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Research article

# Antibodies against prM protein distinguish between previous infection with dengue and Japanese encephalitis viruses.

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## Abstract

**Background:** In Southeast Asia, dengue viruses often co-circulate with other flaviviruses such as Japanese encephalitis virus, and due to the presence of shared antigenic epitopes it is often difficult to use serological methods to distinguish between previous infections by these flaviviruses.

**Results:** Convalescent sera from 69 individuals who were known to have had dengue or Japanese encephalitis virus infection were tested by western blotting against dengue, Japanese encephalitis and West Nile virus antigens. We determined that individuals who had been infected with dengue viruses had IgG responses against the premembrane protein of dengue viruses but not Japanese encephalitis, whereas individuals who had been infected with Japanese encephalitis had IgG specific for the premembrane protein of Japanese encephalitis virus but not the dengue viruses. None reacted with the premembrane protein of West Nile virus. Using the Pearson Chi Square test, it was determined that the difference between the two groups was highly significant with a p value of <0.001.

**Conclusion:** The use of flavivirus premembrane protein in seroepidemiological studies will be useful in determining what flaviviruses have circulated in a community.

## Background

Dengue haemorrhagic fever (DHF) was first described in Southeast Asia half a century ago, and has became increasingly important as a cause of paediatric morbidity and mortality in Southeast Asia over these past decades. Although the dengue viruses have circulated in this and other parts of the world previously, the viruses caused dengue fever (DF) rather than the more severe DHF, which has been for the most part, a Southeast Asian phenomenon since the 1950s. In recent years however, epidemics of dengue outside Southeast Asia have been associated with

DHF and it has become a disease of global importance. Dengue viruses are not the only flaviviruses which have spread beyond their traditional ecologies. This is evident from the establishment and maintenance of West Nile virus (WNV) in the eastern United States over the last 3 summers. Japanese encephalitis virus (JEV) is another example of a flavivirus extending its boundaries. First isolated in Japan in 1935, JEV had been causing extensive outbreaks of encephalitis in parts of East Asia until the introduction of a vaccine in the middle of the last century. Although Japanese encephalitis has now been controlled

in Japan it is increasingly an important public health problem in countries west and south of Japan, and more recently has spread as far as northern Australia. Under these circumstances, it is not unusual to find that more than one flavivirus co-circulates in an area. In Southeast Asia, the most important of these are the dengue viruses and JEV. In South America it is likely that the dengue viruses may co-circulate with yellow fever virus (YFV) while it is becoming likely that in parts of eastern North America we should be concerned about co-circulating St Louis encephalitis (SLE) virus and WNV [1,2].

All these flaviviruses share antigenic epitopes, which elicit antibodies commonly referred to as cross-reacting antibodies. These flavivirus cross-reactive responses can confound the interpretation of serological tests, and it is often impossible to determine with certainty the infecting virus without resorting to performing neutralization tests. This characteristic was most clearly noticed in the days when serological confirmation of dengue and JEV infection was based on the demonstration of a four-fold seroconversion of antibody titre by the haemagglutination inhibition test. It was standard practice to test two fold serial dilutions of paired sera from each patient against haemagglutinins prepared from all 4 dengue serotypes as well as JEV to determine recent infection retrospectively. While IgM capture ELISAs using antigens prepared from different flaviviruses may today be used with care to determine recent infection, ELISA methods to differentiate between IgG responses to different flaviviruses are not as straightforward.

Although the determination of the presence of specific IgG may no longer be useful for the diagnosis of acute infection, seroepidemiological studies are best carried out using ELISAs to detect specific IgG. There is today an expanded effort to develop vaccines against various flaviviruses, including DENV, JEV and WNV. There is thus clearly a need for an ELISA based test for the determination of flavivirus infection history during field site preparation when these vaccine candidates are taken to phase III trials.

