Comparison of a dipstick enzyme-linked immunosorbent assay with commercial assays for detection of Japanese encephalitis virus-specific IgM antibodies

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Received : 12-02-08
Review completed : 30-04-08
Accepted : 30-04-08
PubMed ID : 18626163
J Postgrad Med 2008;54:181-5

ABSTRACT

Background: Japanese encephalitis (JE) is a major public health concern in Asia including India. Objectives: To evaluate an in-house developed dipstick enzyme-linked immunosorbent assay (ELISA) test vis-à-vis two commercial kits for detection of JE virus-specific IgM antibodies. Setting and Design: Comparative study carried out in Research and Development centre. Materials and Methods: A total of 136 specimens comprising 84 serum and 52 CSF samples were tested by in-house dipstick ELISA, Pan-Bio IgM capture ELISA (Pan-Bio, Australia) and JEV CheX IgM capture ELISA (XCyton, India). Results: The overall agreement among all three tests was found to be 92% with both serum and cerebrospinal fluid (CSF) samples. The sensitivity of the dipstick ELISA was found to be 91% with serum and 89% with CSF samples respectively. The specificity of the dipstick ELISA with reference to both commercial assays was found to be 100% in serum and CSF samples in this study. Conclusions: The in-house dipstick ELISA with its comparable sensitivity and specificity can be used as a promising test in field conditions since it is simple, rapid and requires no specialized equipment.

KEY WORDS: Antibody, diagnostic test, dipstick enzyme-linked immunosorbent assay, Japanese encephalitis, virus

Japanese encephalitis (JE), an arthropod-borne viral infection is found throughout the temperate and tropical zones of Asia including India. This virus is a member of the JE serogroup of the genus Flavivirus, family Flaviviridae, and is transmitted between vertebrate hosts by mosquitoes, principally by *Culex tritaeniorhynchus*. Humans are an incidental host. Approximately 45,000 JE cases with 10,000 deaths were notified annually from a wide geographic area.[1] There have been JE outbreaks and epidemics in large parts of Southeast Asia.[2] In India, the first case was reported from the southern state of Tamil Nadu in 1950.[3] Since then, there have been outbreaks of the disease affecting different parts of India.[4-7] Clinical features of infection with JE virus range from nonspecific febrile illness to a severe meningoencephalitis, often associated with seizures or flaccid paralysis.[1] Laboratory diagnosis of JE virus relies on virus isolation, detection of genomic RNA and virus-specific antibodies by RT-PCR and serodiagnosis, respectively. Even with the best laboratory facilities, the success rate in isolation of JE virus from clinical specimens remains less, probably because of low levels of viremia and rapid development of neutralizing antibodies.[8] Viral antibody in cerebrospinal fluid (CSF) and serum can be detected by a wide variety of conventional techniques, such as viral neutralization, hemagglutination inhibition (HI), complement fixation and immunofluorescent staining. All these techniques have limitations to be used as a routine diagnostic test as they are labor-intensive, expensive, cumbersome and not sensitive enough to detect antibodies in CSF.

Tests such as serum neutralization and HI were replaced by simpler enzyme-linked immunosorbent assays (ELISA).[8-9]

The immunoglobulin M (IgM) antibody capture ELISA (MAC ELISA) for serum and CSF has become the accepted standard for diagnosis of Japanese encephalitis.[8-10] It is often positive for clinical specimens collected at the time of admission and distinguishes between antibodies to JE virus and dengue virus, which are serologically cross-reactive.[8] These ELISAs require sophisticated equipment and their use has been confined largely to a few laboratories or referral centers. As majority of JE virus infected patients report to rural hospitals with limited facilities, there is a need for a simple and reliable field-based diagnostic test, appropriate for such settings. This prompted us to compare the commercially available expensive immunodiagnostic systems with an in-house developed JE detection system. The correlation and comparative evaluation among the available test systems gives us the clue for early diagnosis of patients and their management. In this paper, we report the evaluation of an in-house developed dipstick ELISA for detection of JE virus-specific IgM antibodies and its comparison with other commercial assays.

