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# Serodiagnosis of Japanese encephalitis among Nepalese patients by the particle agglutination assay

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## SUMMARY

Japanese encephalitis (JE) is a serious health problem in the southwestern region of Nepal. Serological diagnostic kits for routine diagnostic use in this region have not been available. This study was performed to examine if the particle agglutination (PA) assay for Japanese encephalitis virus (JEV) IgM could be applicable to the samples collected in Nepal and also to evaluate the accuracy of clinical diagnosis of JE. One hundred and ninety-three blood samples were collected from the patients clinically diagnosed with JE or other infectious diseases in the JE-endemic, southwestern region of Nepal, in 2000. The PA assay was performed on these 193 serum samples and the results were compared with those by IgM-capture ELISA. Eighty-six samples were IgM-positive by the PA assay, and 71 of 86 were also positive by IgM-capture ELISA (sensitivity, 99%; specificity, 88%; positive predictive value, 0·82; negative predictive value, 0·99). These results suggest that the PA assay is a simple, reliable and useful diagnostic test to support clinical diagnosis in rural hospitals of Asia including Nepal.

## INTRODUCTION

Japanese encephalitis virus (JEV), a mosquito-borne flavivirus, is a leading cause of viral encephalitis in Asia. JEV is prevalent in India, China, Nepal, Sri Lanka and across southeast Asia. Approximately three billion people live in the area where JEV is endemic and poses a risk of infection. The transmission of JEV has intensified recently in new locations and the disease has extended its geographical range to previously unaffected areas of Asia and to northern Australia [1]. As many as 50000 cases of JEV infection with 10000 associated deaths are reported annually, and up to 50% of the survivors have serious neurological and psychiatric sequelae [2].

JE is endemic and a major public health problem in the southern part of Nepal [3–8]. JE is generally diagnosed on the basis of clinical symptoms in the rural area of Asia including Nepal. JEV is rarely isolated from clinical specimens, probably because of short and low levels of viraemia and rapid development of neutralizing antibodies [9]. The laboratory diagnosis of JE, therefore, usually relies on serology. The haemagglutination inhibition (HI) test has been long employed, but this method has several practical limitations. Most importantly, it is time consuming, requires paired serum samples and cannot give an early

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diagnosis [10]. IgM-capture enzyme-linked immunosorbent assay (IgM-capture ELISA) has become the most reliable technique for the diagnosis of JE [11]. This assay is sensitive and specific, if done and interpreted properly. However, IgM-capture ELISA requires relatively sophisticated equipment, and has been confined largely to a few academic and referral centres in developing countries.

Recently, a simple particle agglutination assay (PA) system, which does not require specific laboratory facilities, has been developed for detecting JEV IgM [12, 13]. In the present study we validated the PA assay using the Nepalese samples. We applied the PA system to serum samples collected from patients clinically diagnosed as JE, or other infectious diseases in the southwestern region of Nepal. We compared the results with those obtained by IgM-capture ELISA and also evaluated the clinical diagnosis of JE.

## METHODS

### Serum samples

A JE epidemic has been reported in the Terai region bordering to India at the altitude of 100–600 m in Nepal [6, 7]. About 80% of the cases were concentrated in the western part of Nepal. One hundred and ninety-three blood samples were collected in 2000 from patients clinically diagnosed as JE or pyrexia with unknown origin (PUO) in the JE-endemic areas: Banke, Bardiya and Dang districts in the southwestern Nepal. Before collecting blood specimens, demographic information was recorded and informed consent was obtained from each of the parents or guardians. The samples were kept in the ice compartment of a refrigerator, brought to Kathmandu and subsequently transported to Institute of Tropical Medicine, Nagasaki University, Japan and stored at $-80\ ^{\circ}C$ until use.

### IgM-capture ELISA

The IgM-capture ELISA was performed as described previously with modifications [11]. The reaction was done with 50 $\mu$l/well at room temperature. The flat bottom 96-well ELISA plate (Nunc, Denmark) was coated with anti-human IgM goat antibody (Cappel, USA) at 10 $\mu$g/ml in phosphate buffer saline (PBS) at 4 $^{\circ}C$ overnight. The plate was blocked with Block Ace (Yukijirushi, Japan) for 1 h and washed three times with PBS containing 0·05% Tween 20. The test sera were twofold serially diluted from 1 in 100 to 1 in 12 800 in Block Ace in the microplates and kept for 30 min. Positive control and negative control sera were similarly diluted. After washing, 50 $\mu$l of JEV antigen, Beijing strain, vaccine grade (Biken, Japan) at 4 $\mu$g/ml was added and kept for 30 min. After further washing, HRP-conjugated anti-JEV rabbit IgG was subsequently added. After washing, a colour reaction was initiated by adding substrate solution (0·5 mg/ml of OPD (*o*-phenylenediamine dihydrochloride) and 0·02% $H_2O_2$ in 0·05 M citrate phosphate buffer in dark). The reaction was stopped by adding 100 $\mu$l/well of 1 N $H_2SO_4$. The $OD_{492}$ on each well was recorded and 630 nm as a reference wavelength by using Microplate ELISA Reader Model 3550 (Bio-Rad, USA). The samples were defined as positive, when the positive to negative (P/N) ratio was $\geqslant 2$.

