Differential expression of NADPH oxidase-2 (Nox-2) and nuclear factor-erythroid 2-related factor 2 (Nrf2) transcripts in peripheral blood mononuclear cells isolated from dengue patients

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Abstract The role of oxidative stress in the pathogenesis of dengue infection is not completely known. A recent study reveals the involvement of oxidative stress responsive molecules in the generation of host immune responses to dengue virus in vitro. Objective of the present study was to analyse the changes in the expression of oxidant-antioxidant genes Nox-2 (NADPH oxidase) and Nrf2 (nuclear factor-erythroid 2-related factor 2) in patients with dengue during the early phase of infection compared to other febrile illness (OFI) cases and healthy controls using Real-time qPCR assay. The study enrolled 88 dengue patients, 31 OFI cases, and 63 healthy individuals as controls. Out of 88 dengue cases, 32 were classified as severe dengue cases (SD) and remaining 56 patients as non-severe dengue (NSD). Blood samples were collected firstly at the time of

admission and a second sampling was done from the available individuals (38 dengue and 13 OFI cases) at the time of defervescence. Total RNA was extracted from the Peripheral blood mononuclear cells and the transcripts level of Nox-2 and Nrf2 were analysed by qPCR. On DOA, both Nox-2 and Nrf2 expression was found to be down regulated in dengue and OFI cases ($P < 0.05$) compared to healthy controls. Interestingly at defervescence, the transcript levels were found to be significantly increased in dengue cases unlike OFI, where no such increment was evidenced. From DOA to DOD, the study observed a significant increase in the levels of Nox-2 transcripts ($P < 0.05$) both in SD and NSD cases. But a significant Nrf2 activation was not observed in SD cases as we found in NSD cases. Thus a steady and significant increase in Nox-2 transcript level in severe, non-severe and secondary dengue infected groups observed in the current study supports the earlier reports on the involvement of anti-oxidant response in dengue severity. However further studies on its protein levels and mechanistic action would decipher the exact role of these potential molecules in the disease virulence.

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Introduction

Dengue is a mosquito-borne acute viral disease affecting two-thirds of the world's population predominantly in tropical and sub-tropical areas of the world. Around 3.6 billion people are living under the risk of dengue infection [1] (<http://www.who.int/denguecontrol/9789241504034/en/>). Average annual incidence of 20,474 dengue cases has reported in India during the tenure of 2006–2012 [18]. The febrile period lasts for 4 or 5 days of infection. The disease

may either worsen or subside followed by the defervescence [6]. Pathogenesis of the illness has not unveiled so far. However, antibody-dependent enhancement (ADE) is widely accepted mechanism behind the ailment [12]. There is no precise treatment regimen available for dengue infection, but the treatment strategy is supportive and symptomatic only. Prompt diagnosis and access to specific medical attention lower the fatality rate below 1% [19] (<http://www.who.int/tdr/publications/training-guideline-publications/dengue-diagnosis-treatment/en/>). Nox-2 or gp91phox is a catalytic subunit of a membrane-bound multicomponent protein NADPH oxidase found in phagocytes. During the microbial invasion, Nox-2 generates superoxide anion in the presence of NADPH by reducing oxygen molecules. ROS generated during the process will help to eliminate foreign bodies invaded in the host [11, 25]. So the pro-oxidant state triggered during the event will activate antioxidant defensive system tries to nullify the deleterious effects of ROS [2]. ROS-sensitive transcription factor Nrf2 dissociates from Keap-1 and translocate to nucleus and binds to ARE (antioxidant response element) of genes encoding antioxidant proteins and induce transcription [7, 26]. Severe and non-severe dengue patients express similar clinical signature during the febrile period of infection. The diagnosis generally made after the patients have presented with severe dengue symptoms. So a prognosis test which helps to distinguish the patients who are more prone to develop the disease severity could be able to safeguard by providing better medical attention [3, 6] (<http://www.cdc.gov/dengue/clinicalLab/>). Earlier we have reported that various biochemical oxidant-antioxidant markers in the circulation of dengue infected study subjects [16, 23]. Olinger et al. [14] studied the involvement of NADPH oxidase-mediated ROS generation and Nrf2 activation in vitro. However, knowledge on gene expression pattern of Nrf2 and Nox-2 in dengue patients is limited. Hence, our objective was to quantify Nox-2 and Nrf2 mRNA transcripts from PBMCs of dengue patients in the various clinical spectrum of dengue compared to control and OFI cases. In addition to that, to interrogate the existence of transcriptional variation as well as correlation between the study genes with respect to the day of admission (DOA) and the day of defervescence (DOD).