During the course of our routine service for the serodiagnosis of recent dengue infection we noticed that in immunoblot analysis of serum from dengue patients, IgG was able to recognize the envelope as well as the NS1 proteins of DENV as well as JEV. However, antibodies against DENV prM, were not reactive with JEV prM. In this study we present data to show that antibodies against prM of DENV and JEV can reliably identify which flavivirus or flaviviruses an individual has previously been infected with. We believe that this specificity of antibodies against prM may be useful in the development of simple methods for determining the flavivirus infection history of an individual as well as for seroepidemiological studies of flavivirus

infections in situations where more than one flavivirus co-circulates.

## Results and Discussion
### Western blots probed with dengue or JEV positive sera

Two equivalent SDS PAGE gels were transferred to nitrocellulose membranes and the resulting blots were probed with two serum pools: a high titred positive reference dengue sera (HPR) and JEV positive sera from swine (PPJ). HPR had been pooled from more than 50 specimens obtained in the Malaysian states of Penang and Perak between 1989 and 1994. The swine sera had been pooled from more than 50 specimens collected during a period of JEV activity in Penang in 1989. Figure 1 (A) shows that the envelope and NS1 proteins of the dengue viruses and JEV were strongly reactive with HPR. NS1 is seen here in its dimeric form because the samples were not heated and were run under non-reducing conditions. What is remarkable however, is that the prM band of each of the 4 dengue serotypes was recognized by HPR while the prM band of JEV was not picked up at all. In the complementary blot in Figure 1 (B), PPJ can be seen to have reacted strongly with the envelope proteins of all the dengue virus serotypes in addition to the envelope protein of JEV. There was some weak cross reactivity with the dengue virus NS1 dimer but PPJ did not recognize any of the prM bands in the lanes containing dengue virus serotypes. Since dengue viruses do not infect swine, PPJ can be confidently considered to be homotypic JEV serum and this specificity for JEV prM was explored further by probing western blot strips with individual IgG positive swine sera.

A group (JS) of 31 JEV positive sera from swine in a highly JEV endemic area were tested separately on strips containing JEV antigens and DENV3 antigens. DENV3 was selected to represent the 4 dengue virus serotypes because it was the most prevalent serotype circulating in the human population during the period of collection of the swine specimens. To our surprise, only 29 of the 31 sera were positive for JEV prM, although these had been scored strongly positive in a dot enzyme immunoassay using JEV antigens. However, none of the 31 sera recognized the prM of DENV3, although the envelope band was reactive in these strips. It is intriguing that in this sample, 2 (6.5%) of the strongly positive swine sera recognized JEV and WNV envelope protein but not prM. It is possible that these sera were from animals with more recent infection and had not yet had raised antibodies to prM. Alternatively, there might be another flavivirus circulating locally which can infect swine.

Next we tested a group (JH) of convalescent sera from 16 children who had had JEV infection proven by IgM capture ELISA (MAC ELISA) using JEV and DENV3 western blot strips. Here again, DENV3 was chosen because it was
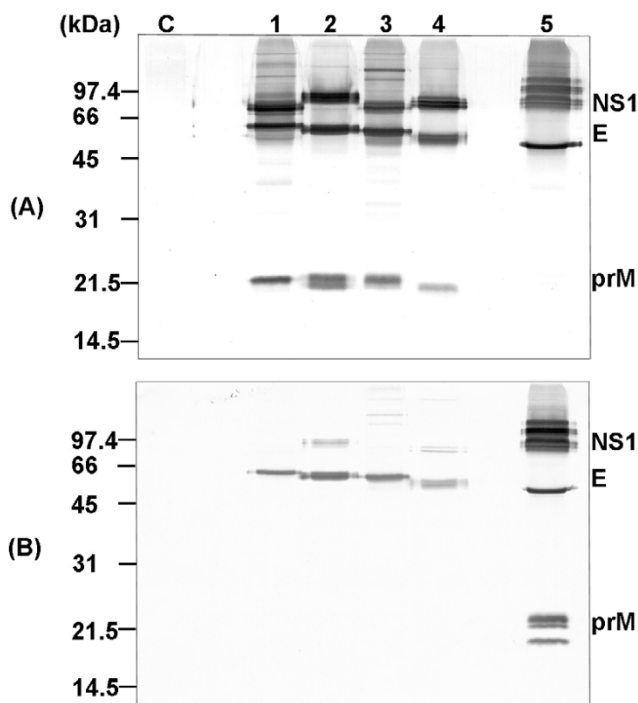
**Figure 1**
**Immunoblots Probed With High Titred Antiserum Pools.** SDS PAGE was run with samples which were not reduced with 2-mercaptoethanol and not heated before electrotransfer to nitrocellulose membranes. Immunoblots were probed with (A): convalescent dengue serum (HPR) and (B): JEV positive swine serum (PPJ). Lane C contains uninfected cell lysates while Lanes 1, 2, 3, 4 and 5 contain lysates of DENV1, 2, 3, 4 and JEV respectively. The position for bands of NS1 dimer, E and prM are labeled on the right.

