The association of lipopolysaccharide and inflammatory markers with severe dengue infection

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Abstract

Background Although serum lipopolysaccharide (LPS) was shown to associate with development of severe dengue, the reasons for high LPS and its subsequent involvement in disease pathogenesis are not known.

Methods: We assessed LPS, lipopolysaccharide binding protein (LBP), CRP, IL-18, procalcitonin in patients with acute dengue fever (DF=129) and dengue haemorrhagic fever (DHF=64) and correlated these observations with the presence of comorbid illnesses, concurrent bacteraemia and clinical disease severity.

Results: LPS levels were significantly (p=0.01) higher in patients with DHF, compared to those with DF. 45 (70%) of those with DHF and 63 (49%) of those with DF had detectable LPS and therefore, presence of LPS was significantly associated with DHF (p=0.005, OR=2.48, 95% CI: 1.29 to 4.64). Those with metabolic diseases, 22/29 (75.9%) and those with atopic diseases 17/22 (77.3%) were significantly more likely to have detectable LPS (p=0.025, OR=2.9, 95% CI- 1.17 to 7.59) and (p=0.039, OR=3.06, 95% CI-1.07 to 7.81) respectively, than others. LPS, LBP and CRP levels were high at the febrile phase, before onset of plasma leakage and reduced towards to the critical phase. The CRP levels were significantly higher (p=0.03) in early illness (≤3 days of illness) in those who progressed to develop DHF when compared to those who developed DF. Those who had detectable serum LPS also had a significantly higher CRP (p=0.01). Although there was no difference in procalcitonin (PCT) levels in patients with DF and DHF, the PCT levels were significantly higher in those who had detectable serum LPS (p=0.02).

Conclusions: LPS levels were higher in patients with DHF and associated with high levels of other inflammatory markers. Since LPS levels were highest during early infection and were significantly more likely to be present in those with comorbid illnesses, the possible role of LPS in disease pathogenesis, should be further investigated.

Background

Dengue viral infections represent one of the most important emerging mosquito borne viral infections, resulting in approximately 100 million symptomatic infections annually [1]. Over 70% of these infections occur in Asia [1]. Although the mortality rates due to dengue have declined in South Asia, the incidence has markedly increased from 285.3 per 100,000 individuals in 1990 to 1371.1 in 2013 [2]. The estimated annual global cost of dengue is $8.9 billion [3], which is a huge burden to resource poor developing countries. As there is no specific treatment for dengue, intense monitoring for complications and meticulous fluid management is currently the only option in the management of dengue.

While the majority of individuals who are infected with the dengue virus (DENV) develop asymptomatic or an undifferentiated febrile illness, it can cause severe clinical disease manifestations such as dengue

haemorrhagic fever (DHF) and organ involvement in 10 to 25% of individuals [4, 5]. Concurrent bacteraemia has been reported in 0.18 to 7% of those with acute dengue infection and is associated with higher mortality rates [6]. Among those who were admitted to intensive care units for the management of severe dengue, in Singapore and Taiwan, concurrent bacteraemia was seen in 15.5% of patients [7] and in 14.3% to 44.3% of those who succumb to their illness [6]. Higher C-reactive protein (CRP) levels on admission, along with higher leucocyte counts, lower serum albumin levels (indicating more leakage), a prolonged activated partial thromboplastin time (APTT) and an advancing age have been shown to be risk factors for development of concurrent bacteremia[7, 8]. Two studies conducted in general medical wards of Singapore revealed that bacteria which are frequently isolated in such patients are *Staphylococcus aureus*, and gram negative bacteria such as *Enterococcus faecalis*, *E. coli*, *Klebsiella pneumonia*, Acinetobacter species and *Pseudomonas aeruginosa*[8, 9].

