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ETIOLOGY OF ENCEPHALITIS SYNDROME AMONG HOSPITALIZED CHILDREN AND ADULTS IN TAKEO, CAMBODIA, 1999–2000

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Abstract. Whether or not Japanese encephalitis virus (JEV) is an important causative agent of acute encephalitis in Cambodia remains unclear. This study was carried out to determine the cause of encephalitis syndrome among children and adults admitted to Takeo Provincial Hospital from October 1999 to September 2000. Ninety-nine cases were included in the study: 52 pediatric cases (12 were fatal) and 47 adult cases (10 were fatal). A causative agent such as human herpesvirus (HHV-3 or HHV-4), *Cryptococcus neoformans,* or *Mycobacterium tuberculosis* had been identified in 8 of the 11 adults who had human immunodeficiency virus type 1 (HIV-1). An infectious agent was identified in 35 (40%) of 88 HIV-1–seronegative patients (60% of the causes remains unidentified). These comprised 11 bacterial infections, 1 fungal infection, and 23 viral infections. The viral infections were 1 fatal HHV-4 infection, 5 dengue virus infections (2 fatal), 1 coinfection with flavivirus and alphavirus, and 16 presumptive infections JEV (no virus detected), one case of which was fatal. Infection with JEV, the principal cause identified in the 99 encephalitis syndromes, concerned 16 (31%) of 52 children.

INTRODUCTION

Japanese encephalitis virus group belongs to the genus *Flavivirus* (family Flaviviridae) and includes various viruses responsible for central nervous system (CNS) infections in humans. Japanese encephalitis virus (JEV) is the virus of the group found in Asia, where it is the most common cause of viral encephalitis. Virus detection remains rare from serum and cerebrospinal fluid (CSF). Consequently, only a presumptive diagnosis of JEV infection can be established by serological tests, including the hemagglutination inhibition test and immunoglobulin (Ig) M–capture enzyme-linked immunosorbent assay (MAC-ELISA). These tests cannot distinguish between different types of flavivirus infections in endemic settings where exposure to more than one flavivirus is common.[1]

Data on JEV in Cambodia are lacking. This virus was isolated for the first time in Cambodia in 1965 from mosquitoes.[2] The country, a lowland tropical area (like southern Vietnam and southern Thailand), is considered to be endemic country where cases occur at low rates year round.[1] In 1993–1994, two pediatric hospitals in Phnom Penh city reported numerous hospitalized encephalitis syndrome (ES) cases, with high case-fatality rates. These cases were attributed to JEV according to clinical criteria (laboratory diagnosis was not available), although the epidemiological patterns (season, median age of patients) did not fit with JEV infection.[3] Laboratory diagnosis of Japanese encephalitis (JE) has been available at Pasteur Institute of Cambodia (PIC) since 1997.

To assess the importance in Cambodia of JEV as an infectious cause of ES, the Ministry of Health and PIC conducted a study in a rural provincial hospital to investigate bacterial, fungal, and viral etiologies in hospitalized ES cases. The results of that study are reported here.

MATERIALS AND METHODS

Patients and specimens. The study was conducted in Takeo Provincial Hospital, 80 km south of Phnom Penh, for 1 year (October 1, 1999, to September 30, 2000). Patients included in the study were those admitted to the hospital with fever, or with history of fever and clinical ES defined by alteration of consciousness (lethargy, confusion, stupor, coma, behavioral changes, or hallucination) and/or focal neurological signs (seizure, motor weakness, increased deep tendon reflexes, extensor plantar responses, tremor, choreiform movements, dysphasias dysphonia, or paralysis). Patients with evidence of intoxication (drugs or others substances), clinical diagnosis of rabies or tetanus, history of seizure, cranial traumatism, or slide-positive cerebral malaria were not included in the study.

Serum and CSF were sampled at admission and, when possible, at discharge. Samples were processed in the laboratory within 2 hr. Patients from whom no CSF samples were obtained were excluded from the study.

Laboratory testing in Takeo Provincial Hospital. Admission CSF was taken for cell count and differential. Gram, Ziehl, and China ink stains were performed. Admission CSF was also used for bacterial culture on GC base, Sabouraud, and Lowenstein-Jensen agars, and, when the CSF was turbid, on Schaedler agar. Bacterial cultures were sent the next day to PIC for eventual growth and identification. The remaining admission CSF and discharge CSF were stored in liquid nitrogen containers and sent weekly to PIC for viral detection and serology.

