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Research Article

Diagnosis of Dengue Infection Cases by Serum Lactate Dehydrogenase Profiling Compared to Dengue NS1 Antigen and Anti-dengue IgM Antibody Detection: A Retrospective Study

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Abstract

Dengue is an acute infection with potential fatal complications; the specific antibody detection for which is an indirect method, and the novel parameter NS1 is reliable for diagnosis of dengue infection, while the serum LDH has been reported to be elevated in severe dengue. This study attempts to evaluate the association of serum LDH with NS1 antigen and IgM in dengue. The serum NS1 and IgM of dengue cases, by ELISA, were estimated from index values by dividing absorbance at 450 nm with cut-off values. Total LDH was measured using autoanalyzer. Electropherotyping of LDH was done in 7.5% polyacrylamide gels. The NS1 and IgM parameters were compared with LDH levels, and analyzed statistically. The NS1 antigens were 35.23 ± 5.37 U (normal < 9 U) with 634.67 ± 350.55 U/L LDH (normal ≤250 U/L) (r = -0.57) among 32.33 ± 7.13 years patients and IgM antibodies were 52.38 ± 7.68 U with 451.67 ± 119.74 U/L LDH (r = -0.13) among 25.92 ± 5.01 years patients. The electropherotype showed intense and greater band pattern when IgM was detected compared to NS1 antigen. Appearance of dengue NS1 antigen as well as anti-dengue IgM in the early phase of infection was inversely related to the level of serum LDH, and increased LDH activity was found when IgM was detected. Thus, LDH might be useful as predictive tool in the early course of dengue infection.

Keywords: Dengue; NS1 Antigen; Anti-DENV IgM Antibody; ELISA; Serum Lactate Dehydrogenase; PAGE Analysis

Abbreviations

DENVs: Dengue Viruses; DF: Dengue Fever; DHF: Dengue Haemorrhagic Fever; DSS: Dengue Shock Syndrome; ELISA: Enzyme-Linked Immunosorbent Assay; GAS: Group A Streptococci; IgM: Immunoglobulin M; LDH: Lactate Dehydrogenase; NS1: Non-Structural 1; PAGE: Polyacrylamide Gel Electrophoresis; WHO: World Health Organization

Introduction

Dengue, which is a mosquito-borne disease vectored principally by *Aedes aegypti* and secondarily by *Aedes albopictus*, is caused with the infection of dengue viruses (DENVs; 4 serotypes: DENV1, DENV2, DENV3 and DENV4) belonging to the *Flavivirus* genus under Flaviviridae family [1]. The disease is prevalent in tropics and sub-tropics over the world and is associated with endemic cycle and in epidemic transmission cycle too. The DENV infection imposes a huge burden 'to health services, to families and to the economic systems' in endemic areas, including India [1-3]. According to 1997 WHO (World Health Organization) classification [4], the clinical spectrum caused due to the infection of DENVs (dengue viruses) ranges from asymptomatic to DF (dengue fever), DHF (dengue haemorrhagic fever) and DSS (dengue shock syndrome). However, as per the 2009 WHO classification [5], dengue illness is categorized into three distinguished phases, such as the 'acute phase, the critical phase and the convalescent phase [1].

The clinical features of dengue are mostly non-specific and imitate many other infections, and hence there have been the chances of misinterpretation of the disease. In addition, no specific treatment protocol is on hand and no vaccine is available for against the infection of dengue. Only, the early and accurate diagnosis of DENV infection, thus, might help in prompt patient management as well as in controlling the disease. Among many, the serological means of detection of DENV infection, dengue NS1 ELISA has been the more useful assay during acute phase, whereas dengue specific IgM ELISA remains a reliable option during the convalescent period [1,6].

