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Sero-diagnosis of *Japanese encephalitis* and *Dengue virus infection* from clinically suspected patients of Nepal

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ABSTRACT

***Japanese encephalitis* (*JE*) and other viral encephalitis are usually not confirmed in the laboratory in Nepal. However, every year during rainy and post-rainy season in Southern Nepal, from east to west, *JE* and other viral disease suspected patients become prevalent. The currently available methods for the laboratory diagnosis of *JE* and *dengue virus infections (DVI)* (*e.g.,* serology, and virus isolation) are difficult and often tedious to carry out as well as time consuming. So, there is a need for sero-diagnosis of viral infections to identify the type of infection (*JE, DVI* or other viral diseases). The use of conventional methods, however, causes confusion due to the cross-reactions between *JE* and *DVI.* In our study, the detection of virus-specific IgM has been proved to be useful for early diagnosis of *JE* and *DVI* even with the use of a single specimen. We applied ELISA to differentiate *JE* from *DVI* by detecting IgM specific to *JEV* antigen and IgM specific to dengue antigen. From the 279 patients, 244 serum and 35 cerebrospinal fluid (CSF) samples were collected and examined with ELISA using two different antigens for *JE* and *DVI.* The results showed that 28.7% were positive for *JE*- IgM antibodies and 10.4% positive for *DVI*-IgM antibodies.**

In addition, *JEV*-specific IgM antibody detection by Indirect IgM-ELISA and IgM-capture ELISA was also compared in the study.

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INTRODUCTION

Dengue virus infection (*DVI*) and *Japanese encephalitis (JE)* have been recognised as a great public health problem in many areas of tropical and sub-tropical countries. Transmission is by mosquito, principally *Aedes aegypti* and *Aedes albopictus* and *Culex tritaeniorhynchus* and *C. fuscocephalus* (Pradhan *et al.,* 1991). In Nepal, the epidemics of encephalitis have been reported annually particularly in the Far-western region since 1978 (Joshi, 1983; 1986; Khatri *et al.,* 1983; Pradhan *et al.,* 1991). Since the causative agents of these diseases belong to the same family *Flaviviridae* (Westaway *et al.,* 1985), the routine hemagglutination- inhibition (HI) tests often detect only *flavivirus* infection, especially among patients living in the areas where multiple *flavivirus* infection coexists, and among those patients showing secondary type of antibody response due to the cross-reactivity between both viruses. Because IgM antibodies were reported to be more specific than total immunoglobulins (Westaway, 1975; Morita *et al.,* 1988; Kubo, 1996), HI tests on IgM class of antibodies isolated by sucrose gradient sedimentation could be used to differentiate *JE* from other *flavivirus* infections, including *DVI*. We have also been trying to apply ELISA to assay IgM antibodies for the serodiagnosis and seroepidemiological studies on *JE* and develop our own system (Sherchand *et al.,* 1998; Bajracharya and Sherchand, 2001). In this report, we extended the method to use IgM capture ELISA for the differential diagnosis of *DVI* and *JE*. In addition, we applied ELISA to differentiate *JE* from *DVI* by detecting specific IgM to *JE* virus (*JEV*) antigen.

MATERIALS AND METHODS

Test specimens

A total of 279 samples (35 CSF and 244 serum samples) were collected between July 1999 and September 2000 from Bheri Zonal hopital, Bankey and Bardiya Health posts and Infectious and Tropical Disease Research and Prevention Centre/ Nepal with the clinical diagnosis of *JE, meningitis* and suspected *DVI.* All the sera and CSF were divided into two aliquots and stored at –30° c in deep freezer until use.

ELISA procedures for JE IgM assay

The indirect IgM-ELISA was performed based on the method previously described by Igarashi *et al.,* 1981 with some minor modifications. The IgM-capture ELISA procedure was slightly different from indirect IgM-ELISA in the steps of immunological reaction of the test. Briefly, the microtiter plates were coated with goat anti-human IgM *(U*-chain specific) and then the patient sera or CSF were introduced to the plate to react with anti-IgM. The *JEV* antigen was then added and allowed to react with IgM anti-*JEV* antigen before adding peroxidase-labeled antibody to *JEV* antigen and peroxidase substract. Formalin-inactivated purified *JE* vaccine concentrate was used as *JEV* antigen (kindly supplied by NIH Japan and Research Foundation for Microbial Disease of Osaka University).

ELISA procedure for Dengue IgM assay

Dengue antigen: Dengue type 2 (D2) New Guinea B strain were inoculated to *Aedes albopictus* clone C6/36 cells (Igarashi, 1978), and the infected cells were maintained at 28° C with 2% heat-inactivated fetal calf serum in Eagle's medium (Eagle, 1959) supplemented with 0.2 mM each of nonessential amino acids. The infected fluids were harvested 7 days later, diluted 1:2 in PBS-Tween and used as ELISA antigen.