Admission blood samples were taken for assessment of malaria parasites and for viral detection and serology. Serum was stored in liquid nitrogen containers after centrifugation of blood. Discharge bloods were also sampled for serology and treated in the same manner.

Virological and serological studies at PIC. *Arbovirus isolation and identification.* Isolation was performed on AP 61 and Vero E6 cells with admission serum (diluted 1:10) and CSF (diluted 1:2) as previously described.[4] Dengue virus (DENV) type-specific, JEV, and flavivirus monoclonal antibodies, and Chikungunya virus (CHIKV) and Langat virus (LGTV) immune ascitic fluids were used for identification.

Arbovirus serological methods. Hemagglutination inhibition (HI) titers were determined for sera by means of the method of Clarke and Casals[5] adapted to microtechnique, with 4 hemagglutinating units (this technique is not suitable

TABLE 1

Pathogens identified in 47 adult patients with encephalitis syndrome in Takeo Provincial Hospital, Cambodia, October 1999–September 2000*

Variable	Duration of illness before admission				
	≤15 days		> 15 days		
	HIV negative	HIV positive	HIV negative	HIV positive	Total (%)
Streptococcus sp.	2				2 (4.2)
Mycobacterium tuberculosis			1		1 (2)
M. tuberculosis + HHV-4	1	1	1		3 (6.4)
Cryptococcus neoformans		2	1		3 (6.4)
C. neoformans + HHV-4		1		3	4 (9)
HHV-4	1				1 (2)
HHV-3				1	1 (2)
DENV	1				1 (2)
Flavivirus + alphavirus	1				1 (2)
Nonflavivirus + nonalphavirus	15		1		16 (34)
Flavivirus + Alphavirus inconclusive	8	2	3	1	14 (30)
Total	29	6	7	5	47 (100)

* HHV = human herpesvirus; HIV = human immunodeficiency virus; DENV = dengue virus.

for CSF). Specific IgM against DENV, JEV, CHIKV, and LGTV were measured in sera and CSF by means of MAC-ELISA for arboviruses.[6] We determined sample titer to be the reciprocal of the last dilution that was equal or greater than the optical density cutoff value (serial dilutions started 1:10 for CSF and 1:100 for serum, with 10-fold dilutions).

Detection of DENV and JEV RNA in admission serum and CSF. One hundred microliters of each sample was mixed with 600 μL of Trizol LS reagent (Life Technologies, Gaithersburg, MD), and RNA was extracted according to the manufacturer's instructions. Dry RNA was redissolved in 25 μL of RNase-free water, incubated for 10 min at 55°C, used for reverse transcription, and stored at −70°C. Reverse transcription and amplification were performed for DENV according to Lanciotti and others[7] and for JEV according to Raengsakulrach and others.[8] Routine DENV and JEV assays were able to detect 10^1 tissue culture infective dose $(TCID)_{50}$/mL and 10^1 plaque-forming units per milliliter of specimen, respectively.

Case definition for an arbovirus infection. Hemagglutination inhibition antibody responses were defined by means of the World Health Organization criteria[9] of ''acute infection,'' ''recent infection,'' or ''absence of infection'' by a flavivirus or an alphavirus. Furthermore, acute infection was demonstrated by ≥ 4-fold rise of IgM titers between admission and discharge, positive MAC-ELISA associated with an HI titer equal to 0, or direct detection of the virus. No change or decrease of IgM titer associated with elevated HI titers constituted a recent infection. Low ratios of the serum antibody titer to the CSF antibody titer, such as 8–32, were suggestive of *in situ* synthesis of antibody and were defined as probable CNS infection,[10] although the integrity of the blood-brain barrier was not established in our study. We defined DENV or JEV confirmed infection by a positive direct detection of the virus. Last, DENV or JEV presumptive infection was defined by a negative direct detection of the virus, associated with DENV- or JEV-specific IgM responses of an acute or a recent infection.

Enterovirus RNA detection in admission CSF. Reverse transcription and amplification seminested polymerase chain reaction (PCR) were performed according to the method of Lina and others.[11] Routinely, this assay, performed with poliovirus 1, poliovirus 2, and human coxsackievirus B3 viruses, was able to detect 10^1 $TCID_{50}$/mL of specimen.

