AN APPRAISAL OF SOME RECENT DIAGNOSTIC ASSAYS FOR JAPANESE ENCEPHALITIS

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Abstract. A study was undertaken in South Arcot district of Tamil Nadu, India to assess relative merits of selected diagnostic techniques for Japanese encephalitis. During the transmission seasons of 1993-1995, a total of 85 patients (mostly pediatric) clinically diagnosed as acute encephalitis or other related central nervous system (CNS) disorders were examined; in 53 (62.4%) a laboratory diagnosis of JE was established. In terms of diagnostic value, immunoglobulin M (IgM) antibody capture ELISA (MAC ELISA) on convalescent serum had the highest sensitivity (89%) and negative predictive value (NPV) (50%). This was followed by MAC ELISA on acute serum and CSF which had similar sensitivity (84%) and NPV (40%). The hemagglutination inhibition test and *Toxorhynchites splendens* inoculation technique for virus isolation were also similar in sensitivity (68%) and NPV (25%). The virus antigen detection technique by IFA in cells of cerebrospinal fluid (CSF) was the least sensitive (58%). The distinct advantage of the acute serum ELISA is that it can be carried out on a single finger-prick blood specimen. The IFA on CSF cells is the most rapid diagnostic test since it requires only 2-3 hours to complete. Therefore, both these tests also offer potential tools for JE surveillance programs.

INTRODUCTION

Japanese encephalitis (JE), a mosquito borne viral zoonosis, continues to be a serious public health problem in India. Since 1973, several major outbreaks have occurred in the country (Rodrigues, 1984) and the first outbreak in Kerala in early 1996 shows that the disease is spreading to newer areas. Each year large numbers of acute encephalitis cases occur among children in endemic areas coinciding with monsoon season and period of high vector prevalence. At peripheral hospitals due to lack of adequate laboratory facilities diagnosis of JE is based primarily on clinical manifestations and therefore disease rates are underestimated. With the concept of disease adjusted life years (DALY) emerging as a new measure of the disease burden (Murray, 1994), reliable estimation of JE specific morbidity and mortality rates is essential to a wide variety of purposes including correct epidemiological record, resource mobilization for control interventions and as a component of surveillance system. Further, specific monoclonal antibodies (Ma *et al*, 1992) and antiviral agents (Cardosa *et al*, 1995) such as acyclovir and gangcyclovir are being tried recently for management of JE patients.

Conventional mouse inoculation and tissue culture methods for virus isolation and neutralization, complement fixation and hemagglutination inhibition tests for antibody assay have been invaluable in laboratory diagnosis of JE. However, more sensitive, specific and rapid diagnostic tests have been described recently for arboviruses. Mosquito inoculation techniques have been used for isolation of dengue viruses from human patients (Lam *et al*, 1986) and JE virus from mosquito vectors (Gajanana *et al*, 1995a). But, virus isolation from JE patients using these techniques has not been documented. Methods for detection of JE virus antigen in cells of CSF (Asha Mathur *et al*, 1990) and IgM antibodies to JE virus (JEV-IgM) in CSF and serum (Burke *et al*, 1985a) offer rapid and specific diagnostic alternatives to cumbersome conventional serological techniques.

A study was therefore carried out in South Arcot district, Tamil Nadu, India to: 1) get a reliable estimation of proportion of JE among cases of CNS diseases reported during transmission seasons, 2) assess relative merits of selected diagnostic tests for JE and 3) identify tests which could be included in JE surveillance programs.

MATERIALS AND METHODS

Background information

South Arcot district, with approximately 700,000 persons of pediatric age group is one of the worst

affected endemic areas in Tamil Nadu. After the major JE epidemic in 1981 (Risbudh *et al*, 1991), each year large numbers of cases of acute CNS diseases have been reported during monsoon and post-monsoon seasons viz: August to December. Hospital records showed that they were clinically diagnosed variously as: meningitis, acute encephalitis, Japanese encephalitis, viral encephalitis, acute meningoencephalitis, viral meningoencephalitis, acute viral encephalitis, CNS infection, transverse meningitis, brain-stem encephalitis and encephalopathy. The numbers of such cases, majority of whom were pediatric patients, recorded for the district for the years 1993, 1994 and 1995, were respectively 132, 158 and 61 (Tamil Nadu Public Health Department, personal communication). In a recent study we found that the seroconversion rate against flaviviruses in a cohort of school children in the 5-9 age group was 46-77% and the majority of them was due to JE. The estimated ratio of overt to inapparent infection was 1 : 270 (Gajanana *et al*, 1995b). Studies on vector infection frequency showed a minimum infection rate of 0.32 per 1,000 (Gajanana *et al*, 1996).