Materials and methods

Patient recruitment and sample collection

The study subjects recruited from Jawaharlal Institute of Post Graduate Medical Education and Research (JIPMER) Hospital, Puducherry, India. The prospective cohort study consist of 88 dengue patients, 31 other febrile illness (OFI)

subjects and 63 controls (20 non-febrile illness subjects (NFI) and 43 healthy volunteers). Out of all 88 dengue cases 38 dengue cases who reported till defervescence stage were involved in the study. Remaining 50 cases were not willing to participate in the study or discharged against medical advice. Similarly, 13 OFI cases who shown up till defervescence were involved. The subjects recruited for the current study categorized into three groups. Group I included 88 dengue infected cases (59 adults and 29 children, with ages varying from 1 to 72 years) whose infection was confirmed by NS1 antigen detection by dengue early ELISA kit or dengue IgM Capture ELISA Kit, (Pan Bio, Inverness Medical Innovations, Australia). To classify the patients into primary and secondary infection dengue IgG capture ELISA kit was used (Pan Bio, Inverness Medical Innovations, Australia). A dengue virus infection has been defined as primary if the capture IgM/IgG ratio is greater than 1.2, or as secondary, if the ratio is less than 1.2 [9]. The samples collected from the patients from the regions of Puducherry and Tamilnadu, India during the dengue fever outbreak in the year of 2012–2014.

The World Health Organization grading system used to classify patients like our earlier studies [24] (<http://www.who.int/tdr/publications/training-guideline-publications/dengue-diagnosis-treatment/en/>). Dengue fever divided into ‘dengue without warning sign, dengue with warning sign and severe dengue.’ Dengue without warning sign consists of fever with two of the following criteria: ‘nausea, vomiting, rash, aches and pains, leukopenia and Positive tourniquet test.’ In addition to the above dengue with warning sign includes any of the following clinical condition: ‘abdominal pain, persistent vomiting, clinical fluid accumulation, mucosal bleeding, lethargy, restlessness, liver enlargement >2 cm, increase in hematocrit concurrent with rapid decrease in platelet count’. The classification of SD was considered for cases with any of the following criteria: ‘severe plasma leakage leading to shock or fluid accumulation with respiratory distress, severe bleeding evaluated by clinician and severe organ involvement such as Liver (AST or ALT $\geq$ 1000), CNS (impaired consciousness), failure of heart and other organs’. Group II included 31 age and sex-matched patients with other febrile illness (OFI). OFI defined as patients with short febrile illness (<2 weeks), negative for dengue NS1 antigen, anti-IgM/IgG dengue antibodies, and there was no evident bacterial, rickettsial or protozoan etiology [5]. Group III is control group further divided into two groups. First is non-febrile illness (NFI) which defined as patients belong to pediatric age group (1–12 years) with illness other than febrile illness who come to JIPMER for minor surgical, skin or eye problem without systemic disease [10]. This group will serve as controls for comparison of pediatric cases. The second one is healthy adult Volunteers of age >13 years.