Herpesvirus DNA detection in admission CSF. A standard method[12] was used to obtain DNA from 100 μL of CSF. Human herpesvirus (HHV)-1, HHV-2, HHV-4, and HHV-5 were detected by PCR according to the method of Rozenberg and Lebon.[13] Routinely, this assay, performed with HHV-1 and HHV-2 viruses, was able to detect 10^0 $TCID_{50}$/mL of specimen. We detected HHV-3 via PCR with specific primers (HHV-3 + 5′-CATACGTCGAGGCGGTTGAC-3′ and HHV-3 – 5′-AAATCGCGACATCGTACTAC-3′) and specific biotinylated probe (5′-GAGGCGGACGAGGCATGT GCAAACTTATTTCATACGCGTA-3′) (Poveda JD, unpublished data).

Serological screening for human immunodeficiency virus (HIV). We tested for HIV in admission or discharge serum via Genscreen ELISA (Sanofi Diagnostics Pasteur, Marnes La Coquette, France).

RESULTS

During the 1-year period of the study, 99 patients were included in the study. Of these, 52 were children (< 15 years old), 12 of whom died, and 47 were adults, 10 of whom died (the reported deaths are those observed at the hospital).

Laboratory diagnosis in adults. Pathogens could be identified in 17 (36%) of the 47 adult patients and included fungi, bacteria, and viruses (Table 1). Eleven of the adult patients (23%) were HIV seropositive. Pathogens were identified in CNS in 8 (73%) of the 11 HIV-seropositive patients, whereas pathogens were identified in only 9 (25%) of the 36 HIV-seronegative patients. Twelve (26%) of the 47 ES infections started > 15 days before admission, and 5 (42%) of these 12 patients were HIV seropositive. Thirty-five (74%) of the 47 ES infections started ≤ 15 days before admission, and 6 (17%) of these 35 patients were HIV seropositive. Only two arbovirus infections (Patient T057, with acute infection, and Patient T070, with recent infection) were diagnosed (Table 2). The definitive implication of the virus in these two patients with ES could not be confirmed because the direct detection of arboviruses in CSF tested negative, the IgM antibody in sera and CSF tested negative

TABLE 2

Summary of patient features and laboratory data for the patients infected by an arbovirus*

Patients no.	Laboratory diagnosis	Age	Sex	Day after onset**	Samples	CSF cells count			Direct diagnosis		Serological diagnosis						
											HI					IgM Elisa***	
						WBC/mm^3	RBC/mm^3	L %	RT-PCR-Nested DENV and JEV	Arbovirus isolation	DENV-2	DENV-3	JEV	LGTV	CHIKV	DENV	JEV
T027	JEV	11m	F	2	S1				N	N	640	640	640	80	<10	N	0
				8	S2						20	40	160	<10	<10	N	3200
				24	S3						20	20	640	<10	<10	N	1600
					CSF1	8	70	ND	N	N						N	10
					CSF2											N	20
T030	JEV	5y	M	5	S1				N	N	1280	1280	640	160	<10	N	N
				12	S2						10240	10240	10240	1280	<10	N	N
					CSF1	40	20	ND	N	N						N	N
					CSF2											N	10
T041	JEV	6y	M	4	S1				N	N	80	160	160	10	640	N	N
				11	S2						2560	1280	1280	320	640	N	N
					CSF1	30	350	ND	N	N						N	N
					CSF2											N	10
T044	JEV	5y	M	6	S1				N	N	<10	<10	<10	<10	<10	N	N
				13	S2						<10	10	640	<10	<10	N	6400
					CSF1	30	20	ND	N	N						N	160
					CSF2											N	≧640
T046	JEV	5y	M	7	S1				N	N	<10	<10	<10	<10	<10	N	1600
				14	S2						10	20	160	<10	<10	N	≧6400
					CSF1	40	20	ND	N	N						N	160
					CSF2											N	160
T047	JEV	5y	F	6	S1				N	N	320	640	320	80	<10	N	N
				11	S2						5120	5120	5120	2560	<10	N	N
					CSF1	10	4	ND	N	N						N	N
					CSF2											N	40
T073	JEV & Alphavirus	9m	M	6	S1				N	N	<10	<10	<10	<10	<10	N	400
				13	S2						<10	10	5120	<10	40	N	≧6400
					CSF1	22	4	65	N	N						N	160
					CSF2											N	320
T083	JEV	6y	M	4	S1				N	N	2560	5120	2560	640	2560	N	N
				21	S2						2560	5120	2560	640	2560	N	N
					CSF1	130	100	82	N	N						N	10
					CSF2											N	10
T086	JEV	9y	M	1	S1				N	N	80	640	40	10	<10	N	N
				8	S2						2560	2540	1280	320	<10	N	N
					CSF1	10	300	ND	N	N						N	N
					CSF2											N	80
T091	JEV	10y	M	7	S1				N	N	1280	1280	320	80	5120	N	N
				9	S2						2560	2560	1280	320	5120	N	N
				24	S3						20480	10240	10240	1280	5120	N	N
					CSF1	80	20	94	N	N						N	N
					CSF2											N	40
T095	JEV	16m	F	2	S1				N	N	<10	<10	20	<10	<10	N	N
				8	S2						<10	<10	40	<10	<10	N	≧6400
					CSF1	8	3	ND	N	N						N	40
					CSF2											N	40

