Cytokine profiling of *Plasmodium* infection in population of two different regions

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Certificate

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ABBREVIATIONS

- PCR : Polymerase Chain Reaction
- IL-4 : Interleukin 4
- HLA : Human Leukocyte Antigen
- TNFα : *Tumor necrosis factor α*
- TAE : Tris-acetate-EDTA
- EtBr : Ethidium bromide
- DNA : Deoxyribonucleic acid
- RNA : Ribonucleic acid
- ELISA : Enzyme Linked Immuno Sorbent Assay
- COX III : Cytochrome Oxidase III
- PBS : Phosphate Buffered Saline
- PC : Positive Control
- NC : Negative Control
- NH : Normal Healthy
- NA : Negative Sample
- µg : Micro Gram
- ng : Nano Gram
- RBC : Red blood cell
- CD4+ :Cluster of Differentiation 4
- Th1 : Type 1 T helper cell
- Th 2: Type 2 T helper cell
- IFN γ : Interferon γ
- NK cell : Natural killer cell
- IL 5: Interleukin 5
- CM : Cerebral malaria
- NO : Nitric Oxide
- IL 12 : Interleukin 12
- TGF β : Transforming growth factor β
- KO mice : Knockout Mice
- Ig- E : Immunoglobulin E
- PBMCs : Peripheral blood mononuclear cell
- MHC II : Major Histocompatibility Complex
- IRF 1 : Interferon regulatory factor 1
- HRP conjugate : Horseradish peroxidase
- BSA : Bovine serum albumin

- pmol : Picomole
- µl : Micro liter
- CT value : Threshold cycle value
- *Eco* RI : E. coli restriction enzyme
- IL 10 : Interleukin 10

Abstract

Cytokine profiling has been carried out to see the different immune response in the Ahmedabad and Nadiad population. The levels of circulating cytokines depends on many factors including disease severity and the level of immune challenge. The ability of the immune system to establish a precise balance between Th1 and Th2 responses can be crucial to guarantee effective clearance of the pathogen without excessive damage to the tissues. In this study we measured the levels of TNF-α and IL-10 in the plasma of patients suffering from malaria. IL-10 levels were found to be high in the malaria infected individuals having high parasitic load exception was found in P. falciparum infected sample. There was a positive correlation between IL-10 and TNF-α levels in the plasma of malaria patients. Our study suggests with increase in parasite density IL-10 expression go up in contrary it down-regulates TNF-α expression. Clinical outcome of the disease is a consequence of the complex interaction between the pathogen and the host and survival of pathogen largely depends on the type of cytokine (Th1/Th2) being produced by host's immune cells on encounter.

Introduction

Malaria is caused by the protozoan *Plasmodium*, which is transmitted to humans by female Anopheles mosquitoes. Malaria accounts for 85% of global infectious diseases. The epidemiology of malaria is complex in India because of geo-ecological diversity, multi-ethnicity and wide distribution of 9 anopheline vectors transmitting 3 plasmodial species *P.falciparum*, *P.vivax*, and *P.malariae*. During 1947, 75 million malaria cases in a population of 330 million were estimated. It gradually decreased to 100,000 during late 1950s and early 1960s. Then malaria again staged a comeback by 1980s. Malaria cases touched to 6.4 million during 1980. As per world malaria report 2014, 22% of India population reside in high transmission areas, 67% in low transmission areas and 11% live in malaria-free areas(National malaria eradication programme, NMEP). In 2013, 0.88 million cases have been recorded with 47% *P.vivax* infection and 53% *P.falciparum* infection. Malaria is contributed the most by Orissa state in India, contributing 25% of the total cases (NMEP).

Life-cycle of a malaria parasite

Female Anopheles feeds on blood to nourish her eggs, while she injects sporozoites into the bloodstream of the individual. Liver cells rapidly take up sporozoites. In most of the species these parasites develop to form schizonts which further develops to several thousand merozoites. The portion of liver-stage parasites (hypnozoites) remain dormant into the hepatocytes in *P.vivax* and *P.ovale*, thus responsible for relapse. As the liver cells rupture, the merozoites are released into the blood stream and rapidly invade RBCs. They replicate asexually rapidly attaining a high parasitic density and destroying every single RBC they infect, leading to the clinical symptoms of malaria. A small percentage of merozoites differentiate into male and female gametocytes, which are again taken up by a mosquito in her blood meal. Thus the transmission cycle continues back to the mosquito. Male and female gametocytes fuse within the mosquito and forms diploid zygotes, which further differentiates to ookinetes. Ookinetes further migrate to the midgut of the insect and passes through the gut wall to form oocysts. Mitotic division of the oocysts leads to the formation of sporozoites,

which migrate to the salivary gland of the female anopheles rendering it to be capable to transmit it to humans.

