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Short Communication

A Preliminary Study of Dengue Infection in Brunei

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SUMMARY: The purpose of this study was to examine the extent of dengue infection in Brunei and to determine the predominant serotype circulating in the country. The study generated useful epidemiological data on dengue infection in Brunei. A total of 271 samples from patients suspected of having dengue infections were selected and analyzed. All patients were seen in clinics and hospitals in Brunei. The samples were collected from April 2005 to April 2006 and transported to the WHO Collaborating Centre for Arbovirus Reference and Research, University of Malaya, Malaysia. The following tests were used to achieve the objectives: in-house IgM-capture enzyme-linked immunosorbent assay, virus isolation in mosquito albopictus cell line (C6/36), and viral RNA detection and serotyping by reverse transcriptase-polymerase chain reaction (RT-PCR). The results show that 45 people were positive for dengue-specific IgM (27 males and 18 females), while RT-PCR detected dengue viral RNA in 12 patients, 3 identified as DEN-1 and 9 as DEN-2. Dengue virus was isolated from 6 patients using the C6/36 cell line; 3 were DEN-2 isolates and 3 were DEN-1 isolates. These data show that dengue virus is circulating in Brunei and the predominant infecting serotype for that period was DEN-2 followed by DEN-1. This study is the first to report the detection and isolation of dengue virus from Brunei using RT-PCR and culture in the C6/36 albopictus mosquito cell line.

Dengue is still one of the most important tropical diseases. It causes an economic burden in more than 100 countries mainly in the tropical belt around the world, where 2.5 - 3 billion people are at risk (1). Annually, it is estimated that 50 - 100 million people worldwide are infected with the virus that causes uncomplicated dengue fever (DF), and 250,000 to 500,000 developed dengue hemorrhagic fever (DHF) resulting in 25,000 deaths (2-5). Dengue has been endemic and epidemic in almost all ASEAN countries. Brunei, with a total area of 2,226 square miles, is located on Borneo Island flanked by the Malaysian States of Sarawak to the west and Sabah to the east. Dengue is endemic in these countries, and as the rate of people traveling in and out of Brunei increases in combination with the increased usage of the country as a link between Sarawak and Sabah, it is anticipated that outbreaks may be on the rise. In 2005, Sabah had 1,730 DF and 40 DHF cases while Sarawak had 706 DF and 13 DHF cases (6). Brunei is divided into four districts: Brunei-Muara, Tutong, Temburong and Kuala Belait. The capital, Bandar Seri Begawan, lies in the Brunei-Muara district, where more than half of the total population of 383,000 lives, 2006 est. (Fig. 1). Serological data compiled by the Public Health Department in Brunei showed that from 1992 to 2006 a total of 398 people were positive for dengue IgM (Fig. 2). The year 2003 had the highest number with 163 dengue IgM positives. The serological test mainly involved the detection of IgM and IgG using the PanBio rapid strip test. No further confirmatory tests were performed due to a lack of specialized facilities. To date, no DHF had been reported in the country. DHF is seen mainly in patients with secondary infection. From our preliminary hemagglutination inhibition (HI) investigation on single samples, the highest titer found was ≤640. This showed probable primary infection; therefore, the risk of DHF may be low. Hypoendemicity is another factor