### Detection of anti-JEV IgM by the IgM-detection particle agglutination kit

Titres of anti-JEV IgM were assessed by the PA assay system as previously reported [12, 13]. Serum samples were twofold serially diluted with PBS containing 0·4% blocking reagent from 1 in 200 to 1 in 25 600 in anti-human IgM-coated V bottom 96-well microplates, and reacted for 30 min at room temperature. Serum samples were removed from the wells and wells were washed three times with PBS. One hundred $\mu$l of JEV antigen-coated, hydroxyapatite-coated nylon (Ha-Ny) beads were added to the wells and left for 2 h at room temperature. When the Ha-Ny beads formed a button pattern at the bottom of the well, the reaction was defined as negative. Adhesion of Ha-Ny beads on the wall of the well was defined as positive reaction. The JEV-specific IgM titre was determined as the highest dilution which gave a positive reaction pattern. The serum samples were considered to be positive for anti-JEV antibody when the titres were $\geqslant 400$.

### Statistical analysis

Differences between values were checked by Student's *t*-test using SPSS version 10, Statistical Analysis Software 2000.

## RESULTS

### Comparison of the PA assay and IgM-capture ELISA results

The 193 serum samples collected from the hospitalized patients with the clinical diagnosis of JE,

Table 1. *Comparison of the result between PA assay and IgM-capture ELISA*

| | | IgM-capture ELISA | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| Particle agglutination assay* | Positive | 71 | 15 | 86 |
| | Negative | 1 | 106 | 107 |
| Total | | 72 | 121 | 193 |

* Sensitivity was 99 % and specificity was 88 %.

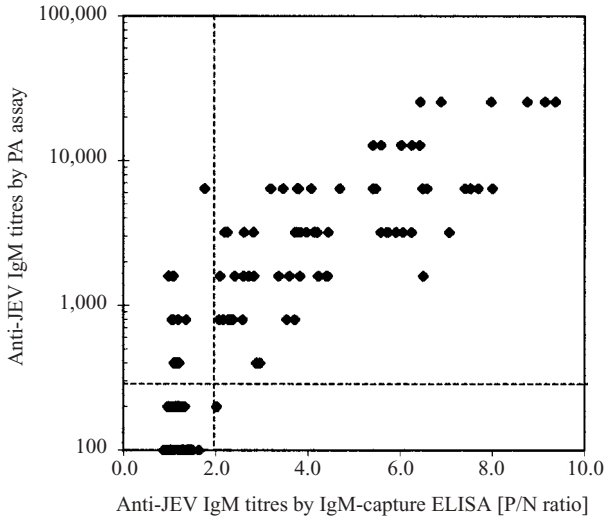


**Fig. 1.** Comparison of JEV IgM titres with the PA assay and the IgM-capture ELISA. The vertical dotted line is the cut-off for positive by IgM-capture ELISA (P/N ratio $\geqslant 2$) and horizontal dotted line is the cut-off for positive by the PA assay (titres $\geqslant 400$).

PUO, malaria and enteric fever in the JE-endemic area of the southwestern Nepal were tested for JEV-specific IgM by two serological methods, IgM-capture PA and ELISA. Eighty-six (44·6 %) of the 193 samples were IgM-positive by the PA assay (Table 1) and 71 (82·6 %) of the 86 PA IgM-positive samples were also IgM-positive by IgM-capture ELISA (Fig. 1, Table 1). Thus, the PA assay had a sensitivity of 99 %, a specificity of 88 %, a positive predictive value of 0·83 and a negative predictive value of 0·99 in comparison with IgM-capture ELISA. Fifteen samples were positive by the PA assay, but negative by IgM-capture ELISA. One hundred and six cases were JEV IgM negative by both IgM-capture ELISA and PA. These results suggest that there is a high level of compatibility between the PA assay and IgM-capture ELISA, using these Nepalese serum samples.

