NS 1 lasts longer than the dengue virus nucleic acid in the clinically suspected patients with dengue fever and dengue haemorrhagic fever

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Abstract The objectives of the study were to compare the DENV NS1 and nucleic acid positivity in dengue fever (DF) and dengue haemorrhagic fever (DHF) patients and to evaluate the usefulness of these parameters in severe dengue. Blood samples were collected from 91 patients with DF or DHF (fever days 3–9) from Gampaha and Negombo General Hospitals, Sri Lanka and tested for DENV NS1 antigen and nucleic acid. On fever day 3, 67% of the DF and 33% of the DHF patients had DENV nucleic acid whereas 50% of the DF and 67% of the DHF patients had NS1. On fever day 4, 67% of the DF and 100% of the DHF patients had DENV nucleic acid whereas 83% of the DF and DHF patients had NS1. From fever day 5 in the DHF group, DENV nucleic acid declined but NS1 remained detectable even on day 9 ($p < 0.0001$). NS1 positivity did not correlate with the decrease in the WBC count in DF and DHF patients ($p > 0.05$) whereas NS1 positivity significantly correlated with the decrease in the platelet count in DF and DHF patients ($p < 0.0001$). NS1 is a useful marker to detect DENV infection even in the latter stages of DHF.

Keywords Dengue fever · Dengue haemorrhagic fever · DENV nucleic acid · NS1 antigen

Dengue virus (DENV) infections results in mild to severe clinical disease in populations living in the dengue endemic

tropical and subtropical regions of the world. All four DENV types namely DENV-1, -2, -3 and -4 have been co-circulating in Sri Lanka since the early 1960s and established in the island causing regular outbreaks especially from the early 1990s [1]. The vectors for DENV transmission, *Aedes aegypti* and *Aedes albopictus* are also well established and adapted even to brackish waters in the country [2].

Clinically apparent DENV infection is categorized into dengue fever (DF) dengue haemorrhagic fever (DHF), the latter will have a critical period of plasma leakage usually lasting for 24–48 h with or without haemorrhagic manifestations (http://www.wpro.who.int/mvp/documents/handbook_for_clinical_management_of_dengue.pdf). A large majority of the dengue associated deaths occurs in the DHF group. The aetiological diagnosis of DF/DHF includes the detection of DENV antigen or antibodies in the serum, virus isolation by culture and virus nucleic acid detection using reverse transcriptase-polymerase chain reaction (RT-PCR) [3]. However, the diagnosis of DF/DHF using each of these methods depends on the disease phase in which the patient is at the time of sample collection, the availability of the test/s and the cost associated with the test/s [3]. Moreover, testing for more than one viral marker would improve the detection of DENV infections in severe cases.

The most common approaches used to test for DENV infection include RT-PCR to detect the viral nucleic acid in the early phases and serological assays to detect anti-DENV IgM/anti-DENV IgG in the latter phases of the disease [4]. However, DENV NS1 antigen has been used to detect DENV infection widely since the last decade [1]. DENV genome encodes seven non-structural proteins, NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5, which are responsible for viral replication and modulation of cellular functions [5]. NS1 is a highly conserved glycoprotein that

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is present at high concentration in the sera of DENV-infected patients during the early clinical phase of the disease and it can serve as an antibody target for DENV [5]. NS1 antigen is found from the first day to up to day 9 after the onset of fever in primary or secondary DENV infections [6]. Use of NS1 detection for the diagnosis of DENV infections in clinically suspected DF/DHF is simple and cost effective with high detection sensitivity and specificity through different immunological platforms like ELISA and immunochromatography based rapid assays to suit the number of samples under evaluation [1]. In the present study we compared the DENV NS1 and nucleic acid positivity in clinically suspected DF/DHF patients and assessed the usefulness of NS1 as a diagnostic marker in the latter stages of DHF.

Ethical approval for the study was obtained from the Ethics Review Committee of the Faculty of Medicine, University of Peradeniya, Sri Lanka (2011/EC/49). Blood samples were collected once from clinically suspected DF/DHF patients ($n = 91$) from fever day 3 to 9 from Gampaha and Negombo General Hospitals in Sri Lanka soon after the admission of the patients to the respective wards. Sera were separated by centrifugation at 2000 rpm for 5 min followed by collection with disposable Pasteur pipettes into an eppendorf tube, which were labeled and stored at -80°C until tested.