TABLE 2
Continued

Patients no.	Laboratory diagnosis	Age	Sex	Day after onset**	Samples	CSF cells count			Direct diagnosis		Serological diagnosis						
											HI					IgM Elisa***	
						WBC/mm³	RBC/mm³	L %	RT-PCR-Nested DENV and JEV	Arbovirus isolation	DENV-2	DENV-3	JEV	LGTV	CHIKV	DENV	JEV
T096	JEV	7y	M	5	S1				N	N	<10	<10	20	<10	<10	N	400
				7	S2						<10	<10	40	<10	<10	N	400
					CSF1	180	10	74	N	N						N	80
					CSF2											N	80
T097	JEV	5y	F	2	S1				N	N	<10	<10	<10	<10	<10	N	N
				9	S2						<10	10	80	<10	<10	N	3200
				16	S3						20	20	160	<10	<10	N	3200
					CSF1	300	100	30	N	N						N	N
					CSF2											N	80
T099	JEV	5y	M	5	S1				N	N	5120	2560	2560	640	<10	N	N
				10	S2						10240	10240	10240	2560	<10	N	N
					CSF1	5	numerous	ND	N	N						N	N
					CSF2											N	20
T101	JEV	12y	F	5	S1				N	N	<10	<10	<10	<10	<10	N	N
					CSF1	50	30	ND	N	N						N	80
T104	JEV	3y	F	4	S1				N	N	<10	<10	<10	<10	<10	N	800
				10	S2						20	20	40	<10	<10	N	≧6400
					CSF1	3	9	ND	N	N						N	80
					CSF2											N	40
T009	DENV-2	6y	M	6	S1				DENV-2	N	40	80	160	0	1280	N	N
				14	S2						20480	20480	20480	10240	1280	800	N
					CSF1	140	170	18	DENV-2	N						N	N
					CSF1											N	N
T025	DENV-3	8y	F	8	S1				DENV-3	N	20	80	40	0	0	N	N
				17	S2						160	320	320	0	0	N	N
					CSF1	150	30	44	N	N						N	N
					CSF2											N	N
T066	DENV-3	3m	F	3	S1				DENV-3	ND	0	0	0	0	0	≧6400	400
					CSF1	20	numerous	ND	DENV-3	N						40	0
T070	DENV	15y	M	5	S1				N	N	320	80	160	0	0	≧6400	N
				11	S2						640	80	320	10	0	≧6400	N
					CSF1	7	25	ND	N	N						80	N
					CSF2											10	N
T072	DENV-3	3y	M	3	S1				DENV-3	DENV-3	640	0	0	0	0	N	N
				10	S2						10240	2560	1280	320	0	1600	N
					CSF1	14	150	ND	DENV-3	N						N	N
					CSF2	0	1	ND								N	N
T057	Flavivirus & alphavirus	42y	M	3	S1				N	N	0	10	320	0	0	N	N
				7	S2						320	160	320	20	80	N	N
					CSF1	10	250	ND	N	N						N	N
					CSF2											N	N

* CSF = cerebrospinal fluid; WBC = white blood cells; RBC = red blood cells; L = lymphocytes; RT-PCR = reverse transcription–polymerase chain reaction; HI = hemagglutination inhibition; LGTV = Langat virus; CHIKV = Chikungunya virus; N = negative; ND = not done; S = serum; y = year; m = month, F = female; M = male.
** CSF1 and S1(admission), CSF2 and S2 (discharge) are sampled on the same day respectively.
*** All the samples tested negative for CHIKV and LGTV IgM.

