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Kinetics of NS1 and anti-NS1 IgG following dengue infection reveals likely early formation of immune complexes in secondary infected patients

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Dengue virus (DENV) is a major health concern throughout the world infecting up to 390 million people globally each year. Infection with any one of the four DENV serotypes produces a spectrum of clinical illness ranging from a mild undifferentiated febrile disease through to severe dengue involving fever and haemorrhage. There is currently no antiviral treatment for dengue and only one licensed vaccine with limited distribution. This study characterises the kinetics of the serological dengue biomarker, NS1, and the appearance of anti-NS1 IgG, anti-E IgM and anti-E IgG responses in patients with primary and secondary infections. Blood samples were collected daily from a cohort of 52 Vietnamese patients during the acute phase of disease. NS1 was detected in 85% of patient samples from disease onset with detection decreasing throughout the acute phase of disease. Anti-NS1 IgG detected from the fourth day of illness and anti-E IgM and IgG from the third day of illness, were all observed to increase throughout the course of the disease. During secondary infection, NS1 levels rapidly decrease with the increasing levels of anti-NS1 IgG, suggesting the possibility of NS1 immune complex formation and a potential role in the pathogenesis of the severe forms of disease associated with secondary infections.

Dengue virus (DENV) is a major public health concern throughout the tropical regions of the world, infecting up to 390 million people each year[1]. DENV is a single stranded RNA virus belonging to the genus *Flavivirus* of the family *Flaviviridae* and is spread primarily by mosquitoes to humans by a bite of the domestic mosquito *A. aegypti*. Infection with any of the four DENV serotypes (DENV1–4) can produce a spectrum of clinical disease, ranging from a mild undifferentiated febrile illness through to the life-threatening forms of disease associated with secondary infection, characterized by increased vascular permeability, thrombocytopenia and hemorrhage[2,3].

There is currently no antiviral therapy for dengue infection. Treatment is purely supportive, such as fluid replacement for patients. During the natural course of infection, an immune response is generated against viral antigens such as the DENV non-structural protein, NS1 which is often observed to be at elevated levels in patient plasma during the early stages of disease, particularly in secondary infection[4]. As a result of these observations, NS1 has been proposed as a surrogate marker for viremia and a predictor of progression to severe disease in secondary infected patients[5]. Furthermore, studies have identified NS1 as a viral toxin that contributes to an overactive innate immune response and endothelial cell dysfunction early in infection. The presence of NS1 in the serum of infected patients has been exploited as a useful early diagnostic biomarker[6–8].

Primary dengue infection is characterized by rising levels of IgM appearing at day 3–5 and persisting for up to a period of months. Patients undergoing a secondary infection typically experience an anamnestic IgG antibody response with peak detection 6–9 days after the onset of symptoms. The DENV NS1 protein is secreted from infected cells during the natural course of infection with elevated levels of circulating secreted NS1 in patient plasma often observed during the early stages of disease[9,10].

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The kinetics of these dengue biomarkers have been intensively researched, and many studies have investigated various combinations of biomarkers in either primary, secondary infections or both. Several key studies by Papa et al. and Koraka et al. characterized NS1 and anti-NS1[11,12], while the study by Hu et al. determined the kinetics of NS1, anti-dengue E IgM and IgG[13]. While these studies provide solid evidence for the kinetics of the appearance of these biomarkers, the combined story has to be inferred by outbreaks from different geographic locations, outbreaks, dengue serotypes and times using various methods. However, no single prospective study has been performed to characterize the kinetics of NS1, anti-NS1, anti-E IgM, and anti-E IgG in a single patient cohort. In the study presented in this manuscript, we investigated the kinetics of these biomarkers in a single prospective study of primary and secondary infected patients from a single dengue outbreak occurring in Vietnam where dengue virus types 1 and 2 were circulating. Here we characterise the kinetics of serological dengue biomarkers during the acute phase of disease. Levels of free NS1 circulating during the course of infection in patient sera were determined by quantitative capture ELISA. Titres were determined for anti-NS1 antibodies circulating in sera by indirect ELISA. Anti-E IgM and IgG levels were assessed by capture IgM and IgG ELISA, respectively[9].