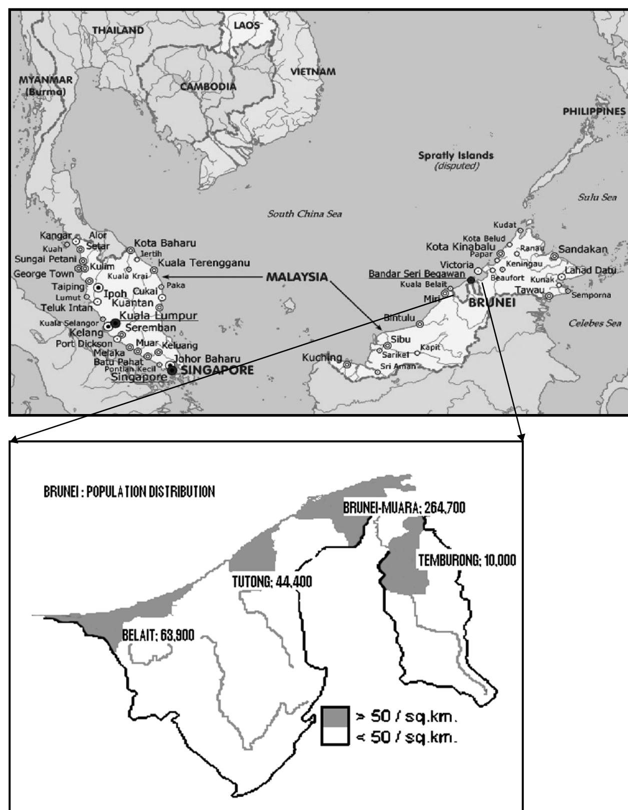

Fig. 1. Map of Brunei showing the population distribution of the country. Figures from Brunei Darussalam Key Indicators 2006-Updated 8th November 2006, Department of Statistics, Department of Economic Planning and Development, Prime Minister's Office.

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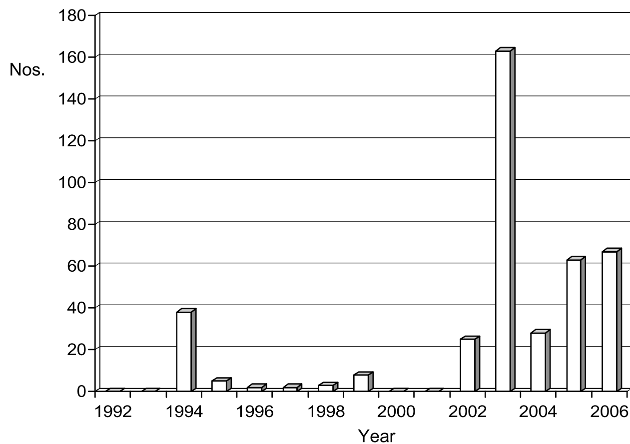

Fig. 2. Number of patients positive for dengue IgM antibody from the year 1992 to 2006. Data from Public Health Department, Ministry of Health, Brunei.

that could explain the absence of DHF in the country; so far our study showed that only two serotypes were found circulating at different times. The presence of a less virulent serotype also plays an important role in determining the outcome of the disease. A low mosquito index could also contribute by reducing the rate of transmission of the virus and thus this rate can reflect the effectiveness of mosquito control in the country. The practice of appropriate and timely fluid therapy and therapy for other symptoms in dengue patients could also reduce the development of DF to DHF.

A total of 271 single serum samples were selected for the study. These were drawn from patients suspected of having dengue infection and for whom dengue serology was requested to confirm the diagnosis. All patients were seen in clinics and hospitals in Brunei. The collection period was from April 2005 to April 2006. All samples were stored at −20°C at the Virology Laboratory, Department of Laboratory Services, RIPAS Hospital, Ministry of Health, Brunei until they were transported to the WHO Centre at University of Malaya, Malaysia for the study analysis. All demographic data were recorded.

