A SEROLOGICAL STUDY OF JAPANESE ENCEPHALITIS VIRUS INFECTIONS IN NORTHERN PENINSULAR MALAYSIA

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Abstract. This study describes the status of viral encephalitis in Perak, Malaysia during the year 1990. In addition, 14 cases selected from Penang and Perak during the years 1989 and 1990 are presented, with data showing titers of neutralizing antibodies against Japanese encephalitis virus (JEV) and dengue 2 virus, titers of antibodies against JEV and dengue virus antigens as determined by DEIA, and a comparison of these with the presence of IgM to JEV and dengue virus. These data show that there probably is far more viral encephalitis due to JEV in Malaysia than the national figures reflect.

INTRODUCTION

First recognised in Japan in 1871, with isolation of the etiologic agent in 1925, acute viral encephalitis caused by the arthropod borne flavivirus Japanese encephalitis virus (JEV) has long been endemic in Asia. In the more temperate of subtropical countries in this region, the occurence of the disease is seasonal, resulting in occasional epidemics, while in the more tropical areas, disease incidence appears to be sporadically distributed throughout the year.

Recent reports of large outbreaks of JEV infection in Nepal, China and elsewhere in the less developed countries of Asia have underlined the need to determine the status of viral encephalitis in Malaysia, where very little attention has been given to assessing the incidence of JEV infection. Indeed, apart from Fang's account of a suspected Japanese encephalitis outbreak in Pulau Langkawi in June 1979 (Fang *et al,* 1980), JEV in Peninsular Malaysia has been addressed seriously only by a few studies in the fifties (Cruikshank, 1951; Peterson *et al,* 1952; Pond *et al,* 1954; McCrumb, 1955) while the situation in Sarawak has been studied somewhat more recently by Simpson *et al,* 1970; 1974).

This study is an attempt to fill this vacuum, and although the number of cases described is not large, the data show that JEV infection does continue to occur at the very least in northern Peninsular Malaysia although the national figures indicate an annual mean incidence of only 20.6 cases of confirmed Japanese encephalitis in the whole country during the 12 year period 1977 to 1988. During the same period a mean of 2 cases per year was reported for the state of Perak (Sinniah, 1989).

MATERIALS AND METHODS

Serum specimens from patients with clinical suspicion of viral encephalitis were obtained for routine serological determinations from Ipoh General Hospital and Penang General Hospital. It was rarely possible to obtain cerebrospinal fluids (CSF). All specimens were stored at -40°C until tested.

IgM capture ELISA was carried out on all specimens exactly as described by Innis *et al* (1989) using JE virus hemagglutinin and a cocktail of dengue virus hemagglutinins as described. Briefly, 96 well microtiter plates (Nunc maxisorb, Denmark) were sensitized with a rabbit anti-human mu chain antibody (Behring, Germany). Patients' sera were diluted 1:100 in phosphate buffered saline (PBS), added to the wells and incubated at 4°C overnight. The plate was then washed with PBS containing 0.05% Tween 20 and 50µl of viral hemagglutinin (JE or dengue cocktail)

diluted in PBS containing 20% acetone extracted normal human serum was then added to the wells. After a two hour incubation at room temperature, the plate was washed again and antigen bound was detected using 25 µl per well of an optimal dilution of horseradish peroxidase conjugated human anti-flavivirus IgG prepared as described by Burke *et al* (1982). After a one hour incubation at room temperature color was developed using the o-phenylene diamine/hydrogen peroxide system as described. Absorbance was read on a plate reader (Anthos, Austria) at 492 nm and results were calculated and interpreted as described by Innis *et al* (1989).

Dot enzyme immunoassay (DEIA) was carried out essentially as described previously (Cardosa *et al*, 1988) using antigens prepared from C6/36 cells infected with JE virus or the 4 dengue serotypes. The JE antigen coated nitrocellulose membranes contained JE antigens and uninfected cell controls, while the dengue coated membranes contained a cocktail of dengue virus antigens and uninfected cell controls. Unbound sites on the nitrocellulose membranes were blocked with 5% skimmed milk in PBS. Patients' sera were serially diluted and incubated with antigen coated membranes for 60 minutes at room temperature. Antibodies bound to the membranes were detected by incubating for 60 minutes with a conjugate of Protein A-horseradish peroxidase while visualization was accomplished using a 4 chloro-1-naphthol/hydrogen peroxide chromogenic substrate.

Plaque reduction neutralization test (PRNT) was performed in 24 well cluster plates (Costar, USA) using a carboxymethyl cellulose overlay following the method of Porterfield (personal communication). 100 µl of serum dilution (heat inactivated at 56°C for 30 minutes) was mixed with 100 of virus dilution (to give approximately 30 to 40 plaques per well) in individual wells of a 24 well cluster plate. Controls included wells containing no virus (diluent only) and wells containing virus with no serum (virus only). Plates were incubated at 37°C for 60 minutes before adding 0.5 ml PS Clone D cells (3×10^5 cells/ml) suspended in Leibovitz 15 medium containing 10% tryptose phosphate broth and 3% heat inactivated fetal bovine serum. Cells were allowed to adhere to the bottom of the wells at 37°C for 2 to 3 hours, after which 0.4 ml of 1.5% carboxymethyl cellulose in Leibovitz 15 medium was added to each well to form a semisolid overlay. Plates were incubated stationary at 37°C for 3 days (JE virus) or 5 days (dengue 2 virus) and stained with naphthalene black to visualize the plaques. Plaques were counted and endpoint antibody dilutions which inhibited at least 50% of the plaques in the virus-only controls were determined for JE and for dengue 2 viruses for each serum.

