Original Article

Early Dengue Infection and Outcome Study (EDEN) – Study Design and Preliminary Findings

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Abstract

Introduction: Dengue is a major public health problem in Singapore. Age-specific dengue morbidity rates are highest in the young adult population, unlike in many other Southeast Asian countries where dengue is mainly a paediatric disease. Hence, the World Health Organization (WHO) guidelines on dengue diagnosis and management which were developed using the paediatric experiences, may not be suitable for the management of adult dengue infections. Materials and Methods: The Early DENgue (EDEN) infection and outcome study is a collaborative longitudinal study to investigate epidemiological, clinical, viral and host-specific features of early dengue-infected adults, in an effort to identify new early markers for prognostication. Patients presenting with early undifferentiated fever were included in the study. We carried out an interim analysis to look for early indicators of severe disease. Results: During the period of this interim study analysis, 455 febrile patients were recruited. Of these, 133 were confirmed as acute dengue cases based on dengue-specific polymerase chain reaction (PCR) results. There were significant clinical and epidemiological differences between dengue and febrile non-dengue cases. Nine per cent of the dengue cases experienced persistent tiredness, drowsiness and loss of appetite beyond 3 weeks of illness. Quantitation of viral loads using the crossover (Ct) value of real-time RT-PCR correlated with the duration of symptoms. More than half of both primary and secondary dengue cases were hospitalised. There was no dengue-related mortality in this study. Conclusion: The duration of illness and prolonged symptom duration in 9% of the subjects indicate that the burden of dengue illness is substantially different from other non-dengue febrile illness in our study cohort. Our study also highlights the paucity of early prognostic markers for dengue fever in adults.

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Key words: Adult, *Aedes*, Singapore, WHO guidelines

Introduction

Dengue fever/dengue haemorrhagic fever (DF/DHF) is a re-emerging disease that is endemic in the tropical world. It is caused by 4 closely-related dengue viruses which are transmitted by the *Aedes* mosquitoes, principally the *Aedes aegypti* mosquito.[1] DHF and dengue shock syndrome (DSS) are complications of the disease that lead to significant morbidity and mortality. The pathophysiology of severe DHF and DSS is characterised by plasma leakage as a result of alteration in microvascular permeability.[2] As yet,

there is no specific treatment for DF and management remains largely supportive, although progress has been made in drug and vaccine development.[3] Prevention of this infection is thus imperative.

In Singapore, DHF appeared in the 1960s and quickly became a major cause of childhood mortality. With the implementation of a vector control programme, the incidence of DHF declined starting in the early 1970s and Singapore experienced a 15-year period of low DF/DHF incidence.[4] However, since the beginning of the 1990s, Singapore has

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experienced a resurgence of dengue despite the continued emphasis on vector control. This resurgence peaked in the year 2005 where the combined incidence of DF and DHF was 335 cases per 100,000 population.[5] In contrast to other dengue-endemic countries where the burden of the disease is in children and early teenagers,[4] the young adult population represent the largest group affected by dengue in Singapore. The reason for this disparity in disease demography may be a result of differences in the age-specific exposure risk and clinical manifestation of the disease or other epidemiological factors. [4]

Dengue infection in adults is not well-studied. Most of our current knowledge and understanding of dengue is based on paediatric data. However, adult disease may differ significantly with respect to epidemiology and clinical outcomes.[6] Accordingly, the current World Health Organization (WHO) classification of dengue infection may not be fully applicable to adult infections.[7] Furthermore, although dengue fever is not a life-threatening condition, its symptoms can be extremely debilitating[8] and the disease is a significant contributor to the loss of economic productivity.[9] As such, a better understanding of dengue infection and its pathophysiology in adults is urgently needed.

To address this goal, we undertook a multi-centre longitudinal study of early adult dengue infection to characterise epidemiological, clinical, viral and host-specific features of adult disease. The Early Dengue Infection and Outcome Study (EDEN) was launched in 2005 and involved researchers from 7 Singapore agencies – Tan Tock Seng Hospital (TTSH), Genome Institute of Singapore (GIS), Novartis Institute for Tropical Disease (NITD), Environmental Health Institute (EHI), Singapore Tissue Network (STN), DSO National Laboratories (DSO) and the National Healthcare Group (NHG) Polyclinic. The specific aims of the study were to 1) identify early markers of the disease that are predictive of outcomes such as DF and DHF, 2) identify pathways that lead to severe disease that may be amenable to therapeutic intervention, 3) study the epidemiological features of adult dengue infection, 4) develop robust molecular tools for epidemiological investigation, 5) correlate virus virulence with their sequences and their replication properties, and 6) refine early dengue clinical and laboratory diagnostic indicators. The study is expected to enrol over 500 dengue patients and be completed by February 2007.