the dominant dengue virus serotype circulating at the time these patients presented. These sera had previously been shown to have seroconverted with respect to IgG against JEV antigens, and in the western blot analysis all these convalescent sera were reactive with JEV prM but not DENV prM, confirming our observations with the swine sera that antibodies elicited against JEV prM do not cross react with DENV prM.

Since there are 4 different serotypes of dengue viruses, and in Southeast Asia, these viruses are commonly co-endemic with JEV, it is not possible to be certain that a patient with a proven dengue infection did not previously have a JEV infection, thus effectively having a secondary flavivirus immune response. In Malaysia this is entirely possible because dengue can occur in older patients in contrast with JEV which is mainly a paediatric disease. We addressed this problem by testing 2 different groups of patients with dengue proven by MAC ELISA. In the first group (DP) of sera collected in Penang where the prevalence of JEV is

low, there were 10 convalescent sera and all were found to be reactive with prM of at least one dengue virus serotype, but only 1 was also reactive with the prM of JEV. The second group (DS) of sera was collected in Sarawak where the prevalence of JEV is very high. Of the 12 convalescent sera tested, 11 were reactive with prM of at least one dengue virus serotype and 5 were reactive with the prM of JEV. Interestingly, the single specimen which was not reactive with dengue prM was reactive with prM of JEV raising the possibility that this patient had had a JEV infection in the past, and a recent dengue infection which had not yet elicited antibodies against dengue prM. Indeed this patient had neutralizing antibodies against both dengue viruses as well as JEV at titres of 1:640 and 1:160 respectively.

The data from the individual serum groups are summarized in Table 1. The JS and JH groups were combined to form a JEV positive subgroup and the DP and DS groups were combined to form a dengue positive subgroup. There were a total of 69 specimens tested and the Pearson's chi-square test was applied, showing a significant difference between the two groups with a p value of <0.001 (JMP Start Statistics, SAS Institute, Inc., USA). In this study, it was not possible to determine if antibody responses to the prM protein of each DENV serotype was associated with prior infection with that particular serotype. This is because there is currently no convincing method to determine infecting dengue serotype retrospectively since even DENV neutralizing antibodies can be cross reactive.

### JEV specific sera do not recognize WNV prM
JEV and WNV belong to the same flavivirus antigenic group, and with the heightened interest in WNV in the United States, we decided to determine if JEV specific sera would discriminate between JEV and WNV prM. We tested the same 16 human JEV positive sera from group JH on western blot strips prepared from WNV lysates. None of the JEV positive sera reacted with WNV prM although there was substantial cross reactivity with WNV envelope protein. Some representative western blot strips are shown in Figure 2.

### Conclusions
Antibodies against the prM protein of JEV do not react with the prM of dengue viruses or WNV. Conversely antibodies against the prM protein of dengue viruses do not react with JEV prM. It is conceivable that prM is a useful viral protein to investigate as a tool in differentiating antibody responses to different flaviviruses and thus provide a method to investigate specific flavivirus seroepidemiology in a community.

