Serological evidence for the co-circulation of multiple dengue virus serotypes in Singapore

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SUMMARY

We did a seroepidemiological study to determine the circulating dengue virus serotypes and the extent to which the Singapore population has been exposed to multiple dengue virus serotypes, using the plaque reduction neutralization assay (PRNT). Of 164 enrolled subjects aged between 18–30 years, 49 subjects (29·8%) were PRNT positive for at least one dengue serotype. The seroprevalence was 39 (23·8%) for dengue virus serotype 1, 37 (22·6%) for type 2, 43 (26·2%) for type 3, and 30 (18·3%) for type 4. Of the 49 subjects with PRNT-positive dengue virus results, 28 (57·1%) were positive to all four virus serotypes, seven (14·3%) to three serotypes, two (4%) to two serotypes, and 12 (24·5%) to a single serotype. All four dengue virus serotypes circulate in Singapore, and a substantial proportion of the adult population in Singapore had exposure to more than one dengue virus serotype. In spite of multiple circulating types, the rate of dengue haemorrhagic fever is low in Singapore.

INTRODUCTION

Dengue is an arthropod-borne acute infectious disease caused by the enveloped, single-stranded RNA dengue virus of the family Flaviviridae [1]. There are four serotypes which share genetic and antigenic features, but infection with one serotype does not provide long-term protection against the other serotypes [2]. Infection by any of the four serotypes results in a wide spectrum of clinical manifestations, including undifferentiated fever, classical dengue fever (DF), dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) [3]. The risk of developing DHF/DSS is increased in patients secondarily infected with a different serotype and this is thought to be due to immune enhancement [4]. Severity of the disease also depends on the serotype of the infecting virus, in addition to host factors, age and degree of viraemia [5, 6].

Dengue is endemic in Southeast (SE) Asia with outbreaks reported since the 18th century [7]. In Singapore, an industrialized island state in SE Asia with approximately 3·6 million inhabitants, a resurgence of dengue has been reported since 1986 [8]. This occurred despite Singapore's well-established integrated nationwide *Aedes* mosquito control programme which has incorporated source reduction,

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public health education and law enforcement since the 1970s [9], but is in tandem with the resurgence of regional dengue activity [10].

We previously documented a high seroprevalence of Dengue IgG in the adult population of Singapore [11]. Here we report the circulating dengue virus serotypes and the extent to which the Singapore population has been exposed to multiple serotypes.

METHODS

Healthy adult volunteers comprising staff and visitors to a large public hospital were recruited over 2 days for a study to determine the seroprevalence of dengue that had previously been reported [11]. Age, gender, race, nationality and medical history were recorded. Those with a history of vaccination against yellow fever or Japanese encephalitis (JE) were excluded. We selected subjects aged 18–30 years for serotyping.

Serotyping of dengue viruses and JE virus was determined by the plaque reduction neutralization (PRNT) assay [12]. Briefly, the plaque count was determined by using the LLC-MK2 plaque assay single overlay technique, using dengue virus serotypes 1, 2, 3 and 4 (DEN-1, DEN-2, DEN-3, DEN-4), as well as JE (Beijing) virus.

A positive serodiagnosis for JE was defined as a positive PRNT for JE, but negative to all dengue viruses.

Dengue antibodies levels were measured with PanBio Dengue IgG ELISA and results interpreted according to the manufacturer's instructions.

Data analysis was carried out in Stata version 7.0 (StataCorp., TX, USA). Logistic regression analysis was used to determine risk factors associated with being positive for at least one dengue virus serotype, and for seroprevalence of one $vs.$ all four serotypes. Odds ratios and their 95% confidence intervals were provided as estimates of the effect sizes. All tests were conducted at the 5% level of significance.

The study was approved by the Ethics Committee of Tan Tock Seng Hospital.

RESULTS

Demographic data

Of the 164 subjects enrolled, the median age was 24 years (range 18–30 years) and 115 (70%) were female. Of the 164 subjects, 137 (84%) were Singaporean, 11 (7%) permanent residents (a foreign national living
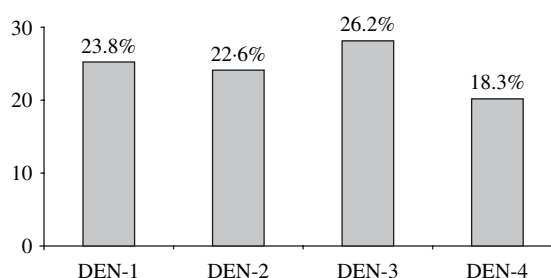

Fig. 1. Seroprevalence of dengue virus types in the adult population of Singapore.

permanently in Singapore) and 16 (10%) foreigners working in Singapore (all of whom originated from SE Asian countries). The ethnic distribution was as follows: 106 (64·6%) were Chinese, 24 (14·6%) Indian, 23 (14%) Malay and the remainder were of various ethnicities from SE Asia. A total of 154 (94%) subjects had no history of medical problems. Eight subjects (5%) had a history of dengue fever, none had a history of JE. All were asymptomatic at the time of blood sampling.

