Development of Immunoglobulin M Capture Enzyme-Linked Immunosorbent Assay To Differentiate Human Flavivirus Infections Occurring in Australia

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We report the development of a flavivirus immunoglobulin M (IgM) capture enzyme-linked immunosorbent assay (MAC-ELISA) which improves the determination of an infecting flavivirus serotype over that by current serological methods. A panel of 165 IgM-positive sera from flavivirus patients with specific diagnostic results was tested by the flavivirus MAC-ELISA using a panel of 10 antigens. For 134 of these sera (81.2%), the highest reactivity was demonstrated against the infecting virus, which was consistent with the original diagnostic result. Specific antibody reactions inconsistent with the original diagnosis were found for six sera (3.6%). In our experience, the flavivirus-serotyping ELISA provides a rapid and accurate alternative to other serological tests, such as hemagglutination inhibition, for the specific diagnosis of flavivirus infections.

Flaviviruses endemic to Australia include Murray Valley encephalitis virus (MVEV), Kunjin virus (KUNV), Alfuy virus (ALFV), Kokobera virus (KOKV), and Stratford virus (STRV) (6). Dengue virus is regularly imported by overseas travelers, often resulting in local transmission and outbreaks in tropical regions of the country (3). Since its first incursion into Australian territory in 1995, Japanese encephalitis virus (JEV) has caused occasional disease and has been regularly detected in sentinel animals in the Torres Strait (10). While sera taken from patients early in a flavivirus infection may be valuable in detecting a specific infecting virus by molecular methods or by isolation of the infecting agent by culture, patients often present later in the infection, when the virus may no longer be detectable. In such cases, the serum may be screened for the presence of flavivirus-specific immunoglobulin M (IgM), which may indicate recent infection. Confirmatory tests, such as the hemagglutination inhibition test (HAI) (1), the ultracentrifugation-hemagglutination inhibition test (UCHI) (14), and the plaque reduction neutralization test, may then be performed in order to attempt to differentiate the infecting flaviviruses or serotypes. These test procedures are expensive and time-consuming and may take several days to complete. Until recently, in our laboratory, flavivirus infections were confirmed by screening patient sera by enzyme-linked immunosorbent assay (ELISA) and testing IgM-reactive sera or sera with equivocal results by the HAI assay. Recent infections were confirmed by testing paired sera and illustrating a fourfold or greater rise in antibody titer. A determination of a specific flavivirus response was made if the titer of antibody against a specific antigen was at least fourfold higher than those for the other viruses in the panel.

Elsewhere in the world, laboratories have reported serospecific results obtained by using multiple viruses in the IgM capture ELISA (MAC-ELISA) format for dengue virus serotyping (8) and to distinguish West Nile virus from other JEV serocomplex viruses (7). We report the use of a similar technology, but we have expanded the test panel to include all flaviviruses likely to be encountered in Australian patients and in overseas travelers who have acquired infections elsewhere and are seeking treatment in this country. It was found that for most sera tested, a specific IgM response could be determined by this ELISA, allowing for more specific and more rapid diagnosis in flavivirus-infected patients.

MATERIALS AND METHODS

Flavivirus serum panel. Sera were selected on the basis of specific results for flavivirus serology or a PCR or culture result indicating a specific serotype of the infecting virus. Sera were excluded if the results suggested a secondary infection (HAI titers of ≥2,560) (12), as it is known that IgM in these cases may be directed to the original infecting virus, a phenomenon known as “original antigenic sin” (2). The panel included the following: 89 sera positive for a specific dengue virus type which had been confirmed by PCR (13), isolation by culture (in the C6/36 cell line, followed by direct fluorescence using specific monoclonal antibodies, UCHI or HAI); 29 JEV-positive sera, 25 KUNV-positive sera, 5 ALFV-positive sera, and 15 KOKV-positive sera, as determined by UCHI or HAI; and 2 sera from a patient with MVEV. No sera from patients with a confirmed Stratford virus infection were available for inclusion in the assay. All sera were originally tested in our laboratory, and, where possible, sera from patients meeting the case definition for flavivirus infection were used. Sera from dengue patients represented more than 50% of the total sera, which reflects the fact that dengue cases are referred to our laboratory at a higher frequency than cases of other flavivirus infections.