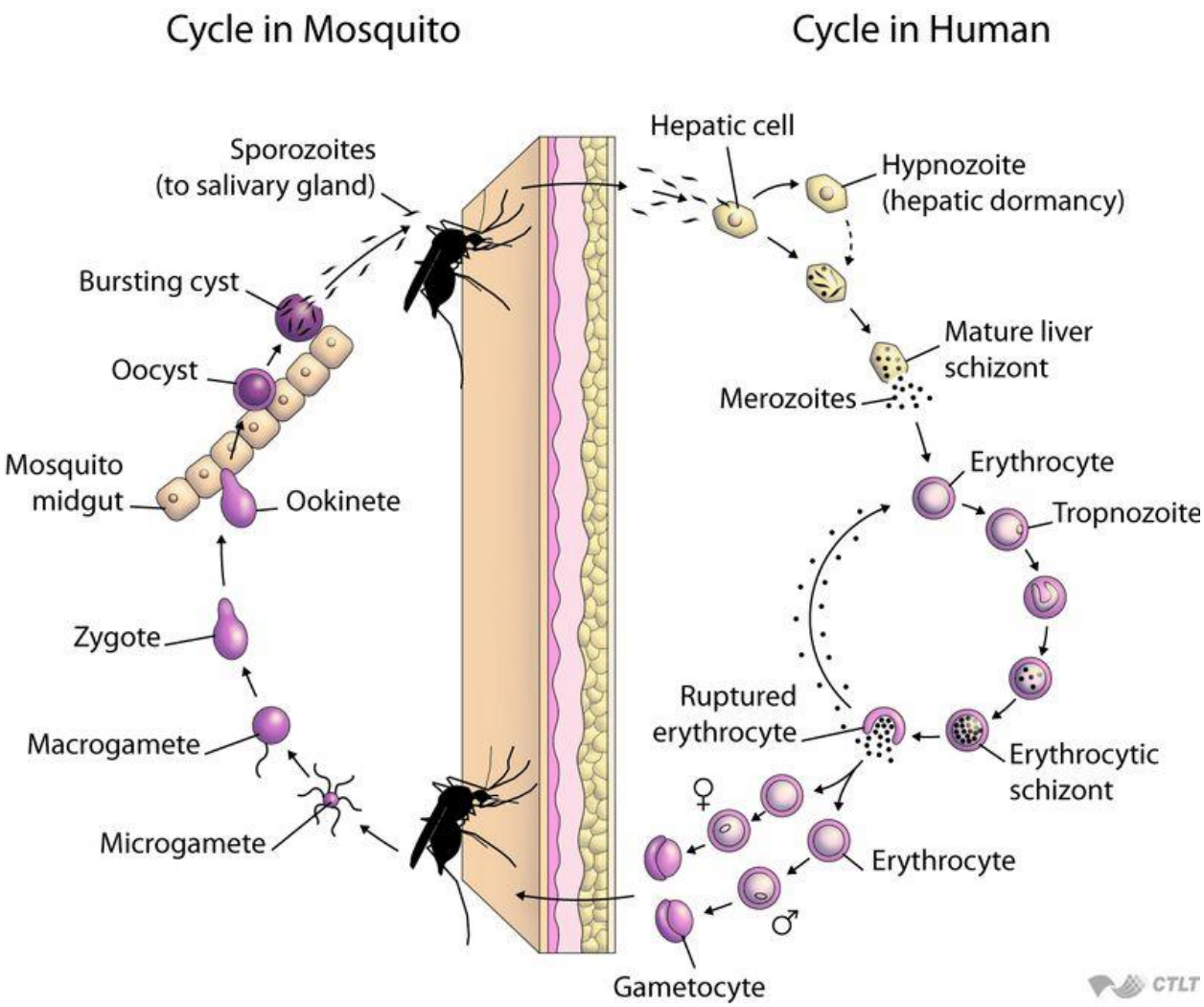


Fig 1: Malaria life cycle.