We report here the interim findings of EDEN after an initial 8-month study period.

Materials and Methods

Patients and Clinical Methods

Ethical considerations: The study protocol was approved by the various individual organisational institutional review boards (IRBs). The collection of patient clinical and epidemiological data within the National Healthcare Group was approved by the NHG IRB (DSRB B/05/013). Enrolment of study participants is conditional on appropriate informed consent administered by a study research nurse. All biological material collected is made anonymous after the completion of demographic and clinical data collection.

Screening and recruitment: Adult patients (age >21 years) presenting with acute onset fever (≥38.0°C for less than 72 hours) without rhinitis or clinically obvious alternative diagnoses are eligible for inclusion in the study. Study recruitment centres have been established within community primary care polyclinics in identified regions of high dengue activity.

Investigations: Upon consent, demographic, clinical and epidemiological information were collected on a standardised data entry form. Initial blood and saliva samples were taken on this occasion (fever day 1 to 3: visit 1). Persons who scored positive for dengue virus on polymerase chain reactions (PCRs) testing were entered into the study as presumptive dengue cases. Blood and saliva samples were also taken at fever day 4 to 7 (visit 2) and 3 to 4 weeks (visit 3) from these individuals. A volume up to 1 mL of saliva and 20 mL of blood was removed on each occasion into separate tubes that were identified by a coded number. The blood and saliva samples which were made anonymous were subsequently stored at the STN where archived samples will be made available for future research, on IRB approval. All reverse-transcription polymerase chain reaction (RT-PCR) results were then reported to the referring physician for necessary follow-up within 1 working day. All those who were RT-PCR negative for dengue were contacted 3 to 4 weeks after the initial sampling for an outcome interview. Patients who were RT-PCR negative were used as controls in this study.

Laboratory Methods

Blood collection: Blood samples were taken by the research nurse in the polyclinic or hospital. Venous blood (up to 20 mL) was collected in vacutainer tubes and centrifuged upon arrival at STN. Two mL of serum was immediately sent to EHI for RT-PCR and ELISA analysis. The remaining serum was aliquoted and stored at -80°C at STN until further use.

Saliva collection: Saliva was collected from patients with a saliva collection device (Oracol, Malvern Medical, UK), with guidance from the research nurse and analysed for IgA antibodies as a potential tool for non-invasive serological diagnosis at a later date.

Haematology: A full blood count was performed on anticoagulated whole blood collected at all time points.

Table 1. Real-time RT-PCR Primers and Conditions Used to Detect Dengue Virus RNA in Serum

Primer	Sequence (5'-3')	Cycling conditions	Fluorescent dye
Forward	TTGAGTAAACYRTGCTGCCTGTAGCTC	10 min RT step at 60°C; 1 min at 95°C; 35 cycles	SYBR green I; fluorescence
Reverse	GAGACAGCAGGATCTCTGGTCTYTC	at 95°C for 0 s, 60°C for 3 s and 72°C for 10 s	detected at 530 nm

RNA: ribonucleic acid; RT-PCR: reverse-transcription polymerase chain reaction
Conditions were optimised for use on the Roche Lightcycler system. Primers were adapted from those reported in a previous study[10] which was given to participants of an Asia Pacific Economic Congress (APEC) workshop on molecular epidemiology of dengue viruses held in Taipei in December 2002. Modifications had been made to the reverse primer which was designed to be compatible with the 4 pairs of FRET probes (Ng LC – unpublished data) that would position internally to the amplified fragments.

Haematocrit, thrombocyte and leukocyte counts were correlated to disease outcome and serve to standardise processes for the cell-based assays. A benchtop Food and Drug Administration (FDA)-approved hematocytometer was used for this application (iPoch-100, Sysmex, Japan). Calibration by internal and external QC controls was also performed on a regular basis. Data were processed and stored by a commercial software application before being exported to the study relational database.