The serum LDH (lactate dehydrogenase) levels have been reported to be increased in DF, and studies showed that LDH levels are higher in DHF and DSS cases [7], while an early elevation of LDH was recorded as an independent predictor of DHF [8]. As per the report of Perveen., *et al.* [9], at the time of hospital stay, patients having DHF had higher level of LDH, compared to the patients with DF. The LDH level was found to be increased in dengue cases (563 U/L), compared to normal individuals (160 - 320 U/L), as has been reported by Shameemunnisa and Jenith [10]. Thus, the higher serum LDH levels have been considered to be the diagnostic markers

of dengue infection cases, but the report on LDH isoforms analysis, by PAGE, has not been made, based upon our knowledge and belief, at least from our part of the globe.

The facts mentioned herein prompted us, in the current study, to perform ELISA assays for dengue NS1 protein and anti-DENV IgM antibody detection, in addition to the serum LDH assays as well as profiling of the LDH, in order to diagnose the early DENV infection. Therefore, the aim of this study was to verify the correlations, if any, between the serum LDH levels and dengue NS1 antigen as well as anti-dengue IgM antibody in dengue cases.

Materials and Methods

Serum samples

The serum samples (n = 12) were retrospectively collected from randomly selected blood samples from suspected cases (adults: 22 - 65 years of age, both males and females) of dengue, in terms of clinical representations (in between day 1 and day 3). The serum samples (n = 4) from normal individuals were utilized as the controls. The separated serum samples were refrigerated (2 - 8^{o}C) and/or stored frozen (≤ -20^{o}C) for further analysis. Since, there was no direct involvement of the subjects in the current study, informed consent from cases and controls were not applied [11], and no ethical clearance was made because of the retrospective procurement of samples utilized in the current study [12].

Detection of NS1 antigen and IgM by ELISA

The serum samples (n = 12) were grouped into two, each having equal number of samples, and were studied for dengue NS1 antigen ELISA (n = 6) as well as anti-dengue IgM ELISA (n = 6). For the detection of dengue NS1 antigen and anti-dengue IgM, the serum samples were processed as per the instruction given in the leaflet from Panbio Dengue NS1 antigen ELISA protocol (Panbio, Australia). The serum NS1 antigens and IgM antibodies of dengue cases, by ELISA, were estimated from index values by dividing absorbance at 450 nm with cut-off values. For NS1, an index value of > 1.1 (equivalent to > 11 units) were indicative of positive for dengue infection. The dengue IgM capture ELISA determined the level of IgM antibodies to dengue in serum samples of dengue cases.

Estimation of serum LDH

The serum samples separated from blood of cases (DENV infection) and controls (DENV negative) were subjected to the enzymatic estimation of total serum LDH using standard protocol (Biochemistry assay kit, ELITech, France), in fully automated analyzing machine (Selectra Pro S, ELITech, France). The LDH levels were compared among dengue cases between NS1grroup and IgM group. The estimated LDH values were expressed in U/L, and the serum LDH levels of > 250 U/L were defined as elevated, since this is the upper limit of normal in the laboratory.

Profiling of serum LDH isoforms by PAGE

The LDH profiling by PAGE was done following standard protocol, for randomly selected serum samples: (n = 6, from cases, and n = 1, from normal), as has been mentioned elsewhere [12]. Briefly, a measured volume of the procured serum samples, each containing an equal quantity of LDH, were loaded on to the 7.5 % native PAGE system to resolve the LDH isoforms in dengue cases and control (DENV negative persons). After electrophoresis (at 120 V for 4 h), the gel was developed for distinct staining of LDH. The gel, on electrophoresis (at 120 V for 4 h), was developed in LDH staining dye [NAD (10 mg), NBT (10 mg), PMS (1 mg) and lithium lactate (5 ml)] for 30 minutes, and kept in the dark, at room temperature, for ≈ 20 minutes, till LDH bands were seen in visible light. After washing and destaining, the gel was photographed, stored in a solution prepared glacial acetic acid (7%) and glycerol (10%) in distilled water (83%) [13].

Statistical analysis

The unit values from dengue NS1 antigens and anti-dengue IgM antibodies ELISA tests, as well as, the serum LDH levels between the two groups (NS1 antigen: n = 6 and IgM antibody: n = 6) were compared and analyzed statistically, following t-test [14]. Pearson's correlation coefficients were determined to assess the association between the serum LDH levels and NS1 antigens values as well as between serum LDH levels and IgM antibodies values [14]. The p values ≤ 0.05 were considered significant.