Results

Analysis of NS1 protein and anti-NS1 IgG kinetics in infected patients

In primary patients, NS1 could be detected in blood from the onset of illness through to 9 days post infection, with peak NS1 levels occurring between day 1–4. In secondary patients, NS1 could be detected from day 1 to 8 post onset of illness. However, lower NS1 levels were observed later in infection when compared to primary patients with NS1 peaking on days 3 and 4. NS1 levels ranged from undetectable to 7.8 μg/ml. Patients with DENV-1 had higher levels of NS1 during primary infection, than those patients with primary DENV-2 infection whose NS1 levels were consistently low (Fig. 1a).

NS1 was successfully detected throughout the acute phase of the disease with detection above 80% for the first four days of illness in primary patients. However, detection was lower in secondary infections, ranging from 50 to 63% during the first 4 days of infection. NS1 was detected in 100% of primary DENV-1 patients during the first 3 days of illness and above 80% through the rest of the acute period. However, the results for primary DENV-2 patients revealed lower detection rates ranging from 50 to 67% during the first 4 days of illness. During DENV-1 secondary infection, NS1 was detected from day 1 for only one patient, with detection decreasing through to day 8 where no NS1 was detected. DENV-2 secondary infection patients had no detectable NS1 on day 1 ($n = 1$), with detection rising to 83% on day 2 and peaking at 85% detection on day 3 before steadily decreasing throughout infection until NS1 was undetectable by day 8 (Fig. 1b).

Primary DENV patients had poor early anti-NS1 IgG responses, as expected for an initial antigen exposure with NS1 specific IgG only detected in one patient on day 4 during acute illness and at 8% detection in the follow-up samples (Fig. 1c). In contrast, and as expected of an anamnestic response, secondary patients showed a rapid anti-NS1 IgG response. Anti-NS1 IgG was detected as early as 4 days post onset of illness (Fig. 1d). Anti-NS1 IgG titers and detection increased during infection, with 88% of secondary patients having detectable anti-NS1 IgG levels in the follow-up samples. Primary patients had high levels of free circulating NS1 while having undetectable anti-NS1 IgG titers, whereas secondary infected patients had decreasing levels of NS1 at the same time that anti-NS1 IgG levels were rising (Fig. 1e). The appearance of anti-NS1 IgG was inversely correlated (Pearsons two tailed t test, 95% CI) with NS1 levels in secondary patients, suggesting the formation of NS1/IgG immune complexes or IgG-mediated NS1 clearance from circulation (Fig. 1f).

Appearance of anti-E IgM and IgG in patient serum samples

The virion surface E protein is a major target of an infected patient's immune response. The presence of anti-E IgM and IgG was determined using IgM and IgG specific capture ELISAs (Fig. 2). The ELISAs were performed as per the manufacturer's instructions. None of the samples from the 3 negative patients tested positive for anti-E IgM or IgG antibodies. IgM was detected from the onset of symptoms with detection levels increasing throughout infection, reaching 100% of patients by 7 days post onset of illness (Fig. 2a). In secondary infected patients, IgM was detected from the third day post onset of symptoms (26.67%) with detection rapidly rising on day 4 (78.95%). Detection of IgM in secondary patients was maintained at ≥ 80% for the remainder of the study period. The anti-E IgM response in DENV-2 patients was delayed by 2 days compared to DENV-1 primary infected patients, however in secondary infection the anti-E IgM kinetics were similar for both DENV-1 and 2 infected patients (Fig. 2b).

