

# Assay of Proinflammatory Cytokines (IL-6, IFN $\gamma$ , TNF $\alpha$ ) and its Correlation with Disease Severity in Dengue Fever

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## ABSTRACT

**Introduction:** Dengue fever is the most common mosquito borne viral illness in humans. Study was done to determine the serum levels of pro-inflammatory cytokines (IL-6, IFN  $\gamma$  and TNF  $\alpha$ ) in patients with dengue infection and to determine the correlation of level of cytokine with disease severity, platelet count and SGOT values.

**Material and methods:** Preserved samples of patients with the clinical features compatible with dengue infection and NSI antigen positivity admitted during the month of May and June 2016 in Pushpagiri Institute of Medical science and Research Centre, Thiruvalla were analysed for levels of pro-inflammatory cytokines (IL-6, IFN  $\gamma$ , and TNF  $\alpha$ ). Disease severity of Dengue patients was assessed from the clinical details obtained from medical records. Total samples used in this study were 80 of which 49 were patients with dengue fever, 11 were patients with severe dengue, 10 were patients with other febrile illness and 10 are from healthy individuals.

**Results:** The levels of all three cytokines were found to be elevated in the infected groups (dengue and severe dengue) when compared to the control groups (healthy and non-dengue). The level of interleukin 6 and TNF  $\alpha$  were found to be significantly elevated (p value <0.05) in the severe form of dengue. However no statistically significant difference (p value <0.05) between the infected and control group was found in Interferon gamma although it showed an elevation in the infected group. It was observed that all the three cytokines showed a negative correlation with platelet count.

**Conclusions:** IL6 and TNF  $\alpha$  correlated with disease severity suggesting that these cytokines can be used as a marker to predict the severity of the disease. Thus, necessary treatments and precaution can be taken in advance. TNF  $\alpha$  correlated with the degree of thrombocytopenia and IL6 correlated with level of SGOT.

**Keywords:** Dengue Fever, Cytokines, Dengue Haemorrhagic Fever, IL6.

confers life long immunity to that particular serotype and a brief period of partial immunity to other serotypes.<sup>2</sup> The viral replication occurs in reticulo-endothelial cells. The spectrum of clinical presentation include –asymptomatic disease, mild undifferentiated febrile illness, classic dengue fever, dengue haemorrhagic fever and dengue shock syndrome. Dengue haemorrhagic fever and dengue shock syndrome are fatal with high mortality rate. Dengue haemorrhagic fever is characterized by severe bleeding manifestations and capillary leak syndrome. Dengue shock syndrome manifests as hypotension and shock. Laboratory investigations may show –increase in hemoglobin, leucopenia, thrombocytopenia and elevated transaminases. In clinically suspected cases, diagnosis is based on serological tests. In the initial period of illness, diagnosis is based on the detection of NSI antigen and later by IgM antibody. Presence of IgG antibody indicates past infection (secondary Dengue).<sup>3</sup> Infections with one serotype produces neutralizing and non neutralizing antibodies. Neutralizing antibodies are protective and prevent further infection with the same serotype. When an infection with a different serotype occurs later, the previously formed non neutralizing antibody forms complexes with the new serotype virus. This complex enters the monocytes where the virus multiplies.<sup>4</sup> The monocytes releases various cytokines which damages the capillary endothelium and lead to capillary leak. The manifestations of severe dengue infections are proposed to be due to cytokines (Cytokine storm). So it is speculated that the levels of various pro inflammatory cytokines will be more marked in severe dengue infections.

Study objectives were to determine the serum levels of proinflammatory Cytokines in patients with documented dengue infection and to determine the correlation between level of Cytokines with disease severity, platelet count, and SGOT.