Ninety-one patients' blood samples were tested to detect DENV NS1 using ELISA (SD Diagnostics). Following the manufactures instructions anti-dengue NS1 coated plate was used to capture dengue NS1 antigen from the patients' sera. Absorbance reading was taken at 450 nm with a reference optical density of 620 nm using the ELISA reader (Model-SN0393004373-Tecan, Austria). Viral RNA was extracted from the sera using a validated extraction system according to the manufacturer's instructions (QIAmp Viral RNA mini kit, Hilden, Germany, Cat No 52906). DENV nucleic acid by RT-PCR was performed using primers (Forward DV1-TCAA-TATGCTGAAACGCGCGAGAAAACCG, Reverse DV2-TTGACCAACAGTCAATGTCTTCAGGTTC, amplifying a 511 bp product) targeting the capsid gene of the DENV following the methods described by Lanciotti et al. [7]. The statistical analysis for the study was performed using the Student's t test for comparison between different groups with the help of statistical software, Graphpad Prism, Version 7. The differences in the mean WBC and platelet counts were considered significant at p value ≤ 0.05 .

Of the 91 patients tested, 45 (49.5%) had DF and 46 (50.5%) had DHF based on the clinical classification using the plasma leakage and haemorrhagic manifestations during the clinical illness. Patients who developed DHF showed high NS1 positivity even in the latter stages of

illness (Fig. 1). DENV nucleic acid positivity by RT-PCR was high in DF on fever day 3 and in DHF on fever day 4 compared to NS1 antigen as detected by ELISA. From fever day 5 onwards, NS1 antigen positivity increased in both DF and DHF patients, however, NS1 positivity was significantly higher in the DHF group than that in the DF group ($p < 0.0001$) (Fig. 1). In contrast, DENV nucleic acid positivity declined with time in both DF and DHF patients. On fever day 5, 50% of the DF patients showed DENV nucleic acid positivity whereas only 29% of the DHF patients were positive for DENV nucleic acid on fever day 5. Conversely, the DENV nucleic acid positivity in DF patients was higher in fever day 5 and 6 compared to DHF patients.

DENV NS1 positive DF patients had a mean WBC count of 3450 cells/mm³ and DHF patients had a mean WBC count of 2967 cells/mm³ as compared by the unpaired t test ($p = 0.235$). Mean platelet count of NS1 positive DF patients was 84,800 mm³ and the mean platelet count of DHF patients was 42,945/mm³ ($p = < 0.0001$). The association between the drop in platelets and NS1 positivity was statistically significant in DF and DHF patients (Fig. 2).

DENV NS1 protein is involved in RNA replication as it resides at the site of RNA replication, however, the exact role of NS1 is not clear [6]. In this study we have evaluated the association between the NS1 positivity and severe dengue and the usefulness of detecting NS1 in patients who present late in DHF. In the current study, similar to many other studies, NS1 antigen positivity was detected from fever days 3 to 9 [6]. However, NS1 negativity in latter stages does not eliminate an acute DF/DHF, as the negativity might be due to undetectable levels of NS1 antigen in the blood at the time of testing.

In our study, DENV NS1 was detected when DENV nucleic acid was negative by RT-PCR in both DF and DHF patients at the latter part of clinically apparent DENV infections. DENV nucleic acid positivity was higher in DF patients on fever days 3 and 4 when compared to NS1 at the same time of the illness. RT-PCR detection of DENV nucleic acid declined with time indicating the suitability of RT-PCR during the viraemic febrile phase. But the test is not useful around the time of defervescence, a time when the clinical complications of vascular leakage are most likely to manifest with a decrease in viral burden [8–10]. Moreover, detection of DENV nucleic acid by RT-PCR is expensive and not readily available in resource poor dengue endemic regions.