Flavivirus strains. Dengue virus serotypes 1 (DENV-1) through 4 were isolated in our laboratory from patients infected with dengue virus in East Timor in 2000. The Nakayama strain of JEV was used, and all other flaviviruses were type strains which formed part of the arbovirus culture collection maintained by our laboratory (MVEV strain MRM66, KUNV strain MRM16, ALFV strain MRM926, KOKV strain MRM32, and STRV strain C338).

Antigen production. The dengue virus and STRV strains were cultured in the Aedes albopictus cell line C6/36. All other flaviviruses were cultured in porcine-stable equine kidney (PSEK) cells. Antigens were prepared as described (9). Uninfected cell controls were produced for both C6/36 and PSEK cell lines.

Antigens were titrated as follows: Nunc-Immuno module 16-well U-bottom Maxisorp strips (product no. 469264; Nunc, Roskilde, Denmark) were coated with 0.05 ml of flavivirus monoclonal antibody (WR/DEN2/4G2 ascitic fluid; TroBio Pty. Ltd., Townsville, Australia) (4) at a 1:10,000 dilution in carbonate buffer, pH 9.6. The plates were incubated for 2 h at 37°C, after which they were...
Flavivirus MAC-ELISA. A serum screening dilution of 1:400 was chosen as the dilution at which the infecting viral serotype was best differentiated (data not shown). A specific control serum for each flavivirus was used in the ELISA run. Where available, these sera were from patients with confirmed human flavivirus with a specific diagnostic result by serological or molecular methods. Five flavivirus-negative sera were pooled to produce a negative control serum for use in the test.

The ELISA was performed as follows. Maxisorp strips in plate frames were coated with 0.05 ml of 1:1,600 chain-specific rabbit anti-human IgM (DakoCytomation, Carpinteria, Calif.) per well. The plates were incubated at 37°C for 2 h and then stored at 4°C before use. Specific control sera were selected for each flavivirus used in the ELISA. Test and control sera were diluted to 1:400 in 0.05 ml of each dilution was transferred to the wells of the ELISA plates. The plates were incubated at 37°C for 1 h and washed as described above. The bound virus-negative sera were pooled to produce a negative control serum for the same antigen. Excel spreadsheets were added, and the plate was developed as described for the antigen titration. Positive/negative (P/N) ratios for each patient serum against each virus were calculated by dividing the absorbance of the patient serum for a particular antigen by the absorbance of the negative control serum for the same antigen. Excel spreadsheets were used to calculate the P/N ratios and to determine the highest

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**TABLE 1. P/N ratios for representative sera for 10 flavivirus antigens compared with original diagnostic results**

| Serum | Original diagnostic result | Method | DENV-1 | DENV-2 | DENV-3 | DENV-4 | JEV | MVEV | KUNV | ALFV | KOKV | STRV |
|-------|---------------------------|--------|--------|--------|--------|--------|-----|------|------|------|------|------|
| 1     | DENV-1 PCR                | 1.12   | 1.50   | 0.75   | 1.90   | 1.40   | 2.00 | 1.65 | 1.40 |
| 2     | DENV-1 HAI                | 1.50   | 1.00   | 0.75   | 1.90   | 1.40   | 2.00 | 1.65 | 1.40 |
| 3     | DENV-1 HAI                | 1.50   | 1.00   | 0.75   | 1.90   | 1.40   | 2.00 | 1.65 | 1.40 |
| 4     | DENV-1 HAI                | 1.50   | 1.00   | 0.75   | 1.90   | 1.40   | 2.00 | 1.65 | 1.40 |
| 5     | DENV-1 HAI                | 1.50   | 1.00   | 0.75   | 1.90   | 1.40   | 2.00 | 1.65 | 1.40 |
| 6     | DENV-1 HAI                | 1.50   | 1.00   | 0.75   | 1.90   | 1.40   | 2.00 | 1.65 | 1.40 |
| 7     | DENV-1 HAI                | 1.50   | 1.00   | 0.75   | 1.90   | 1.40   | 2.00 | 1.65 | 1.40 |
| 8     | DENV-1 HAI                | 1.50   | 1.00   | 0.75   | 1.90   | 1.40   | 2.00 | 1.65 | 1.40 |
| 9     | DENV-1 HAI                | 1.50   | 1.00   | 0.75   | 1.90   | 1.40   | 2.00 | 1.65 | 1.40 |
| 10    | DENV-1 HAI                | 1.50   | 1.00   | 0.75   | 1.90   | 1.40   | 2.00 | 1.65 | 1.40 |