**Table 1: prM Reactivity of Different Serum Groups**

| SERUM GROUP | DESCRIPTION | NUMBER TESTED | NUMBER DENV prM POSITIVE (%) | NUMBER JEV prM POSITIVE (%) |
|---|---|---|---|---|
| JS | JEV positive swine | 31 | 0 (0) | 29 (93.5) |
| JH | Convalescent JE, human | 16 | 0(0) | 16 (100) |
| ALL JEV | SUBTOTAL OF JEV SERA | 47 | 0 | 45 (95.7%) |
| DP | Convalescent dengue, Penang (low incidence JE) | 10 | 10 (100) | 1 (10) |
| DS | Convalescent dengue, Sarawak (high incidence JE) | 12 | 11 (91.7) | 5(41.7) |
| ALL DENV | SUBTOTAL OF DENV SERA | 22 | 21 (95.5%) | 6 (27.3%) |

## Materials and Methods

### Source of virus and monoclonal antibodies

Four prototype dengue virus serotypes, Japanese encephalitis virus, Nakayama strain and West Nile Virus, Egypt 101 strain, were all adapted to culture in the C6/36 *Aedes albopictus* cell line. All the flaviviruses used in this study were passaged from suckling mouse brain preparations obtained originally from the collection of Dr James S Porterfield while he was at Oxford University. The mouse monoclonal antibodies, MF4/5/A5/C3-3/D4/C6 (dengue group reactive) and MV12/1/C2-2/1 (JEV specific), were provided by Venture Technologies, Penang, Malaysia.

### Preparation of virus antigens

C6/36 cells were grown in 75 mm³ tissue culture flasks (Nunc, Denmark) in Leibovitz 15 medium containing 5% heat inactivated foetal bovine serum and antibiotics (Penicillin, 50 U/ml and streptomycin sulphate 50 mg/ml). Virus stocks were inoculated onto confluent monolayers after removal of growth medium and allowed to adsorb to the cells by rocking the flasks at room temperature for one hour, after which the flasks were replenished with 20 ml of maintenance medium (Leibovitz 15 medium with 3% heat inactivated bovine serum and antibiotics), then incubated stationary in an incubator set at 30°C. Maintenance medium was changed after 2 to 4 days (depending on the virus) and the culture supernatants and infected cells were harvested when cytopathic effect was apparent throughout the monolayer. The culture supernatants were clarified by centrifugation for 10 minutes at 1000 rpm at 4°C in a Beckman Avanti J-25 with a JS7.5 rotor, and stored in aliquots at -80°C until use. The infected monolayers were washed with phosphate buffered saline (PBS) and lysed in 2 ml of a hypotonic buffer containing 1% TX-100. Intact nuclei were removed by brief centrifugation at 14,000 rpm in a microcentrifuge (Eppendorf, 5415C) and the lysate supernatants (referred to as "lysates") were aliquoted and stored at -80°C until use. Culture supernatants were used for two purposes: as infectious virus in plaque assays as well as virus antigen source in the IgM or mouse immunoglobulin capture ELISAs. When used as antigens in an IgM capture assay, the culture supernatants of DENV1, 2, 3 and 4 were pooled in a ratio which was designed to ensure equal antigen titres for all serotypes. The lysates were used for the immunoblots as well as for coating 96 well plates for indirect ELISAs. Control antigens were prepared in an identical manner using mock infected cells harvested at the same time as the oldest virus cultures.

### Serum specimens

Three pools of sera were used as controls: High Positive Reference (HPR) contained pooled convalescent serum from patients with dengue. Negative Reference Serum (NRS) was serum pooled from individual healthy donors who were negative for dengue and JEV IgG when tested in a dot enzyme immunoassay at 1:250 dilution. Pooled Positive JEV (PPJ) serum was pooled from swine slaughtered in the Penang state abbatoir in 1989 after a JEV outbreak in the state. The sera had been tested by haemagglutination inhibition test and dot enzyme immunoassay for antibodies to JEV [3].

Four groups of individual serum were used in this study and are described here as follows:

Group JS: JEV IgG positive serum from 31 individual domestic swine from JEV endemic areas, as determined by dot enzyme immunoassay.

