in both serum and CSF specimens, in all samples on the day of the admission, the 8th hospital day, and the day of discharge, leading to the diagnosis of JE.

The 3rd case showed a positive P/N ratio (over 2.0) of anti-JE IgM-ELISA on both sera and CSF on the day of admission, the 8th hospital day, and the day of discharge, leading to diagnosis of JE. However, the P/N ratio on admission was relatively low (4.587 and 5.932), compared with the other 3 cases 1st, 2nd, and 4th, whose clinical symptoms were not so severe.

The 5th case showed unique serological reaction compared with the other 4 cases, but was diagnosed as JE based on the positive P/N ratio (4.1 to 7.7) of anti-JE IgM ELISA in 3 CSF samples. On the other hand, her serum IgM-ELISA indicated dengue type 3 virus infection rather than JE. All 3 serum specimens showed a negative P/N ratio of anti-JE IgM-ELISA, but marginally positive P/N ratio of anti-dengue type 3 IgM-ELISA (Table 3). The data indicated that this case may have been recently or concomitantly infected with type 3 dengue virus.

It is noteworthy that all serum and CSF specimens from these 5 cases tended to react more with dengue virus type 3 rather than type 2 antigen.

Series 2: Clinical severity and blood-level of TNFα and IL-6

Ravi *et al* (1997) reported that TNFα levels of serum and CSF can be used as possible prognosticators of a fatal outcome in JE infection, that is, high TNFα levels tends to be associated with a fatal outcome. In the present study, serum levels of TNFα and IL-6 were measured using 3 samples from each of the 5 cases, and compared with the clinical severity of the patients (Table 4). TNFα levels in the most severe case (the 3rd) on the 1st and 8th hospital days were much higher compared with other cases, except that the 1st case showed the highest level on the 8th day. The 3rd case also showed much higher level of IL-6 on the 1st day compared with other specimens (Table 4).

Hematological findings of the 5th case

Discrepancy between the serological diagnoses using the serum and CSF specimens in the 5th case has been described above, although the case are eventually diagnosed as JE. In order to support this diagnosis, the results of blood examination were

Table 4
Clinical severity of the patients and levels of TNFα and IL-6 in their blood.

TNFα	Clinical severity	Case	Samples collected on the hospital day		
			1st day	8th day	Discharge
	Moderate	1st case	31.9	655.9	33.6
		2nd case	32.2	40.3	48.7
		4th case	5.6	19.8	15.6
		5th case	27.7	29.1	37.1
	Severe	3rd case	357.3	346.7	29.1
IL-6					
	Moderate	1st case	16.2	46.0	106.0
		2nd case	151.5	127.8	246.5
		4th case	53.9	136.0	136.5
		5th case	0	135.1	28.9
	Severe	3rd case	935.3	51.0	166.4

Table 5
Hematological findings of 5th case.

Parameters	1st hospital day	8th hospital day	Discharge day
Hemoglobin (g/dl)	12.0	10.4	11.1
Hematocrit (%)	33.8	32.4	36.0
White blood cells /μl	6×10^3	9.7×10^3	10.83×10^3
Red blood cells /μl	4.76×10^6	4.21×10^6	4.6×10^6
Platelet /μl	229×10^3	812×10^3	127×10^3
CRP(mg/dl)	5.1	<0.5	<0.5

shown in Table 5. This case did not show any hematological characteristics of DHF, such as thrombocytopenia, followed by its rapid recovery, increased hematocrit value, and alteration of hemoglobin level. In addition, similar findings were not obtained for the remaining 4 cases which had definitely been diagnosed as JE (data not shown).

DISCUSSION

Prevalence of JE has been suspected in Indonesia based on the ecological conditions favorable for JE virus transmission and indirect evidence of seroepidemiology among humans and animals (Wuryadi and Suroso, 1989). Prevalence of JE antibodies among humans in Indonesia was examined by neutralization or hemagglutination-inhibition tests, and was significantly high in some areas, for example 52% in Bali. The prevalence of anti-JE neutralizing antibodies among pig populations in several localities in Indonesia is distributed in a great range, but is 27% in Bali. When sentinel sero-negative pigs were placed in an area near Jakarta, a 4-fold or greater rise in antibody titer to JE was observed some time later. JE virus was isolated from both the sentinel pigs and some mosquito species, such as *Cx. tritaeniorhvnchus*, a representative JE vector.

Our results in Series 1 also indicated the presence of human JE cases in Bali, Indonesia. The patients No. 7 to 12 in Table 1 were diagnosed as DHF, because of their clinical symptoms, and their IgM-ELISA P/N ratios >2.0 against dengue type 2 virus antigen. Their high total Ig-ELISA titer to JE antigen is reflecting an anamnestic antibody response to cross-reacting flaviviruses including JE and dengue viruses. Increase in the total Ig- antibody titers to JE from the acute to convalescent phase was also observed in patients No. 1, 2, 3, 5, and 6 who were clinically diagnosed as viral encephalitis or meningitis. These results were compatible with the serodiagnosis of JE by IgM-ELISA (patients No. 1 and 4).The presence of human JE in Bali, Indonesia, was further confirmed by our observation in Series 2, in which serodiagnosis of JE was given by

IgM-ELISA using both serum and CSF specimens to 5 cases showing typical clinical symptoms of acute encephalitis. According to the report of Burke *et al* (1985a), the demonstration of anti-JE IgM ELISA antibodies in CSF rather than in serum is a better method for laboratory diagnosis on JE. It was suspected that the 5th case was simultaneously or recently infected with dengue virus, probably type 3, as suggested by the IgM-ELISA. The findings in blood examination of the 5th case were not characteristic of DHF, and similar to those of the 1st to the 4th cases who were diagnosed as JE. Thus, JE remains as a possible diagnosis in the 5th case.

Most of the specimens from the 1st to the 4th cases showed high JE IgM-ELISA P/N ratio over 15 even in the admission specimens except for the 3rd case who showed most severe clinical symptoms but whose JE IgM-P/N ratio was relatively low (4.587 and 5.932). These findings are compatible with the reports of Burke *et al* (1985b) that low anti-JE IgM antibody titer in CSF resulted in a poor prognosis. Furthermore, the high levels of TNFα on the 1st and 8th hospital days of the 3rd case are also compatible with the report of Ravi *et al* (1997, in which a correlation of TNFα levels in serum and CSF with a clinical outcome of JE was presented.

Regarding the environmental conditions of the residential areas of the patients, the 3rd to the 5th cases inhabited Denpasar where there are rice fields and pig farms, while the 1st and the 2nd cases lived in rural areas distant from Denpasar and surrounded by rice fields and pig farms. The rice fields in the study areas were dry without rainfall and irrigation during the study period.

Wuryadi and Suroso (1989) commented that the increased mosquito population, for example *Culex tritaeniorhynchus*, does not always correlate with rainfall, but with agricultural practices. However, it is likely that mosquitos could have bred and transmitted JE virus from pig to human in Denpasar and nearby at that time.

Our serological confirmation of 5 JE cases in Indonesia supports the warning of Wuryadi and Suroso (1989) that we have to be alert, and it is only a matter of time until JE will become a health problem in Indonesia.

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