As expected, anti-E IgG detection in primary patients was low in comparison with IgM with a positive response detected in only 3 patients during the acute disease phase. Anti-E IgG was detected on days 6 and 7 in 19% and 17% of patients, respectively. Higher rates of detection were observed in the follow-up samples with IgG detection in 44% of patients (Fig. 2c). In secondary infected patients, anti-E IgG was detected from day 3 in 6.67% of patients, with detection rapidly rising to ≥ 87% from day 5–10 (Fig. 2d). Follow-up samples (day 16–45) showed that all patients mounted a detectable anti-E IgG response. In summary, primary patients infected with either DENV-1 or DENV-2 had similarly low levels of anti-E IgG responses during the acute phase of disease. In contrast, secondary responses to each DENV serotype had higher detection rates with increasing detection from day 4, reflecting a strong anamnestic antibody response to cross-reactive epitopes.

Discussion

In this prospective study, we examined the kinetics of appearance of NS1, anti-NS1 IgG, anti-E IgM and IgG in the serum of 51 patients infected with dengue during epidemics in Vietnam from 2005 to 2007. Patients enrolled in this study either experienced DENV-1 or DENV-2 infections (both primary and secondary), with levels of
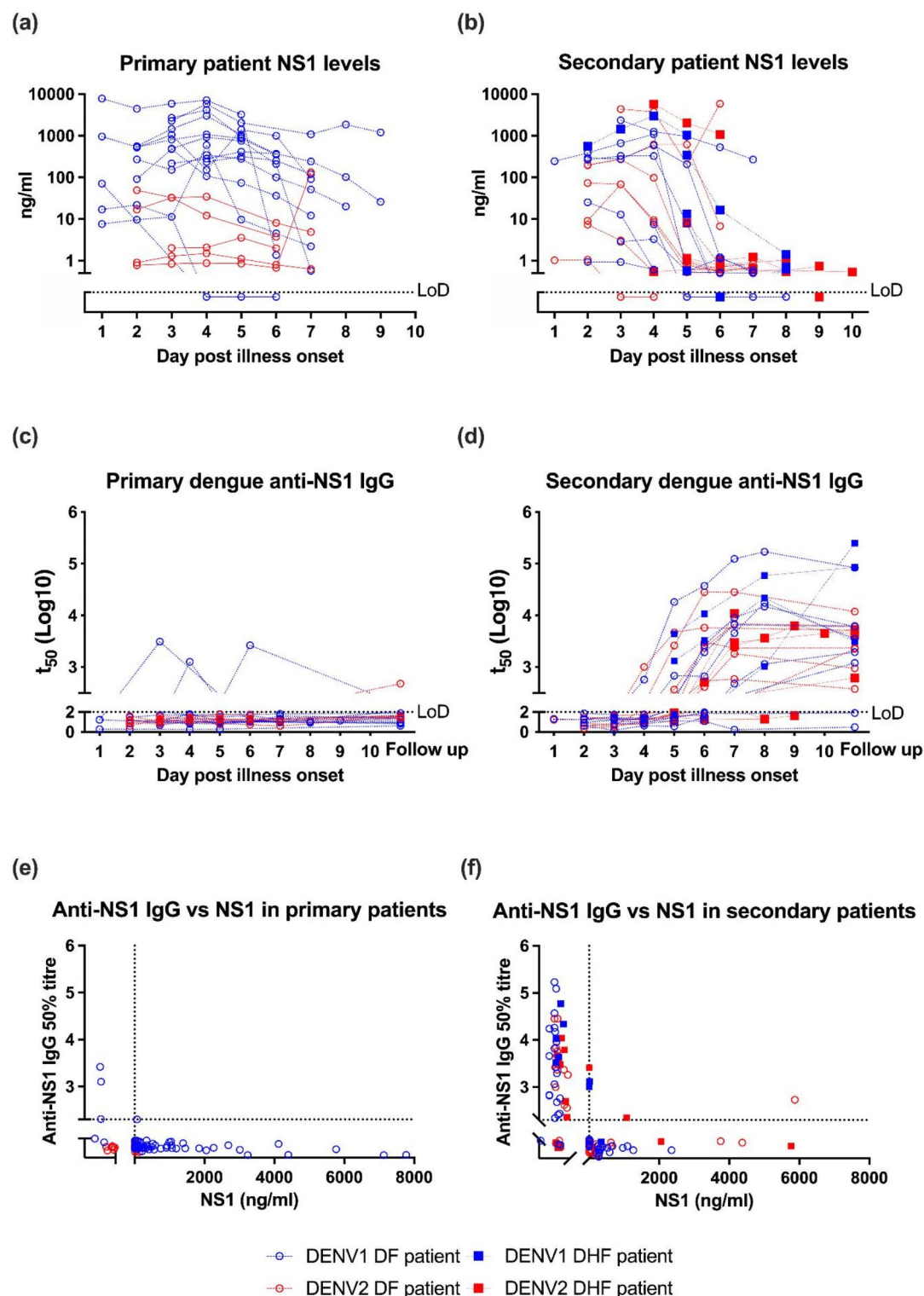
Fig. 1. Daily patient samples tested for NS1 and anti-NS1 levels. (**a**) Primary patient NS1 levels and (**b**) Secondary patient NS1 levels during the acute phase of illness. (**c**) Primary patient anti-NS1 IgG responses. (**d**) Secondary patient anti-NS1tested by indirect ELISA and plotted as 50% titres. Anti-NS1 IgG titre plotted against NS1 levels in (**e**) primary and (**f**) secondary patients. Blue circles: DENV-1 DF (Dengue Fever) patient, Red circles: DENV-2 DF patient, Blue squares: DENV-1 DHF (Dengue Haemorrhagic Fever) patient, Red squares: DENV-2 DHF patient, *LoD* limit of detection.
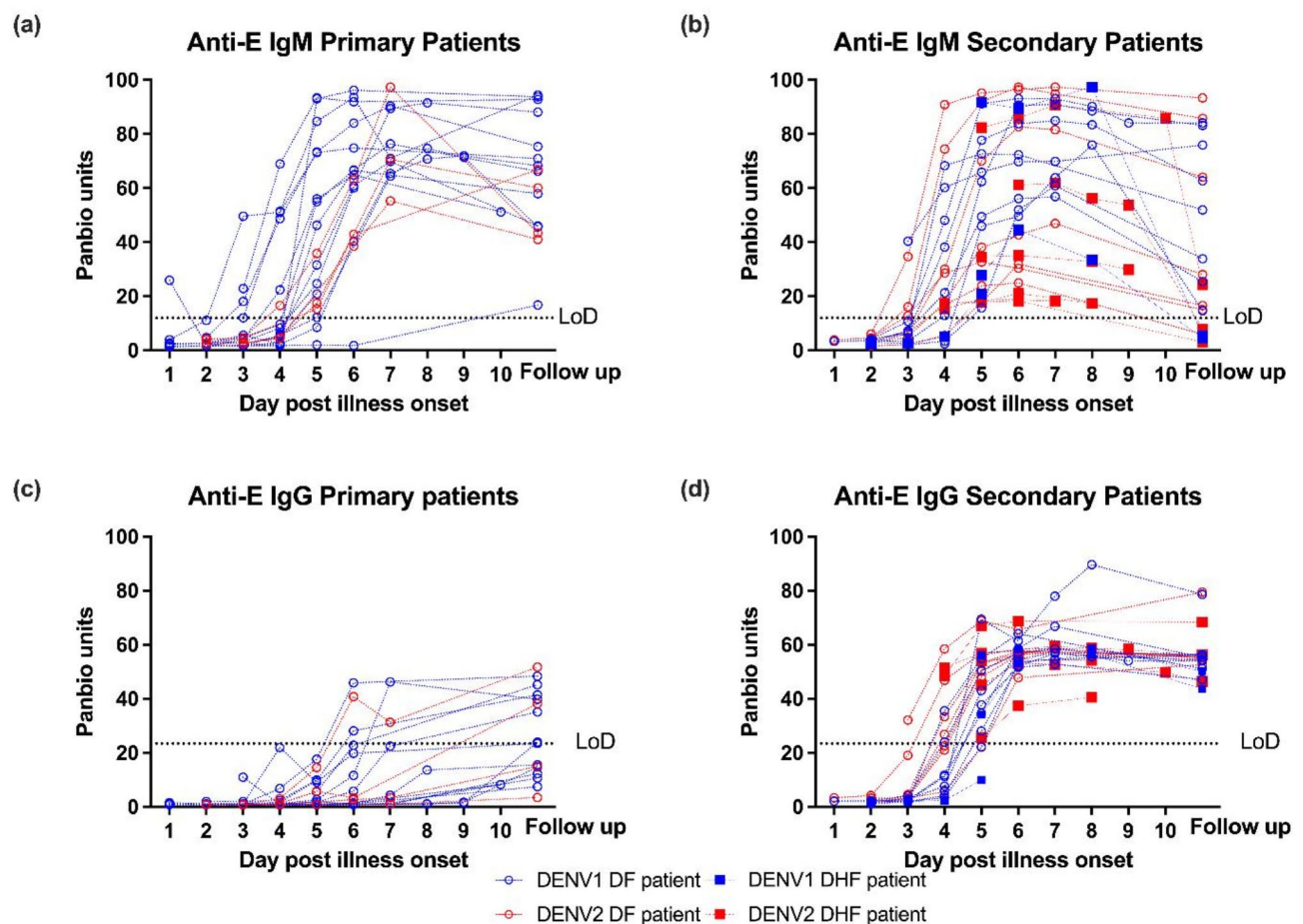
Fig. 2. Anti-dengue IgM and IgG responses. (**a**) Daily primary patient anti-E IgM responses. (**b**) Daily secondary patient anti-E IgM responses. (**c**) Daily primary patient anti-E IgG responses. (**d**) Daily secondary patient anti-E IgG responses. Blue circles: DENV-1 DF patient, Red circles: DENV-2 DF patient, Blue squares: DENV-1 DHF patient, Red squares: DENV-2 DHF patient. Limit of detection (LoD) set at 12 and 23.4 Panbio units for IgM and IgG respectively.

