A Seroprevalence Survey of Dengue Virus Infection in Healthy Singapore University Undergraduates by Enzyme Immunoassay and Plaque Reduction Neutralization Test

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Abstract

A seroepidemiological survey was conducted from 1998 to 2000 to determine the seroprevalence of dengue virus infection in a cohort of healthy Singapore university undergraduates aged 19-26 years using a commercial dengue indirect IgG enzyme immunosorbent assay (ELISA) kit (PanBio). Out of 184 volunteers, 41 (22%) tested seropositive, suggesting that 78% of this young cohort would be susceptible to primary dengue infection. This relatively low dengue seroprevalence in this age group appeared to be predictive of the increasing dengue incidence in Singapore for the period 2001-2004, which witnessed a mean incidence of 5141 cases per year. In addition, the dengue incidence rate in Singapore for 2004 was the highest in the age group of 15-24 years. The general distribution pattern of the seropositive subjects matched well with the geographical distribution of confirmed dengue cases in Singapore for 1998. In order to ascertain the level of protective immunity against the virulent DENV-2 in this young adult population, 38 ELISA-seropositive and 18 ELISA-seronegative sera were subjected to dengue plaque reduction neutralization test (PRNT). Approximately 60% of ELISA-positive samples but none of the ELISA-negative sera showed DENV-2 neutralizing antibody activity. Thus, this implies that only a small proportion of the Singapore young adult population in 1998-2000 had protective antibodies against DENV-2. Intriguingly, DENV-2 was the predominant circulating serotype in Singapore in 1990-1991, 1993, 1998 and 2001-2003. There was no correlation between the ELISA PanBio index and PRNT titre. However, a negative correlation between the percentage of reduction of plaque-forming units (PFU) against serum dilution factor was observed, with a Pearson correlation coefficient of 0.479 (P<0.01). Interestingly, several samples subjected to PRNT exhibited increased percentages of PFU, thereby alluding to the presence of infection-enhancing antibodies to DENV-2.

Keywords: DENV-2, neutralizing antibodies, PanBio dengue IgG ELISA, plaque reduction neutralization test, predictive value, seroepidemiology, Singapore university undergraduates.

Introduction

The dengue virus exists as four serotypes (DENV-1 to -4) belonging to the family Flaviviridae. All four serotypes are causative agents of dengue fever (DF), dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS), which constitute a major public health problem in tropical and subtropical regions of the world where the main mosquito vectors, Aedes aegypti and Aedes albopictus, are responsible for transmission of the virus[1,2].
In Singapore, dengue was first recognized as a public health menace in the 1960s, and a nationwide Aedes mosquito control programme was established in 1969\cite{1,2}. This programme was based on a combination of source reduction, public health education and law enforcement measures. As a result of these measures, the national annual Aedes House Index (HI) has been kept below 2% since 1979. From 1974 to 1985, the incidence of dengue infection was relatively low. However, from 1986, the annual number of reported dengue cases has increased progressively. From an annual average of 153 cases in 1981–85, 742 in 1986–90 and 1850 in 1991–95, the dengue incidence subsequently rose to 2943 cases per year in 1996–2000. Most dengue cases were reported in the younger population aged between 16 and 25 years. It was suggested that successful mosquito control had resulted in a population of young adults with a low level of herd immunity to dengue infection\cite{3-9}.

Therefore, a seroprevalence survey was carried out from 1998 to 2000 in a cohort of healthy Singapore university students aged 19 to 26 years in order to determine their immune status against dengue. Their serological data and residential addresses were matched with the geographical distribution of confirmed dengue cases in Singapore in 1998. Dengue antibody levels were measured by a commercial enzyme-linked immunosorbent assay (ELISA), while protective neutralizing antibodies against the virulent DENV-2 were determined by plaque reduction neutralization test (PRNT). This seroprevalence survey can provide a better understanding of the dengue sero-epidemiology in the young adult population in Singapore, and help in the prediction of subsequent dengue outbreaks in the country.

### Materials and Methods

#### Serum samples

From 1998 to 2000, 184 healthy medical and bioscience undergraduates (99 males and 85 females), aged between 19 and 26 years, volunteered to participate in this study. About 5 ml of venous blood were collected from each student and left to clot overnight at 4 °C. Sera were separated by centrifugation at 3600 rpm for 15 minutes and stored at −20 °C.

#### Enzyme immunoassay

Sera were assayed for the presence of dengue IgG antibodies using a commercial dengue indirect IgG ELISA test kit DEG-100 (PanBio, Brisbane, Australia). Based on detection by horseradish peroxidase-conjugated anti-human IgG, the assay was performed according to the manufacturer’s protocol. Absorbances were read using an ELISA reader (Tecan, Mannedorf, Switzerland) at a wavelength of 450 nm. The colour intensity is directly related to the dengue antibody concentration in each test sample.