Group JH: Convalescent sera from 16 individuals with encephalitis due to JEV as determined by IgM capture ELISA.
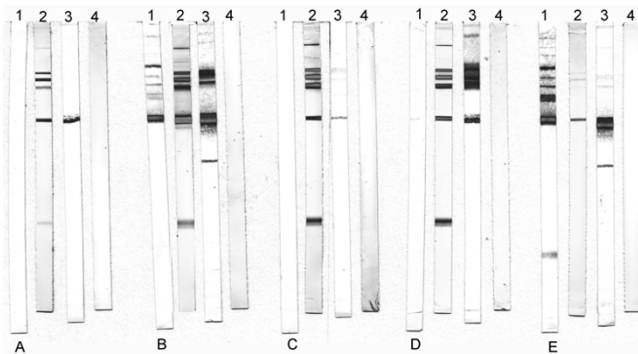
**Figure 2**
**Representative Immunoblot Strips Probed With Individual Sera.** Strips contain viral antigens separated by SDS PAGE under non-reducing conditions without heating: (1) DENV3, (2) JEV, (3) WNV, (4) uninfected cell control. Each set of strips were probed with a single convalescent serum specimen (1:1000, overnight) from an individual with a JEV infection (A – D) while the set labeled E was probed with HPR.

Group DP: Convalescent dengue IgG positive sera from 10 patients with dengue fever or dengue haemorrhagic fever where recent dengue was determined by IgM capture ELISA. Patients were from Penang in West Malaysia where the incidence of Japanese encephalitis is low.

Group DS: Convalescent dengue IgG positive sera from 12 patients with dengue fever or dengue haemorrhagic fever where recent dengue was determined by IgM capture ELISA. Patients were from Sarawak in Malaysian Borneo, where the incidence of Japanese encephalitis is high.

### IgM Capture ELISA (MAC ELISA) to distinguish recent dengue and JEV infection

When Innis and coworkers described the IgM capture ELISA for dengue in 1989, they included JEV antigens to differentiate dengue and JEV infections [4]. This detail is important in settings where both dengue and JEV co-circulate and we have adapted this basic assay to utilize culture supernatants instead of mouse brain derived antigens and monoclonal instead of polyclonal antibodies. We validated the use of culture supernatants as the antigen source previously [5] and the current assay uses in addition an equal mixture of the monoclonal antibodies MF4/5/A5/C3-3/D4/C6 and MV12/1/C2-2/1, which together can recognize all 4 dengue serotypes as well as JEV. Table 2 shows adjusted OD readings of a mouse immunoglobulin capture ELISA (MIC ELISA) to show the specificities of these 2 monoclonal antibodies. Thus MF4/5/A5/C3-3/D4/C6 is a dengue group reactive antibody and MV12/1/C2-2/1 is JEV specific.

Briefly, 96 well flat bottom plates (Maxisorb, Nunc, Denmark) were coated overnight at 4°C with rabbit anti-human μ chain (Dako A425) at 1:2000 dilution in carbonate-bicarbonate buffer pH 9.6 before blocking with PBS containing 1% casein. Washed plates were loaded with patients serum (in triplicate) at 1:100 dilution in PBS containing 1% casein and incubated at room temperature for 2 hours before washing with PBS containing 0.5% Tween 20 (PBS-Tween). Each specimen was then tested against 3 different antigens: DENV pool (of all 4 serotypes), JEV and the uninfected cell control. These antigens were loaded into the wells for an overnight incubation at 4°C and then washed with PBS-Tween prior to the antigen detection step, which uses a mixture of the monoclonal antibodies MF4/5/A5/C3-3/D4/C6 and MV12/1/C2-2/1 for one hour at room temperature. After washing with PBS-Tween the bound monoclonals were detected using an anti-mouse immunoglobulin conjugated with HRP (Dako P260) at 1:2000 dilution for one hour at room temperature. Colour development was achieved after washing using the substrate, o-phenylenediamine – hydrogen peroxide for 30 minutes at room temperature. The reaction was stopped with sulphuric acid and the optical density (OD) was read at 490 nm wavelength using 650 nm as the reference.