TABLE 3

Pathogens identified in 52 pediatric patients with encephalitis syndrome in Takeo Provincial Hospital, Cambodia, October 1999–September 2000*

Variable	Duration of illness before admission		Total (%)
	≤ 15 days	> 15 days	
Haemophilus influenzae	3		3 (5.8)
Staphylococcus sp.	1		1 (1.9)
Mycobacterium tuberculosis	1		1 (1.9)
DENV-2	1		1 (1.9)
DENV-3	3		3 (5.8)
JEV	15		15 (28.9)
JEV + alphavirus	1		1 (1.9)
Nonflavivirus + nonalphavirus	10	2	12 (23.1)
Flavivirus + alphavirus inconclusive	15		15 (28.8)
Total	50	2	52 (100)

* DENV = dengue virus; JEV = Japanese encephalitis virus.

(Patient T057), or the ratio of the serum DENV IgM titer to the CSF DENV IgM titer was > 40 (Patient T070). Patient T057, who was 42 years old, was diagnosed with an acute flavivirus and alphavirus coinfection. Patient T070, who was 15 years old, was diagnosed as a presumptive recent dengue infection. No JEV or enterovirus infections were diagnosed. Acute or recent infection by a flavivirus or an alphavirus was dismissed in 16 patients (34%). Arbovirus laboratory diagnosis was inconclusive in 14 (30%) of the 47 patients with ES.

Laboratory diagnosis in children. Pathogens were more often identified among children (48%) than among adults (36%). Table 3 shows that 20 of the 25 laboratory diagnoses were viral infections, and all were caused by arboviruses. Sixteen (31%) of the 52 children with ES had ES that was caused by presumptive JEV infections, and 4 (8%) infections were caused by DENV infections. One patient with presumptive JE was coinfected with an alphavirus. No enterovirus or herpesvirus infection was detected. All children were HIV seronegative, and 50 of them (96%) had been sick for ≤ 15 days before admission. Acute or recent infection by a flavivirus or an alphavirus was dismissed in 12 cases (23%). Arbovirus laboratory diagnosis was inconclusive in 15 patients (29%).

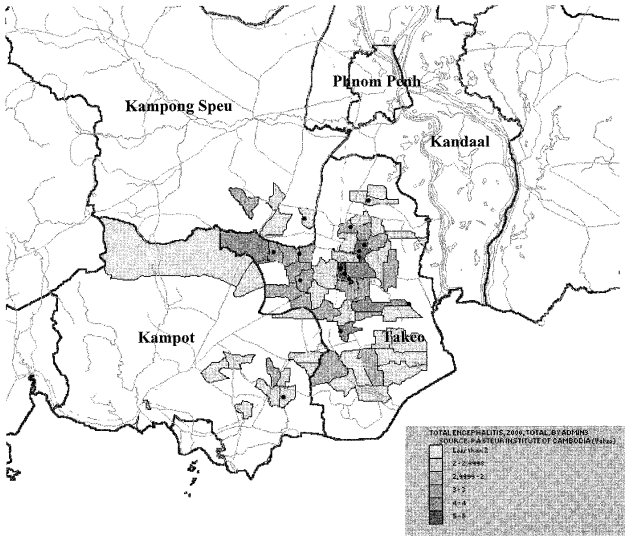

FIGURE 1. Spatial distribution of 99 encephalitis syndrome cases and 16 presumptive Japanese encephalitis (JE) cases. Patients were admitted to Takeo Provincial Hospital, October 1999–September 2000. One dot represents one presumptive JE case. 1 cm = 30 km. Location was unknown for 3 patients (one of whom had presumptive JE).

Arboviruses infections in children. Four dengue infections were diagnosed among children (Table 2): three DENV-3 infections (Patients T025, T066, and T072) and one DENV-2 infection (Patient T009). One patient with DENV-3 and the patient with DENV-2 died. Patient T025 presented an hemorrhagic manifestation (hematuria). In this case, DENV involvement in the CNS was not established because specific DENV IgM antibody tested negative in CSF and the virus was detected only in serum taken at admission. The DENV involvement in the CNS was also not established in the three other patients: viruses were detected in both admission CSF and serum, but specific DENV-IgM antibody tested negative in all CSF samples (Patients T009 and T072), or ratios of serum antibody titers to CSF antibody titers were > 40 (Patient T066).