This purified D2 antigen was coated onto the surface of microtitration wells. Diluted test sera were then applied; specific antibodies bind to the antigens in the well. Unbound material was washed away and peroxidase conjugated anti-human IgM was applied. If antibodies were bound to the wells, the conjugate would bind to these antibodies. Unbound material was again washed away. On addition of the substrate, stabilised 3.3', 5.5', Tetramethyl Benzidine (TMB), a colour would develop only in those wells in which enzyme was present, indicating the presence of human anti-Dengue antibody. Adding of sulfuric acid then stopped the enzyme reaction and the absorbance was measured. The optical density (OD) greater than the cut off level was considered positive.

RESULTS

Sera and JE-IgM ELISA

A total of 279 samples of 244 patients, sera (116 male and 128 female) from suspected cases of *JE, meningitis* and *DVI* were examined for *JEV*- specific IgM antibody by indirect IgM-ELISA and IgM-capture ELISA. As Table 1 shows 36.3% of *JE,* 20.0% of meningitis and 10.7% of *DVI* patients were positive for IgM and anti-*JEV*. The data also indicates that 36.3% of viral encephalitis was caused by *JEV.* The same data shows that indirect IgM-ELISA and IgM-capture ELISA was highly specific because only 10.7% *DVI* cross-reacted with *JEV* antigen. Based on the current serological findings *JEV* and *DVI* were prevalent, it is possible that this may not really be a cross reactivity between dengue and *JE* virus antigen, rather it may be the result of inapparent infection with *Japanese encephalitis virus*.

Table I: The distribution of positivity for *JE*-IgM antibodies in serum in different groups of patients:

Clinical diagnosis	*Number of patients*	*Serodiagnosis (%)*	
		Positive	*Negative*
Encephalitis (*JE*)	146	53 (36.3)	93 (63.7)
Meningitis	70	14 (20.0)	56 (80.0)
Dengue virus infection (*DVI*)	28	3 (10.7)	25 (89.3)
Total	244		

CSF and *JE*-IgM ELISA

Of the 279 specimens, 35 CSF (22 male and 13 female) were collected from 19 clinical suspected *JE* patients, 13 meningitis and 3 *DVI suspected patients*. The CSF samples were examined as for specific IgM antibodies by indirect IgM-ELISA and IgM-capture ELISA (Table 2) 36.8% of *JE* and 23.1% of meningitis patients gave positive result for JE-IgM antibodies. The result is tabulated as below. The study revealed that indirect IgM ELISA and IgM-capture ELISA were found highly specific because no (0%) cross-

reaction was found between *JEV* and *DV* antigens.

Table II: The distribution of positivity for *JE*-IgM antibodies in CSF in different groups of patients:

Clinical diagnosis	*Number of patients*	*Serodiagnosis (%)*	
		Positive	*Negative*
Encephalitis (*JE*)	19	7 (36.8)	12 (63.2)
Meningitis	13	3 (23.1)	10 (76.9)
Dengue virus infection (*DVI*)	3	0 (0.0)	3 (100.0)
Total	35		

Overall positivity for *JE*-IgM antibodies in serum and CSF of different groups of patient

Two hundred and seventy nine sera and CSF were examined by *JE*-IgM ELISA using *JE* virus antigen and found positive 80 (28.7%) antibodies against *JE* as shown in Table III.

Table III:

Clinical diagnosis	*Number of patients*	*JE-IgM ELISA Positive (%)*
Encephalitis (*JE*)	165	60 (36.4)
Meningitis	83	17 (20.5)
Dengue virus infection (*DVI*)	31	3 (9.7)
Total	279	80 (28.7)

Overall positivity for *DVI*-IgM antibodies in serum and CSF of different groups of patient

Two hundred and seventy nine sera and CSF were examined by *DVI*-IgM ELISA using *DV* antigen and found positive (10.4%) antibodies against *DVI* as shown in Table IV.

Table IV:

Clinical diagnosis	*Number of patients*	*DVI-IgM ELISA Positive (%)*
Encephalitis (*JE*)	165	5 (3.0)
Meningitis	83	6 (7.2)
Dengue virus infection (*DVI*)	31	18 (58.1)
Total	279	29 (10.4)

Comparative titration of anti-*JE* antibodies in serum and CSF by indirect IgM-ELISA and IgM-capture ELISA

The sensitivity of indirect IgM ELISA and IgM-capture ELISA for the detection of specific IgM antibodies to JEV antigen were compared and assessed with 4 standard scores as strongly positive (++), positive (+), weakly positive (±) and negative (-), by comparing the colour density with those of the standard sera. Table 5 shows that 164 sample out of 279 were negative, 19 were (±) 31 were (+)and 42 were (++) by both the methods.

The results obtained by both standard techniques were up to 91.8% in better agreement. There were 2 samples positive by indirect IgM ELISA but negative or (±) by IgM-capture ELISA and 1 serum which was positive by IgM-capture ELISA but plus-minus by indirect IgM-ELISA. Although the results obtained by both methods were 91.8% agreeable, IgM-

capture ELISA was more sensitive than the indirect IgM-ELISA for the detection of IgM antibodies in sera and CSF specimens.