It has been postulated that bacteraemia may occur due to intestinal barrier dysfunction that occurs in severe dengue, leading to the translocation of gut microbes [10, 11]. Weg de Van et al. showed that serum lipopolysaccharide (LPS) levels were significantly higher in those with plasma leakage and the LPS levels were associated with immune activation [10]. Although it was suggested that high serum LPS was due to microbial translocation, it was not clear how many patients with severe dengue had elevated LPS levels, its association with the presence of comorbid illnesses, how many patients with LPS in their sera developed concurrent bacteraemia and if so the type of bacteria isolated. LPS is a potent stimulus for production of platelet activating factor (PAF) and other inflammatory cytokines from monocytes[12, 13]. We found that LPS appeared to act synergistically with the DENV by inducing significantly higher levels of PAF in DENV infected monocytes than in uninfected monocytes[14]. Since PAF was found to be an important mediators of vascular leak [15], it is possible that LPS acts with the DENV in acute dengue, initiating or potentiating PAF induced vascular leakage. This could further worsen intestinal barrier dysfunction, possibly leading to concurrent bacteraemia.

We previously reported that the activity of secretory phospholipase A2 (sPLA2) was significantly higher in patients with DHF compared to those with DF[16]. LPS is one of the most potent mediators that potentiate sPLA2[17]. Since sPLA2 activity was found to be highest during early illness, it is possible that pre-existing LPS in patients with comorbid illnesses such as diabetes and obesity, possibly due to metabolic endotoxaemia, could lead to activation of sPLA2 and also contribute to generation of PAF. As patients with metabolic diseases have been shown to have low grade endotoxaemia [18], presence of LPS at the time of infection with the DENV could aggravate disease severity. The presence of metabolic diseases such as diabetes and hypertension have been shown to be risk factors for the development of severe dengue [19, 20] and also carrying a higher risk of fatalities [21]. In addition, patients with metabolic diseases are shown to be more likely to develop concurrent bacteraemia during dengue which also increased the likelihood of fatal dengue [22]. Therefore, it would be important to determine if low-

grade endotoxaemia seen in patients with metabolic diseases [23], contribute to severe dengue and subsequently concurrent bacteraemia.

In this study, we sought to investigate if presence of serum LPS was a risk factor for severe dengue in a Sri Lankan cohort, and also investigated whether high serum LPS levels preceded vascular leakage or followed vascular leakage. We further proceeded to determine the association of lipoprotein binding protein (LBP), CRP and procalcitonin with severe dengue infection.

Methods

Patients

We recruited 193 adult patients with confirmed acute dengue infection who were admitted to the National Institute of Infectious Diseases Sri Lanka during the years 2016 to 2018. In 103, only one blood sample was collected on a mean day 4 of illness (SD± 1), whereas in 90 of these patients, blood samples were collected daily in the morning (9.00am) from the time of admission to discharge from the hospital. Informed written consent was taken from all patients. Day one of illness was considered as the first day of fever. Clinical features such as fever, abdominal pain, vomiting, bleeding manifestations, hepatomegaly, blood pressure, urine output were recorded several times a day in all patients. The full blood counts were monitored several times a day (three to four times) and liver function tests and ultrasound examination to detect the presence of fluid was carried out once a day. The clinical disease severity was classified according to the 2011 World Health Organization (WHO) guidelines [24]. Accordingly, patients who had ultrasound evidence of fluid leakage or a rise in the haematocrit of $\geq$ 20% from their baseline level were classified as having DHF. Based on this classification, of the patients in whom daily samples were obtained, 41/90 patients had DHF and 49/90 patients had DF. Of the 103 patients in whom only one blood sample was obtained 23 patients were classified as having DHF and 80 patients as DF. All clinical disease outcomes such as the extent of fluid leakage based on ultrasound scans and the number of fluid boluses required, type of bleeding manifestations, extent of liver injury as measured by the rise in liver transaminases and whether blood was given was recorded. In those patients who were found to have severe leakage, serum electrolytes, serum creatinine and coagulation parameters were recorded.

Quantification of serum LPS levels

Serum was separated under sterile conditions in a laminar air flow *hood and stored at -80^0 C and repetitive freeze-thaw cycles were avoided. The LPS levels were determined with a commercially available ELISA assay according to the manufacturer's instructions (Human LPS ELISA kit, CUSABIO, China). Serum*

samples were diluted to 1:2 with sample diluent and heat inactivated at 70^0C for 20 min prior to use in the assay according to manufacturer's instructions.