Ethical apporoval

The study was approved by institutional ethics committee (Human studies) meeting of the year 2012 held on 03/11/2012 (Ref No.IEC/SC/2012/4/101). After taking written informed consent, three mL of blood were collected within 24 h of admission (Febrile period), and another sample during the follow-up coinciding with the day of defervescence. Day of defervescence defined as the day of which fever is subsiding indicated by a decline in body temperature which commences nearly 3rd or 4th day of admission and at the same time points for OFI patients.

PBMC isolation, RNA extraction and cDNA synthesis

PBMCs were separated by using HiSep LSM 1077 (Hi-media, Mumbai, India), washed twice with PBS, stored in 1 mL RNAiso Plus reagent (Takara Bio Inc., Shiga, Japan) and kept at -80 degree. Total RNA was extracted using RNA easy minikit (Qiagen, GmbH, Hilden, Germany) based on the manufacturer's protocol. The purity and concentration of RNA were assessed using a NanoDrop spectrophotometer (ThermoScientific, Waltman, MA, USA). The complementary DNA (cDNA) was synthesized from 1 micro gram of RNA using high capacity cDNA reverse transcription Kit with RNase inhibitor (Applied Biosystems, Foster City, CA, USA). The cDNAs thus obtained were tested for integrity by amplification of β -actin transcripts in a 40-cycle PCR. Standard curves were constructed using the mean cycle threshold (Ct) value.

RT-PCR analysis of Nox-2 gene

Primers and probes used in the assays are given in the Table 1. A total of 20 μ L of the reaction sample consisted of 2 μ L of 1:30 diluted cDNA, 10 μ L of Taqman master mix (Ampliqon, Denmark), 0.4 μ L of each primer at 0.2 μ M, 0.5 μ L of probe at 0.25 μ M (Sigma-Aldrich,

Australia), and 6.8 μ L of nucleic acid free water (Sigma-Aldrich St. Louis, MO, United States). Amplification were monitored using CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, USA). Reaction conditions were 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 60 s and 72 °C for 30 s.

RT-PCR analysis of Nrf2 gene

A total of 20 μ L of the reaction sample consisted of 2 μ L of 1:30 diluted cDNA, 10 μ L of SYBR Premix Ex TaqII (Takara Bio Inc., Shiga, Japan), 0.4 μ L of each primer at 0.2 μ M (VBC-Biotech Service GmbH, Vienna, Austria), and 7.2 μ L of nucleic acid free water (Sigma-Aldrich St. Louis, MO, United States). Amplification were monitored using CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, USA). Reaction conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 55 °C for 30 s. The melting curve analysis was used to determine the specificity of each primer set. Specific PCR products confirmed by melting curve analysis. PCRs were performed in triplicate, and the reproducibility of the syber green real-time PCR was assessed by running control samples independently on different days. The relative expression level of Nox-2 and Nrf2 were determined using the comparative Ct method, also known as 2-delta (Ct) method. Quantitative PCR method enables to analyze the expression of genes relative to the expression of the housekeeping gene. The relative copy number (RCN) of the target gene allows direct comparison of mRNA expression across individuals, time points, and groups standardized against constitutively expressed housekeeping genes.

Statistical analysis

Variables were analysed by descriptive statistics to evaluate the clinical characteristics of the cases. Results of all parameters are presented as Mean (SD) or median with

Table 1 Primers and probes for Nox-2, 18srRNA, Nrf-2 and Beta actin

Gene primers	Sequence
Nox-2 forward primer	GGAGAATTAACCTCCTGCCA
Nox-2 reverse primer	GGCTAGCTGGAGAAGACCAC
Nox-2 probe	FAM-TGGCTCATCTAGTCCTGCTCCTTGTG-TAM
18srRNA-forward primer	TAAAGGAATTGACGGAAGGG
18srRNA-reverse primer	CTGTCAATCCTGTCCGTGTC
18 s rRNA probe	FAM-CGCAGGCTCCACTCCTGGTG-TAM
Nrf-2 forward primer	GTTGCCCACATTCCCAAATC
Nrf-2 reverse primer	CGTAGCCGAAGAAACCTCAT
β -actin—forward primer	5'-ATCTGGCACCAACACTTCTACA-3'
β -actin—reverse primer	5'-GTTTCGTGGATGCCACA-3'