disease outcome varying from mild infection to severe disease with or without vascular leak. Samples were collected daily during the acute disease period then at convalescence.

NS1 was detected in both primary and secondary DENV patients during the acute phase of disease from the first day of illness. In primary infections detection was retained through to at least 9 days post illness, while detection in secondary cases was found to decline by days 6–7. Peak detection for both primary and secondary patients was on days 3–4[14–16]. NS1 was detected in ≥72% of samples during the first 4 days of illness ranging from undetectable to 7.8 µg/ml. By day 5, detection dropped to 50%, then decreased throughout the remainder of the disease period. These results are consistent with the findings of others who have used in-house and commercial NS1 capture ELISAs to detect NS1[17–28]. As with the current analysis, these studies found that NS1 capture ELISAs had high sensitivity and specificity early during the acute period with detection decreasing during infection.

Primary DENV-2 patients had lower NS1 levels overall with 133 ng/ml the highest NS1 level recorded, as opposed to DENV-1 primary patients which peaked at 7.8 µg/ml. The lower levels of NS1 associated with DENV-2 infection has also been observed in other studies in Vietnam[29]. While the use of dengue 2 virus NS1 as the sole standard for NS1 quantification for all samples likely impacts absolute values, the trends in relative levels of NS1 within individual patients, the main focus of this study, will not be impacted. Interestingly, secondary DENV-2 patients showed comparable levels of NS1 to secondary DENV-1 patients, with peak levels detected at 5.5 µg/ml. The lower levels of NS1 in primary DENV-2 patients may simply reflect an overall lower viral load[14]. The higher relative levels of NS1 in secondary DENV-2 infected patients may be a consequence of antibody-dependent enhanced infection, with these higher levels shown to be a predictive marker of progression to more severe disease[4].