Patients and clinical specimens

Patients with acute CNS diseases admitted to the Government General Hospital, (GH) and Danish Mission Hospital (DMH), Vridhachalam and Raja Muthia Chettiar Medical College Hospital (RMMCH), Chidambaram formed the main group. A few cases from private medical practitioners were also enrolled. On admission day, finger-prick blood (200-300μl in heparinized vials) and CSF specimens were collected from patients admitted to GH and DMH and only blood specimens from those admitted to RMMCH. Wherever possible second blood samples were collected from those who were alive beyond 2-weeks of admission. In the field laboratory at Vridhachalam, blood and CSF samples were centrifuged at low speed for a brief period and plasma was separated and stored at 4°C. The sediment of CSF was spotted on Teflon coated microscope slides (duplicate smears on separate slides), air dried, fixed in chilled acetone, wrapped in clean tissue paper and stored at 4°C. The supernatant of CSF was stored in liquid nitrogen. Later these materials were transported to Madurai; plasma samples and CSF smears on ice and CSF supernatant in liquid nitrogen. In Madurai, plasma samples and CSF smears were stored at -20°C and CSF supernatants at -80°C. Plasma and CSF samples were used for serological studies. Additionally, CSF samples were used for virus isolations. CSF smears were used for detection of JE virus antigen in cells by IFA.

Diagnostic techniques

JE virus antigen detection in cells of CSF by immunofluorescence technique (CSF Cell-IFA) (Asha Mathur *et al*, 1990): Out of the two smears of each CSF sample one was first stained by broadly reacting rabbit anti-JE virus serum (RIS) raised by CRME against P 20778 strain of JE virus (provided by Dr K Banerjee, National Institute of Virology, Pune) followed by FITC-conjugated swine anti-rabbit immunoglobulins (Dakopatts) and examined under an Aristoplan Leitz Fluorescence microscope. If the smear was positive, then the second smear was stained with JE virus-specific monoclonal antibody, MAB 112 (Kimura-Kuroda and Yasuri, 1983) (provided by Dr Junko-Kimura-Kuroda, Tokyo Metropolitan Institute of Neurosciences, Japan) followed by FITC-conjugated rabbit anti-mouse immunoglobulins (DAKO) for confirming JE virus identity.

JE virus isolation by inoculation of *Toxorhynchites splendens* larvae (Toxo-IFA) (Gajanana *et al*, 1995a): Eight late third instar larvae of *Toxorhynchites splendens* from a laboratory cycling colony were inoculated intracerebrally with approximately 0.17μl of CSF. After 7 to 8 days of incubation at 29°C, head-squeeze smear from each larva was prepared in duplicate on separate slides, fixed in acetone and used for indirect immunofluorescent technique. The thorax-abdomen portions were stored at -80°C for further work if needed. One set of smears were first stained with RIS followed by anti-rabbit FITC conjugate and if positive, a second set of duplicate smears were stained with JE virus-specific MAB-112 followed by anti-mouse FITC conjugate for confirmation. Even if one smear was unambiguously positive, the test CSF was considered positive for JE virus. If all the smears were negative either after staining by RIS or MAB, sub-passages of the thorax-abdomens were made in fresh larvae and examined as before. At least 2 sub-passages were carried out before declaring a CSF specimen negative.

IgM antibody assay by MAC ELISA (Burke *et al*, 1985a): Virus-specific IgM antibodies were deter-

mined against JE, WN and DEN-2 virus antigens by a modified isotypic capture ELISA. Briefly, microtiter plates were coated with anti-human μ-chain specific antibody and blocked with 1% bovine serum albumin (BSA). After washing, the test samples (1:10 of CSF/1:100 of plasma) were added followed by 2 hours incubation during which time the IgM in the sample was captured and bound to the solid phase. Plates were washed and 50 HA units of JE/WN/DEN-2 mouse brain derived antigens were added and kept overnight at 4°C. Bound antigens were in-turn detected by the addition of a mixture of two biotinylated monoclonal antibodies [(Flavivirus group specific (D14G2) and encephalitis group specific (J311G) (kindly supplied by Dr Mary K Gentry, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington DC, USA)] for 2 hours at 37°C. The plates were then washed and 1:10,000 streptavidin-HRPO (Dako) was added and incubated at 37°C for 1 hour. After a series of washes, substrates O-phenylenediamine (OPD) and urea peroxide were added to detect the enzyme activity, and optical density (OD) was measured at 490 nm in a Biotek ELISA reader. Known negative and positive samples were included.