interquartile range. Normality was assessed with the Shapiro–Wilk test. Kruskal Wallis test or One-way analysis of variance was used to estimate the presence of any statistical differences between the study variables. Wilcoxon signed rank test used for comparing febrile and following deferescence day of gene expression analysis. All statistical analyses were performed using SPSS 21 (SPSS, Chicago, IL).

Results

Clinical, haematological and biochemical characteristics

Out of 182 study subjects, average age of controls, dengue infected patients and OFI cases were 20.87 ± 26 , 24.13 ± 15.90 and 23.65 ± 15.66 respectively (Table 2). Age of all the study group were comparable ($P = 0.91$). All dengue infected cases were classified based on the WHO 2009 criteria in which 36 patients were categorized as dengue without warning sign, 20 under dengue with warning sign and 32 under severe dengue. Clinical characteristics such as abdominal pain, third space collection were found to be increased ($P < 0.001$) and platelet count was decreased ($P < 0.01$) in severe dengue cases compared to non-severe dengue (Table 2). Percentage of hepatosplenomegaly was found to be decreased in all dengue cases compared to OFI ($P < 0.05$, Table 2). Haematological parameters such as total count, neutrophils and platelet count were found decreased in all dengue cases compared to OFI counterpart ($P < 0.05$). Conversely increased levels of haematocrit ($P < 0.05$), lymphocyte count ($P < 0.001$) and aspartate amino transaminase or AST [$P < 0.01$] were seen in all dengue cases compared to OFI, Table 2).

Gene expression of study variables in dengue cases and OFI

Expression levels of study genes were significantly down regulated ($P < 0.05$) in all dengue groups including OFI cases compared to healthy controls during the day of admission as shown in Fig. 1. During DOD expression of Nox-2 and Nrf2 was found to be up regulated except in OFI cases (Fig. 2).

Pattern of gene expression between SD and NSD cases

There were no statistically significant variation of study parameters observed between SD and NSD cases during

the DOA (Fig. 1). From DOA to DOD, the study observed a significant increase in the levels of Nox-2 transcripts ($P < 0.05$) both in SD and NSD cases. But a significant Nrf2 activation was not observed in SD cases as we found in NSD cases (Fig. 2).

Gene expression in primary and secondary dengue cases

There was no significant change observed between primary and secondary dengue cases during DOA (Fig-1). During DOD increased Nox-2 and Nrf2 expression change was found in secondary dengue compared to admission as shown in Fig. 2. But the same was not appreciated in primary dengue cases (Fig. 2).

Discussion

Abundance of viral molecules will result impaired clearance of misfolded proteins due to diminished proteasome activity leads to redox imbalance in the host [8, 15]. The ROS generated during the innate immune response will induce the redox homeostasis mechanism through the activation of antioxidant defence [17]. We have earlier reported that increased levels of inflammatory cytokines along with lipid peroxides in various clinical spectrum of dengue disease [21]. The present study shows that Nox-2 as well as Nrf2 gene expression was attenuated in all study groups including OFI during DOA. Earlier Olainger et al. reported that NADPH oxidase-dependent intracellular increase of ROS in DENV-2 infected monocyte-derived dendritic cells accompanied by Nrf2 mediated transcriptional activation of antioxidant genes. Whereas NADPH oxidase inhibition by chemical/genetic silencing impaired the ROS production, lead to viral persistence [14]. In dengue infection viremia usually persisting for 4–5 days of infection [6]. It has been reported that Nox-2 derived ROS were induced the transcription factor IRF-3 which mediates the antiviral response through the activation of interferon genes [20]. So it presumes that initial decrease in the levels of Nox-2 observed in the current study may be due to viral defense strategy to bypass the innate immune mechanism for its survival in the host. This is not surprising as similar results were seen in OFI group, but in contrast, during DOD the Nox-2 gene expression was found to increase in dengue cases whereas no such hike was observed in OFI, which makes us to assume if the increase in Nox-2 during DOD is ‘specific’ for dengue infection. Monica et al. [4] studied that during Hepatitis C virus infection delocalization of sMAF proteins caused the formation of Nrf2-sMAF complex which in turn blocked Nrf2 translocation and ultimately caused transcriptional inactivation of