Results and Discussion

The increasing trend of transmission of DF as well its most severe forms: DHF and DSS, remain the major public health problem around the globe, especially in developing countries, where there are limited access to preventive and diagnostic resources, including India [3]. In the current study, the suspect cases of dengue (n = 6) had clinical manifestation of fever (Figure 1), headache with retro-orbital pain, rashes and thrombocytopenia. Yang., *et al.* [15] reported the prominent dengue features with clinical relevance: thrombocytopenia (mean platelet count 85.90 × 10^{9}/L, compared to a mean platelet count 81.88 × 10^{9}/L in DHF cases), fever (mean temperature 39.2^{0}C) and rash in most of the DHF cases.

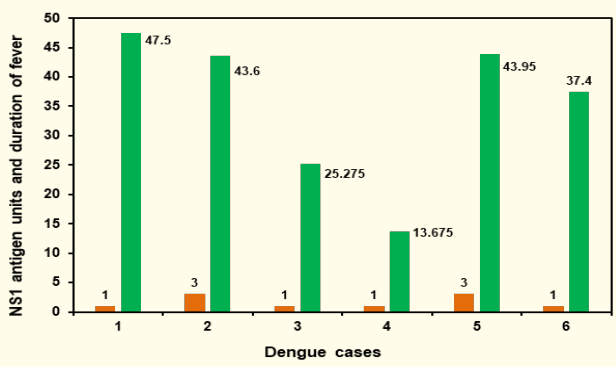

Figure 1: Dengue NS1 antigen unit values (digits as represented with green columns) in cases at two different days of onset of fever (values as represented with orange columns).

The ELISA unit values in detecting dengue NS1 antigens and anti-dengue IgM among dengue cases along with their serum LDH levels are represented in Table 1. The dengue has been recognized as a single but dynamic disease with varied clinical representations ranging from asymptomatic condition to severe DHF and DSS courses leading to high morbidity and mortality [16], and as such in the dearth of anti-dengue antiviral drug, the clinicians face great challenges to determine the disease severity at the early stage of infection, for proper intervention and effective management, in reducing the disease complication and death [17]. The IgM antibod¬ies specific to DENV are detectable on 4 to 6 days after the onset of fever, or after viremia ends [18], and hence, basically the serologic test that detects IgM by ELISA includes the most usual method in the diagnosis of DENV infection. On the other hand, the dengue NS1 antigen has currently been regarded as an important biomarker for early detection of DENV infection, because of its abundance in patient serum at early stage of infection [19]. Thus, detection of dengue NS1 antigen signifies a novel approach for acute dengue diagnosis. In the current study, the mean values of dengue NS1 antigens among the dengue cases were 35.23 ± 5.37 U (normal < 9 U) with 634.67 ± 350.55 U/L LDH (normal ≤ 250 U/L) (r = -0.57), among 32.33 ± 7.13 years patients (Table 1).

Detection criteria	Age (Year)	Absorbance (450 nm)	ELISA Unit value	LDH level (U/L)
NS1 antigen (n = 6)	32.33 ± 7.13	1.41 ± 0.22	35.23 ± 5.37	634.67 ± 350.55
IgM antibody (n = 6)	25.92 ± 5.01	1.05 ± 0.15	52.38 ± 7.68	451.67 ± 119.74
p value	0.48	0.21	0.1	0.66

Table 1: Assessment of dengue cases by NS1 ELISA (n = 6) and IgM ELISA (n = 6) and the LDH values among the cases.