The IgM titers of the 271 serum samples were determined by in-house IgM-capture enzyme-linked immunosorbent assay (ELISA) (7) developed at the WHO Centre using the 96-well microtiter plate coated with polyclonal rabbit anti-human IgM (μ-chain; Dakocytomation, Copenhagen, Denmark) diluted in 0.05 M carbonate-bicarbonate buffer, pH 9.6, and kept overnight at 4°C. Prior to the addition of samples and controls, the wells were washed 4 times at room temperature with PBS-T (0.05% Tween 20 in phosphate buffer saline, pH 7.2). Test sera and controls were diluted 1:100 (10 μl in 1,000 μl) in 0.5% bovine albumin phosphate saline (BAPS) and 100 μl was added to each well. The plate was incubated at 37°C for 1 h and was washed as described above. Dengue type 2 strain Tr 1751 (National Institute of Health, Nonthaburi, Thailand) was used as the assay antigen. The antigen was diluted 1:100 in 0.5% BAPS and 100 μl/well was dispensed. The plate was incubated at 37°C for 1 h and washed as above. One hundred microliters of the monoclonal antibody, Dengue type 2-MHIAF (Centers for Disease, Control and Prevention, Atlanta, Ga., USA; 1: 5,000 dilution in 0.5% BAPS), was added to each well and incubated at 37°C for 1 h. After another washing as before, 100 μl of Goat Anti-Mouse IgG (H+L) Horse Radish Peroxidase Conjugate (diluted 1:50,000 in 0.5% BAPS) was added to each well and incubated at 37°C for 1 h. After an additional washing as before, 100 μl of the substrate *o*-phenylendiamine dihydrochloride was added and incubated at room temperature in the dark for color development for 30 min. The reaction was stopped by adding 50 μl 4N H_2SO_4 and absorbance at a wavelength of 490 nm with a reference filter of 630 nm measured. A P/N (Sample OD_{490}/Negative control OD_{490}) ratio equal to or greater than 2.0 was taken as positive. A negative sample was defined as having a ratio of less than 2.0 (7).

The *Aedes albopictus* mosquito cell line (C6/36) was employed for the isolation of dengue virus by standard methods (8). Cells were infected with 100 μl of patient serum and incubated for 2 h to allow for virus adsorption at 37°C. The cells were replenished with L-15 (Leibovitz; GIBCO, Grand Island, N.Y., USA) medium supplemented with 2% heat-inactivated fetal calf serum (FCS) and 10% tryptose phosphate broth (maintenance medium). The culture flask with the infected cells was incubated at 28°C and subcultured every 10 days. All samples were subjected to three successive passages. The infected cells and the culture fluid were harvested 10 days after the third passage. The infected cells were washed with PBS and fixed in acetone at 4°C on Teflon-coated slides. The infected culture fluid (ICF) was centrifuged at 3,700 g (3,000 rpm) for 10 min at 4°C, and the supernatant was stored at −80°C until use. The presence of viral antigen was verified by the indirect immunofluorescence antibody test (IFAT) using dengue-specific monoclonal antibodies [DEN 1(MAB D2-1F1-3), DEN 2(MAB 3H3-1-21), DEN 3(MAB D6-8A1-12) and DEN 4(MAB 1410-6-7)] to detect the antigen in fixed infected cells. The presence of the virus in the tissue culture supernatant was determined by reverse transcriptase (RT)-PCR. RNA was extracted from all samples using a commercial kit, namely the QIAamp® viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Viral RNAs (70 μl) were extracted from 140 μl of serum and cell culture supernatants and stored at −70°C. The one-step RT-PCR amplification of Kong et al. (9) was carried out using the QuantiTect® SYBR® Green RT-PCR reagent kit (Qiagen). The amplification process was done in the iCycler system (Bio-Rad, Hercules, Calif., USA). Samples were assayed in a 25-μl reaction mixture. The thermal profile consisted of a reverse transcription step at 50°C for 30 min and *Taq* polymerase activation at 95°C for 10 min, followed by 35 cycles of PCR at 94°C for 30s-denaturation, 60°C for 40s-annealing, and 72°C for 50s-extension.

In 2006, the population of Brunei was estimated to be 383,000 (10). The gender distribution was as follows: 53.08% were male while 46.92% were female, making a sex ratio of 1.13:1.0. The ethnic group distribution was as follows: Malay 67%, Chinese 11% and others 22%. The age structure of the population was as follows: 12.8% were 0 - 4 years old, 26.7% were 5 - 19 years old, 54.3% were 20 - 54 years old, 3.5% were 55 - 64 years old and 2.7% were 65 years old and over (10). From a total of 271 suspected cases of dengue infection, 57 were confirmed by dengue IgM antibody and RT-PCR. Of these 57 cases of dengue, 45 (79%) had dengue IgM antibodies while 12 (21%) had the viral RNA in their blood. Thirty-four (60%) were males and 23 (40%) were females. The mean age of the 57 confirmed dengue cases was 38.35 ± 14.14 years, and the male:female ratio was 1.5:1.