### Interpretation of MAC ELISA readings

Every individual serum specimen included an internal cell control antigen. The OD reading of the well containing the cell control antigen was subtracted from each of the wells containing the virus antigens (DENV in one and JEV in the other) to give an adjusted OD which took account of any reaction the patient might have had against mosquito antigens. Each plate also contained 3 sets of positive and negative serum controls. A specimen was considered to be IgM positive when the ratio:

adjusted OD of virus / mean adjusted OD negative control >= 5.

It is often the case that patients will have IgM which react with both DENV and JEV. This should be expected since flaviviruses have shared epitopes, and in our experience it is not difficult to determine which is the most likely infecting virus by comparing the adjusted OD of DENV with that of JEV. Normally this would be much higher for one than the other, and would clearly indicate the recent infecting virus. It is thus critically important when using MAC ELISAs to run DENV and JEV antigens in parallel as first described by Innis and coworkers[4] in order to avoid misdiagnosis in countries where both viruses co-circulate. In this current assay we have not attempted to define units as described by Innis and coworkers since to be useful, this would require standard reagents made freely available to all, a resource not yet in existence. In a few instances

**Table 2: Specificities of Monoclonal Antibodies MF4/5 and MV12/1 in a Mouse Immunoglobulin Capture ELISA**

| ANTIGEN | MF4/5/A5/C3-3/D4/C6 ADJUSTED OD | MV12/1/C2-2/1 ADJUSTED OD |
| --- | --- | --- |
| DENV1 | 1.906 | 0.293 |
| DENV2 | 1.813 | 0.055 |
| DENV3 | 1.847 | 0.184 |
| DENV4 | 0.746 | 0.056 |
| JEV | 0.051 | 1.735 |

there is no substantial difference in the adjusted OD of both DENV and JEV and in such cases it would be difficult to determine with confidence whether the recent infecting virus was a DENV, JEV or some other flavivirus. A spreadsheet (see Additional File) is provided to demonstrate what OD readings are seen in a clinical situation where both dengue and JE viruses circulate in the same location.

### Mouse Immunoglobulin Capture ELISA (MIC ELISA) to identify monoclonal antibody specificity

Essentially this was similar to the MAC ELISA with the following differences: The plates were coated with rabbit anti-mouse immunoglobulin (Dako Z412) at 1:2000 dilution in order to capture mouse immunoglobulins from undiluted hybridoma culture supernatants overnight at 4°C. The bound mouse antibodies were incubated with various cell culture derived antigens as described above, and the antigens bound were detected using high titred human anti-dengue IgG (HPR) followed by anti-human γ conjugated to HRP (Dako P214) at 1:5000 dilution.

### Western blotting

Lysates from infected and uninfected cells were run on 12% discontinuous SDS polyacrylamide gels and electroblotted onto nitrocellulose membranes. The membranes were then blocked with PBS containing 5% non-fat skimmed milk (PBS-SM) for 30 minutes. Membranes were then washed and probed with serum diluted 1:1000 in PBS-SM with 10 mM sodium azide by incubating overnight on a rocker at room temperature. The following morning the membranes were washed extensively with PBS (5 washes consisting of a 20 minutes soak with rocking) and then incubated with the relevant HRP conjugate for 4 hours with rocking. For human serum, we used anti-human IgG HRP (Dako P214) and for swine serum we used Protein A-HRP (KPL 14-50-00). Reactive bands were visualized by incubating with 4-chloro-1-naphthol and hydrogen peroxide for 30 minutes. The reaction was stopped with water.

## Authors' contributions

MJC and PHT made the initial observations and conceived the study. SMW and MSHS prepared the immunoblots and ran all the immunoassays. MJC performed the statistical analysis and drafted the manuscript. All authors participated in producing and approved the final manuscript.

## Additional material

### Additional File

*In order to illustrate the kind of OD readings which can be expected in a situation where patients are likely to be exposed to both dengue as well as Japanese encephalitis viruses, we have provided an excel file which shows representative data of IgM capture ELISA readings for real life examples of patients from dengue and JEV endemic areas.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2180-2-9-S1.xls]

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