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*a* Homologous reactions are shown in bold.

*b* The MVEV case was diagnosed clinically in conjunction with flavivirus serology results.
response for each serum across the panel of antigens. Ratios for the viral antigens were required to be at least twice the values for the control antigens or else the result for that serum was determined to be nonspecific. Nonspecific sera were excluded from the validation analysis. Results of the MAC-ELISA were not confirmed by the plaque reduction neutralization test, as the low volumes of sera did not allow for this test to be performed.

RESULTS

A total of 165 flavivirus patient sera were selected for validation of the serotyping ELISA. These sera were tested as described above, and the results were compared with the results previously obtained for the same patients. All sera were from patients who had been tested in our laboratory, and a specific flavivirus serotype was determined by various methods, including PCR, isolation, UCHI, and HAI, except for two sera from a single MVEV patient who was diagnosed based on both flavivirus serology results and clinical grounds (5).

Eighty-nine IgM-positive sera from patients diagnosed specifically with DENV-1 (n = 22), DENV-2 (n = 34), DENV-3 (n = 27), or DENV-4 (n = 6) infections were tested. Seventy-six sera from non-dengue flavivirus infections were included in the study, and these comprised sera from patients with JEV (n = 29), MVEV (n = 2), KUNV (n = 25), ALFV (n = 5), or KOKV (n = 15) infections.

P/N ratios of representative sera against each of the 10 flavivirus antigens are given in Table 1. Results for all sera were charted by using Microsoft Excel to compare antibody responses across the panel of antigens. In most cases, the response to the homologous antigen was significantly higher than those to the heterologous antigens (Fig. 1A and C). Some dengue patient sera showed cross-reactivity with different dengue virus antigens, but the results could be differentiated from those for non-dengue virus antigens (Fig. 1B), whereas some

FIG. 1. Examples of charted P/N results obtained by flavivirus MAC-ELISA. (A) Serum from a patient originally diagnosed as being infected with DENV-1 by isolation culture and showing specific IgM antibody response to DENV-1. (B) Serum from a DENV-1 patient, originally diagnosed by both PCR and isolation, showing an unspecified dengue virus IgM response. (C) Serum from a JEV patient, diagnosed by UCHI, showing a specific antibody response to JEV. (D) Serum from a KUNV patient, diagnosed by HAI, showing a cross-reactive IgM response to both KUNV and ALFV.