IgM antibody was detected in 45 (16.6%) out of 271 suspected cases of DF. There were 27 males and 18 females with ages ranging from 7 to 74 years. The male:female ratio was

1.5:1. IgM was negative in the 12 and 6 patients that were positive for RT-PCR and virus culture, respectively. Of the 271 acute sera processed for viral culture, dengue virus was isolated from 6 patients (Table 1). All isolates were detected by IFAT and confirmed by RT-PCR. RT-PCR detected and identified dengue virus RNA from the sera of 12 patients in this study. Nine isolates were identified as DEN-2 and three as DEN-1 (Table 1). Among the 57 patients, the highest number of dengue infections was observed in the 31- to 40-year-old age group (24.6%) followed closely by the 21- to 30-year-old age group (22.8%). This was followed by 19.3% in the 41- to 50-year-old age group, 10.5% in the 51- to 60-year-old age group, 10.5% in the ≥61-year-old age group, 7.0% in the 0- to10-year-old age group and 5.3% in the 11- to 20-year-old age group (Fig. 3). The age group from 21 to 30 years of age was reported to be the most susceptible age group in the study done during the 2002 dengue outbreak in Bangladesh (11), while the 31- to 40-year-old age group was the most susceptible age group reported in Malaysia in 2005 (6). Our study indicates that in Brunei, people in the 21- to 40-year-old age group were exposed to dengue infection at a higher rate, as they were involved in more outdoor activities and traveling. These activities and their life styles increased the risk of contracting the infection by being bitten by infected mosquitoes. The results also indicate that children and young adults in the 0- to 10-year-old and 11- to 20-year-old age groups were the least affected by the infection. This may be due to the lower vector numbers in homes and schools, where people in these age groups spend most of their day.

The male:female ratio of 1.5:1 in the confirmed dengue cases showed a predominance in males as compared to females. This may have reflected the fact that in Brunei, more men than women work in outdoor occupational activities, and thus have greater exposure to infected mosquitoes. The geographical distribution of the 57 dengue confirmed cases (Table 2) showed that 29 (51%) patients were living in the Brunei-Muara district, followed by 26 (46%) in Temburong and another 3% in Tutong and Kuala Belait. Brunei-Muara has the largest population (264,700) in the country while Temburong (10,000), which is bordered by Sabah and Sarawak, is the location of the road link for travelers to these two Malaysian states, and therefore the risk for the transmission of dengue to the locals is increased in this area (Fig. 1). Throughout the study period, it was observed that dengue infection peaked from October to December in 2005 (Fig. 4). During these months, the country experienced heavy rainfall, which may increase the population of vectors and hence cause an increase in the rate of infection. The other 214 patients suspected of dengue infection were found to be negative in all the laboratory investigations used in this study. This low positivity rate of the IgM-capture ELISA could be due to single sampling in the initial phase of the disease when patients first came in for clinical examination. In our setting, as most of these patients were out-patients, we experienced difficulty in obtaining the appropriate second samples. Multiple samplings in the acute and convalescent stages might increase the number of finding of positive cases. Another reason could be that sample storage, i.e., at −20°C while in Brunei, before transportation to the WHO Centre, was not adequate to keep the virus viable for analysis, leading to the low detection rate in RT-PCR and viral cell culture. High levels of IgM will neutralize the virus and degrade the RNA, thus rendering the virus non-viable. This finding indicates the importance of timely sample collection or collection before the titer reaches the neutralizing level. In our study, RT-PCR was able to detect more dengue viral RNA than the mosquito

Table 1. List of patients sera that show positive for dengue IgM, RT-PCR and virus isolation

Serum no.	IgM	RT-PCR	Virus culture in C6/36 cell line
DS6/210505	–	DEN-1	DEN-1
DS10/160805	–	DEN-2	–
DS14/260905	–	DEN-1	DEN-1
DS31/291005	–	DEN-2	DEN-2
DS4/121105	–	DEN-2	–
DS11/221105	–	DEN-2	DEN-2
DS12/171105	–	DEN-2	–
DS19/211105	–	DEN-2	–
DS3/291205	–	DEN-2	–
DS4/221205	–	DEN-2	DEN-2
DS8/190106	–	DEN-2	–
DS212/110306	–	DEN-1	DEN-1