Immune response by the human body

The cellular and humoral arms of the adaptive immune system are important elements for eradication of plasmodium from the body. They both are critically dependent on α/β CD4+ lymphocytes. It is known that CD4+ T cells are comprised of at least two functionally different subsets, differentiated on the basis of lymphokine secretion in Th1 (IFN-γ producing) and Th2 (IL-4/IL-5 producing) cells. Both Th1 and Th2 type have the regulatory functions in malaria.

They are the crucial requirement for controlling the infection. Surface antigens of malaria parasite leads to the formation of malaria-specific antibodies which mediate no. of anti-parasitic effector mechanisms including cytoadherence inhibition, antibody dependent cytoxicity and cell inhibition. Cellular responses involve activation of macrophages by NK-cell, Th1 derived IFN-γ or γ-δ T cell for enhanced phagocytosis and removal of infected erythrocytes[1]. The $CD8^+$ T cells inhibits the growth and development of parasite inside hepatocytes. Nitric oxide (NO) from macrophages and IFN-γ from T cells can have antiparasitic effects. At high concentration, NO is even known to kill the *P.falciparum* parasites. As NO interferes with the neurotransmission, it is implicated with the pathogenesis of CM. Severe malaria is found to be associated with high circulatory levels of inflammatory cytokines like TNF-α, IL-10, IL-6. TNF-α is substantially higher in plasma from children having CM or severe anaemia. IFN-γ has clearly been linked to the onset of pathology in mice as well as in humans. The pool of cross-reactive malaria Th1 memory cells is likely to increase with age, resulting in stronger inflammatory responses and increased risk of severe pathology in adults as compared to children. The pool of cross-reactive memory cells is likely to be small in infants and young children and therefore malaria infection is of limited pathogenicity, but serve to prime T cells for pro-inflammatory role during subsequent infection. The higher risk of severe disease in non-immune adults may be due to the higher frequency of cross-reactive primed T cell. The Humoral and Cellular responses of adaptive immune system both are pivotal elements for the eradication of the parasite from the body, and both are critically dependent on α/β CD4+ lymphocytes. CD4+ are comprised of at least 2 different subsets, which differentiates on the basis of lymphokine secretion in Th1 (IFN-γ producing) and Th2 (IL-4/IL-5 producing) cells. Th1 and Th2 both responses have the regulatory functions in malaria and are crucial requirement for the control of infection, but a strict balance between those responses should be finely-tuned in intensity and time.

Cytokines in early protection

IFN-γ production in the early stage helps in resistance against infection. It is also likely to have mechanisms of resistance independent of IFN-γ and NO. Although IL-12 may have a role in making a mice resistant or susceptible, it is possibly involved in pathology. It is critically linked

to or to act through the production of IFN-γ. IL-12 also extends the antibody response by producing IgG2a Abs. A very interesting role for IL-18 indicates that IL-18 along with IL-12 enhances Th1 responses, but in absence of IL-12 it can potentiate Th2 response. Some results suggest that IL-18 plays a protective role by enhancing in vivo production of IFN-γ. A similar role for TNF-α is depicted to that of IFN-γ in early response against malaria infection. Accelerated cure and improved prognosis is found to be associated with high levels of TNF-α. The switch from Th1 to Th2 response is attributed to IL-10. Early productions of IL-10 has been associated with the susceptibility to the infection and has a prominent anti-inflammatory effect, thus limiting in some way the damage to the normal tissues by excessive Th1 response. TGF-β may have crucial role in the regulation of the activation of different effector mechanisms.

Immunopathology of malaria and cytokines

Malaria pathogenesis is complex and likely to comprise of immunologic and non-immunologic mechanisms. It is now generally accepted that malaria is the consequence of alterations in various tissues and organs, which can further lead to metabolic acidosis and localized and ischemia. Ample of evidences says that glycosylphosphatidylinositol is important factor having potential to induce TNF-α and IL-10. For the development of cerebral Malaria (CM) [13]. Not all pro-inflammatory cytokines are equally relevant. The best documented evidences comprises IFN-γ, TNF-α and IL-12, and no evidence has been found for IL-6. IFN-γ and IL-12 are strongly involved in pathogenesis of Cerebral Malaria. The role of anti-inflammatory cytokines is much less clear in control of CM. Although a data based on KO mice indicates that IL-12 and IFN-γ display a dominant role in pathology while IL-4 and IL-10 are not required for the pathogenesis. A protective role has been demonstrated of IL-10 and the role of TNF-α seems to be well established. Levels of cytokine IL-12, which boosts erythropoiesis are correlated with anaemia.