#### Mapping of the residential addresses of subjects compared with geographical distribution of dengue cases in Singapore

The map of residential addresses of the subjects who were tested for dengue antibodies by ELISA was compared with the geographical distribution of confirmed dengue cases in Singapore for 1998 (data kindly provided by Ministry of the Environment, Singapore). The maps were generated using the Geographical Information System (GIS) Arcview 3.2A software system.
Cell culture, DENV-2 strain and neutralizing antibody assay

Baby hamster kidney (BHK) cells were cultured at 37 °C in RPMI medium supplemented with 10% fetal calf serum (FCS). Confluent BHK cells were inoculated with DENV-2 New Guinea C (NGC) strain, and incubated at 37 °C for 1 hour with rocking at 15-minute intervals. Following virus adsorption, maintenance medium (RPMI containing 3% FCS) was added and incubated at 37 °C for 3–4 days. Infected cell culture fluid was harvested, and the virus stocks were titred by plaque assay on BHK cells and stored at –80 °C for the neutralization assay.

PRNT were performed on selected samples, i.e. 38 ELISA-positive and 18 ELISA-negative human sera. Sera were heat-inactivated at 56 °C for 30 minutes and serial dilutions (from 1:10 to 1:2560) were prepared in RPMI containing 2% FCS. Each serum dilution (150 µl) was incubated with an equal volume of DENV-2 NGC strain (90 plaque-forming units or PFU) at 37 °C for 1 hour, with rocking every 15 minutes. Each respective serum-virus mixture (100 µl) was then transferred to wells containing confluent BHK cells cultured in 24-well plates and incubated at 37 °C for 1 hour with rocking every 15 minutes. The mixture was decanted and each well was overlaid with 1 ml of 1% carboxymethylcellulose (CMC) containing RPMI with 3% FCS. After 6 days of incubation at 37 °C, CMC was discarded and cells were fixed with 20% formaldehyde solution for 30 minutes. The plaques were visualized by staining with 1% crystal violet solution. The percentage of plaque reduction was calculated relative to the virus control without serum. Titres were expressed as the highest serum dilution yielding ≥50% reduction in the number of plaques (PRNT50).

Results

Out of the 184 serum samples collected from the medical and bioscience undergraduates between 1998 and 2000, 41 (22%) tested positive for the presence of dengue antibodies by indirect IgG ELISA (PanBio) according to the criteria set by the manufacturer. The PanBio index was obtained by calculating the ratio of the sample absorbance to the mean cut-off absorbance and multiplying by 10. For a sample to be positive, it should have a PanBio index of greater than 11. A result of ≥40 PanBio units is associated with secondary dengue infection.

The topographical distribution of confirmed dengue cases in Singapore for 1998 is displayed in Figure 1A. Most of the cases in 1998 were clustered around the central-eastern and south-eastern coastal regions with scattered cases in the northern and south-western regions. Areas within the north-western part of Singapore are sparsely populated and less developed, accounting for the scattered incidence of dengue. The residential addresses of students who were tested for dengue antibodies were superimposed on the map of Singapore. It is noteworthy that the general distribution pattern of the residences of the seropositive subjects (Figure 1B) matched well with the pattern of confirmed dengue cases in 1998.

In order to test for neutralizing antibodies against DENV-2 (NGC strain) in vitro, PRNT was conducted on 38 ELISA-seropositive and 18 ELISA-seronegative samples. Three of the ELISA-seropositive samples were not subjected to PRNT due to insufficient sera. A result with ≥50% plaque reduction at a serum dilution of ≥1:10 was considered to be positive for neutralizing antibodies against DENV-2. None of the ELISA-negative sera exhibited neutralizing antibody titres, whereas 23 (60.5%) of ELISA-positive samples revealed neutralizing antibody titres ranging from 1:10 to 1:1280.
Figure 1. (A) Map of Singapore showing the geographical distribution of confirmed dengue cases in 1998. (B) Geographical distribution of the residential addresses of the cohort of Singapore university undergraduates (1998–2000) classified according to their dengue immune status (by ELISA)
However, the ELISA PanBio indices did not correlate positively with the PRNT titres (Figure 2). Furthermore, 15 sera that tested positive for anti-dengue IgG by ELISA did not show neutralizing antibody titres. When the percentage of PFU reduction was plotted against the PRNT serum dilution factor, we observed a negative correlation with a Pearson correlation coefficient of 0.479 ($P<0.01$) as depicted in Figure 3.

**Discussion**

Since 1989, annual dengue outbreaks in Singapore have occurred more commonly compared to the five-year cyclical epidemic pattern observed in the 1970s[3-5]. In general, increasingly more adults were infected with time and the age of fatal cases shifted from 10 years in 1973 to 17 years and above in 2000–2002[9]. The dengue seropositivity rate of only 22% in our seroprevalence survey of healthy university students aged 19–26 years conducted from 1998 to 2000 suggests that 78% of this young adult cohort would be susceptible to primary dengue infection. It also forecasts the likelihood of increasing dengue infection associated with a low seroconversion rate in the young adult population. The dengue surveillance in Singapore for 2004 confirmed that the incidence rate was highest in the age group of 15–24 years[10].