Materials and Methods

A panel of 136 specimens comprising 84 serum and 52 CSF samples previously collected for serological and virological investigation from 136 different patients with clinical symptoms of encephalitis, admitted to the Pediatrics and Medicine wards of B.R.D. Medical College, Gorakhpur, India during July-November, 2005 was included in this study.[7] All the specimens were stored at −80°C till further investigation.

Japanese encephalitis virus (Strain JaOArS982) kindly provided by Prof K. Morita, Nagasaki University, Japan was adapted to grow in C6/36 cells following standard protocol.[11] C6/36 [cloned cells of larvae of *Aedes albopictus*] cells initially obtained from the National Center for Cell Science (NCCS), Pune, were maintained at 28°C by regular sub-culturing at periodic intervals of three to four days in Eagle's minimum essential medium (Sigma, USA) supplemented with 10% Tryptose phosphate broth (Difco, USA), 10% Fetal Bovine serum (Sigma, USA), 3% L-glutamine (Sigma, USA) and gentamicin (80mg/l) (Nicholas Piramal, India).[12] The preformed monolayer of C6/36 cells was infected with JE virus following the techniques of standard adsorption. On the fourth to fifth post-infection day, the cells were harvested by low-speed centrifugation. The clarified viral lysate was heat inactivated at 56°C for 30 min and purified by the method described earlier.[11] Briefly, precipitation was carried out by slow addition of 7% (w/v) polyethylene glycol (PEG) 8,000 and 0.5% NaCl over a period of 2 h at 4°C under continuous stirring. After overnight settling, the precipitate was pelleted by centrifugation at 10,000 rpm for 30 min. The obtained pellet was dissolved in a 1/100th volume of Glycine-Tris-Sodium chloride-EDTA (GTNE) buffer. The PEG-pelleted antigen was purified by discontinuous sucrose density gradient centrifugation with 60% and 30% cushions at 100,000 g for 2 h in ultracentrifuge. The band obtained from the interface was dialyzed and used as semi-purified antigen. To obtain a purified viral antigen the semi-purified viral antigen was subjected to the second round of ultra-centrifugation as described above.

The in-house developed indirect dipstick ELISA test was carried out as follows. The nitrocellulose (NC) membranes on the tips of plastic combs were coated with purified viral antigen (0.8 µg/ tip) in carbonate-bicarbonate buffer (Na_2CO_3 0.1M, $NaHCO_3$ 0.2M; pH-9.6) and incubated for 1 h at 37°C. The unoccupied sites of the NC membranes were then blocked with 2% BSA in PBS overnight at 4°C. These coated NC projections were washed with PBS-T washing buffer (0.05% Tween-20 in PBS) and stored at 4°C. Patient serum samples were diluted (1:100) and CSF (1:10) with serum diluents (0.01 % Tween-20 in PBS) and dispensed at the rate of 200 µl/well. The antigen-coated combs were dipped in the wells of this microtiter plate and incubated for 1 h at 37°C. Combs were then washed with washing buffer (0.05% Tween-20 in PBS) three times, each for a duration of five min. After washing, the combs were dipped in Goat antihuman IgM HRP conjugate (Sigma, USA), diluted (1:2000) in conjugate diluent (PBS with 2% BSA), and incubated for 1 h at 37°C. The combs were then washed as above and developed with phosphate-citrate buffer, pH 4.5 containing 3, 3'-diamino benzidine (Sigma, USA) and H_2O_2. Appearance of brown color dot indicated presence of IgM antibodies. Positive and negative controls were included each time when the test was performed [Figure 1]. All the specimens were tested along with known positive and known negatives. When all the six authors interpreted the result independently, a consensus was taken and the result was interpreted. In addition, after an initial study, at random some of the specimens were coded by senior authors and the test was performed again for its validity. Brown color dots were always clear with the known positive specimen. Test sample with clear brown dots as compared to known positive were considered positive.