In a clinical setting, timing and choice of what diagnostic test to employ is critical, as each method described in this study has an ideal window in which it is at its most effective. The NS1 capture ELISA is at its most effective early in infection, i.e. from the onset of symptoms, whereas IgM and IgG ELISAs rely on the patients induced immune response, which in most cases appears from day 3 or 4[30,31]. By the time IgM ELISAs are performing

at a high diagnostic rate, NS1 detection may be rapidly declining, particularly in those patients experiencing a secondary infection. This highlights the need for complementary testing of both NS1 and antibody levels, as this testing regime was found to cover the disease period with greater than 80% successful diagnosis (in this study day 2 was an exception with 73% detection)[32,33]. Performing concurrent tests on patient sera for NS1 capture as well as anti-E IgM and IgG capture would allow additional determination of primary versus secondary infection[30].

Anti-NS1 IgG 50% titers were determined by indirect ELISA, with both detection and titers varying in correlation with the infection status of the patient. Previous studies have suggested that NS1 specific antibodies may play a role in the development of severe disease and vascular leak, as anti-NS1 antibodies are seldom seen in primary infection but detected in most secondary infected patients[32,34–37]. All primary infected patient titers were below the negative sample cut-off values, with the exception of a small number of patients ($n=3$). In secondary infected patients, specific anti-NS1 IgG was detected from day 4, with titers and sensitivity of detection rising throughout the course of the infection to ≥ 81% detection in convalescent sera. All secondary infected patients with positive anti-NS1 IgG had no or rapidly decreasing NS1 levels. It was observed in most patients that decreasing levels of NS1 correlated with rising levels of anti-NS1 IgG. In other patients with no NS1 detected, high levels of anti-NS1 IgG were present. The logical interpretation of these observations is the formation of immune complexes, which render the bound NS1 unable to be detected in the capture ELISA.

One limitation of the NS1 capture ELISA employed in this study is that detection is limited to free-circulating NS1. In this study and others, it has been noted that the ability to detect NS1 diminishes from day 4–5. It has been hypothesized that this loss of NS1 detection in secondary infection is due to the presence of NS1 specific IgG removing NS1 from circulation and leading to the formation of immune complexes. These immune complexes are then presumed to be cleared from the circulatory system or deposited onto the surface of endothelial cells possibly contributing to vascular leak. In this study we have confirmed an inverse correlation between decreasing NS1 levels and increasing anti-NS1 IgG over the course of infection within individual patients. Note added in proof: while this manuscript was under review, our findings were confirmed by similar observations reported by Papa et al.[12].

Speculation on the role of antibodies directed against NS1 has, at times provided a somewhat confusing and contradictory narrative. Historically, NS1 has been viewed as a potential vaccine candidate that would give rise to a protective immune response that does not have the same intrinsic ADE risks associated with E-based vaccines[10]. Our group identified a role for an anti-dengue NS1 monoclonal antibody in protection of mice from dengue virus or Zika virus challenge[38] and there is a wealth of literature demonstrating that NS1 vaccination elicits protective antibodies by targeting dengue-infected cells for destruction via complement-mediated lysis and other mechanisms[39–45]. However, NS1 vaccination has also been shown to produce inappropriate immune responses with antibodies that are host reactive via self-mimicry apparently enhancing disease[46–50]. Furthermore, the protective efficacy of the anti-NS1 response needs to be balanced against its role in pathogenesis. As demonstrated in our manuscript and others, NS1 is rapidly cleared from circulation during a secondary infection by anti-NS1 IgG. The loss of free circulating NS1 through the formation of immune complexes aligns with the onset of severe dengue disease. We speculate that the formation of immune complexes and deposition onto endothelial cell walls rather than clearance, likely plays a role in the vascular leak characteristic of severe dengue. Based on our observations, this hypothesis is limited by the relatively small number of patients experiencing secondary dengue and severe disease. Therefore, the sample size needs to be expanded in future studies to conclusively determine the role that immune complexes may be playing in severe disease.

While the kinetics of dengue biomarker appearance has been intensively investigated, to our knowledge, this is the first prospective study to examine the kinetics of NS1, anti-NS1, anti-E IgM and IgG in a single dengue patient cohort. Overall, our findings confirm what has otherwise been inferred by numerous previous studies reporting the kinetics of dengue biomarkers in various patient cohorts. The identification of a viral toxin activity for NS1 that impacts vascular integrity of the endothelial barrier 3 days post intravenous injection in mouse studies further highlights the importance of early NS1 detection[6,7]. Taken together, these results support the suggestion that both soluble NS1 and its antibody bound immune complex may be contributing factors in the pathogenesis of severe disease.