Previous reports had shown that the level of herd immunity of the general population to dengue virus infection declined from 47% during the period 1990–1991 to 39.6% in 1993, and to 29.4% in 1998[7]. This trend was despite the increase of dengue infection during these periods. From our study, this relatively low dengue seroprevalence in the young adult population in Singapore appeared to be predictive of the increasing incidence of dengue from 2001 to 2004. The mean annual dengue incidence over this period escalated to 5141,
with 2372, 3945, 4788 and 9459 cases in 2001, 2002, 2003 and 2004 respectively. In 2005 alone, a record number of over 14 000 dengue cases were reported in Singapore.

Owing to ever-increasing global travel and movement of people across countries, the number of dengue cases in Singapore has risen in tandem with global and regional dengue activity\(^{11-14}\). For example, in 2001, WHO reported a total of 211 039 dengue cases in the South-East Asia Region and 132 949 in the Western Pacific Region\(^{9}\). It is noteworthy that ~10% of dengue cases in Singapore in 2002 were imported, the majority (~90% of imported cases) being acquired from neighbouring Malaysia and Indonesia\(^{9}\).

From the distribution of confirmed dengue cases in Singapore for 1998, we observed that the majority of cases clustered in the south-eastern and central-eastern regions of Singapore. These areas reflected the acquisition of infection in places where the Aedes vector was circulating\(^{2}\). These areas are mainly residential areas, which are more heavily populated compared to the northern and south-western parts\(^{2}\). Another dominant feature in these areas was the high level of construction activities, which represent common breeding grounds for Aedes mosquitoes. In 2002, reports indicated that residents in government Housing and Development Board flats, compound houses and condominiums accounted for 57.4%, 24.0% and 11.5% of confirmed cases respectively, totalling 92.9%. The other 7.1% of cases were scattered over the northern and western regions that represented mainly industrial zones and upcoming small towns\(^{8}\). Thus, a higher
incidence of dengue infection is observed in heavily populated residential areas than in industrialized areas. Significantly, the distribution pattern of the residential addresses of the seropositive subjects corresponded to those of the confirmed dengue cases.

All the four dengue serotypes are detected in Singapore in any one year. Monitoring dengue virus serotypes and strains circulating in the community is useful for identifying the changes in predominant serotypes, which may account for the emergence of new outbreaks and more severe cases\textsuperscript{15}. In this study, 60\% of ELISA-seropositive subjects tested positive for protective neutralizing antibodies against DENV-2, representing only a small proportion of the Singapore young adult population with immunity against the virulent DENV-2\textsuperscript{16}. Interestingly, DENV-2 was the predominant circulating serotype in Singapore in 1990–1991, 1993, 1998 and 2001–2003\textsuperscript{8,10}.

Subjects with ELISA-positive sera but without DENV-2 neutralizing antibodies would likely to be prone to subsequent secondary infection with DENV-2 which is linked with complications of DHF and DSS\textsuperscript{17}. However, these individuals may harbour neutralizing antibodies against other dengue virus types since PRNT against DENV-1, -3 and -4 were not performed. Thus, the ELISA positivity of such sera could be indicative of cross-reactive IgG against DENV-1, -3 and -4, or other related flaviviruses such as Japanese encephalitis virus (JEV)\textsuperscript{18-20}. Previous studies revealed that 25\% of JE patients demonstrated ELISA results of ≥40 PanBio units. Serological cross-reactivity across related members of the flavivirus group is common at the level of IgG antibodies measured by ELISA.

There was no correlation between the PanBio units by ELISA with neutralizing DENV-2 antibody titres. Despite high dengue IgG levels of ELISA-seropositive volunteers, only three fifths had protective antibodies against DENV-2. However, a negative correlation between the percentage of PFU reduction against the serum dilution factor was observed with a statistically significant Pearson correlation coefficient, thus supporting the reliability of testing serial dilutions of serum samples by PRNT. Furthermore, several samples subjected to PRNT displayed negative percentages of reduction of PFU (i.e. increased percentages of PFU) as shown in Figure 3, implying the presence of infection-enhancing, non-neutralizing antibodies to DENV-2\textsuperscript{21}.

Our data corroborate existing reports that protective immunity to dengue virus in young adults has been declining, contributing to the increased incidence of dengue in the Singapore population\textsuperscript{22,23}. The successful vector control in Singapore implemented since 1969 reduced the overall Aedes HI from over 25\% to the current 1–2\%\textsuperscript{9}, paradoxically resulting in a highly vulnerable population. In addition, the behaviour and modes of dispersal of Aedes mosquito vectors may also facilitate dengue transmission\textsuperscript{24}. Consequently, dengue outbreaks tend to occur more easily, which may explain the increased incidence rate. Until effective tetravalent dengue vaccines become available for mass immunization\textsuperscript{25}, sustained vector control should remain the mainstay for the prevention and control of dengue.

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Seroepidemiology of Dengue in Singapore University Undergraduates

References


Seroepidemiology of Dengue in Singapore University Undergraduates


