

USE OF IgM-CAPTURE ELISA FOR CONFIRMATION OF JAPANESE ENCEPHALITIS INFECTIONS IN THE PHILIPPINES

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Abstract. In 2001, the Research and Biotechnology Division (RBD) of St Luke's Medical Center, in collaboration with the Institute of Tropical Medicine of Nagasaki University in Japan, initiated a long-term study of Japanese encephalitis in the Philippines. Laboratory confirmation of acute cases of Japanese encephalitis was done by IgM-capture ELISA, which detects anti-JEV immunoglobulin M in cerebrospinal fluid (CSF) samples. In the period 2002-2004, a total of 614 CSF samples were submitted to RBD, and of these, 11.7% were positive for anti-JEV IgM: 17 in 2002, 18 in 2003, 32 in 2004, and 5 in 2005. Positive cases came from patients aged 2-77 years. In the 72 positive cases where gender was identified, 44 were male and 28 female. Possible co-infections with dengue virus were also detected by separate testing for anti-dengue IgM by ELISA in 17 CSF samples positive for JE.

INTRODUCTION

Japanese encephalitis virus (JEV) belongs to the Genus *Flavivirus*, Family *Flaviviridae*, and is the causative agent of Japanese encephalitis, a mosquito-borne disease transmitted by *Culex* spp. (*C. tritaeniorhynchus* and *C. vishnui* groups) breeding mostly in flooded rice fields (Kurane, 2002). Pigs are an important vertebrate amplifying host, facilitated by significant viremia following JE infection, large population size, high turnover rate and preferential feeding of the vectors (Burke *et al*, 1986). Humans are infected through the bite of an infected mosquito. The mosquito vector for JEV is widely distributed across Southeast Asia and the Western Pacific. The Philippines is endemic for JE virus (Vaughn and Hoke, 1992).

The Japanese encephalitis virus affects the membranes of the brain. Pathologic changes, such as perivascular congestion and hemorrhage, may be diffuse or focal, but are seen predominantly in the cortical gray and deep gray matter (WHO, 1988). In an epidemic of Japanese encephalitis, up to 10% of the susceptible human population may be infected. Most JE cases are mild infections accompanied by fever and headache, or without apparent symptoms. However, severe disease exhibits a rapid onset of headache, neck stiffness, disorientation, coma, seizure, and spastic paralysis, and may lead to death (WHO, 2005). One in 300 infected develops clinical encephalitis, and 20-40% of these cases become fatal, while half of the

survivors develop neuropsychiatric sequelae (Burke *et al*, 1985).

Few studies have documented the occurrence of JE in the Philippines (Barzaga, 1989) and there has been no surveillance for this disease. In 2001, the Research and Biotechnology Division (RBD) of St. Luke's Medical Center (SLMC), in collaboration with the Institute of Tropical Medicine of Nagasaki University in Japan, initiated a long-term study of Japanese encephalitis in the Philippines. Initial findings included confirmation of high levels of JEV infection in farm-bred pigs and Philippine monkeys in a rural area near Metro Manila (Inoue *et al*, 2001).

This paper reports on the usefulness of IgM-capture ELISA for confirming acute cases of Japanese encephalitis in the Philippines.

MATERIALS AND METHODS

Collection of CSF samples

Six hundred and fourteen (614) cerebrospinal fluid samples extracted by lumbar puncture from patients showing clinical encephalitis and/or meningitis were submitted by St Luke's Medical Center, San Lazaro Hospital, and the Department of Health, for confirmation.

IgM-capture ELISA for dengue and Japanese encephalitis

The IgM-capture ELISA protocol used was modified from the procedure of Bundo and Igarashi (1985). A 96-well flat-bottom microtiter plate was coated with 100 µl of affinity-purified goat anti-human IgM overnight at 4°C. Each well was blocked with 100 µl of Blockace at room temperature for 60 minutes,

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washed 3 times with PBS-0.5% Tween20 for 3 minutes each time. After this, 100 μ l of the test and control sera, each diluted to 1:1,000 with PBS-0.5% Tween20, were added to each duplicate well and incubated for 60 minutes. The wells were washed and 100 μ l of assay antigen (Tetravalent dengue virus antigen: 25 ELISA units per serotype; JEV antigen: 160 ELISA units, both prepared at the Research and Biotechnology Division, St Luke's Medical Center) were added and incubated for 60 minutes. After washing, the wells were reacted with 100 μ l of horseradish peroxidase (HRPO)-conjugated anti-flavivirus immunoglobulin G, prepared from anti-dengue high-titered human sera (selected from the St Luke's Dengue Serum Bank), at 1:4000 dilution for 1 hour. The plate was washed, and 100 μ l of substrate solution containing o-phenylenediamine dihydrochloride (OPD) and 30% hydrogen peroxide were added to each well and kept at room temperature in the dark for 60 minutes. All the preceding steps were done at 37°C unless otherwise indicated. The reaction was stopped by adding 100 μ l of 1N hydrochloric acid, and absorbance at 492 nm was measured by ELISA plate reader. A positive-to-negative ratio (P/N) was obtained by dividing the A_{492} of the test specimen by the A_{492} of the negative control. Samples showing a P/N ratio greater than or equal to 2.0 were considered positive.