PRNT assay

Forty-nine subjects (29·8%) were positive on the PRNT assay for at least one dengue serotype. The prevalence of DEN-1 was 39 (23·8%); DEN-2, 37 (22·6%); DEN-3, 43 (26·2%), and DEN-4, 30 (18·3%) (Fig. 1). On univariate analysis, the prevalence of dengue virus serotypes increased significantly with age, was higher in Indians and other races compared to Chinese and was also higher for those of SE Asian nationality compared to Singaporeans (Table 1). There was no difference between males and females. On multivariate analysis, the only independent factor associated with a higher prevalence of dengue virus serotypes was nationality.

Of the 49 PRNT-positive dengue virus results, 28 (57·1%) were due to previous exposure (infection) to all four viruses, seven (14·3%) to three, two (4%) to two, and 12 (24·5%) to a single serotype (Fig. 2). The most frequent combination of co-exposure was DEN-2 and DEN-3 in 36 subjects (21·9%). Serological evidence for exposure to all four serotypes occurred in 28 of the 164 subjects (17·1%). On univariate analysis to assess risk factors associated with exposure to all four serotypes $vs.$ just one, neither age, gender, nor nationality were found to be risk factors.

Six subjects had a positive JE result (3·7%), of which four were females and two males. Four (2·9%) were Singaporeans, one (9%) a Singapore resident

Table 1. *Univariate factors associated with prevalence of at least one dengue virus serotype in the adult population of Singapore*

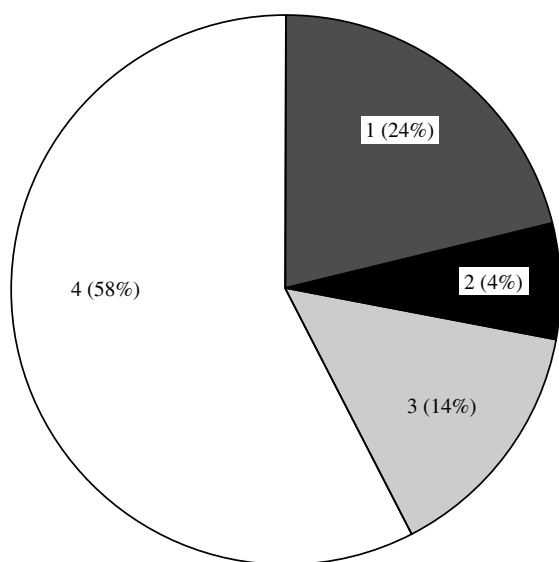| Covariates	OR	95% CI	*P* value
Age (every year increase)	1·12	(1·02–1·23)	0·016
Ethnicity			
Chinese	1		
Malay	0·42	(0·12–1·51)	0·184
Indian	1·67	(0·66–4·25)	0·280
Others	12·54	(2·55–61·59)	0·002
Nationality			
Singaporean (by birth)	1		
Singaporean resident	1·18	(0·30–4·71)	0·813
Foreign worker (from SE Asia)	13·66	(3·67–50·87)	<0·001

OR, Odds ratio; CI, confidence interval.

Fig. 2. Seroprevalence of exposure to multiple dengue virus serotypes. 1, Exposure to one dengue serotype; 2, exposure to two dengue serotypes; 3, exposure to three dengue serotypes; 4, exposure to four dengue serotypes.

and one (6·3%) a foreigner from SE Asia. Two of the six (33%) who were positive for JE on the PRNT assay had a positive dengue IgG but were negative on PRNT.

ELISA assay

Of the 164 subjects, 39 (23·8%) had a positive dengue serology. Of the 49 subjects positive on the PRNT assay for at least one dengue serotype, the ELISA dengue IgG was positive in 37 (75·5%), and for those 115 subjects without evidence of any dengue

Table 2. *Sensitivity and specificity of dengue IgG*

Dengue virus serotype (at least one)	Dengue IgG		
	Negative	Positive	Total
Negative (%)	113 (98·3)	2 (1·7)	115 (100)
Positive (%)	12 (24·5)	37 (75·5)	49 (100)
Total	125 (100)	39 (100)	164 (100)

serotypes, the dengue ELISA was positive in two cases (1·7%). This results in a sensitivity of dengue ELISA IgG (using the PRNT assay as gold standard) of 75·5%, and a specificity of 99% (Table 2). The two cases that were positive on dengue IgG but negative on the PRNT assay were positive for JE. Of the 12 false-negatives, 11 had exposure to just one dengue virus serotype and one had exposure to three dengue virus serotypes.