Table V: Comparative titration of anti-*JE* antibodies in serum and CSF by indirect IgM-ELISA and IgM-capture ELISA.

Serum/CSF		*IgM- capture ELISA*				*% Agreement*
		-	±	+	++	
Indirect IgM- ELISA	-	**164**	11			164+19+31+42 / 279 x 100 = 91.8%
	±	2	**19**	1		
	+	1	2	**31**	1	
	++			6	**42**	

Dengue IgM antibodies in sera

Anti- dengue virus IgM was measured from 244 seurm samples of clinically suspected *JE* (147), meningitis (70) and *DVI* (28) patients. The frequency distribution of dengue-IgM antibodies positive cases were found to be 2.7% from *JE*, 8.6% from meningitis and 57.1% from *JEV* suspected patients as depicted in Table 6. The indirect IgM ELISA test showed high specificity because of very low cross-reactivity between *DV* and *JE* antigens.

Table VI: The distribution of positivity for Dengue-IgM antibodies in serum in different groups of patients:

Clinical diagnosis	*Number of patients*	*Serodiagnosis (%)*	
		Positive	*Negative*
Encephalitis (*JE*)	146	4 (2.7)	142 (97.3)
Meningitis	70	6 (8.6)	64 (91.4)
Dengue virus infection (*DVI*)	28	16 (57.1)	12 (42.9)
Total	244		

Dengue-IgM antibodies in CSF

Of the 35 CSF samples collected, Dengue IgM antibody was found positive in (5.3%) of *JE* suspected patients and 66.7% from *DVI* patients as shown in Table VII.

Table VII: The distribution of positivity for Dengue-IgM antibodies in CSF in different groups of patients:

Clinical diagnosis	*Number of patients*	*Serodiagnosis (%)*	
		Positive	*Negative*
Encephalitis (*JE*)	19	1 (5.3)	18 (94.7)
Meningitis	13	0 (0.0)	10 (100.0)
Dengue virus infection (*DVI*)	3	2 (66.7)	1 (33.3)
Total	35		

DISCUSSION

The ELISA described in this study provides serodiagnosis of 2 flavivirus: *Japanese encephalitis* and *Dengue virus* infections. There are two principles which form the basis of this test. First, IgM antibody specific for an infecting agent appears shortly after infection and wanes after several weeks. Second, this IgM antibody may be specific enough to differentiate between closely related etiologic agents (Gadkari *et al.,* 1984; Innis *et al.,* 1989). Subsequent infections by related agents may trigger an anamnestic immune response characterized by the increased synthesis of IgG reactive with shared epitopes, but IgM reactive with epitopes unique to the infecting agent may also appear.

Burke *et al.,* (1982) showed that IgM assay was diagnostic for *JE* by using antibody capture radioimmunoassay on patient's sera or cerebrospinal fluid. Later, they extended the method to use ELISA (Burke *et al.,* 1983) reporting that acute *DV* and *JE* infections showed stronger reaction with the specific antigens by IgM- ELISA and we obtained the same results in the study. In the past a study using ELISA by Innis *et al* (1989); Ruechusatsawat *et al* (1993) have reported the IgM titre in units, the ratio of sample OD/ weak positive control OD. And in their ELISA procedure, they used 20% normal human serum in antigen diluent in order to remove nonspecific binding of enzyme conjugate with the anti-human IgM antibodies. The large number of antibody negative human serum is not always easy to obtain. In our preliminary test, nonspecific binding of enzyme-conjugate to the catching antibody was observed when IgM was not completely removed from the high-titred *DVI* patient's sera before conjugation. This problem was overcome by careful separation of IgG from IgM and other proteins through DEAE-Sephacel column chromatography. Such preparation, after conjugation with peroxidase, did not bind to the antihuman IgM antibodies and we could get low background without adding any normal human serum in the antigen diluent.

We propose the diagnostic criteria on *JE* and *DVI* by IgM-capture ELISA that can be applied to the serodiagnosis of *JE* and *DVI,* when both *JE* and *DVI* coexist or the type of infection is not known.

In Nepal the epidemics of these virus infections appear in rainy and post rainy seasons. Although safe, effective and economical vaccines exist for these infections, they have been not widely used in Nepal due to lack of resources. It is experienced that population growth in the country has led to unplanned and uncontrolled urbanization, which in turn has led to deterioration of housing and of water, sewage and waste management system in many part of Southern Nepal. The crowded human population living in intimate contact with increasingly higher densities of mosquito populations create ideal conditions for increased mosquito-borne diseases like *JE* and *DVI.*

Hence, the discussion underline some of our views: the Ministry of Health must keep Flaviviral diseases control as a priority

and support prevention-oriented programmes, including active laboratory-based surveillance, education of the public and the medical community, and effective mosquito control. Support must be given to developing new academic centres where professionals could be trained to deal with these diseases, and to conduct researches on new methods of prevention and control.

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