HI test: Plasma samples were extracted with acetone, adsorbed with goose erythrocytes and tested for HI antibodies by the method described by Clarke and Casals (1958), adapted to microtiter plates (Sever, 1962). Each sample was tested against JE, WN and DEN-2 virus antigens. Known positive and negative controls were included in each day's work. The diagnostic criteria described by Prasada Rao *et al* (1982) were followed.

The antigens used in ELISA and HI test were provided by National Institute of Virology (NIV), Pune. Paired samples were tested simultaneously in ELISA and HI tests. A patient was diagnosed as having JE if any of the diagnostic tests employed was positive. Relative merits of diagnostic tests were assessed using sensitivity and negative predictive value (NPV) of individual tests (Rifky *et al*, 1993). For this purpose only data on those samples which were subjected to all the tests were used.

RESULTS

Human patients

Between August 1993 and February 1996 a total

of 85 patients with acute symptoms of encephalitis or other related CNS diseases were enrolled into the study. These cases occurred mainly between August and December with a peak in October coinciding with paddy cultivation and abundant mosquito populations. The main clinical signs and symptoms were fever, headache, vomiting, fits, neck rigidity, altered sensorium and convulsions. All except two (aged 30 and 32 years) were pediatric patients aged from < 1 to 12 years. The male to female ratio was 1:0.8. Duration of illness in 58 cases ranged from 1-14 days (mean 3.5 days), in two it was 25 and 45 days and in 25 this information was not available. There were 14 deaths among 42 patients (33%) who were subsequently diagnosed as JE by laboratory tests.

Diagnostic tests

In 1993 and 1994, all the 6 diagnostic tests were employed. In an extended study in 1995, only the HI test and MAC ELISA were compared.

Out of 46 patients enrolled during the transmission seasons of 1993 and 1994 a diagnosis of JE was established in 43 (93.5%) (Table 1). In 15 out of 31 (48.4%) patients, Cell-IFA was positive when stained with RIS but none gave positive staining with JE specific MAB-112. Virus isolation was made in 24 out of 45 (53%) CSF samples inoculated to *Toxorhynchites splendens* larvae and 22 (91.6%) of these were confirmed as JE virus by MAB-112. Of the 24 isolations 7 were from fatal and 17 from nonfatal cases.

CSF samples from 45 patients were tested by MAC ELISA and 27 contained JE-IgM antibodies alone and 7 contained JE - IgM and WN-IgM antibodies, but, the JE IgM titers were 1.2 to 1.6- fold higher than WN-IgM titers and therefore they were diagnosed as JE as per National Institute of Virology criteria. Thus 34 out of 45 (75.5%) patients were diagnosed as JE by MAC ELISA on CSF. Acute plasma samples from 45 patients were tested by MAC ELISA and JE-IgM antibodies were found in 30 (66.7%). In 9 of these WN-IgM and in one DEN-IgM antibodies were also present, but the JE-IgM titers were 1.2 fold higher than others. Convalescent plasma samples from 29 patients were tested and 24 (82.7%) were positive for JE IgM antibodies.

Paired plasma specimens from 29 patients were

Table 1

Comparison of diagnostic tests for Japanese encephalitis in South Arcot district, Tamil Nadu, 1993 and 1994.

Cerebrospinal fluid			Plasma		
			MAC ELISA		HI Test
Cell-IFA	Toxo-IFA	MAC ELISA	Acute	Convalescent	
15/31 (48.4)	24/45 (53.3)	34/45 (75.6)	30/45 (66.7)	24/29 (82.7)	13/29 (44.8) *18/29 (62.1)

Numerator : No. positive.
Denominator : No. of encephalitis patients examined.
*5 presumptive positives.
Figures in parantheses are percentages.

tested for HI antibodies to flaviviruses and 13 (44.8%) showed a 4-fold rise in JE antibody titers in convalescent specimens. Additionally in 5 samples both acute and convalescent samples showed high JE antibody titers (160-1,280) and if these are diagnosed as presumptive JE, a total of 18 (62%) patients could be diagnosed as JE based on the HI test. None of the patients showed WN or DEN HI antibodies with diagnostic titers.