Detection of IL-18, Procalcitonin (PCT), LBP, PAF and CRP

IL-18, PCT and LBP levels were determined in serum samples obtained daily from the time of admission to discharge, using the enzyme linked immunosorbent assay (ELISA) human IL-18 (Abcam, UK), LBP assay (Abcam, UK) and PCT assay (Abcam, UK). PAF levels were assessed in patients in whom only one blood sample was obtained, using the ELISA human PAF (CUSABIO, China). The ELISAs were performed and results were interpreted according to the manufacturer's instructions. *The CRP levels were assessed by a quantitative immunoturbidimetric method using the Thermo Scientific^{TM} Indiko^{TM} system. CRP levels were expressed as mg/l and levels <10 mg/l considered as normal.*

Confirmation of acute dengue infection

The samples were initially screened by using rapid strip tests (SD diagnostics, South Korea) for NS1 antigen and dengue IgM and IgG antibodies. Samples tested positive for either NS1 antigen or antibody was included in the study. Acute dengue infection was confirmed in serum samples using a quantitative real-time PCR (see below).

Serotyping of the DENV and quantifying the viral loads

Serotyping of the DENV and quantifying viral loads were carried out as previously described [25]. Extraction of viral RNA in serum was carried out using QIAamp Viral RNA Mini Kit (Qiagen, USA) and transcribed to cDNA using High Capacity cDNA reverse transcription kit (Applied Biosystems, USA) according to manufacturer's protocol. Quantitative real time PCR was performed using the CDC real time PCR assay for detection of the dengue virus and Oligonucleotide primers and a dual labeled probes for DEN 1-4 serotypes were used (Life technologies, USA) based on published sequences[26]. The reaction was performed in an Applied Biosystems 7500, 96-well plate detection system for 40 cycles of 3 sec at 95ºC and 30 seconds at 60ºC. The threshold cycle value (Ct) for each reaction was determined by manually setting the threshold limit. A multiplex method was optimized to quantify the four serotypes in a single reaction and viral loads of unknown samples were determined using the optimized multiplex real-time protocol.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 7. As the data were not normally distributed, non-parametric statistical methods were used for data analysis. The differences in LPS, CRP, PAF and PCT in single samples were done using the two tailed Mann-Whitney U-test. The degree of association between serum LPS levels and other markers was analyzed using Spearman correlation coefficient test. Changes in the LPS, CRP, IL-18 and LBP levels throughout the course of illness was compared using the Holm-Sidak method. Corrections for multiple comparisons were completed using the Holm-Sidak method and the significance value was set at 0.05 (alpha). Degree of association between the presence of metabolic disease, the presence of LPS and clinical disease severity was expressed as the odds ratio (OR), which was obtained from standard contingency table analysis by Haldane's modification of Woolf's method.

Results

Patient characteristics

Of the 193 patients, 64 (33%) had DHF and 129 (67%) had DF based on the 2011 WHO dengue disease classification[24]. The mean age in those with DHF was 30.5 years (± SD 13.3) and 32.8 years (± SD 14.9) in those with DF. The average day of recruitment of patients to the study was on day 4 (SD±1) of illness. The clinical and laboratory features of these 193 patients are shown in table 1. There were no fatalities and only 7 (3.6%) developed shock.

Serum LPS levels in patients with acute dengue

In this study, we initially measured LPS in serum samples of all 193 patients. We found that serum LPS levels on day 4 (SD± 1) of illness were significantly (p=0.01) higher in patients with DHF (median- 11.36, IQR- 0 to 28.5 pg/ml), when compared to those with DF (median-0, IQR- 0 to 17.63 pg/ml). LPS was detected in 45 (70%) of those with DHF and 63 (49%) of those with DF. Therefore, patients with DHF were significantly more likely to have detectable LPS in their sera compared to those with DF (p=0.0055, OR=2.48, 95% CI: 1.29 to 4.64). Among the 64 patients with DHF, there were 7 patients who developed

dengue shock syndrome (DSS). There was no significant difference (p=0.62) between the LPS levels in patients with DHF who did not develop shock (median-11.28, IQR- 0 to 29.23 pg/ml) compared to those who developed DSS (median-13.99, IQR 8.25 to 22.91 pg/ml) (Fig 1A).