Table 2 Clinical, hematological and biochemical characteristics of the study groups

Characteristics	OFI (n = 31)	All dengue cases (n = 88)	NSD (n = 56)	SD (n = 32)
Gender				
Male N (%)	13 (58.06)	51 (57.95)	29 (51.79)	22 (68.75)
Female N (%)	18 (41.94)	37(42.05)	27(48.21)	10 (31.25)
Age in years mean (SD)	24.13 (15.90)	23.65 (15.66)	25.43 (15.07)	20.69 (16.39)
Pulse rate mean (SD)	101.6 (19.77)	90.98 (19.38)	88.82 (18.77)	93.35 (20.24)
Systolic BP mean (SD) mmHg	110 (20.92)	105.02 (14.19)	105.42 (12.97)	104.57 (15.78)
Diastolic BP mean (SD) mmHg	69.76 (12.29)	70.44 (11.07)	70.83 (11.39)	70 (10.95)
Positivity for dengue				
NS1 Ag N (%)	0	45 (51.14)	32 (57.14)	13 (40.63)
Anti-dengue IgM antibody N (%)	0	85 (96.59)	54 (96.43)	31 (96.88)
Anti-dengue IgG antibody N (%)	0	66 (75.00)	38 (67.86)	28 (87.50)
Clinical characteristics				
Third space collection (%)	2 (6.45)	18 (20.45)	0	18 (56.25)###
Abdominal pain N (%)	9 (29.03)	40 (45.45)	16 (28.57)	24 (75.00)###
Myalgia N (%)	16 (51.61)	38 (43.18)	27 (48.21)	11 (34.38)
Headache N (%)	13 (41.94)	36 (40.90)	23 (41.07)	13 (40.63)
Retro-orbital pain N (%)	5 (16.13)	16 (18.18)	11 (19.64)	5 (15.63)
Arthralgia N (%)	2 (6.45)	13 (14.77)	11 (19.64)	2 (6.25)
Maculopapular rash N (%)	1 (3.23)	14 (15.90)	7 (12.5)	7 (21.88)
Gingival bleeding N (%)	3 (9.68)	12 (13.64)	4 (7.14)	8 (25.00)
Vomiting N (%)	17 (54.84)	43 (48.86)	26 (46.43)	17 (53.13)
Hepato-spleno-megaly N (%)	6 (19.35)	4 (4.54)*	2 (3.57)	2 (6.25)
Haematological parameters				
Total Count Mean(SD) cells/mm ³	7234.07 (3207.20)	5137.19 (2508.31)*	4976.84 (2443.95)	5371.53 (2630.37)
Neutrophils Mean (SD) %	63.84 (14.62)	47.40 (13.33)***	47.34 (11.30)	47.48 (16.19)
Lymphocytes Mean (SD) %	31.64 (13.57)	47.56 (12.82)***	47.95 (10.66)	46.96 (15.77)
Hct mean (SD)	36.10 (5.62)	42.09 (7.57)*	40.85 (5.36)	44.05 (9.96)
Platelet count mean (SD) cells/mm ³	139,576.92 (84,038.64)	67,906.25 (62,866.63)***	82,923.08 (67,858.36)	44,480 (46,320.54)##
Biochemical parameters				
Albumin mean (SD) g/dL	3.26 (0.49)	3.46 (0.56)	3.56 (0.44)	3.23 (0.71)
Total protein mean (SD) g/dL	6.34 (0.95)	6.376 (1.10)	6.58 (0.84)	5.92 (1.47)
ALT mean (SD) IU/mL	67 (41.94)	92.02 (67.90)	91.78 (63.32)	92.53 (78.90)
AST mean (SD) IU/mL	91.13 (88.13)	154.87 (98.97)**	158.14 (105.96)	147.94 (84.85)