DISCUSSION

Using serotyping, we have documented that all four dengue virus serotypes circulate in Singapore. We also documented that a substantial proportion of the adult population of Singapore has serological evidence of having been infected with more than one dengue virus serotypes in the past. This is consistent with the detection of all four serotypes in field-caught *Aedes aegypti* and *Aedes albopticus* in Singapore by type-specific PCR, with DEN-1 most frequent in *A. aeygpti*, and DEN-4 in *A. albopticus* [13]. The sero-prevalence was similar for all dengue serotypes in this age group, with DEN-4 associated with the lowest prevalence. This is in keeping with previous findings from Singapore where all four dengue serotypes have been detected from infected persons, with DEN-2 predominating in 1990, 1991 and 1993, DEN-3 in 1992 and 1994 and DEN-1 in 1995 and 1996 [9]. The co-circulation of all four dengue virus serotypes in the same community, has been common since the 1950s in SE Asia [14]. Our documented seroprevalence of exposure to multiple serotypes in Singapore is far higher than that in the Americas (5·5%) [14]. In fact, seroevidence of exposure to all four serotypes was the most frequent constellation in our cohort.

Secondary infection is thought to be the main risk factor for DHF and DSS [2, 15]. A high national

incidence of DHF and DSS would, therefore, be expected based on the documented high rate of secondary, tertiary and even quaternary infection in Singapore. However, surprisingly, Singapore has a relative low incidence of DHF (1·5% of all dengue cases in 2000 [16]), which is far lower than that of other countries in SE Asia [17]. The explanation for this phenomenon remains unclear. One possibility is the shift of dengue to an older age group in Singapore: the resurgence of dengue in Singapore has been associated with an adult predominance and a very low incidence in children [8]. Unlike most other dengue endemic countries where the highest incidence of DHF is in childhood [2], in Singapore the highest incidence of DF/DHF cases is in the 15–24 years age group, followed by the 25–34 years age group [10]. Parallel with the increase in the age of cases of dengue over the past 20 years, there was a decrease in cases presenting as DHF in Singapore [9], and this may be partially due to the fact that adults present less frequently with DHF than children [6]. Our observations in Singapore of the lack of DHF despite exposure to multiple dengue serotypes suggest that other factors may be responsible in the pathogenesis in addition to the immune enhancement theory [4, 18, 19]. An absence of DHF despite hyperendemic co-circulating dengue virus transmission has also been observed in Haiti [20].

In addition to secondary infection, severity of disease also depends on the strain and serotype of the infecting virus [5]. Epidemic DHF has appeared in association with DEN-3 [21]. DEN-3 may have greater epidemic potential or virulence in the Americas [21]. In Asia, increased dengue disease severity correlated with DEN-2 [5]. DEN-1 infection followed by DEN-2 has been associated with DHF epidemics, although in hyperendemic areas it is not easy to define the virus producing the primary infection [22]. The predominance of DEN-1, DEN-2 and DEN-3 in our population may have contributed to the resurgence of dengue in Singapore since 1986 [8].

The risk of JE in Singapore is very low [23]. In recent years there were only a few sporadic cases, most of them imported [24]. Our findings show that 2·9% of Singaporeans were positive for JE. This is most likely due to exposure in early childhood as JE was more prevalent in the 1980s [25]. However, the risk for JE in Singapore has not been totally eliminated, as JE virus was still detected in pigs on Singapore's offshore islands [26]. Serological cross-reactivity exists between dengue and JE virus and this needs to be taken into account when performing assays for dengue antibody prevalence [27–29]. In this study, 33% of JE cross-reacted with the indirect ELISA dengue assay.

The sensitivity of the dengue indirect IgG ELISA was 75%, but the specificity was 98%, which is in keeping with that reported [30]. Relying on the ELISA indirect IgG assay in seroepidemiological studies may, therefore, underestimate the seroprevalence.

In summary, our findings show that dengue is still a major problem with all four serotypes circulating in Singapore despite a successful vector control programme [9]. This indicates that vector control programmes cannot be the only strategy in the control of dengue and that it needs to be paired with other strategies including the development of a tetravalent dengue vaccine.

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