Diagnosis and prognosis of malaria and cytokines involved

Due to predominance of Th2 over Th1 helper cells, the elevated levels of IgE is being found in the blood of malaria patients. Highest levels of TNF-α was directly correlated in the children with severe anemia. In response to malarial antigen, the PBMCs of patients with Mild Malaria produce IFN-γ. The TNF-α levels in the serum can predict the fatal outcome in CM cases.

Majority of the clinical symptoms of this disease are due to the parasite at stages in which it outnumbers asexually in red blood cells. *P.falciparum* infection is most severe in children, although few of the infected children develops severe complications. The reason behind it can be the genetic makeup of host, social and geographic factors. Malaria infected population is characterized by excessive inflammation, and the effectors response to it involves a fine-tuned interplay between varied cell types and cytokines. It is believed that if a precise balance between pro- and anti-inflammatory cytokines is established, control of parasite and host survival is guaranteed. From several studies it was noted that the control population from endemic region exhibited a lower pro- to anti-inflammatory cytokine ratio, which indicates a shift towards a high basal Th2 response. And IL-10 levels mostly contributed towards the region-specific difference in basal cytokine response, and was also the strongest predictor of the disease in endemic region. A key regulatory cytokine IL-10 has been shown as a protection to mice against pathologic conditions in acute *P.chabaudi chabaudi* AS malaria model. Still, the picture remains unclear about the critical cellular source of IL-10. A low mean IFN-γ/IL-10 ratio is found to be associated with the severity of the disease in the endemic region. And the low mean of IL-12/IL-10 ratio denoted the disease outcome in non-endemic region. TNF-α significantly increases with the disease severity, particularly in Cerebral Malaria. The role of Anti-inflammatory cytokines in severity of malaria remains controversial, as different studies reports high IL-10 levels associated either with severe malaria or as protection against severe malaria in humans. It is also noted that the balance between IL-10 and TNF-α concentrations determines the severity of anaemia in the infected children. Higher levels of TNF-α was recorded in serum and cerebrospinal fluid of children with cerebral malaria.