OFI other febrile illness, ADC All dengue cases, NSD Non-severe dengue, SD Severe dengue, Hct hametocrit, ALT Alanine amino transferase, AST Aspartate amino transferase

* indicates $P < 0.05$; ** indicates $P < 0.01$ and *** indicates $P < 0.001$ when compared OFI

indicates $P < 0.05$ and ## indicates $P < 0.01$ when compared to NSD

detoxifying enzymes. This could be a possible mechanism for down regulated Nrf2 expression during day of admission. The increased Nrf2 gene expression during DOD might be to neutralize the enhanced oxidative stress in dengue severity reported in previous studies [22]. From the current study, it is clear that during the same period Nox-2 augmented oxidant state could not able to trigger a compensatory activation of Nrf2 mediated antioxidant response in severe dengue cases (Fig. 2). This results is an extension of earlier reports of higher levels of lipid peroxidation and

depleted levels of sialic acid and sulfhydryl groups in severe dengue cases compared to non-severe cases [16, 23]. It has been evident that patients with secondary dengue infection are more prone to develop severe dengue [12]. During DOD there was significant increase of Nox-2 and Nrf2 were observed in secondary dengue infection compared to DOA (Fig. 2). But the same was not found in primary dengue cases for both genes remains unknown.

The study did not investigate the levels of gene expression in a cell-specific manner as well as based on the

Fig. 1 Gene expression of Nox-2 and Nrf2 in all the study groups at the day of admission (DOA). The amount of mRNA of each tested gene was measured by qPCR assay and expressed as relative copy number (RCN) with median and interquartile range. *** indicates $P < 0.001$; ** indicates $P < 0.01$; * indicates $P < 0.05$ when compared to controls. DOA Day of admission, CTRL Controls, OFI

other febrile illness, ADC All dengue cases, NSD Non-severe dengue, SD Severe dengue, PDC Primary dengue cases, SDC Secondary dengue cases. CTRL (n = 63), OFI (n = 31), ADC (n = 88), NSD (n = 56), SD (n = 32), PDC (n = 29), SDC (n = 59). Statistics: Kruskal–Wallis test

Fig. 2 Comparison of gene expression levels of Nox-2 and Nrf2 in various study groups between the day of admission (DOA) and the day of defervescence (DOD). ** indicates $P < 0.01$; * indicates $P < 0.05$ when compared DOA. DOA Day of admission, DOD Day of defervescence, OFI other febrile illness, ADC All dengue cases, NSD Non-severe dengue, SD Severe dengue, PDC Primary dengue cases, SDC Secondary dengue cases. OFI (n = 13), NSD (n = 22), SD (n = 16), ADC (n = 38), PDC (n = 9), SDC (n = 29). Statistics: Wilcoxon signed rank test

dengue serotype though prevalence of all serotypes has been reported in regions which were patients were recruited [13]. A discrete data analysis was not performed for juvenile or adult cohort because of the limited sample size. Drop out of study subjects caused less sample size during DOD. Because of the limited sample volume, we could not able to study the levels of protein associated with the study genes in the circulation.

Gene expression of Nox-2 and Nrf2 gene were found to be down-regulated in dengue disease and OFI during the DOA. During DOD Nox-2 gene up regulation were

observed in dengue cases but a compensatory up-regulation of Nrf2 gene was not found specifically in severe dengue cases. Further studies on regulatory pathways of study variables are prerequisite to validate the findings of the current study.

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