Viruses used were dengue 2 (16681 strain) and JE virus (Nakayama strain) which were gifts of Dr JS Porterfield at the Universiy of Oxford. Both viruses were propagated in *Aedes albopictus* C6/36 cells.

RESULTS

Laboratory confirmation

Table 1 shows that there was a total of 60 cases of viral encephalitis seen in Perak during 1990. Laboratory confirmation was carried out by determination of IgM specific for JEV (usually these cases did not have IgM to dengue virus) in serum or CSF. Paired sera were requested but as shown in Table 2, we received paired sera from only 17 of the 60 cases. If IgM to JEV was absent in the first serum it was not possible to confirm JEV infection without the second serum. As shown in Table 2, 3 cases with paired sera which did not have IgM in the firse serum specimen showed seroconversion to positive IgM in the second serum specimen, while 3 cases with IgM in the first serum specimen did not have IgM to JEV in the second serum specimen.

Thus, returning to Table 1, it was not possible to be certain of JEV infection status of the 37 cases with single specimens which did not have IgM to JEV. Only 7 of the 17 or 41.2% of the cases with paired sera were IgM negative for JEV in both sera, suggesting a clinical accuracy of 58.8% for confirmable cases (that is, cases with paired specimens).

Table 2 shows that 43 of the 60 cases (71.7%) had only single specimens and that only 6 (14%) of these had IgM to JEV. It is unclear why only single specimens were available for such a large majority of the cases. One possible reason is that the clinical diagnosis was changed and the follow-

Table 1

Viral encephalitis in Perak during 1990: laboratory confirmation.

Month	J	F	M	A	M	J	J	A	S	O	N	D	Total
Positive	2	4	0	2	2	1	0	1	1	1	0	2	16
Negative	0	0	0	1	0	1	1	1	2	0	0	1	7
NEG singles	2	1	3	3	4	0	6	1	4	6	4	3	37
Total	4	5	3	6	6	2	7	3	7	7	4	6	60

Table 2

Comparison of cases with single and paired specimens : Perak 1990.

	Single	Paired
1st specimen:		
IgM positive	6	7
IgM negative	37	10*
2nd specimen:		
IgM positive	-	7
(Seroconversion)*		(3)
IgM negative	-	10
Total positive cases	6 (14.0%)	10 (58.8%)
Total cases	43	17

*3 cases seroconverted

up specimen was thus not taken. Some of these cases may also have died before a second specimen was taken, but we do not routinely receive any feedback on this.

In summary then, only 16 of the 60 viral encephalitis cases in Perak during 1990 could be confirmed as JEV infections. As mentioned earlier, the annual mean number of laboratory confirmed JEV infections recorded for Perak has been 2 for the period 1977 to 1988 (Sinniah, 1989).

Case studies: single specimens

Fourteen cases positive for JEV infection were selected from cases in Penang and Perak during the years 1989 and 1990. Seven of these had single specimens (Table 3) and 7 had paired specimens (Table 4). Each serum was titered for antibodies to JE and Dengue 'viruses by DEIA and by PRNT (see Table 5). The first case in Table 3 (AM) was reported as presumptive negative for JE because the single specimen did not have IgM to JE. It was later determined that this patient had high titer DEIA antibodies to JE and the reciprocal PRNT titer for JEV antibodies was 640 as compared to only 40 for dengue. This is very suggestive that this patient who presented with high grade fever and CNS signs had had a JEV infection, and that if a second serum had been obtained, laboratory confirmation could have been possible. However, since this patient was 26 years old, the high titer

Table 3

JE case studies : single specimens.

Case	Age/sex	JE IgM	DEN IgM	JE IgG (>1:250)	JE IgG (>DEN)	JE PRNT	JE PRNT (>DEN)
AM	26/M*	0	0	1	1	1	1
ES	3/F	1	0	1	1	1	1
JA	7/M	1	0	1	1	1	1
KA	6/F	1	0	1	1	1	1
LE	10/M	1	0	0	0	1	1
MX	14/F	1	0	0	0	1	0**
SA	6/M	1	0	1	1	1	1

*Presumptive positive **Same titer

Table 4

JE case studies : paired specimens.

Case	Age/sex	JE IgM	1st SPEC positive	DEN IgM	JE IgG (not DEN)	JE PRNT	JE PRNT (>DEN)
BA	18/M	1	1	0	1	1	1
CH	11/F	1	0	0	1	1	1
HA	9/M	1	1	0	0*	1	1
ME	9/M	1	1	1	1	1	1
MS	18/M	1	0	1	1	1	1
NZ	12/F	0	0	0	1	1	1
RU	8/M	1	1	0	1	1	1

*Starting titer high (>1:1000)
1 = Yes
0 = No

JEV specific neutralizing antibodies could conceivably be suggestive merely of a recent JEV infection.