Sensitivity and NPV of each test are given in Table 2. A total of 21 patients were examined by all the 6 tests in 1993-1994. MAC ELISA on convalescent plasma showed the highest sensitivity (89%) and NPV (50%) followed by ELISA on acute plasma and CSF, which had similar sensitivity and NPV (84% and 40%). Similarly, virus isolation technique (Toxo-IFA) and HI test were equal in sensitivity and NPV (68% and 25%). CSF Cell-IFA was the least in sensitivity (58%) and NPV (20%).

Further evaluation of the MAC ELISA in comparison with the HI test was carried out on patients admitted to RMMCH during transmission season of 1995. A diagnosis of JE was made in 9 out of 25 (36%) patients by HI test. By MAC ELISA, diagnosis of JE was established in 16 out of 39 (41%) patients using acute plasma and in 10 out of 25 (40%) patients using convalescent plasma samples.

DISCUSSION

The study reported here showed that in South Arcot district, Tamil Nadu during the transmission season, the proportion of JE among the study group of patients with acute CNS diseases was as high as 94% as in 1993-1994 when a battery of tests was employed. As per the public health records, the number of cases with CNS diseases for the district for the years 1993, 1994 and 1995 were 132, 158 and 61, respectively. Therefore, the estimated number of JE cases for 1993 and 1994 works out to be 124 and 149 (94% of 132 and 158). Even in the third year (1995), when only two tests were employed, about 40% of cases were diagnosed as JE and this would give an estimated 30 (40% of 61). These estimates are at great variance with the recorded numbers of clinically diagnosed JE cases, which were only 7, 7 and 1 respectively for the three consecutive years. This clearly underlines the importance of accurate diagnosis to get a real estimate of disease burden in a community.

Definitive diagnosis of JE can be made by isolation of the virus. Since the viraemic phase in human patients is very brief, virus isolation from peripheral blood is seldom successful. Virus has been isolated from CSF but it is generally opined that isolation from this source is a rare event and suc-

Table 2

Sensitivity and negative predictive values of diagnostic tests for Japanese encephalitis in South Arcot district, Tamil Nadu, 1993 and 1994.

Patient No.	Plasma			Cerebrospinal fluid		
	HI Test	MAC ELISA		Cell-IFA	MAC ELISA	Toxo-IFA
		Acute	Convalescent			
12	+	+	+	+	+	-
13	-	-	-	-	-	-
14	+	-	+	+	+	+
15	+	+	+	+	+	+
16	+	+	+	+	+	-
19	+	+	+	-	+	+
20	-	+	+	-	+	+
21	?+	+	+	-	+	-
22	+	+	+	-	+	-
24	+	+	+	-	+	+
25	?+	+	+	+	+	+
26	-	+	+	-	+	+
27	+	+	+	-	+	+
31	?+	+	+	+	+	+
32	+	+	+	+	+	+
33	-	-	-	+	-	+
35	-	+	+	+	+	+
37	+	+	+	-	-	-
38	-	-	-	-	-	-
40	-	+	+	+	+	-
44	-	-	-	+	-	+
Total positive	13	16	17	11	16	13
Sensitivity (%)	53	84	89	58	84	68
	68*					
Negative predictive value (%)	18	40	50	20	40	25
	25*					

+ = Diagnosed as JE by the test; - = Negative by the test.

$$\text{Sensitivity} = \frac{\text{No. positive by the test}}{\text{No. positive by any of the test}} \times 100; \qquad \text{Negative predictive value} = \frac{\text{No. negative by all the tests}}{\text{No. negative by the test}} \times 100$$

?= Serial numbers 21, 25, 31 had high HI titers in both acute and convalescent sera, but no 4-fold difference between the two. Hence presumptive diagnosis as JE.
*= Sensitivity and negative predictive value if the 3 presumptive cases are included.

cessful only in the early phase of acute encephalitis (Asha Mathur *et al*, 1990). Using mouse inoculation test, Chakraborty *et al* (1980), Mohan Rao *et al* (1983) and Asha Mathur *et al* (1982) were unsuccessful in isolating JE virus from CSF samples collected during epidemics in India. However, using the same system JE virus isolations were made from about 24% (n = 92) (Rashmi Kumar *et al*, 1990) and 30% (n = 17) (Asha Mathur *et al*, 1990) of encephalitis patients in hospital based studies. But Prasad Rao *et al* (1982) reported only 3% (n = 29) isolation rate during 1978 outbreak in