Sixteen of the 52 children were presumed infected by JEV. No JEV strain was isolated or detected by reverse transcriptase nested PCR. According to HI titers in sera and IgM titers in sera and CSF (Table 2), there were 14 acute infections and two recent infections (Patients T083 and T096). Furthermore, in all 16 patients, JEV was probably involved in the CNS infection because specific JEV IgM antibody was present in the admission CSF but absent in admission serum in all patients but five (Patients T046, T073, T096, T097, and T0104). These five patients had specific JEV IgM antibody detected first in both admission CSF and serum (Patients T046, T073, T096, and T0104) or first in both discharge CSF and serum (Patient T097). The JEV involvement in CNS infection is suspected in these five patients because ratios of the serum antibody titer to the CSF antibody titer were no more than 40. Hemagglutination inhibition titers for the sera of Patient T027 decreased and increased. Patient T027 was an infant, and these variations were related to a consumption of maternal antibodies, then to the infant's own production of antibodies. Last, Patient T073 was coinfected by an alphavirus detected by HI test (CHIKV IgM antibody tested negative).

The median age of patients with presumptive JE was 5 years old (range, 9 months–12 years). Ten patients (63%) were boys, and all were admitted to the hospital within seven days after onset of the disease. Only one patient (Patient T101) died. Mean CSF white blood cell count average was 59/mm^3 (range, 3–300/mm^3). There was no apparent spatial cluster of patients with presumptive JE or in the other ES cases. The 16 JE cases represented 12 different municipalities, and the 99 total ES cases represented 25 different municipalities (Figure 1). The short duration of our study (1 year) did not allow us to show any temporal clustering for ES and presumptive JE cases (Figure 2).

No LGTV infection was detected.

DISCUSSION

Our 1-year study in Takeo Province demonstrated that arboviruses were an important cause of ES (22% of cases),

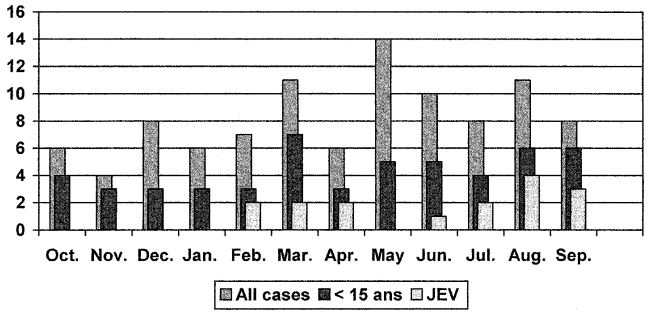

FIGURE 2. Monthly distribution of 99 encephalitis syndrome cases and 16 presumptive Japanese encephalitis cases. Patients were admitted to Takeo Provincial Hospital, October 1999–September 2000.

especially among children (38% of cases). Presumptive JEV infection was the primary cause of ES among children (31%) but was absent among adult patients. This situation is similar to that in neighboring countries where JE is endemic. Antibody prevalence is almost 100% in northern Thailand by age 14 years[14] but is slightly lower in southern Thailand, where cases occur sporadically among children[2] (as was found in our study). A study in northern Vietnam during the summer epidemic encephalitis season found 31 JE cases among 46 children with ES (67%) and found two cases of JE among 33 adults with ES (6%).[15] The median age and the sex ratio of the presumptive JE cases in Takeo Provincial Hospital were similar to those usually reported.[16] Despite no pediatric intensive care unit in Takeo Provincial Hospital, the case fatality rate was low (6%) among the presumptive JE cases in our study (one fatal case with negative virus detection), as compared with higher case-fatality rates reported elsewhere (extreme, 8 and 25%).[1,16] This low case-fatality rate could be because eight of the 12 children who died were inconclusively diagnosed (death occurred rapidly within four days after onset of the disease for five of the eight patients) and could have been cases of JE. However, JEV RNA in admission CSF tested negative in the undiagnosed patients who died, even though a positive detection is usually associated with death in JE infection.[17]