The Pan-Bio IgM capture ELISA (Cat # E-JED01C, PanBio, Australia) was performed according to the manufacturer's protocol. The kit is based on JE-DEN IgM combo ELISA. Each sample was tested in duplicate with one well using JE-Mab compex and the other well using Dengue-Mab complex. Lyophilized JE and dengue antigens were reconstituted in reconstitution buffer and mixed well with an equal volume of Mab tracer, separately. Patient serum sample diluted 1:100 and CSF 1:10 in the diluent provided, were added at 100 µl to each well of the assay plate containing bound anti-human IgM. The plates were incubated for 1 h at 37°C. After incubation the assay plate was washed six times with washing buffer, and 100 µl/well of JE antigen Mab tracer mix and dengue antigen-Mab mix were added into the respective wells and incubated for 1 h at 37°C. The plate was again washed and the bound complex was stained with tetramethylbenzidine substrate (100 µl/well), and absorbance read at 450 nm in an ELISA reader. The cutoff value was determined from the average absorbance of triplicates of calibrator provided by manufacturer, multiplied by calibration factor of the batch. The index values in respect of each sample were calculated and converted into Panbio units as given in product insert. Those samples giving JE Panbio units >11 and dengue Panbio units <11 were considered as JE-positive. When both JE and dengue Panbio units were >11, a JE/ dengue ratio was calculated and when this ratio was ≥1 a presumptive diagnosis of JE infection was made.

The JEV Chex IgM capture ELISA (XCyton Diagnostics, Bangalore, India) was performed according to the manufacturer's protocol. The test procedure was almost similar to that described for PanBio IgM capture ELISA except that serum sample was diluted 1:20 and CSF samples 1:10; biotinylated Mab was used in place of JE antigen-Mab tracer, and streptavidin conjugate in place of Mab tracer. The OD_{450} values obtained with JEV-CheX

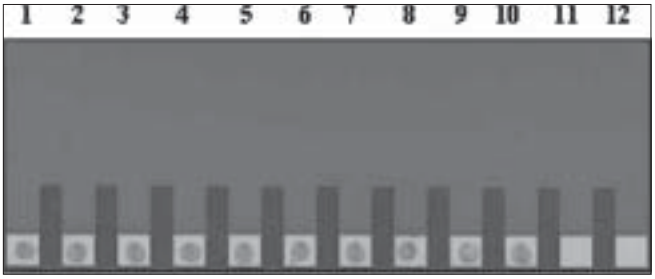

Figure 1: Dipstick ELISA results. Lane 1-9, IgM positive sample; Lane 10, Positive control; Lane 11, Negative sample; Lane 12, Negative control

were expressed in terms of ELISA units as per product insert. All samples with ELISA units ≥100 were considered positive for CSF as well as serum samples.

Results

The referred epidemic of JE occurred during July-November 2005 affecting 5737 persons with more than 1344 deaths spanning over seven districts of eastern Uttar Pradesh.[13] Rural populations within the age groups of three months to 15 years were affected but nearly 50% of the cases belonged to 6-10 years of age followed by 35% between 0-5 years, with an overall 23% case fatality. The clinical history revealed that all the patients had suffered from fever ranging from 38.5° to 40°C. The prominent clinical symptoms included severe headache (75%), convulsions, vomiting, paralysis, coma and death. The epidemic affected males and females in a ratio of 1.9 to 1.

In the present study, a total of 136 clinical specimens comprising 84 serum and 52 CSF samples was included. All the above samples were tested by in-house Dipstick ELISA, Panbio JE-DEN IgM combo ELISA and JEV-CheX IgM capture ELISA. The results of the three tests have been presented in Table 1. In addition, a panel of well-defined negative serum samples collected from 20 healthy volunteers as well as 10 PCR-positive dengue serum samples were included in this study. None of them gave positive result thereby confirming its specificity.