Dengue NS1 levels differed in individuals ranging from a couple of nanograms per milliliter to 50 μml^{-1} during defervescence [6, 13]. Thus, detection of NS1 antigen might allow a virological diagnosis of DENV infection in the early stages as well as latter stages even

Fig. 1 DENV nucleic acid and DENV NS1 antigen positivity in relation to fever days in DF and DHF patients ($n = 91$) sampled once between fever days 3 to 9. DENV NS1 antigen detected by ELISA and DENV nucleic acid detected by RT-PCR are given and the number of patients tested for each marker is given in parentheses, *DF-DENV nucleic acid*—DENV nucleic acid in DF patients; *DF-NS1 antigen*—DENV NS1 antigen in DF patients; *DHF-DENV nucleic acid*—DENV nucleic acid in DHF patients; *DHF-NS1 antigen*—DENV NS1 antigen in DHF patients

Fig. 2 DENV NS1 positivity in clinically suspected DF and DHF patients, **A**—WBC counts in DF patients who were NS1 positive and negative; **B**—WBC counts in DHF patients who were NS1 positive and negative; **C**—Platelet count in DF patients who were NS1 positive and negative; **D**—Platelet count in DHF patients who were NS1 positive and negative ***($p < 0.0001$). In all cases the bar represents the mean

when PCR is not useful. DENV NS1 antigen levels correlate with disease severity early in the dengue illness [11], suggesting NS1 tests might also have a prognostic value. Based on the findings of the current study, NS1 antigen positivity in DF patients declined with time and fever days 7, 8 and 9 showed 57, 29 and 29% positivity, respectively. In DHF even in fever days 7, 8 and 9, NS1 antigen was positive in 71, 71 and 57% of patients, respectively. Recent studies have also shown the positive correlation between NS1 positivity and antigenaemia in severe dengue [12, 13].

Low WBC count was observed in NS1 positive DF and DHF patients, however, no significant difference was noted in the WBC count between these two groups in the present study. Previous research findings have shown leucocytopenia in DF patients possibly caused by virus-induced inhibition/destruction of myeloid progenitor cells [14]. Thrombocytopenia is a criterion for the diagnosis of DF according to WHO guidelines (http://www.wpro.who.int/mvp/documents/handbook_for_clinical_management_of_dengue.pdf). The current study showed a significant decrease in platelet counts in NS1 positive DF and DHF

patients ($p = <0.0001$). The association between NS1 levels and clinical severity is thought to be related to the cross-reactivity of anti-DENV NS1 antibodies with platelets and endothelial cells leaving sufficient levels of NS1 for detection [15, 16].

In this study we have compared DENV NS1 and nucleic acid profiles in DF and DHF patients. DENV NS1 antigen positivity is high when compared to DENV nucleic acid in patients with clinically apparent DENV infection. Some patients with absence of DENV nucleic acid showed NS1 positivity from fever day 3 to 9, suggesting the usefulness of NS1 to detect DENV infection even in the latter stages of DHF.

Moreover, NS1 antigen can be used as a viral marker to detect DENV antigenaemia helping a cost effective diagnosis even in latter stage of DF/DHF in resource poor settings. With the availability of rapid immunochromatography based tests for DENV NS1 detection, rapid and early diagnosis of dengue is possible contributing to stringent patient management. Further investigations are needed to understand the relationship between DENV NS1 antigen and DHF to predict clinical severity in patients with severe dengue. A few limitations of the present study include testing both adult and paediatric patients in a relatively small sample size and not using quantitative tests for detecting DENV nucleic acid and NS1 antigen profiles. With an increase in the sample size by another 50% including a reasonable number of children and adults, the study would have evaluated the changes in DENV nucleic acid and NS1 antigen in paediatric and adult DF and DHF patients. Use of quantitative tests to detect these markers would have helped to assess the changes in the concentrations of these viral markers in DF and DHF patients in different days of fever.