## INTRODUCTION

The first epidemic of dengue fever was reported in the year 1635 from West Indies. In 1953, another epidemic of dengue haemorrhagic was reported in Manila.<sup>1</sup> 50-100 million cases are reported every year of which around 5 lakh cases are dengue haemorrhagic fever. It is estimated that around 22,000 death occur every year due to dengue fever. 2.5 – 3 billion people in the tropical and subtropical countries are at the risk of developing infection with dengue virus. Dengue virus belong to the genus Flavivirus. It is a single stranded non segmented RNA virus. There are four serotypes-DENV 1,2,3 and 4. Infection with one serotype

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**MATERIAL AND METHODS**

The samples used in this study were preserved samples of patients with the clinical features compatible with dengue infection and NSI antigen positivity admitted during the month of May and June 2016 in Pushpagiri Institute of Medical science and Research Centre. The serum samples were allowed to clot for 2 hours at room temperature after which it was centrifuged for 15 minutes at 2000rpm. The supernatant was collected and it was stored at -70 degree Celsius.

Hospital records of all the patients with dengue infection was collected from medical records department and the necessary details were entered in a detailed proforma for clinical assessment. Severity of the disease was assessed by 2009 dengue classification by WHO. The proforma was one for 180 patients out of which 60 were selected for the study. Dengue specific IgM and IgG antibodies were detected by IgM and IgG capture ELISA respectively. Total samples used in this study were 80 of which 49 were patients with dengue fever, 11 are patients with severe dengue, 10 are patients with other febrile illness and 10 are from healthy individuals. Serum levels of IL6, TNF alpha and IFN gamma were detected by ELISA according to manufacturer’s instructions. Standards were included in each assay and standard curves obtained were used for the estimation of cytokine concentration.

**STATISTICAL ANALYSIS**

The data was analyzed and the levels of cytokines were presented as mean and standard deviation. The mean difference of these values between the categories of dengue fever was compared using one –way ANOVA. The statistical significance of difference in the levels of IL6, TNF alpha, IFN gamma between various groups (healthy, non dengue, dengue, severe dengue) were analyzed using post hoc test. P –value <0.05 was considered to be statistically significant. The correlation between cytokine levels and SGOT and platelet count were analyzed using pearsons correlation test. All statistical analyses of the data were done using SPSS program

**RESULTS**

Out of 60 cases studied, 49 patients had dengue fever and 11 patients had severe dengue (DHF). It was also found that 27 cases belonged to primary infection (IgM Positive, IgG Negative) and 33 case belonged to secondary infection (IgM

Cytokines	Type of infection	Total no	Mean+/-SD
IL 6	primary	27	90.46+/- 57.97
IL 6	secondary	33	94.40+/- 55.94
TNF alpha	primary	27	67.68+/-52.90
TNF alpha	secondary	33	81.66+/-75.82
IFN gamma	primary	27	145.35+/-107.68
IFN gamma	secondary	33	163.02+/-106.50

**Table-1:** Level of IL6, TNF alpha and IFN gamma in primary and secondary infection

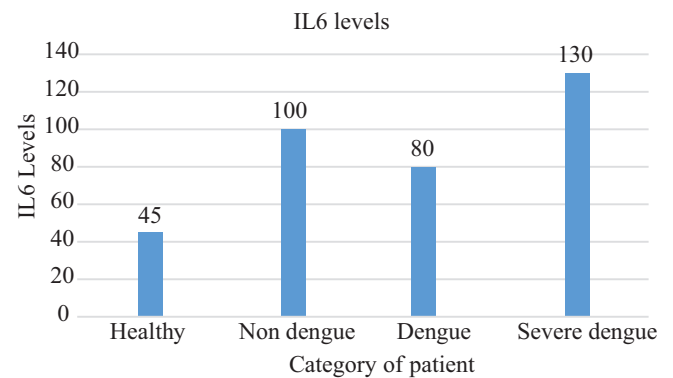
Positive, IgG Positive). The concentration of interleukin 6, TNF alpha and IFN gamma in all the samples were

Cytokines	r value	P value
IL 6	-0.085	0.529
TNF alpha	-0.292	0.028
IFN gamma	-0.128	0.341