Tirunelveli district (Tamil Nadu). Again, Ravi *et al* (1989) reported isolation rate of 3.7% (n = 27) using *Aedes albopictus* and Burke *et al* (1985 b) 7.5% (n = 66) using *Aedes pseudoscutellaris* cell cultures, in hospital based studies. Mosquito inoculation techniques have been shown to be sensitive for isolation of flaviviruses (Gajanana *et al*, 1995a). Inoculation of *Toxorhynchites splendens* larvae is relatively simple and safe and has been employed for isolation of dengue virus from human cases (Lam *et al*, 1986) and JE virus from field-caught mosquitos (Gajanana *et al*, 1996). In the present study the same system was successfully used for the first time for isolation of JE virus from human CSF. Our isolation rate was 53% (n = 45), much higher than the rates so far reported using other host systems. JE virus was recovered from 4 patients with duration of illness as long as 7-14 days. Also, virus recovery was made in the presence of antibodies.

Cultivable virus from CSF is said to be associated with grave prognosis and Asha Mathur *et al* (1990) and Burke *et al* (1985b) observed fatal outcome in all those patients whose CSF specimens yielded JE virus. But Ravi *et al* (1989) did not find a correlation between fatal outcome and virus isolation from CSF. They explained that their failure to isolate virus from fatal cases could have been due to inactivation of virus or to small sample size studied. We also found that fatal outcome and virus yield were unrelated. In fact 70% of our isolations were from nonfatal cases. The probable reasons for divergent observations could be, among other undefined factors, due to differences in virulence of virus strains, sensitivity of isolation techniques and variation in host susceptibility.

Detection of virus-specific IgM antibodies in CSF/serum indicates recent infection and is of diagnostic value (Burke *et al*, 1986). In our study in terms of diagnostic capacity the best test was MAC ELISA for detection of JEV-IgM in convalescent serum (89% sensitivity and 50% NPV). But, the test can be conducted only after 2 weeks of acute clinical manifestations if the patient survived. On the otherhand, MAC ELISA on acute serum could detect JEV-IgM as early as one day after the onset of symptoms and hence is of great value for early diagnosis and patient management. In our study the sensitivity (84%) was almost equal to that of MAC ELISA on CSF. This is comparable to the sensitivity reported by Burke *et al* (1985b, 1986) using their format of MAC ELISA on admission serum specimens.

The CSF Cell-IFA provides the quickest diagnostic technique since result can be obtained in about 2-3 hours. But we found that the test possessed least sensitivity (58%) when compared with other tests. Further, smears which were positive for flavivirus antigen when stained with broadly reacting antiserum, were however negative when stained with JEV-specific monoclonal antibody. In contrast Asha Mathur *et al* (1990) could detect specific JE virus antigen in CSF cells in 15 out of 17 (88%) of confirmed JE patients. Possible reasons for lower sensitivity in our hands could be : 1) we prepared smears in the field and stored them at 4°/ -20°C for 1-2 weeks before examination by IFA, which might have resulted in loss of antigen, whereas Asha Mathur's group used fresh smears; 2) the number of cells in each smear examined by us was much less; 3) our JE-specific monoclonal antibody (MAB 112) may not be compatible with the indigenous strains; 4) the sensitivity of our monoclonal antibody preparation MAB 112 may be less than that used by their group.

In conclusion, our study demonstrated that 1) in South Arcot district, reliance only on clinical diagnosis underestimates the true JE incidence, 2) *Toxorhynchites splendens* is a very sensitive host for isolation of JE virus from CSF and 3) MAC ELISA for detection of IgM antibodies in acute serum and IFA for detection of JE virus antigen in CSF cells are promising tools for surveillance of JE. A distinct advantage of MAC ELISA is that it can be performed on small quantity of fingerprick-blood specimen dried on filter paper under field conditions (Burke *et al*, 1985c). With the availability of a fluorescence microscope objective attachment to a standard laboratory microscope for field use (Polsuwan *et al*, 1992; Makler, 1992), it should now be possible to perform the JE virus antigen detection test even in peripheral hospital laboratories. However there is a need for more studies in other endemic areas to confirm these observations.

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