31/193 (16%) patients with acute dengue had received antibiotics due to clinical suspicion of a concurrent bacterial infection (recurrence of fever, productive cough and sore throat). Of these patients 10/31(32%) had positive bacterial cultures and the predominant organisms identified were *E. Coli, Streptococcus, Staphylococcus species and Pseudomonas aeroginosa.* 15/31(48%) patients with suspected concurrent bacteraemia and 7/10 (70%) patients with culture confirmed bacteraemia had DHF, while 3 (30%) had DF. Of those with DSS, 2/7 (28%) had suspected concurrent bacteraemia and only 1/7 had culture confirmed bacteraemia. Only 16/31 (52%) patients with suspected concurrent bacteraemia and only 5/10 (50%) patients with culture confirmed bacteraemia had detectable levels of LPS in serum.

Kinetics of changes in LPS levels throughout the course of illness

Since we found LPS levels were higher in patients with DHF than in those with DF, we sought to investigate the kinetics of LPS throughout the course of illness in those with DF and DHF in order to understand if rise in LPS precedes or follows the onset of the vascular leakage phase. We were only able to assess the changes in LPS levels throughout the course of illness in 90/193 patients in our cohort. 41/90 patients had DHF while 49/90 had DF. In this subset of patients, LPS was detected in 25 (61%) patients with DHF and 23 (47%) patients with DF. Although we found that LPS levels were significantly higher in patients with DHF when analyzing our larger cohort of patients (n=193), this increase was not observed when analyzing the changes in LPS levels in this sub cohort. In this sub-cohort LPS levels were not significantly higher at any time point of illness, although there was a trend for LPS levels being higher in patients with DHF during early illness. (Fig 1B).

We also proceeded to determine if higher viral loads associate with clinical disease severity and the relationship with the viral loads with serum LPS levels. The viral loads on day 4 (SD± 1) of illness in those with DF (median 302, IQR 0 to 11430 viral copies/ml) were similar to those with DHF (median 324, IQR 0 to 2190 viral copies/ml, p=0.48). Interestingly, we observed a statistically significant but weak correlation between the degree of viraemia and serum LPS levels (Spearmans'r=-0.28, p=0.004).

Association of LPS levels with presence of comorbid illnesses

As patients with metabolic diseases have been shown to have low grade endotoxaemia [18], presence of LPS at the time of infection with the DENV could aggravate disease severity. Therefore, in order to find

out if patients with metabolic diseases had higher LPS and more severe disease we analyzed the association of serum LPS levels with presence of comorbid illnesses.

47/193 (24%) patients in our cohort had co-morbid illnesses. 22/47 (47%) had atopic diseases such as asthma, allergic rhinitis and 29/47 (62%) of them had metabolic diseases such as hypertension, diabetes, ischaemic heart disease and hyperlipidemia. 22/29 (75.9%) patients with metabolic diseases and 17/22 (77.3%) of patients with atopic diseases had detectable LPS in their sera. Therefore, those with metabolic diseases were significantly more likely (p=0.025) to have detectable LPS than those who did not (OR=2.9, 95% CI- 1.17 to 7.59) and those with atopic diseases (asthma and allergic rhinitis) were also significantly more likely (p=0.039) to have detectable LPS in their sera compared to those without atopic disorders (OR=3.06, 95% CI-1.07 to 7.81). However, those with metabolic disease or atopic disease were not more likely to develop DHF in our cohort as this study was not powered to evaluate such associations. There were no significant differences of viral loads with the presence of any comorbid illnesses (p=0.52) or metabolic diseases (p=0.54) or atopic diseases (p=0.98).