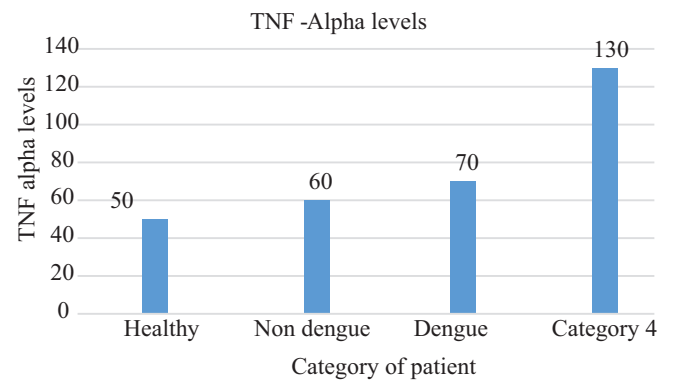
**Table-2:** Cytokines and its respective correlation coefficient

Cytokines	r value	P value
IL 6	0.378	0.006
TNF alpha	0.059	0.676
IFN gamma	0.036	0.797

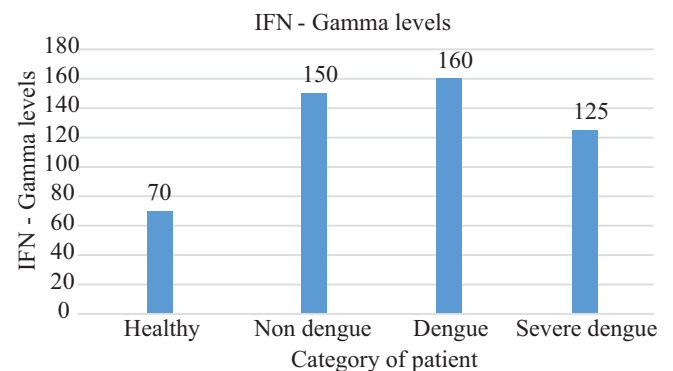
**Table-3:** Correlation between cytokine levels and SGOT and its respective correlation coefficient



**Figure-1:** Level of IL6 in healthy, dengue fever, non-dengue and severe dengue



**Figure-2:** Level of TNF alpha in healthy, non-dengue, dengue fever and severe dengue



**Figure-3:-** Level of IFN gamma in healthy, non-dengue, dengue fever and severe dengue

determined using the standard curve.

The level of all three cytokines were found to be elevated in the infected groups (dengue and severe dengue) when compared to the control groups (healthy and non-dengue). the level of interleukin 6 and TNF alpha were found to be significantly elevated (p value <0.05) in the severe form of dengue when compared to the healthy, non-dengue, and dengue cases suggesting that interleukin 6 and TNF alpha levels are correlated with illness severity. However no statistically significant difference (p value <0.05) between the infected and control group was found in IFN gamma although it showed an elevation in the infected group (As per figure 1,2,3).

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As per table 2 it was observed that all the three cytokines showed a negative correlation with platelet count. However, a statistically significant difference (p value < 0.05) as observed only in the case of TNF alpha suggesting that TNF alpha are correlated with platelet count.

As per table 3 it was observed that all the three cytokines showed a positive correlation with SGOT. However, a statistically significant difference (p value <0.05) was observed only in the case of IL6 suggesting that IL6 are correlated with SGOT.

## DISCUSSION

Dengue virus infection results in the release of various cytokines from infected monocytes, lymphocytes and mast cells. So it is expected that the cytokines level may increase during dengue infections. In severe dengue infection which usually occurs in secondary dengue infection, because of the phenomenon of immune enhancement, more viruses enters the macrophages and multiply with in macrophages. So more release of cytokines (cytokine storm) is expected So the focus of interest has shifted to cell mediated immunity. Dengue virus can infect both CD4+ and CD8+T lymphocytes.

In this study, 60 patients were randomly selected from around 150 patients who got admitted in the department of medicine (Pushpagiri Institute of Medical and Research Centre) during the month of june –july 2016. Out of the 60 patients only 18.3% has features of severe infections.