Table 2. *Clinical diagnosis of the patients who were IgM-positive by the PA assay and/or IgM-capture ELISA*

| | Number of JE IgM-positive cases determined by | | | | | |
|---|---|---|---|---|---|---|
| | PA | | PA and IgM ELISA | | IgM ELISA | |
| Clinical diagnosis | No. | (%) | No. | (%) | No. | (%) |
| JE | 66 | (77) | 60 | (85) | 60 | (83) |
| Pyrexia with unknown origin | 17 | (20) | 8 | (11) | 8 | (11) |
| Malaria | 2 | (2) | 2 | (3) | 3 | (4) |
| Enteric fever | 1 | (1) | 1 | (1) | 1 | (1) |
| Total | 86 | | 71 | | 72 | |

Table 3. *JE IgM-positive rate among patients clinically diagnosed as JE or other diseases*

| | JE IgM-Positive | | | | |
|---|---|---|---|---|---|
| | | PA | | IgM-ELISA | |
| Clinical diagnosis | Numbers | No. | (%) | No. | (%) |
| JE | 97 | 66 | (68) | 60 | (62) |
| Pyrexia with unknown origin | 59 | 17 | (28) | 8 | (16) |
| Malaria | 22 | 2 | (9) | 3 | (14) |
| Enteric fever | 15 | 1 | (7) | 1 | (7) |

**Accuracy of clinical diagnosis of JE in Nepal**

The number of patients who were clinically diagnosed as JE among those who were IgM-positive by the PA assay was analysed. Sixty-six (77 %) of the 86 patients who were IgM-positive had a clinical diagnosis of JE (Table 2). Twenty (23 %) IgM-positive patients were clinically diagnosed as PUO, malaria or enteric fever. The data were also analysed based on the clinical diagnosis (Table 3). Ninety-seven patients were clinically diagnosed as JE. Of these 97 patients, 66 (68 %) and 60 (62 %) were IgM-positive by the PA assay and IgM-ELISA, respectively. These results suggest that the clinical diagnosis of JE was reasonably accurate in Nepal.

## DISCUSSION

In the past 50 years, the number of JE cases has decreased dramatically in Japan, Taiwan, and South Korea. However, many JE cases are still reported annually in the Indian subcontinent, China, southeast Asia, and JE cases were reported recently in Torres Strait, Australia [1, 14]. Nepal reported the first confirmed JE cases in 1978 and the number of cases has been increasing since then [5]. JE occurred mainly in the Southern Terai districts of Nepal during the past two decades. At present, about 1000 cases are reported every year in Nepal with an increasing trend [8]. JE has spreaded to the inner Terai and high hills including Kathmandu Valley (altitude 1300 m) where the first outbreak of JE was reported in 1995 [15]. The Epidemiology and Disease Control Division, Department of Health Services of Nepal reported 1729 cases and 169 deaths due to encephalitis in 2000. Mid and Far Western regions of Terai area were mostly affected by JE with the incidence ratio of 1·4 to 1·8/1000 [16]. Kailali and Banke districts reported 683 and 435 cases, respectively, representing 65% of the total cases. About 30% of the entire inhabitants in Banke, Bardiya and Kailali districts are infected by JEV from July to October each year [16, 17]. The expansion of JE is probably due to agricultural development, increasing irrigation, deforestation, and animal husbandry favouring the breeding of *Culex* mosquitoes. The actual number of JE cases could be underestimated, because most of the patients do not visit hospitals in the rural area. The patients are usually brought to hospitals at the late stage of illness in the rural areas. Most authorities agree that the control of JE requires universal childhood immunization, because 70% of the cases occur in children and vector control is very difficult. The available inactivated vaccine is expensive and requires three doses. It is likely that JE will continue to be an important public health problem in Asia.

At present, JE is diagnosed based on patients' clinical symptoms: high fever, headache and altered consciousness. These clinical features are also common manifestations of typhoid fever, cerebral malaria, bacterial meningitis, measles and some enterovirus infections. In the present study, we observed that 60–70% of the patients who were clinically diagnosed JE were positive for JEV IgM by the PA assay and IgM ELISA. The results suggest that the clinical diagnosis of JE is fairly accurate in those areas of Nepal. Although there is currently no effective antiviral treatment, rapid diagnosis of JE provides the opportunity to treat complications such as convulsions, hyponatraemia, high intra-cranial pressure and avoids unnecessary investigations.

The laboratory diagnosis of JE has advanced considerably in recent years. The commonly used methods for the diagnosis of JE were haemagglutination inhibition and neutralization assays. IgM-capture ELISA has recently been used as the standard and the most reliable technique for the diagnosis of JE. However, these tests require sophisticated instruments and trained personnel, and are more useful in the referral diagnostic centre in the developing countries. The development of the PA assay, which does not require specific equipment and is relatively economical, would be beneficial for the rural areas with limited facilities and where trained personnel are not available. We applied the PA assay to the serum samples collected in Nepal. The sensitivity and specificity of the PA assay is acceptably high and determined to be useful in rural areas of Asia including Nepal. The discrepancies between the PA and IgM-capture ELISA need to be further evaluated by analysing the patient information in depth. Serially collected serum samples could provide more valuable information. Cross-reactivity of anti-flaviviral IgG has been well documented; however, IgM is known to be specific [18]. The data suggest that the PA assay for JEV IgM is quick, easy to perform and specific. This assay system is useful especially in the rural areas of Asia to support the clinical diagnosis, management, and epidemiological studies of JE.

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