Association between Serum CRP and LPS in patients with acute dengue

Since patients with DHF were more likely to have higher LPS levels, we next sought to investigate the possible association of CRP values with serum LPS. We found that although CRP levels were slightly higher in patients with DHF (median 8.67, IQR 5.34 to 20.33 mg/L) than those with DF (median 6.82, IQR 5.27 to 11.85 mg/L), the difference was not significant (p=0.103) (Figure 2A). In addition, there was no significant difference (p=0.21) between the CRP levels in patients with DHF who did not develop shock (median-8.3, IQR 5.32 to 19.34 mg/L) compared to those who developed DSS (median-15.05, IQR 6.49 to 38.15 mg/L). However, patients with elevated CRP levels (>10mg/L) were significantly more likely (p=0.01) to have detectable LPS in their sera compared to those with normal CRP levels (OR=2.261, 95% CI: 1.206 to 4.169). There was no significant correlation between CRP levels and viral loads (Spearman's r=0.06, p=0.56).

As serum CRP levels were found to be higher in patients with DHF although not significant, we sought to investigate the kinetics of CRP in patients with DHF and DF throughout the course of illness in patients with DHF (n=41) and DF (n=51), as a high CRP in early illness was suggested to have a good predictive value in developing severe dengue[27]. We indeed found that the CRP values in early illness (day 3 of illness) was significantly higher (p=0.03) in those with DHF (median 20.3, range 5.1 to 56 mg/dl) compared to those with DF (median 9.4, range 4.8 to 22.9 mg/dl) (Figure 2B). When we analyzed the association in the kinetics of serum LPS and CRP levels throughout the course of illness, we found that

serum CRP levels statistically significantly but weakly correlated (Spearman's r=0.15, p=0.017) with serum LPS levels (Figure 2C).

Association of serum IL-18 and LPS in patients with acute dengue

As we have previously observed that significant changes occur in levels of serum cytokines in patients with acute dengue at different phases of illness, in order to fully understand the changes in IL-18 levels we evaluated the changes in serum IL-18 levels in patients with DHF (n=30) and DF (n=23) throughout the course of illness. We found that although serum IL-18 levels were higher in patients with DHF compared to those with DF, especially during early illness, these differences were not significant at any time point (Figure 3A). The median values of IL-18 seen throughout the course of illness was similar to the values observed previously in patients with DHF (median values 409.5 pg/ml in early illness to 352.3 pg/ml in late illness) and DF (median values 327.1 pg/ml in early illness to 224.2 pg/ml in late illness) (Fig 3A)[28] and were several folds higher than IL-18 levels seen in healthy individuals[28]. However, serum IL-18 levels did show a statistically significant but weak correlation with serum LPS levels (Spearman's r=0.20, p=0.009) (Figure 3B).

Association of serum LBP with LPS in patients with acute dengue

As LBP has shown to be crucial for the immune responses to LPS, we assessed the LBP levels in patients with DHF (n=30) and DF (n=23) throughout the course of illness. We found that LBP levels were markedly high in both group of patients throughout the course of illness. Although the LBP levels were slightly higher in those with DHF there was no significant difference between both groups at any time point during the course of illness (Fig 3C). The LBP levels remained high throughout (4 to 5-fold higher than values detected in healthy individuals[29]) in patients with both DF and DHF. There was no association between serum LBP and LPS levels (Spearman's r=0.112, p=0.16) (Figure 3D).

Association of serum PAF levels with LPS levels in acute dengue

We previously reported that LPS treated DENV infected monocytes produced significantly more PAF compared to uninfected monocytes treated with LPS alone and DENV infected monocytes untreated with LPS [14]. Since we also reported that PAF was an important cause of vascular leakage in acute dengue[15], we proceeded to determine if PAF levels were higher in those with endotoxaemia. Serum PAF levels were assessed in 103 (DHF=23, DF=80) and there was no significant difference of serum PAF levels on day 4 (SD ±1) between patients with DHF (median 11.63, IQR 9.57 to 14.74 ng/ml) compared to DF (median 11.63, IQR 10.17 to 14.14 ng/ml). There was no difference in PAF levels in those who had LPS (median 11.51, IQR 9.76 to 14.11 ng/ml) in their serum compared to those who did not have LPS

(median 12.39, IQR 10.89 to 14.98 ng/ml) (Figure 4A). Serum PAF levels did not correlate with serum CRP, LPS or PCT levels. However, serum PAF levels did statistically significantly but weakly correlate with serum viral loads (Spearman's r=0.23, p=0.02) (Figure 4B)