RT-PCR: RNA extraction. All viral RNAs were extracted from the first serum portion or virus culture supernatant using the QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RT-PCR was carried out to detect dengue viral RNA using a set of generic pan-dengue primers which targets the 3' non-coding region of dengue viruses. The sequence of the primers and the RT-PCR conditions are summarised in Table 1. Melting curve analysis was performed after PCR amplification to verify that the correct product was amplified by examining its specific melting temperature (Tm). Results were analysed with LightCycler software version 3.5. Reactions with high Ct value or ambiguous melting curve results are analysed by electrophoresis on a 2% agarose gel, to confirm the presence of the product of the correct size. RNA from each of the 4 serotypes of dengue viruses cultured in C6/36 cells were included as controls in every PCR run.

Virus isolation: 1 mL of serum was inoculated onto the *Aedes albopictus* mosquito (C6/36) cell line (ATCC: CRL-1660) for virus isolation followed by serotyping using indirect immunofluorescence assay (IFA). Briefly, after a 1-hour adsorption at 37°C, the cell monolayer was incubated at 33°C for up to 10 days and observed daily for cytopathic effects (CPE). At the end of the 10-day period or when 75% of the cell monolayer showed CPE, whichever was earlier, the C6/36 cells were scraped off and spotted onto a Teflon-coated slide. Isolation of dengue virus was confirmed and serotype using indirect immunofluorescence assay (IFA) was performed using dengue virus group-specific and dengue virus serotype-specific monoclonal antibodies derived from the clarified hybridoma culture supernatant

supernatant (ATCC: HB-114, HB-46, HB-47, HB-48, HB-49). Fluorescein isothiocyanate-conjugated goat anti-mouse antibody was used as a secondary antibody. Blind passage was carried out for those samples that scored negative for dengue virus after the first round of culture until a positive result was obtained, up to a maximum of 2 rounds of re-passage.

Serology: Serology testing for anti-dengue IgM antibodies was performed using an IgM capture ELISA kit (PanBio, Brisbane, Australia), according to the manufacturer's instruction. Anti-dengue IgG antibodies were detected with a dot-blot enzyme immunosorbent assay (DB-EIA) using cell-cultured derived, heat-inactivated dengue virus antigen (Sil BK, unpublished data) at a serum dilution of 1:100.

In addition, as part of the study protocol, we will also look at viral genome analysis, viral characterisation and host characterisation, genome and transcriptome upon completion of the study in February 2007.

Statistical Analysis

The Student's *t*-test was used for comparison of continuous variables, and Fisher's exact test was used for comparison of dichotomous variables with an expected sample size of less than 5. For data that was not normally distributed, the Mann-Whitney U test for continuous values was used. Variables found to be statistically significant in univariate analyses were entered into multivariate analysis using a logistic regression model to identify independent risk factors for outcomes of interest. A two-tailed *P* value of <0.05 was considered statistically significant. All statistical computations were performed using the SPSS software (version 13 for Windows; SPSS Inc, US).

Data Analysis

During the 8-months study period from April 2005 to December 2005, 458 patients were interviewed and 455 patients met the inclusion criteria (Table 2). Seventeen patients in the dengue group did not complete all 3 visits and 3 patients in the control group could not be contacted for an outcome interview.

Table 2. Demographics of the Study Population

| | | Disease group (%) | | P |
		Dengue (n = 133)	Controls (n = 321)	
Age	Mean	40.9	37.2	0.008
	Standard deviation	13.0	13.1	
Gender				
	Female	54 (40.6)	140 (43.6)	0.555
	Male	79 (59.4)	181 (56.4)	
Ethnicity				
	Chinese	105 (78.9)	222 (69.2)	0.195
	Malay	7 (5.3)	38 (11.8)	
	Indian	14 (10.5)	44 (13.7)	
	Others	7 (5.3)	17 (5.3)	
Nationality				
	Local	91 (68.4)	223 (69.5)	0.950
	Foreigners	42 (31.6)	98 (30.5)	
Housing Type				
	Landed	20 (15.0)	32 (10.0)	0.084
	Apartment	113 (85.0)	289 (90.0)	
Location by zone				
	North	20 (15.0)	22 (7.2)	
	Northeast	76 (57.1)	208 (67.8)	
	East	2 (1.5)	8 (2.6)	0.510
	West	3 (2.3)	6 (2.0)	
	Central	30 (22.6)	63 (20.5)	
	Missing	2 (1.5)		
Chronic medical illness				
	Diabetes mellitus	14 (10.5)	36 (11.2)	0.244
	Hypertension	4 (3.0)	19 (5.9)	0.871
	Ischaemic heart disease	2 (1.5)	9 (2.8)	0.520
	Dyslipidaemia	8 (6.0)	20 (6.2)	1.000
	Others	9 (6.8)	28 (8.1)	0.702