The dengue specific IgM antibod¬ies are finely demonstrable ≈ 4 - 6 days after the onset of fever. As has been shown by Guzman and Kouri [20], by day 5 of symptomatic phase, 80% cases possessed detectable IgM antibodies, while most (93-99%) of the patients had measurable IgM by day 6 to day 10. Therefore, ELISA in detecting dengue NS1 antigen in serum of DENV infected person, in acute phase of the disease, may provide a supportive method

in association with existing diagnostic tests for dengue infection [21-23]. The combined antigen (NS1)-antibody test might augment the diagnostic effectiveness for early detection of DENV infection, as has been reported by the earlier authors [24,25]. Currently, the commercially available dengue NS1 antigen and anti-dengue IgM detection kits are useful in the diagnosis of acute dengue infection worldwide [26-28]. Herein, the mean values of anti-dengue IgM antibodies were recorded as 52.38 ± 7.68 U/L, with 451.67 ± 119.74 U/L LDH (r = -0.13) among 25.92 ± 5.01 years patients (Table 1).

The current study demonstrates among early dengue cases, an association between increased serum LDH levels (> 250 U/L) and dengue NS1 antigen as well as anti-dengue IgM antibodies (Figure 2 and Figure 3). As has been represented by Yang., *et al.* [15], an increase in LDH level was recorded among DF and DHF cases. The mean LDH levels in febrile phase, critical phase of illness and convalescence stage of dengue were recorded as 550.33 IU/L, 748.68 IU/L and 406.23 IU/L, respectively, while, the mean LDH level on the day of least platelet count was 608.87 ± 228.67 IU/L [29]. Shankar., *et al.* [30] reported the development of dengue severity among patients having higher serum LDH level (> 600 IU/L). Perveen., *et al.* [9] demonstrated higher LDH levels among DHF cases (mean 618.38 U/L), when compared to the DF cases (mean 316.45 U/L). Sirikutt., *et al.* [7] reported LDH levels in dengue patients as > 500 IU/L, while the values in dengue negative subjects was recorded as < 500 IU/L; the authors also noted an increased level of LDH, at the end of febrile phase, among DHF and DSS cases only: the LDH levels as estimated in DHF, DSS, DF and non-dengue individuals were 1,060.7, 1,180.7, 787.2 and 423.8 IU/L, respectively [7]. Mehta., *et al.* [31] reported an average LDH level in non-fatal dengue cases as 4750 IU/L (range: 2500 - 11328 IU/L), while the value was 18750 IU/L (range: 13500 - 75000 IU/L), in fatal dengue cases. Villar-Centeno., *et al.* [8] observed an increase in the level of LDH in DHF patients (612.6 - 810.6 U/L), when compared with DF patients (524.9 - 599.7 U/L). Mittal., *et al.* [28] estimated the date of discharge of patients as calculated based on the levels of LDH on the day of least platelet count using LDH in dengue scale. In the current study, the diagnosis of dengue was established by increased level of serum LDH levels (> 250 U/L), in addition to the dengue NS1 antigen as well as IgM antibody tests.

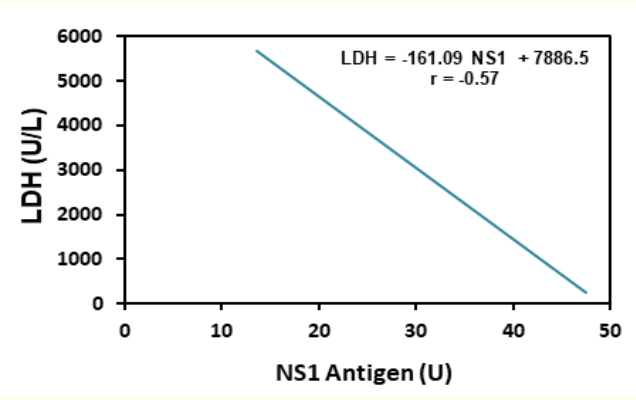

Figure 2: Variation of total serum LDH activity with NS1 antigen level among dengue cases.