Serum procalcitonin (PCT) levels in patients with acute dengue

PCT is considered to be a biomarker that can be useful in differentiating sepsis from other non-infection triggers in critically ill patients[30]. However, it was recently shown that PCT can also be elevated in patients with acute dengue and that PCT levels more than 0.7 ng/ml were associated with DSS [31]. PCT levels above 0.1 ng/ml have been shown to indicate probable sepsis and therefore patients with PCT values over 0.1ng/ml are considered to have an elevated PCT [32, 33]. Therefore, in order to find out the usefulness of PCT in acute dengue to differentiate those who develop complications of acute dengue (DHF, DSS) from those who have concurrent bacteraemia, we evaluated PCT values in our cohort of patients.

Serum PCT levels were not significantly (p=0.24) higher in those with DHF (median-0.11 ng/ml, IQR-0 to 0.30 ng/ml) compared to those with DF (median-0.08 ng/ml, IQR-0.02 to 0.19 ng/ml) (Fig 5 A). Although the PCT was elevated (>0.1ng/ml) in 32 (50%) patients with DHF and 52 (40%) patients with DF, an elevated PCT was not significantly associated (p=0.22) with DHF (OR 1.481, 95% CI: 0.798 to 2.762). There was no significant difference (p=0.29) between the PCT levels in patients with DHF who did not develop shock (median-0.1, IQR 0 to 0.289 ng/ml) compared to those who developed DSS (median-0.2, IQR 0.08 to 0.47 ng/ml) (Fig 5A). In addition, none of the patients in our cohort with DSS had PCT values >0.7 ng/ml. However, the PCT levels were significantly (p=0.02) higher in patients who had detectable LPS in their serum (median-0.1 ng/ml, IQR- 0.03 to 0.27 ng/ml) compared to patients who did not have LPS (median-0.07 ng/ml, IQR 0 to 0.16 ng/ml) (Fig. 5B). Serum PCT levels also statistically significantly but weakly correlated with LPS levels (Spearman's r=0.163, P=0.023) and CRP levels (Spearman's r=0.233, P=0.001) (Fig. 5 C and D).

We also found that 20/29 (69%) of those with metabolic diseases were significantly more likely (p=0.004) to have elevated PCT levels in their sera (OR=3.5, 95% CI- 1.47 to 8.5). However, there was no association between elevated PCT and presence of atopic disorders (asthma and allergic rhinitis).

Discussion

In this study we found that LPS levels were indeed significantly higher in Sri Lankan patients with plasma leakage (DHF) as previously reported in other cohorts [10]. Although LPS was detected at a higher frequency of in patients with DHF (70%), it was also detected in 49% of patients with DF, who did not have any features of plasma leakage. Those who were more likely to have endotoxaemia were those with either metabolic diseases (75.9%) or those with atopic diseases such as allergic rhinitis or asthma (77.3%). As patients with metabolic diseases have been shown to have low grade endotoxaemia and those with endotoxaemia were shown to have a higher risk of developing diseases such as diabetes [18, 34], it is possible that LPS detected in these patients were due to the presence of pre-existing LPS. In fact, there was trend toward LPS levels being higher during early illness rather than during the critical phase in these patients. In addition, there was no difference in the LPS levels in patients with DHF compared to those with DF, especially during the critical phase, which suggests that translocation of gut bacteria due to intestinal barrier dysfunction is unlikely to occur during the critical phase. Although the patients who had metabolic disease were considerably older than those who did not have metabolic disease, numerous studies have shown that endotoxemia does not relate to age but rather to the presence of metabolic diseases [35, 36]. As patients with diabetes and hypertension are known to be at a higher risk of developing severe dengue [19, 20] and also a higher risk of fatalities [21], it is possible that LPS levels in such patients contribute to disease pathogenesis when infected with the DENV.

CRP was shown to be significantly higher in patients with DHF and was a reported as a predictor of shock in acute dengue and was shown that those with dengue shock syndrome had median CRP values of 124.5mg/L, which are values usually only seen in patients with acute bacterial infections [27]. However, only one patient our cohort had CRP values exceeding 50mg/L. This patient who had CRP levels between 120 and 150 during the critical period, did not develop shock, although he did develop acute liver failure. Therefore, CRP levels over 124.5mlg/dL (or any level) was not a predictor of dengue shock syndrome in our cohort.