Japanese encephalitis virus was not detected in CSF or serum during our study. Low sensitivity of isolation or reverse transcriptase-PCR that uses these specimens has been previously reported.[1,10] This lack of JEV detection could be attributed to the implication of another virus found in Asia belonging to the JEV group that was responsible for the 16 JEV presumptive infection. This virus could be responsible for our positive JEV MAC-ELISA test (using antigens from JEV and not from other viruses of the JEV group). On the other hand, the risk of misidentification is reduced by the use for Lanciotti's molecular method for DENV detection, which has been shown to detect in the first round of amplification West Nile virus and St. Louis encephalitis virus, both of which belong to the JEV group (only DENV strains were detected in our study by this method). Misidentification with other groups of flaviviruses was not likely because our test showed specificity between the flavivirus groups (a dengue-positive patient tested negative for LGTV and JEV MAC-ELISA, and patients with presumptive JEV tested negative for DENV and LGTV MAC-ELISA). The JEV IgM antibody appeared first in the CSF for 63% of the patients and was detected concurrently in both matched sera and CSF in the other patients. This result was in agreement with that of Burke and others,[18] as was the finding that all the presumptive JE cases in our study were JEV IgM positive in CSF within seven days after admission. On the contrary, not all of the CSF samples in our study tested JEV IgM positive within seven days after onset, as reported elsewhere.[19,20]

Arboviruses other than JEV were the secondary cause of ES in our study. Two alphavirus infections were detected, one associated with presumptive JEV infection in a child and one associated with a flavivirus infection in an adult. Virus identification and specific CHIKV IgM tested negative. This alphavirus could be Me Tri virus, closely related to Semliki Forest virus, which has been isolated from *Culex tritaeniorhynchus* mosquitoes (vectors of JEV) during the summer epidemic encephalitis season.[21] Dengue infection was detected among five (5%) of the 99 patients we assessed (one adult and four children) and was the secondary cause of ES in HIV type 1 (HIV-1)-seronegative patients. A similar percentage was recently reported in southern Vietnam.[22] The DENV-3 and DENV-2 viruses, involved in the ES cases, were the two viruses most isolated in Cambodia (Horm Srey V, unpublished data). These data were based on 98 patients with pediatric dengue hemorrhagic fever or on patients with dengue shock syndrome, nine of whom died, who were admitted to the same hospital during our study period, a time when intermediate dengue hemorrhagic fever incidence was reported in Cambodia. Analysis of these unpublished data suggests that ES caused by DENV might be more numerous with a high dengue hemorrhagic fever incidence.

The frequency of other pathogens was $< 4\%$ in HIV-1-seronegative patients. Bacteria other than *Mycobacterium* species were found from all the turbid CSF (Horm Srey V, unpublished data). No enterovirus was detected among children. Enterovirus is usually more responsible for meningitis than encephalitis.[23] Herpesvirus infections were found only among adults, mostly HIV-1-seropositive patients. We did not detect HHV-1 and HHV-2 in our study, nor have they been reported as an important cause of ES in other studies.[20,24] Our study detected HHV-4 in eight patients (five HIV-1-seropositive patients) and in association with another pathogen in seven of these eight patients. The role of HHV-4 in the neurological illness of the patient is uncertain when there is concurrent detection of neurotropic infectious agents without HHV-4 antibodies in CSF.[25] Unfortunately, our study did not test HHV-4 antibodies.

There was no laboratory diagnosis in 58% of the patients, a similar value to that reported in other studies.[15,20] In our study, this could be explained in part by the use of antibiotics before admission that prevented the *in vitro* culture of bacteria. Furthermore, computed tomography had not been performed, which would have allowed us to exclude some of the patients; nor was it possible to obtain brain necropsy specimens—necropsy is a known sensitive detection method—from undiagnosed patients who died to look for JEV virus.[1] Furthermore, laboratory investigations

were limited and did not explore all the worldwide causes of aseptic encephalitis, including viruses responsible for postinfectious encephalitis (mumps, measles, or rubella), bacteria, or parasites such as toxoplasma, leptospira, and borrelia.[26]

Last, two emerging pathogens that are known to be responsible for encephalitis have been recently reported in Southeast Asia. The first is Kadipiro virus, which belongs to the coltivirus subgroup B, which has been isolated from *Culex* sp.[27] known from Cambodia.[28] The second is Nipah virus, which belongs to a new genus of the family Paramyxoviridae,[29] isolated from the bat *Pteropus hypomelanus* (Lam KSL and others, unpublished data), and this bat species has been reported from Cambodia.[30] Nipah virus was recently responsible for an outbreak of severe ES in Malaysia and Singapore.[29]

Presumptive JEV infection was found to be a major cause of viral encephalitis in children in Takeo Province. Because there was no diagnosis in 58% of the cases, further investigations for viral causes of ES in Cambodia will be pursued with brain samples from patients who died and through the implementation of a laboratory diagnosis for coltivirus and Nipah virus infection.

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