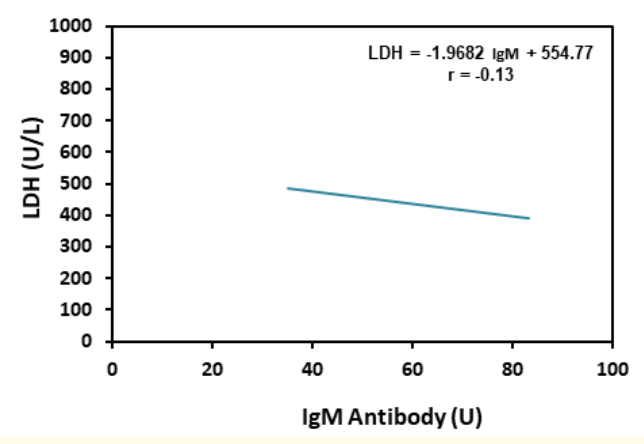

Figure 3: Variation of total LDH activity with IgM antibody level among dengue cases.

The elevated serum LDH levels have been reported to be the diagnostic markers of various illnesses in humans, including the life-threatening bacterial infection [32]. This study demonstrated the serum LDH profiles in dengue cases as well as non-dengue subjects, first time, within and around Malda, India, in order to confirm the intensity of LDH bands in the stained gel (Figure 4), from PAGE system, with the estimated levels of serum LDH in cases as well as non-dengue individuals. Earlier, in GAS infection cases, an increased expression of serum LDH: LDH1, LDH2 and LDH3 isoforms was reported, with their (serum LDH isoforms) usefulness as a 'follow-up marker' of recovery of GAS infection [12]. In the current study, band intensity of LDH1, LDH2 and LDH3 were in the order of LDH2 > LDH3 > LDH1; the band corresponding to LDH4 was found in lane 4, and very weak in lane 3; however, bands in other lanes, corresponding to LDH4 were very feeble. Similarly, LDH5 was very faint in all the lanes. In case of dengue, cathodic increases of band intensity are indicative of skeletal muscle and liver (LDH5) damages, and liver and kidney (LDH4) damages, while anodic increases are associated with damage of heart and RBCs (LDH1); mid zone increases are associated with spleen, platelets and lymphoid tissue damage.

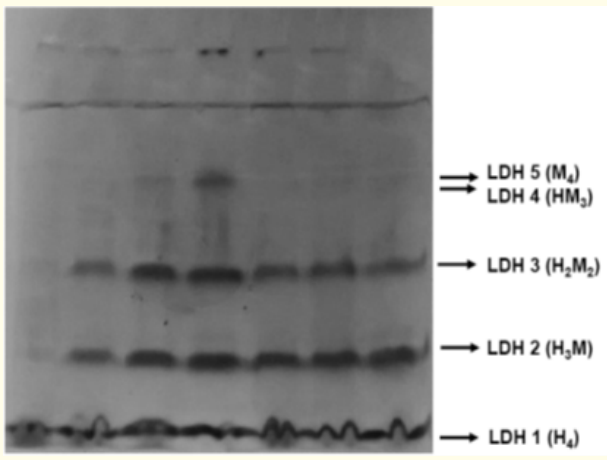

Figure 4: LDH isoenzymes profiles in dengue infection by polyacrylamide gel electrophoresis. Lane 1: serum sample from non-dengue individual; Lane 2 - 7: serum samples from dengue cases. Note the very faint band inensity of LDH isoforms in serum sample from non-DENV person (Lane 1).

Conclusion

This study is the first to demonstrate the serum LDH profiles in dengue illness cases, from our part of the globe. The decrease in level of serum LDH was proportional to the rise of dengue NS1 antigen titers. Appearance of dengue NS1 antigen in the early course of infection was inversely related to the level of serum LDH, indicating the rise of infection being detected with LDH as a predictive tool. However, the appearance of anti-dengue IgM in sera was associated with declining trend of LDH values, although with a weakly negative correlation coefficient and much lower level of LDH values compared with NS1 antigen detection.

The dengue NS1 antigen might be useful in early and precise diagnosis of acute phase of dengue illness, and the combination of dengue NS1 antigen and IgM ELISA on single sample can improve the diagnosis of dengue. The increased levels of serum LDH was found to be associated with DF and sever DF and, thus, serum LDH may be useful as the early predictive marker of dengue illness for the benefit of patients for required and prompt therapies.

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