LBP is crucial in mediating immune responses to LPS and is produced by the liver in response to IL-6, IL-1β and TNFα [37]. LBP levels were several folds higher than reported in healthy individuals in patients with DHF and DF[29]. Although the LBP levels were higher in patients with DHF, it was not significantly higher than patients with DF at any time point of illness. However, the LBP levels were similar or higher than the levels seen in those with gram positive and negative sepsis, especially in early infection. Given that LBP has a very short half-life of a few hours[38], it appears that the stimulants that induce LBP production are highest during early illness. Since LBP levels actually declined rather than increased during the critical phase in patients with DHF, it is unlikely that events that occur during the critical phase, induce LBP production.

PAF is an inflammatory phospholipid, which was shown to be an important mediator of vascular leak[15]. We recently showed that rupatadine, an antihistamine with a PAF-R blocking activity reduced the downregulation in gap junction ZO-1 expression and reduction in trans-endothelial resistance in human endothelial cell lines in vitro, reduced the rise in the haematocrits in a dose dependent manner in dengue mouse models and showed a trend toward reducing vascular leak in patients with acute dengue, when given early [39]. Since the DENV was shown to act synergistically with LPS to produce PAF from monocytes [40], we investigated if LPS could be contributing to increase in the PAF levels in patients with acute dengue. Although we observed a marked difference in the PAF levels in patients with DHF and DF in our previous studies [15, 16], no such relationship was observed in this study, probably due to the timing of the sample collection. In both our previous studies the samples for PAF analysis was collected in the morning at 5.00 a.m. and we showed that while, the PAF levels were markedly high at this time, in the samples collected from the same patients at 1.00 p.m. on the same day, the PAF levels were undetectable or very low and no differences between DF and DHF were seen[15, 16]. In the current study, the samples were collected once a day around 9 to 10 a.m. in the morning and the PAF levels seen, as expected was far lower than those we previously observed in the samples taken at 5.00 a.m. We also observed similar changes in IL-1β and TNFα levels previously[40]. Therefore, we believe that the reasons for low PAF levels in this study, which were similar in patients with DF and DHF were due to the timing of sample collection. Due to the possible diurnal variation in production of these inflammatory lipid mediators and cytokines, it would be important to take into account these natural variations in future studies. Indeed, it was recently shown that mast cells, which are one of the predominant produces of PAF, are driven by circadian rhythms [41].

In this study, we also evaluated the usefulness of PCT in determining the presence of concurrent bacteraemia in acute dengue. Although it was previously reported that PCT levels were significantly higher in patients with DHF and especially those with DSS[31], we did not find such an association, possibly because none of our patients had profound shock. However, PCT levels were associated with the presence of blood endotoxaemia.

Conclusion

In summary, we found that LPS levels were significantly higher in those with DHF compared to those with DF, and that 70% of those with DHF had detectable LPS, compared to 49% of those with DF. Those with LPS were significantly more likely to have either metabolic disease (75.9%) or atopic diseases (77.3%). As the LPS levels were highest in early infection, before the onset of plasma leakage, and since its presence was significantly associated with comorbid illnesses, the LPS levels detected in patients could be due to pre-existing endotoxin in such patients. Since patients with such comorbid diseases are at increased risk

of developing severe and fatal dengue, the role of LPS in disease pathogenesis should be further investigated.

Abbreviations

DF	dengue fever
DHF	dengue haemorrahagic fever
DSS	dengue shock syndrome
LPS	lipopolysaccharide
LBP	lipoprotein binding protein
CRP	C-reactive protein
PAF	platelet activating factor
PCT	Procalcitonin
sPLA2	Secretory phospholipase A2

Declarations

Ethics approval and consent to participate

The Ethical approval was obtained from the Ethics Review Committee of the Faculty of Medical Sciences, University of Sri Jayewardenepura. All patients were recruited following informed written consent.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests

Dr. Gathsaurie Malavige is an Associate Editor for BMC Infectious Diseases.