TABLE 2. Flavivirus MAC-ELISA results with IgM-reactive sera

| Virus identified by original serology (total no. of sera) | No. of sera positive for indicated virus by MAC-ELISAa |
|---------------------------------------------------------|-----------------------------------------------------|
| DENV-1 (22)                                             | 15 DENV-1 DENV-2 DENV-3 DENV-4 JEV MVEV KUNV ALFV KOKV STRV DENVb FLAVIc |
| DENV-2 (34)                                             | 0 29 0 0 0 0 0 0 0 0 0 5 1 |
| DENV-3 (27)                                             | 0 0 26 0 0 0 0 0 0 0 0 1 0 |
| DENV-4 (6)                                              | 0 0 1 5 0 0 0 0 0 0 0 0 0 |
| JEV (29)                                                | 0 0 0 0 22 0 0 1 1 0 0 0 4 |
| MVEV (2)                                                | 0 0 0 0 0 0 0 1 0 0 0 0 1 |
| KUNV (25)                                               | 0 0 0 0 0 0 0 20 0 0 0 0 5 |
| ALFV (5)                                                | 0 0 0 0 0 0 0 0 4 0 0 0 1 |
| KOKV (15)                                               | 0 0 0 0 0 0 0 1 0 12 1 0 1 |

a Sera were tested previously as described in Materials and Methods; boldface values indicate matches between MAC-ELISA results and the original serology results.
b DENV, dengue virus serotype unspecified.
c FLAVI, flavivirus unspecified.
sera from patients with non-dengue virus infections, while showing little reactivity against the dengue virus antigens, demonstrated cross-reactivity with non-dengue virus antigens (Fig. 1D).

Results of the flavivirus MAC-ELISA compared with the original test results with the same specimens are shown in Table 2. For cases in which cross-reactive dengue virus results were obtained, results are listed as “dengue virus serotype unspecified.” For cases in which cross-reactivity against other flaviviruses was determined, results are listed as “flavivirus unspecified.”

Results of the MAC-ELISA matched the original diagnostic results for 134 of the sera (81.2%). Of the dengue patient sera, 75 (84.3%) gave results consistent with the original results, while a further 11 (12.4%) were determined to represent unspecified dengue virus infections, since the reactions to the dengue virus antigens could be differentiated from those of the other flaviviruses. Twenty-two of the JEV patient sera (75.8%) reacted most strongly to JEV antigen, while four (13.7%) could not be differentiated. One of the MVEV patient sera showed a specific reaction to MVEV antigen, while the other was cross-reactive. Twenty of the KUNV patient sera (80%) showed highest antibody responses to KUNV antigen, while the remaining five (20%) were cross-reactive. Four of the ALFV patient sera (80%) reacted most strongly to ALFV antigen, and 12 of the KOKV patient sera (75%) reacted most strongly to KOKV antigen. Of the total panel, six sera (3.6%) displayed antibody responses which were inconsistent with the original diagnoses. These included one DENV-1 patient serum which reacted most strongly to DENV-2 antigen, one DENV-4 patient serum which reacted most strongly to DENV-3 antigen, two JEV patient sera which reacted most strongly to ALFV and KUNV antigens, and two KOKV patient sera which showed highest reactions to KUNV and STRV antigens.

**DISCUSSION**

Australia is home to a wide range of flaviviruses, along with a population which is inclined to travel to areas of the world where other flaviviruses are likely to be encountered and possibly imported by returning travelers or overseas visitors. The range of possible flaviviruses infecting humans in this country is therefore potentially wider than that in any other area of the world. The infecting flavivirus serotype may be determined by various serological methods, such as the HAI test, but in our experience, specific HAI results are rarely obtained. For example, in 2002, 568 patient sera were tested by flavivirus HAI, with 29 (5.1%) giving a specific result. Other disadvantages of the HAI test are that it is expensive, has a relatively low sensitivity, involves a degree of subjectivity in reading results, and is also labor-intensive and can take up to 3 days to produce a result. The flavivirus-serotyping ELISA has been found to differentiate the infecting flavivirus serotypes in most cases by detecting specific IgM in patient serum.

In this work, we have demonstrated that an IgM test using a panel of antigens was able to differentiate infecting flavivirus serotypes for the majority of sera tested.

As a replacement for the HAI test, the flavivirus-serotyping ELISA can reduce turnaround times for results and decrease the cost of testing by significantly reducing the labor component of the test. We believe that the flavivirus-serotyping ELISA greatly improves our ability to provide timely, accurate, and meaningful results in cases of flavivirus infection, in particular, for the diagnosis of dengue virus infections.

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