The overall agreement, sensitivity and specificity of the in-house dipstick ELISA with respect to Pan-Bio IgM capture ELISA and JEV- CheX IgM capture ELISA in serum and CSF samples respectively have been presented in Table 2. The comparison of two commercial assays, JEV-CheX and Panbio IgM capture ELISA with respect to individual samples has been shown in Figure 2. The dipstick has not picked up any false positive

Table 1: Comparison of Dipstick results vis-à-vis Pan-Bio and JEV-Chex enzyme-linked immunosorbent assay

Sample type and Dipstick results	Panbio ELISA		JEV-CheX	
	JEV positive	JEV negative	JEV positive	JEV negative
CSF				
JEV positive	31	0	31	0
JEV negative	3	18	4	17
Serum				
JEV positive	49	0	49	0
JEV negative	2	33	5	30

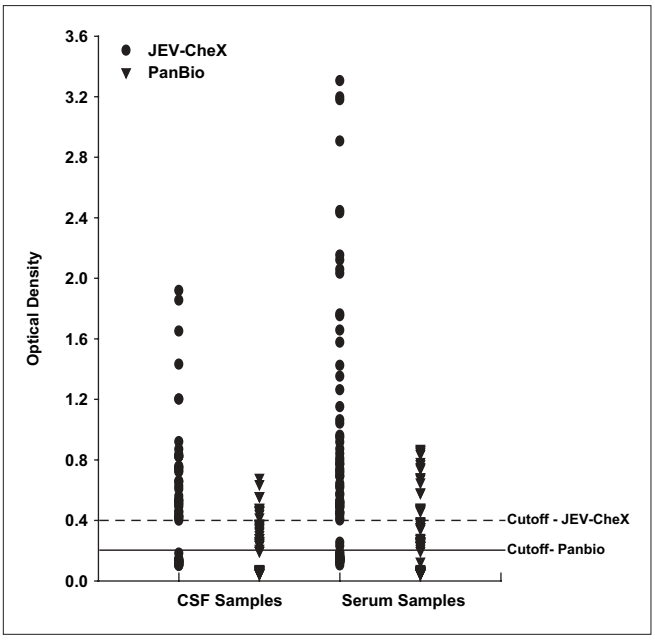

Figure 2: Comparison of JEV-CheX and Panbio IgM capture ELISA

thereby establishing its specificity. Statistical analysis of the data was done employing Sigma Stat Jande Sci, USA program. Value of *P* was 0.70 in case of CSF and 0.73 in case of serum samples. This is not significant ($P < 0.05$ considered as significant) suggesting that all the three tests evaluated in this study were equally specific. The dot pattern of dipstick ELISA test with positive and negative serum sample is shown in Figure 1.

Discussion

The epidemiological trend of JE virus outbreak has changed over the last few years. Though mass vaccination campaigns have been associated with a decrease in the number of encephalitis cases in Japan, Taiwan and South Korea, the geographic area affected by the virus has expanded to India, China, Southeast Asia and the Western Pacific region.[2] The live-attenuated vaccine (SA14-14-2 strain) has been licensed in China since 1988 where currently it is administered to more than 20 million children each year. The vaccine has recently been approved in the Republic of Korea, Nepal, Sri Lanka, and India.[14] In India JE is endemic due to lack of early diagnostic facilities.[4,6-7] A correct diagnosis focuses the physician's attention on the specific complications of JE such as hyponatremia, convulsions, and raised intracranial pressure which avoids irrelevant investigation

Table 2: Comparative evaluation of dipstick enzyme-linked immunosorbent assay with reference to Pan-Bio IgM capture ELISA and JEV-Chex

Type of sample	Agreement‡	Sensitivity§	Specificity‖	PPV¶	NPV**
Serum*	98% (82/84)	96% (49/51)	100% (33/33)	100% (49/49)	94% (33/35)
CSF*	94% (49/52)	91% (31/34)	100% (18/18)	100% (31/31)	86% (18/21)
Serum†	94% (79/84)	91% (49/54)	100% (30/30)	100% (49/49)	86% (30/35)
CSF†	92% (48/52)	89% (31/35)	100% (17/17)	100% (31/31)	81%(17/21)