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Authors' contributions

NLAS carried out the experiments, analysed the data and helped in designing the study and writing the manuscript, SDM recruited the patients and collected the data, LG helped in carrying out the experiments, AW helped in patient recruitment and patient classification, GSO helped in writing the paper, GNM helped in data analysis, designing the study, obtaining funding and writing the paper.

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Table 1

	DHF (n=64)	DF (n= 129)
Clinical features		
Vomiting (%)	15 (23)	21 (16)
Abdominal pain (%)	31 (48)	21 (16)
Diarrhoea (%)	13 (20)	27 (21)
Hepatomegaly (%)	18 (28)	1(0.7)
Bleeding manifestations (%)	3 (5)	0
Pleural effusion (%)	26 (41)	0
Ascites (%)	56 (87)	0
Shock (%)	7 (11)	0
WBC count		
$<4x10^9$/L (%)	41 (64)	94 (73)
Lowest platelet count		
<20000 (%)	36 (56)	5 (4)
20000-50000 (%)	21 (33)	26 (20)
50000-100000 (%)	7 (11)	73 (56)
>100000 (%)	0	25(19)

Table 1: Clinical and laboratory characteristics of patients with DHF and DF recruited for the study.

Figures

Figure 1

Lipopolysaccharide (LPS) levels in patients with acute dengue. LPS levels were measured by ELISA in patients with acute (A) DF (n=129, indicated in red), DHF (n=57, indicated in blue) and DSS (n=7, indicated in green) during on day 4 (SD± 1) of illness. Error bars indicate the median and interquartile range (IQR). *P<0.05 (B) LPS was also measured in patients with DF (n=49) and DHF (n=41) throughout the course of illness. Error bars indicate mean and standard error of mean (SEM).

Figure 2

C-reactive protein (CRP) levels in patients with acute dengue. CRP levels were measured using quantitative immunoturbidimetric method in patients with (A) DF (n=129, indicated in blue) and DHF (n=64, indicated in red) between day 4 and 6 of illness. Error bars indicate the median and interquartile range (IQR). *P<0.05 (B) CRP was also measured throughout the course of illness in patients with DF (n=49) and DHF (n=41) throughout the course of illness. Error bars indicate the mean and the SEM (C) CRP levels significantly and positively correlated with serum LPS levels (Spearmans r=0.15, p=0.01).

Figure 3

Serum IL-18 and lipoprotein binding protein (LBP) levels in patients with acute dengue. (A) Serum IL-18 levels were measured by ELISA in patients with DF (n=23, indicated in blue) and DHF (n=30, indicated in red) throughout the course of illness. (B) Serum IL-18 levels significantly and positively correlated with serum LPS levels (Spearmans r=0.2, p=0.009) (C) Serum LBP levels were measured by ELISA in in patients with DF (n=23) and DHF (n=30) throughout the course of illness (D) Serum LBP levels did not show any correlation with serum LPS levels (Spearmans r=0.11, p=0.16). Error bars indicate the mean and the SEM.

Figure 4

Serum platelet activating factor (PAF) levels in patients with acute dengue. Serum PAF levels were measured by ELISA in patients (A) who had detectable LPS in their serum (n= 56, indicated in blue) and those who did not (n=38, indicated in red) between day 4 and 6 of illness. Serum viral loads (B) positively and significantly correlated (Spearman's r=0.23, p=0.02) with serum PAF levels. Error bars indicate the median and the IQR.

Figure 5

Serum procalcitonin (PCT) levels in patients with acute dengue. Serum PCT levels were measured by ELISA in patients with (A) DF (n=129, indicated in blue) and DHF (n=64, indicated in red) (B) in patients who had detectable LPS in their serum (n= 107, indicated in blue) and those who did not (n=86, indicated in red) on day 4 (SD± 1) of illness. Serum LPS levels (Spearmans r=0.16, p=0.023) (C) and serum CRP levels (Spearman's r=0.23, p=0.001) (D) positively and significantly correlated with serum LPS levels. Error bars indicate the median and the IQR. * (P<0.05)