RESULTS

Clinical specimens, collected from patients admitted for Japanese encephalitis, viral CNS infections, and TB meningitis, were submitted to the Research and Biotechnology Division of St Luke's Medical Center (SLMC-RBD) for testing. These samples, consisting mainly of cerebrospinal fluid, came from areas in North and Central Philippines, including urban areas of Metro Manila and Cebu City. These were kept at -86°C in the Cold Storage Facility of SLMC-RBD for preservation and archiving.

This study involved the development and application of a molecular assay, IgM-capture ELISA, for laboratory confirmation of acute cases of Japanese encephalitis. Anti-JEV immunoglobulin M was detected in sera and cerebrospinal fluid (CSF) samples taken from Filipino patients exhibiting symptoms of CNS infection. Separate testing for dengue virus infection by a similar procedure was also performed.

In the period January 2002-October 2005, a total of 614 CSF samples was submitted, and of these, 11.7% were positive for anti-JEV IgM: 17 of 184 in 2002, 18 of 164 in 2003, 32 of 174 in 2004, and 5 of 92 in 2005 (Table 1). Positive cases came from patients

whose ages ranged from 2-77 years (Table 2). In the 72 positive cases where gender was identified, 44 were male and 28 female (Table 3). The majority of positive samples came from Luzon (30), with the others from the National Capital Region (5) and the Visayas (4); the origins of the rest (33) were not reported (Table 4). Seventeen CSF samples positive for JE were positive for anti-dengue IgM in a separate ELISA assay (Table 5). The occurrence of acute cases of Japanese encephalitis peaked in the period July-September, coinciding with the rainy season (Fig 1).

Table 1
Frequency of Japanese encephalitis cases confirmed by IgM-capture ELISA, 2002-2005.

Year	No. of samples	IgM-positive (%)
2002	184	17 (9.2)
2003	164	18 (11.0)
2004	174	32 (18.4)
2005	92	5 (5.4)
Total	614	72 (11.7)

Table 2
Distribution of JE cases, by age.

Age	IgM-positive (%)
0-5	9
6-10	15
11-17	11
18-25	5
26-40	1
41-50	1
51-60	2
>60	4
ND	27

Table 3
Distribution of JE cases, by gender.

Sex	2002	2003	2004	2005	Total
Male	5	14	11	2	32
Female	0	8	1	3	12
ND	12	0	7	0	44

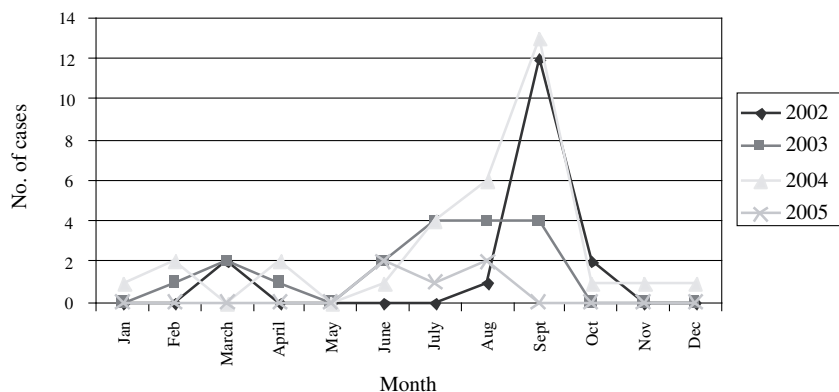


Fig 1- Seasonal trends of Japanese encephalitis in the Philippines, 2002-2005.

Table 4
Areas of origin of JE cases in the Philippines, 2002-2005.

Area	2002	2003	2004	2005
National Capital Region	0	1	5	1
Luzon	1	17	6	4
Visayas	0	3	4	0
ND	16	1	17	0

Table 5
Occurrence of cross-reaction with dengue virus antigen.

Year	No. of JE- and dengue-positive samples
2002	4
2003	5
2004	8
2005	0
Total	17 (24%)

DISCUSSION

In the Philippines, Japanese encephalitis was found to occur in persons of all ages and quite a number of cases developed in school-age children. Japanese encephalitis has been reported to be endemic in all

islands of the Philippines, but this has not been clearly supported by previous data. Published reports have described outbreaks of human cases in Nueva Ecija, Luzon, and Manila (Ksiazek *et al.*, 1980). This study reports cases occurring in other islands, like Cebu and Mindoro in Central Philippines, and several provinces in the northern Philippines.

The detection of antibodies to JEV and dengue virus in 17 samples may be explained as a cross-reaction of the IgM detected by the assay, as the Philippines is endemic for both viruses. Being both flaviviruses, JEV and dengue viruses share common antigenic determinants.

The method of IgM-capture ELISA is a rapid, sensitive, and useful diagnostic test for the early detection and confirmation of acute Japanese encephalitis in patients diagnosed with a viral infection of the central nervous system. This assay is capable of distinguishing Japanese encephalitis cases from other viral encephalitic infections exhibiting similar symptoms. It does not require more than one sampling, unlike the hemagglutination inhibition test, and is superior to culture in terms of speed and ease in getting a result. The use of this method to support a clinical diagnosis will facilitate treatment of the disease and management of potential outbreak events.

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