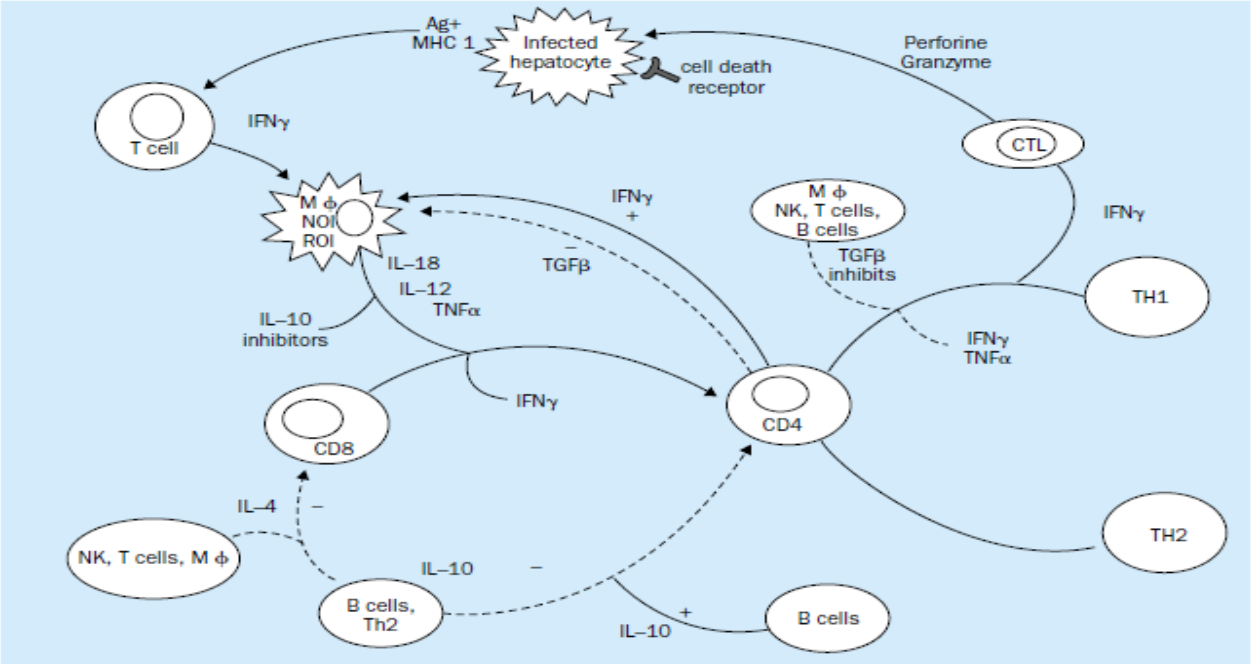


Fig 2: Rapid processing and presentation of Sporozoites and the Immune response

The sporozoites are rapidly processed by the host cell and is presented on the surface of the infected hepatocytes in association with MHC I, it further leads to recognition by CTLs and killing of the infected cells, or leads to the stimulation of NK cells and CD4+ T cells to produce Interferons, which can further trigger a cascade of immune reactions and can lead to the clearance of intracellular parasites. Naive CD4+ T cells can develop into Th1, as they are important for the eradication of the parasite. The hallmark cytokine of Th1 cells is interferon, which promotes the microbicidal activity of the macrophage and cytokine production such as TNF, IL-12 and IL-18. Development of Th1 response can be antagonized directly by production of IL-4 and TGF, and indirectly by IL-10, which inhibits the production of pro-inflammatory cytokines. IL-10 induces B-cell proliferation, which is essential for the development of malarial antibodies.

The difficulties currently faced in designing a successful vaccine against *Plasmodium* is our current incomplete knowledge of protective immunity and its induction. And the pathogenesis of two severe most complications of *P. falciparum* malaria “Several Malarial Anaemia” and

"Cerebral Malaria" both are seemed to involve in dysregulation of immune system. If understood clearly can provide crucial clues to reach the goal of better vaccines.

There is a widespread and remarkable acceptance that cytokines like IL-10 and TNF are most essential mechanisms of systemic disease due to infectious agents. There lies a wide agreement that cell mediated and antibody-dependent responses are required for adequate protection, encompassing various mechanisms finely-tuned in time. And innate immunity is thought to be a crucial system in clearance of *plasmodium* parasites from the host. Along with spleen, liver has been shown to function as an alternate clearing site. Pro-inflammatory cytokines such as TNF-α are thought to play an important role in malaria pathogenesis, especially in CM, by increasing the surface expression of adhesion molecules on cerebral endothelial cells that enhance parasite attachment [2]. Excessive production of TNF-α in patients with CM seems to be a consequence of genetic variation in the host's propensity to produce this cytokine [3]. It has been shown that the balance between IL-10 and TNF-α concentration determines the severity of anaemia in infected children [4]. A recent study in Kenya shows that the lower levels of TGF-β in serum and cerebrospinal fluid from children with CM were found to be associated with higher levels of TNF-α [5]. Pathogenic Th1-type responses have been suggested to be down-regulated by factors such as IL-10 [6], but protective Th1-type immunity can also be downregulated and suppressed in severe forms by malaria by second type of response triggered by plasmodial infection [7] [8]. cytokines IL-1β, TNF-α, IL-10, TGF-β were significantly found in high concentrations in CM patients and in moderate concentrations in the patients suffered from CM within 6 months and IL-10 showed a direct correlation with parasitemia [9]. A control population from endemic region exhibited a lower pro- to anti-inflammatory cytokine ratio, which indicates a shift to a high basal Th2 response, and IL-10 mostly contributed to region-specific difference in basal cytokine response and was also the strongest predictor of the disease [10]. TNF-α significantly increases with the severity of the disease, particularly in CM [10]. It is also noted that balance between IL-10 and TNF-α concentrations, determine the severity of anaemia in infected children. SM is found to be associated with high circulatory levels of inflammatory cytokines like TNF-α, IL-1 and IL-6 [11, 12].