*Dipstick versus Pan-Bio, †Dipstick versus JEV-CheX, ‡(Number of samples positive by both methods + number of samples negative by both methods)/(total number samples) x 100, §True positive/(true positive + false negative) x 100, ‖True negative/(true negative + false positive) x 100, ¶True positive/(true positive + false positive) x 100, **True negative/(true negative + false negative) x 100

and possibly inappropriate treatment of other central nervous system infections.[5,15] In some countries, vaccination campaigns can be mobilized and targeted towards areas where patients with JE originate; in others, vaccination will not be introduced without good epidemiological support.[1]

The JE virus usually cannot be isolated from clinical specimens even with the best laboratory facilities probably because of low level of viremia and the rapid development of neutralizing antibodies.[8] The diagnosis is therefore usually done serologically. The diagnosis of JE has advanced considerably in the last 20 years. The MAC ELISA overcame many of the problems associated with HI tests, namely the need for paired serum samples, acetone extraction of serum and serial dilutions. MAC-ELISA is, however, considered as a valuable diagnostic tool in secondary flavivirus infection, unlike HI.[8] In the present study, we have tested 136 specimens (84 serum and 52 CSF) for the presence of IgM antibodies by Pan-Bio IgM capture ELISA, JEV-CheX IgM capture ELISA and an indigenous dipstick ELISA test. The indirect dipstick ELISA test has shown comparable sensitivity and specificity for detection of anti-JE virus IgM antibodies as compared to both commercial assays. In case of JE, MAC dot ELISA has been found to be sensitive and specific with more than 90% confidence.[8]

In comparison to the other two commercial assays, the in-house dipstick ELISA though slightly less sensitive is equally comparable in specificity. Statistical analysis of the data with *P* value of 0.70 in case of CSF and 0.73 in case of serum samples indicate that all the tests are comparable in detection of IgM antibodies. In the other two tests, the principle involved is the initial capturing of IgM antibodies and its detection by monoclonal antibody conjugate as the secondary antibody. In the indirect in-house dipstick ELISA, since both IgM and IgG antibodies have equal opportunities to bind with the antigen, the use of IgM-specific antibody conjugate is the deciding factor to detect the presence of IgM antibodies in acute infection. The slightly lower sensitivity in this case can be attributed to the presence of interfering IgG antibodies in the patient samples. Though removal of IgG antibodies would have enhanced the sensitivity of the test for IgM antibody detection it was not carried out keeping in the view the fact that this test was developed as a rapid diagnostic test for field conditions for screening a large number of cases during an outbreak. Moreover, the IgM antibody level will also get diminished during IgG antibody removal. Since the specificity of the test is comparable to the other two tests, it has an edge over the other two so far as its utility in field conditions is concerned. The other advantages of this in-house dipstick ELISA test are that it is cost-effective, doesn't require trained personnel, ELISA reader or any high-quality reagents, which are available in only a few laboratories with good financial resources.[8,16] The long shelf-life of reagents (six months at 4°C) makes it appropriate for use in rural healthcare centers with limited financial resources. In addition, this system is faster than MAC DOT and IgM capture plate ELISA.[8]

The in-house dipstick ELISA is a qualitative test meant for field use. In comparison with both commercial assays the dipstick ELISA showed 92% accordance. The common problem with JE serological assays lies in the detection of circulating cross-reactive antibodies against other members of *Flavivirus*. This cross-reactivity was found to be significantly reduced while identifying JE virus infection with dipstick ELISA employing pre-coated cell culture purified JE antigen as compared to mouse brain antigen or crude cell culture antigen.[17] A similar dipstick ELISA test system for dengue has also been extensively used and evaluated in previous studies with field sera samples collected from different parts of India.[16,18-20] The sucrose density gradient purified antigens have shown best results for dengue in terms of signal to noise ratio and distinguished efficiently a panel of positive and negative sera.[16,20]

The present study demonstrates that the in-house developed JE dipstick ELISA test can be used as a promising test due to its comparable sensitivity, specificity and field applicability in developing countries, which will help authorities to undertake effective control measures and adopt management strategies against impending JE menace.

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Source of Support: Nil, **Conflict of Interest:** Not declared.