All the three cytokines (interleukin 6, tumor necrosis factor and interferon gamma) were elevated in the infected group compared to the control group. Statistically significant elevation was found in IL6 and TNF alpha. In the case of IFN gamma, though elevated, the value was not statistically significant. A similar observation was reported in the study by Anita Chakravarthiet. Al<sup>1</sup>, where they noted that interferon gamma level was more elevated in dengue fever

when compared to severe dengue infection. In this study also, paradoxically interferon gamma was noted to be more elevated in dengue fever when compared to severe dengue infection. However in a study by John et al<sup>2</sup>, they noted significant elevation of interferon gamma in infants having dengue fever and dengue haemorrhagic fever. It has been proposed that interferon gamma is secreted by T helper cells, as apro-inflammatory cytokine which in turn may lead to more T cell activation and release of TNF alpha and other interleukins. Therefore interferon gamma on its own may not have a role in the pathogenesis of severe dengue infections.<sup>3</sup> Nguyen et al<sup>5</sup> has observed that although interferon gamma is produced early in the course of illness, peak levels occur much later (on or before the day of defervescence thus coinciding with the disappearance of viremia). The present study being a retrospective study, the serum samples were taken at varying time from the onset of fever. In patients who got hospitalized early in the illness, the serum samples may have been taken early in the illness at which interferon gamma may not have peaked. This could explain the observation of higher level noted in dengue fever patients when compared to those with severe infection.

In the case of TNF alpha, the level showed a statistically significant difference in severe dengue infection when compared to dengue fever. similar observation was noted in various other studies as well. This observation suggests that TNF alpha correlates with disease severity.

The present study also investigated the level of interleukin 6. On reviewing literature, so far there are only limited studies with regard to the level of interleukin 6. It was observed that, statistically significant elevation was noted in severe dengue infection when compared to dengue fever and control groups. Therefore, it can be included that interleukin 6 correlates with illness severity.<sup>6</sup>

Both interleukin 6 and TNF alpha were not significantly elevated in dengue fever compared to the control groups proving the point that cytokine elevation is more significant in severe infection. It has been proposed that TNF alpha is secreted by dengue virus infected monocytes, which induce endothelial cell production of reactive nitrogen and oxygen species, leading to apoptotic cell death and therefore hemorrhage.

Another observation from the study was that all the three cytokines showed increased level in secondary infection (IgM and IgG positive) compared to primary infection (IgM positive). This was expected as secondary infection are supposed to be more severe.<sup>7</sup>

Thrombocytopenia is a common finding observed in most of the dengue infection irrespective of the fact that the disease is severe or not. Normal platelet count ranges from 1.5 to 4 lakhs. Clinical bleeding manifestations occur when the platelet count is very low only. Dengue patients with very low platelet count especially bleeding manifestation may require platelet transfusion. Clinically there is no correlation with the degree of thrombocytopenia and disease severity.<sup>6,8</sup> This study investigated the correlation with the platelet count and cytokine levels. It was observed that all the three

cytokines showed a negative correlation with the platelet count ie when platelet count decreases, the level of cytokine increases. Statistically significant correlation was observed in the case of TNF alpha suggesting that its level correlates with severity of thrombocytopenia.<sup>8</sup>

SGOT and SGPT are liver enzymes which become elevated in various types of liver injury. Most of the patients with dengue fever show mild to moderate elevation of SGOT and SGPT. It has been observed that SGOT is more elevated than SGPT. Levels more than 1000 international units usually indicates bad prognosis in dengue infection. The study investigated the correlation between cytokine level and SGOT levels. All three cytokines showed a positive correlation with SGOT levels. The correlation was noted to be stronger for IL6.

## CONCLUSION

From the study it was observed that IL6 and TNF alpha correlated with disease severity suggesting that these cytokines can be used as a marker to predict the severity of the disease. This helps in early prediction of the severity of the disease thereby helping us to take necessary treatments and precaution in advance. It was also found that TNF alpha correlated with the degree of thrombocytopenia and IL6 correlated with level of SGOT.

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