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References

1. Senaratne TN, Noordeen F. Diagnosis of dengue in Sri Lanka: improvements to the existing state of the art in the island. *Trans R Soc Trop Med Hyg.* 2014;108:685–91.
2. Vijayakumar TS, Chandy S, Sathish N, Abraham M, Abraham P, Sridharan G, et al. Is dengue emerging as a major public health problem? *Indian J Med Res.* 2005;121:100–7.
3. Guzman MG, Halstead SB, Artsob H, Buchy P, Farrar J, Gubler DJ, Hunsperger E, Kroeger A, Margolis HS, Martínez E, Nathan MB, Pelegrino JL, Simmons C, Yoksan S, Peeling RW. Dengue: a continuing global threat. *Nature Rev in Microbiol.* doi: [10.1038/nrmicro2460](https://doi.org/10.1038/nrmicro2460).
4. Senaratne T, Wimalaratne H, Alahakoon DGS, Gunawardane N, Carr J, Noordeen F. Characterization of dengue virus infections in a sample of patients suggests unique clinical, immunological and virological profiles that impact on the diagnosis of dengue and dengue haemorrhagic fever. *J Med Virol.* 2016;88:1703–10.
5. Jacobs MG, Robinson PJ, Bletchly C, Mackenzie JM, Young PR. Dengue virus nonstructural protein 1 is expressed in a glycosyl-phosphatidylinositol-linked from that is capable of signal transduction. *FASEB J.* 2000;14:1603–10.
6. Alcon S, Talarmin A, Debruyne M, Falconar A, Deubel V, Flaman M. Enzyme-linked immunosorbent assay specific to dengue virus type 1 non-structural protein NS1 reveals circulation of the antigen in the blood during the acute phase of disease in patients experiencing primary or secondary infections. *J Clin Microbiol.* 2002;40:376–81.
7. Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase- polymerase chain reaction. *J Clin Microbiol.* 1992;30:545–51.
8. Dussart P, Petit L, Labeau B, Bremond L, Leduc A, Moua D, Matheus S, Baril L. Evaluation of two new commercial tests for the diagnosis of acute dengue virus infection using NS1 antigen detection in human Serum. *PLoS Negl Trop Dis.* doi: [10.1371/journal.pntd.0000280](https://doi.org/10.1371/journal.pntd.0000280).
9. Kumarasamy V, Wahab AH, Chua SK, Hassan Z, Chem YK, Mohamad M, Chua KB. Evaluation of a commercial dengue NS1 antigen-capture ELISA for laboratory diagnosis of acute dengue virus infection. *J Virol Methods.* 2007;140:75–9.
10. Chuansumrit A, Chaiyaratana W, Pongthanapitsith V, Tangnaratthakrit K, Lertwongrath S, Yoksan S. The use of dengue nonstructural protein 1 antigen for the early diagnosis during the febrile stage in patients with dengue infection. *Pediatr Infect Dis J.* 2008;27:43–8.
11. Libraty DH, Young PR, Pickering D, Endy TP, Kalayanarooj S, Green S, Vaughn DW, Nisalak A, Ennis FA, Rothman AL. High circulating levels of the dengue virus non-structural protein NS1 early in dengue illness correlate with the development of dengue hemorrhagic fever. *J Infect Dis.* 2002;86:1165–8.
12. Hang VT, Nguyen NM, Trung DT, Tricou V, Yoksan S, Dung NM, Ngoc TV, Hien TT, Farrar J, Wills B, Simmons CP. Diagnostic accuracy of NS1 ELISA and lateral flow rapid tests for dengue sensitivity, specificity and relationship to viraemia and antibody responses. *PLoS Negl Trop. Dis DrosophilaInfServ.* 2009;3:e360.
13. Thomas L, Najjioullah F, Verlaeten O, Martial J, Brichler S, Kaidomar S, Moravie V, Cabié A, Césaire R. Relationship between nonstructural protein 1 detection and plasma virus load in Dengue patients. *Am J Trop Med Hyg.* 2010;83:696–9.
14. Lin SF, Liu HW, Chang CS, Yen JH, Chen TP. Hematological aspects of dengue fever. *Gaoxiong Yi Xue Ke Xue Za Zhi.* 1989;5(1):12–61.
15. Martina BE, Koraka P, Osterhaus AD. Dengue virus pathogenesis: an integrated view. *Clin Microbiol Rev.* 2009;22(564–581):1.
16. Falconar AK. The dengue virus non-structural-1 protein (NS1) generates antibodies to common epitopes on human blood clotting, integrin/adhesin proteins and binds to human endothelial cells: potential implications in haemorrhagic fever pathogenesis. *Arch Virol.* 1997;142:897–916.