Table 3. Table of Frequency (%) of Symptoms Reported by Patients

| | Disease group | | P | OR |
	Dengue (n = 133)	Controls (n = 321)		
Mean temperature (SD) – ºC	38.4 (0.9)	37.6 (0.9)	0.000	
Symptoms				
Drowsiness	83 (62.4)	157 (48.9)	0.007	1.75
Headache	107 (80.5)	217 (67.6)	0.005	1.99
Myalgia	95 (71.4)	196 (61.1)	ns	1.64
Arthralgia	75 (56.4)	124 (38.6)	0.001	2.11
Loss of appetite	112 (84.2)	180 (56.1)	<0.0005	4.43
Diarrhoea	27 (20.1)	48 (15.0)	ns	1.44
Nausea	63 (47.4)	91 (28.3)	<0.0005	2.31
Vomiting	27 (20.3)	36 (11.2)	0.007	1.98
Red eyes	45 (33.6)	30 (9.3)	<0.0005	4.49
Rashes	15 (11.2)	12 (3.7)	0.003	3.27
Retroorbital pain	35 (26.1)	68 (21.2)	0.012	1.36
Swollen glands	4 (3.0)	8 (2.5)	ns	1.20
Taste alteration	110 (82.7)	177 (55.1)	<0.0005	3.87
Skin sensitivity	44 (32.8)	46 (14.3)	<0.0005	3.07
Bleeding	10 (7.5)	5 (1.6)	<0.0005	5.13

ns: not significant; OR: odds ratio; SD: standard deviation

84.2% of the RT-PCR positive samples concurred fully with the direct serotyping from clinical specimens, using RT-PCR.

Table 3 shows the frequency of symptoms reported by patients in both groups.

The mean number of days that symptoms lasted among dengue RT-PCR positive cases was 10.4 days (95% CI, 9.7 to 11.1) while that for RT-PCR negative cases was 4.5 days (95% CI, 4.2 to 4.8; P <0.05). There was no difference in total duration of symptoms in primary (10.6 days; 95% CI, 9.8 to 11.4 days) or secondary infection (10.0 days; 95% CI, 8.9 to 11.1 days).

The comparison between duration and number of symptoms reported by dengue and non-dengue cases is summarised in Table 4.

The laboratory tests done at recruitment are summarised in Table 5. When these same early parameters were sub-analysed against the hospitalised and non-hospitalised dengue cases, only platelet and lymphocyte count were significantly lower in the hospitalised group compared to the non-hospitalised group (Table 6).

The mean Ct (crossover threshold) value among the dengue RT-PCR cases was lower in the hospitalised cases (17.86; SD = 3.84) compared to non-hospitalised cases (21.04; SD =4.05; P <0.05). Furthermore, linear regression

All 133 dengue RT-PCR positive cases had corresponding positive dengue IgM on their second visit. Among the RT-PCR positive cases, 112 (84.2%) were also positive by virus isolation. There were 204 (44.8%) patients with a prior evidence of dengue infection based on a positive dengue IgG result at recruitment. Fifty-eight (43.3%) dengue patients scored positive for IgG antibodies to dengue, which is indicative of a prior dengue infection. Seventy-five (56.5%) dengue patients were hospitalised versus 4 (1.2%) amongst the controls. Of all the dengue patients who were hospitalised, there was an almost equal number of patients with primary and secondary infections (52% vs 48%). They stayed for an average of 4.6 days (+/-1.6). The length of stay was similar in primary and secondary infections (4.5 vs 4.8 days).