Table 2. Number of dengue IgM, RT-PCR and virus culture positive and their distribution among the districts

District	IgM-capture ELISA	RT-PCR	culture in C6/36 cell line
Brunei-Muara	25	4	2
Tutong	0	1	1
Kuala Belait	1	0	0
Temburong	19	7	3
Total	45	12	6

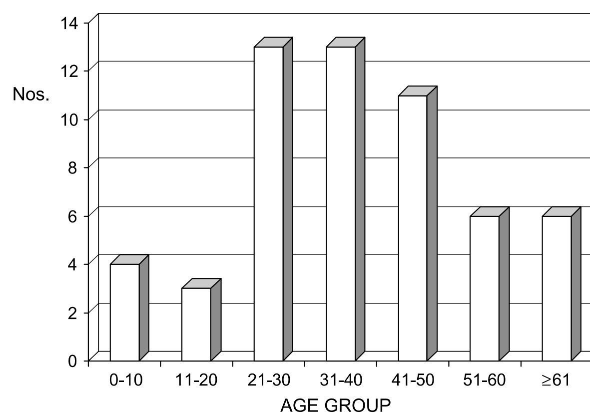

Fig. 3. Age distribution of dengue RT-PCR and IgM-capture ELISA positive cases.

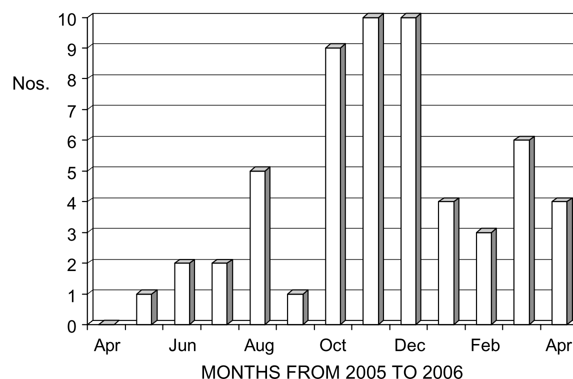

Fig. 4. Number of dengue RT-PCR and IgM-capture ELISA positives from April 2005 to April 2006.

cell line culture (Table 1). This finding showed that RT-PCR is more sensitive than virus culture as it requires only a very small number of viral RNA copies and also is able to pick up non-viable RNA as well as viable RNA. The time required to detect viral RNA is also short in comparison to the period of at least a month (3 passages) required in the C6/36 mosquito cell culture system. In this study, we isolated dengue viruses infecting patients in Brunei for the first time. The isolation of these viruses demonstrated that dengue virus was circulating in the country. The predominant serotype circulating and infecting the people during the study period was DEN-2 followed by DEN-1. From our preliminary study of the molecular characterization of the 5 Brunei DEN-2 isolates, 4 showed sequence homology ranging from 97 to 99% at the nucleotide level to Sarawak DEN-2, strain LF5-99 (GenBank accession no. AF400004), 2 showed 100% homology to each other and 99 and 100% homology at the nucleotide and amino acid level to the Malaysian isolates of 1998 and 1999, respectively (GenBank accession nos. AJ556811 and AJ556813). These were clustered together with other isolates from Malaysia, Indonesia and Thailand in genotype I (unpublished data).

In conclusion, the study achieved its objectives by providing useful epidemiological data regarding dengue infection during the study period, and it determined the predominant circulating dengue virus in the country. Dengue viruses from Brunei were successfully isolated and identified. As this is preliminary work, we are currently collecting appropriate data, i.e., detailed clinical data analysis and a sufficient number of paired samples, to establish the status of dengue infection in Brunei. This would provide further understanding of the impact of the disease. The isolation of the virus and molecular characterization of it to determine its origin, evolution, and geographic diversity and distribution are very important (12-14) and will generate data to be used in monitoring any serotype or genotype shifts that may occur in dengue virus infection in Brunei.

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