All the other cases with single specimens were children, and all had IgM to JEV but not to dengue virus.Two of these(LE and MX) had no detectable IgG antibodies to JE and dengue viruses by DEIA but had neutralizing antibodies to JEV. All the other cases shown in Table 3 showed antibodies to JEV by DEIA and the titers were higher to JEV than to dengue virus. These children also had higher titers of neutralizing antibodies to JEV than to dengue.

Case studies: paired specimens

In the second group of case studies (Table 4, Table 5B), all cases had paired sera and the ages ranged from 8 to 18 years. All these except one (NZ) had IgM antibodies to JEV in at least one of the pair of serum specimens taken. NZ had no IgM to JE or to dengue viruses in either specimen but was considered to have had a confirmed JEV infection because she showed IgG seroconversion by DEIA to JEV but not to dengue virus. She also showed seroconversion by PRNT from a reciprocal titer of less than 10 to 640 for JEV but only seroconverting to a reciprocal titer of 20 for dengue 2 virus.

Two of the other cases in this study (CH and MS) had no IgM to JEV in the first specimen, but had IgM to JEV in the second specimen. One of these (MS) also had IgM to dengue virus although the titer was higher to JEV than to dengue virus. Furthermore, this patient had high titer neutralizing antibodies to JEV but not to dengue virus. Another patient (ME) also had IgM to dengue as well as to JEV, but this dengue IgM was less persistent than the JE IgM, being undetected in the second specimen. The patient ME also had neutralizing antibodies to JEV but not to dengue 2 virus.

All cases consistently showed neutralizing antibodies to JEV as being more marked than neutralizing antibodies to dengue 2 virus. The patient CH had a PRNT reciprocal titer of 160 against dengue in both sera, but seroconverted from no neutralizing antibodies to JEV in the first serum to a reciprocal titer of 160 in the second serum. This case can be deduced to have had a dengue infection prior to this JE infection (she had IgM to JEV not to dengue in this episode of illness).

It should also be noted that in this group of patients with paired sera, all showed IgG seroconversion to JEV by DEIA except when the IgG titer was already high (case HA). Further, none of these patients except CH had detectable IgG antibodies to dengue virus as determined by DEIA.

DISCUSSION

These data show that there are significant numbers of viral encephalitis cases in northern

Table 5

Comparison of IgM capture ELISA with PRNT and DEIA titers for selected cases.

A. Single specimens

Case	Date of onset	Date specimens taken	IgM capture ELISA		PRNT (reciprocal titer)		DEIA (reciprocal titer)	
			JE	DEN	JE	DEN2	JE	DEN
AM	9.12.88	19.12.88	−	−	640	40	1000	500
ES	19.6.90	4.7.90	+	−	160	<10	250	<200
JA	23.2.90	26.2.90	+	−	10	<10	200	<200
KA	4.9.89	19.9.89	+	−	160	20	250	<200
LE	7.11.88	14.11.88	+	−	40	10	<200	<200
MX	21.7.90	25.7.90	+	−	20	20	<200	<200
SA	2.1.90	5.1.90	+	−	640	20	>1000	500

B. Paired specimens

Case	Date of onset	Date specimens taken	IgM capture ELISA		PRNT (reciprocal titer)		DEIA (reciprocal titer)	
			JE	DEN	JE	DEN2	JE	DEN
BA	22.5.90	30.5.90	+	−	80	10	<200	<200
		5.6.90	+	−	160	20	>1000	<200
CH	na*	6.7.90	−	−	<10	160	200	500
		17.7.90	+	−	160	160	500	500
HA	18.1.90	19.1.90	+	−	<10	<10	1000	<200
		16.2.90	−	−	160	<10	1000	<200
ME	22.2.90	26.2.90	+	+	40	<10	500	<200
		12.3.90	+	−	640	<10	1000	<200
MS	1.4.90	16.4.90	−	−	160	20	<200	<200
		3.5.90	+	+	640	40	1000	<200
NZ	18.2.90	22.2.90	−	−	<10	<10	<200	<200
		5.3.90	−	−	640	20	1000	<200
RU	26.4.90	30.4.90	+	−	80	<10	250	<200
		4.6.90	+	−	160	10	1000	<200

*not available

peninsular Malaysia which are caused by Japanese encephalitis virus. The true incidence of encephalitis of JEV etiology is unknown at this time since only single serum specimens are available for the majority of these cases. Determination of IgM to JEV in a single serum specimen is not adequate to confirm that JEV is *not* implicated. A detailed study of some cases in Penang and Perak clearly shows that patients with no IgM in the first serum specimen may show seroconversion either for IgM or neutralizing and other antibodies (DEIA) to JEV.

It is thus necessary to perform a detailed study of the incidence of JEV in Malaysia in order to assess public health policies and to design an effective control program for Japanese encephalitis virus infections in Malaysia.

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