Serotyping of the dengue virus using RT-PCR showed that 66 (48.9%) were infected with DEN-1, 62 (46.6%) with DEN-3 and 5 (3.8%) with DEN-2. DEN-4 was not detected. Serotyping of results from viruses isolated from

Table 4. Comparison Between Duration and Number of Symptoms Reported by Dengue and Non-dengue Cases

	Dengue	Non-dengue	*P*
Mean duration of symptoms	10.5 days	4.4 days	<0.05
Mean number of symptoms reported on direct questioning (maximum of symptoms surveyed = 10)	6.4 (95% CI, 6.1 to 6.8)	4.4 (95% CI, 4.1 to 4.6)	<0.05
Number of cases with persistent symptoms when interviewed on the 3rd week of illness	11 of 118 (9.3%)	13 of 319 (4.1%)	<0.05
Type of persistent symptom	Tiredness (72.7%) Drowsiness (45.5%) Loss of appetite (45.5%)	Cough (77%)	

Table 5. Mean and SD of Results of Laboratory Tests Carried Out on Blood Sample Collected at Recruitment

	Disease group				
	Dengue (n = 134)		Control (n = 321)		*P*
Laboratory results	Mean	SD	Mean	SD	
Platelet x 10^3/mm^3	166.00	71	255	78	<0.005
White cell count x 10^3/uL	3.95	2.20	8.32	4.46	<0.005
Abs neutrophils	3.28	2.12	5.53	4.29	<0.005
Abs lymphocytes	0.53	0.25	1.35	0.75	<0.005
Haematocrit %	42.50	5.69	41.8	7.0	ns

ns: not significant; SD: standard deviation

Table 6. Mean and SD of Laboratory Test Results Carried Out on Blood Samples Collected at Recruitment in Eventually Hospitalised Versus Non-hospitalised Dengue RT-PCR Positive Cases

	Dengue RT-PCR positive cases				
	Hospitalised (n = 77)		Non-hospitalised (n = 56)		*P*
Laboratory results	Mean	SD	Mean	SD	
Platelet x 10^3/mm^3	145	63	193	66	<0.005
White cell count x 10^3/uL	3.59	1.80	4.17	1.80	ns
Abs neutrophils	2.99	1.76	3.41	1.73	ns
Abs lymphocytes	0.46	0.21	0.6	0.26	0.001
Haematocrit %	43.3	5.25	42.6	6.26	ns

ns: not significant; RT-PCR: reverse-transcription polymerase chain reaction; SD: standard deviation
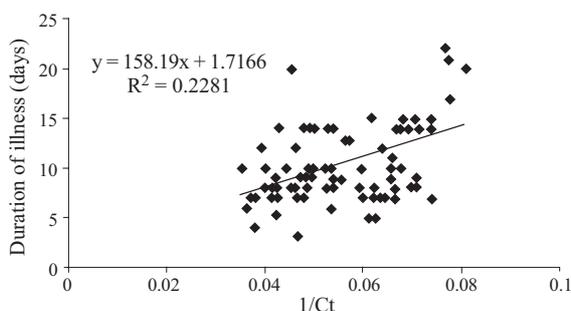

$y = 158.19x + 1.7166$
$R^2 = 0.2281$

Fig. 1. Correlation between 1/Ct value and duration of symptoms.

analysis (Fig. 1) showed a statistically significant relationship between the Ct value of the RT-PCR at visit 1 with total duration of symptoms reported (R = 0.47; R^2 = 0.22; F = 27.13; *P* <0.05). The mean Ct value of primary infection cases was 18.58 (95% CI, 17.72 to 19.44) and that for secondary infection was 19.61 (95% CI, 18.41 to 20.81, *P* >0.05).

Discussion

Dengue infection in adults appears to have 2 effects. Firstly, the proportion of clinical-to-subclinical infection is higher than in children.[4,8] This may explain the overall

observed resurgence of dengue cases in Singapore. Secondly, dengue infection in adults may have different clinical outcomes.[4,6,7] Adults may be at lower risk of developing DHF compared to children due to differences in capillary permeability.[11,12] Taking these 2 factors together, the overall effect would be an increase in the incidence of dengue in the form of DF. This is indeed the observed epidemiological pattern in Singapore. Although the risk of DHF outbreak cannot be excluded given Singapore's 35-year experience, the obvious gap in our knowledge is how to better manage the disease burden of DF in adults.

Out of the 71 hospitalised dengue cases who completed the study, the final diagnosis in 2 cases was DHF while the rest were diagnosed with DF. While a more detailed study of these cases is underway, our observation thus far supports the reported epidemiological pattern of greater DF to DHF case ratio among our dengue cases.[4] Although DF is a self-limiting disease, it can cause considerable incapacitating morbidity.[13,14] Hence while DF is not life-threatening, understanding the development of the debilitating symptoms may offer clues to improved clinical management of this illness, thereby reducing the level of morbidity. Our study shows that the symptoms and duration of illness experienced by dengue cases are different from the non-dengue cases.