Materials and methods
Clinical samples and study objectives
We selected 303 serum samples from 52 dengue patients (confirmed positive by qPCR) enrolled in research studies at the Hospital for Tropical Diseases in Ho Chi Minh City, Vietnam (reviewed by the ethics committee and approved by the internal review board of the hospital), during outbreaks that occurred between 2005 and 2007. Daily serum samples were taken from patients on presentation to healthcare facilities during the course of disease development (1–9 days) and convalescent serum samples were taken several weeks after illness resolution. The patient cohort contained primary and secondary patients experiencing DENV-1 and 2 infections, with symptoms ranging from mild dengue fever through to severe dengue involving haemorrhage and shock (Table 1). Patients were classified as having a primary vs. secondary infection based on RT-qPCR results coupled with IgM and IgG analysis. A negative IgG ELISA result in the first 72 h with no increase in dengue-reactive IgG by day 7 defined a primary infection, and a positive indirect ELISA result within 72 h and/or an equivocal response, with a clear increase in dengue-reactive IgG greater than the IgM response by day 7, defined a secondary infection. This study examines the kinetics of NS1 production, as well as the appearance of anti-NS1 IgG, anti-dengue IgM and anti-dengue IgG in patients with primary and secondary infections.

Dengue	Primary	Secondary		Unknown	Total
		DF	DHF/DSS		
Type 1	14	8	3		25
Type 2	5	7	6		18
Unknown			5		5
Control				3	3
Total	19	15	14	3	51
# of samples	121	102	62	18	303

Table 1. Patient cohort breakdown based on infection and disease status.

Study design

Diagnostic ELISAs: NS1, anti E IgM and anti-E IgG

Evaluation of NS1, anti-dengue IgM and anti-dengue IgG levels were assessed using the Panbio Early dengue ELISA, Panbio Dengue IgM capture ELISA and Panbio IgG capture ELISA IgG kits as per manufacturer's instructions (Abbott). Quantitation of NS1 in patient samples was made possible by the addition of a baculovirus expressed recombinant dengue virus 2 NS1 protein standard curve from which quantitative values were determined using GraphPad Prism. The authors note that the use of a single serotype NS1 will likely impact absolute protein values attributed to other serotypes. However the findings of this study relate to the relative rise and fall in levels of circulating NS1 within patients.

Indirect anti-NS1 IgG

Nunc maxisorp plates were coated with 50 µl of 100 ng/ml of NS1 in carbonate buffer pH 9.6 and incubated at 4 °C overnight. After coating, the plates were blocked with 150 µl of a solution of 1% milk powder, 1% sucrose, 0.3% Tween 20 in PBS at room temperature for 2 h. Following blocking, plates were washed and 50 µl of 1 in 10 dilutions of patient plasma samples were serially diluted in a 1:10 series in blocking solution and incubated for 1 h at 37 °C. After antibody binding, the plates were washed 3 times and anti-human IgG HRP at 1:100 in 0.3% Tween 20 in PBS (PBST-20) was added to the plate for 1 h at 37 °C, then washed a further 6 times. Fifty µl of K-Blue TMB substrate (ELISA systems) was added, and the colour reaction was developed for 5 min in the dark. The reaction was stopped by the addition of 25 µl of 2 M H_2SO_4, with plates read spectrophotometrically at 450 nm.

Data availability

The authors declare that all data supporting the findings of this study are available within the paper.

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Author contributions

Funding

Declarations

Competing interests
The authors declare no competing interests.

Institutional review board statement
The study was conducted in accordance with the Declaration of Helsinki, and the use of serum samples routinely taken from patients as part of ongoing research projects was approved by the Ethics Committee of the Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam.

Informed consent statement
Informed consent was obtained from all subjects involved in the study.

Additional information
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