Our observation has 2 important implications. Firstly, the WHO guidelines on the classification of DHF, although useful for the paediatric population, do not fully serve the management of adult DF cases. Balmaseda et al[7] have previously reported that the strict adherence to WHO guidelines underestimates severe dengue infections in adults. Although we have not completed the analysis of the differences in in-patient management of the hospitalised cases compared to our study parameters, the detection of a long duration of illness supports the need for a more detailed study of DF.

A second implication of our observations is that a long duration of illness has a direct implication on the economic burden to society. Previous studies have suggested that DF may have a considerable disease burden although less than DHF.[9,15] Another report has suggested that the economic burden of dengue in Singapore is small in comparison to SARS (severe acute respiratory syndrome), HIV/AIDS and road-traffic accidents.[16] The number of days lost to work for non-hospitalised and hospitalised cases of dengue has been estimated to be 5 and 10 days respectively. No consideration was made for prolonged symptoms that would affect productivity adversely. Based on our finding of an average of 9.4% of cases with prolonged symptoms, 1299 out of the 13,186 laboratory-confirmed DF cases in Singapore in 2005 would have experienced prolonged illness. Since 90% of dengue cases in Singapore are 15 years and older (70% are 25 years and older), an assumption

of 1000 persons in the workforce having to either stop work or work at reduced productivity rates for a period of 3 weeks or longer would not be unreasonable. The findings of our study indicate that the true burden of dengue in Singapore may have been significantly underestimated. This in turn has tremendous implications on the disease prevention budget.[4]

Apart from the correlation between Ct value and duration of illness, no other clinical or haematological parameter collected at visit 1 showed statistically significant association with the duration of illness. Although we have only limited the analysis to the duration of illness and the number of symptoms reported (with further analysis against hospitalised data presently underway), our findings do suggest that there is a paucity of prognostic markers for dengue infection in adults. Such markers may be particularly useful in determining the necessity for hospitalised care, which would not only reduce the healthcare cost but also aid in the appropriate utility of hospital beds. At the moment, such a decision is made based on the finding of a platelet count of 80,000 cells per mL and below. Since others have shown that platelet count is not indicative of bleeding or hypovolaemic shock,[17] the need for more predictive markers for prognostication is urgently needed.

Our interim analysis of DF in adults also indicates that there is no difference in the rates of primary or secondary infection. Secondary dengue cases also did not experience a longer duration of illness compared to primary cases. This is unlike DHF where epidemiological observations have implicated secondary infections as a risk factor. The reason for this difference in observation is not known. The postulated mechanism to explain the epidemiological pattern of an association between secondary infection and DHF is that cross-reacting or sub-neutralising levels of antibodies developed due to a previous infection, which mediates monocytes and macrophages through the Fc receptors. The resulting effect of this immune enhancement of infection is a larger viral load in the host, leading not only to a greater degree of immunological response to the virus but also damage to the endothelial integrity of capillaries.

In adults, the degree of capillary permeability under normal physiological conditions is less than that for children[11] which may in turn contribute to a reduced risk of DHF in a dengue infection. On the other hand, the immune response may be more robust than children, thus leading to no difference in the outcome of primary and secondary infections when measured in terms of duration and number of symptoms.

Although we had no deaths in our series and only 2 cases were diagnosed as DHF, the true severity of illness in our cohort has yet to be determined. The low number of DHF cases may be due to the adherence to WHO criteria for

diagnosis of DHF/DSS. Although the ELISA that we have used to detect anti-dengue IgG antibodies would also detect cross-reacting antibodies generated by other flaviviral infections such as Japanese encephalitis (JE) virus, most of the ELISA positive samples are likely to be true dengue infections. This assumption is backed both by the low incidence of JE in Singapore following the elimination of pig farming, as well as the observed low positive rate of neutralising antibodies to JE virus in the Singapore human population in a recent serological survey.[18]

In conclusion, our initial evaluation suggests that DF in adults lasts longer than non-dengue febrile illnesses. Nine per cent of the dengue cases also reported a persistence of symptoms up to 3 weeks after the onset of illness. Both these observations indicate that the burden of dengue illness is substantially different from other non-dengue febrile illnesses. Our study also highlights the paucity of prognostic markers for dengue fever in adults.

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