Both Th1 (IFN-γ producnd Th2 (IL-4/IL-5 producing) type of CD4+ T cells have the regulatory function in malaria. They are crucial requirement for controlling the infection, but the balance between these responses should be finely tuned in intensity and time [13]. A prominent role in switching from Th1 to Th2 responses is attributed to IL-10. Early IL-10 production has been associated with susceptibility to infection and it is thought that this cytokine has a prominent anti-inflammatory effect, limiting in some or the other way the damage inflicted to the normal tissues by excessive Th1 response [13]. IL-10 downregulates MHC-II expression on macrophages, hence decreased antigen presentation, preventing T cell priming and proliferation. It also suppress the production of IFN-γ, IL-6, TNF-α and GM-CSF by T cells [1]. An important role for IL-12 has been proposed in early response against *plasmodium* infection that it may have role in making $(IRF\text{-}1)^{-/-}$ mice resistant or susceptible to malaria, it may possibly be involved in pathogenesis. It appears to be critically linked to or act through IFN-γ production. IL-12 extends the antibody responses against *plasmodium* by the production of IgG2a [13]. The basal levels of pro- to anti- inflammatory cytokines between controls of endemic and non-endemic region, the endemic region control exhibited a lower ratio of pro- to anti-inflammatory cytokines, thus indicating a shift towards high basal Th2 response in the endemic region. IL-10 contributed the most to the region-specific difference in the cytokine response [10]. The levels of cytokines circulating, depends on various factors including severity of the disease and the level of immune challenge. The half-life of most of the cytokines is short and are also cleared rapidly by liver and kidneys. It is thought that the population of malaria endemic region are able to control peripheral parasitaemia in such a way that, no clinical symptoms are observed, which includes production of pro-inflammatory cytokines [14] [15]. The elevated levels of TNF in non-endemic patients suggests its role both in resolution of infection and induction of pyrogenic response. IL-12 is regulated in a negative feedback manner by IL-10, as uncontrolled IL-12 production leads to excessive production of pro-inflammatory cytokines resulting in detrimental downstream effects [16]. High TNF-α levels have been associated with severe *falciparum* malaria [16] [17].

Hypothesis

Here we hypothesize that,

Discrete patterns of cytokine profiles may define clinical immunity to malaria in regions of varying endemicity and Levels of Pro- and Anti-inflammatory cytokines and the relative balance between Th1 and Th2 response may illustrate how population residing in areas of varying disease endemicity respond to Plasmodium induced immune challenge.

Objectives

1. To identify malarial population from two different region.
2. Differentiation based on parasitic load.
3. Cytokine profiling of the identified population.

Materials

1) Agarose Gel Eletrophoresis:-

- 50X TAE buffer
- Agarose
- EtBr
- Purple loading dye
- DNA / PCR product
- 100 bp ladder

2) DNA isolation kit [QIAamp DNA mini kit (51104) QIAGEN, Germany]

3) RNA isolation kit [Qiagen (762174)]

4) Q-PCR kit (SYBR Green)

5) ELISA Kit (PeproTech)

For IL-10 (#900 –M21) TNF-α (900-M25)

- 10x PBS
- Wash buffer
- Block buffer
- Diluent
- Detection Antibody
- Avidin-HRP conjugate

- 2,2’- Azino-bis9 (3-ethylbenzothiazoline-6-Sulfonic acid) (ABTS)

6) Total Protein Estimation

- 1x PBS
- Bradford’s reagent
- Sample (Plasma)
- 1% BSA

7) Real Time PCR

- SYBR Green Dye
- ROX Dye
- Template

8) Cloning

- DH5α *E.coli* strain
- Invitrogen TOPO TA cloning kit
- Plasmid isolation kit (Macherey–Nagel (740588.50), Germany)
- Gel and PCR clean-up (Macherey–Nagel (740609.50), Germany)

Methods

Study design, region and population

A cross-sectional study was conducted from November 2016 to January 2017 with Plasmodium patients from Sola Hospital from Ahmedabad and Nadiad, a pre-Amazon region of the state of Gujarat, which presents a high prevalence of plasmodium infections from Ahmedabad is low endemic region and Nadiad is high endemic region for plasmodium infection.

Before collection of blood samples from malaria patients, we have taken ethical approval from civil hospital, Sola and prior consent was taken from Director of NIMR, New Delhi. It took almost 6-7 months. Then patient samples were collected from both Nadiad and Ahmedabad. A total of (n = 41) malaria +ve samples, (n = 39) malaria –ve samples and (n = 4) healthy donor samples were collected from Ahmedabad and (n = 3) +ve samples and (n = 3) malaria –ve samples were collected from Nadiad. All the blood samples were microscopically examined by the physicians. The methods were carried out in accordance with approved guidelines.

Blood collection and Plasma isolation

Approx. 2-3 ml Peripheral venous blood was collected in EDTA vials. Plasma was obtained by centrifuging the blood samples at 4000 rpm for 10 min and was stored at - 80ºC for further usage.

Genomic DNA isolation

Whole DNA was isolated from blood sample by using QIAamp DNA mini kit (QIAGEN, Germany) according to manufacturer's instruction, and then eluted in to 200µl of Nuclease free water. DNA was stored at -20ºC for further use.

DNA quantification

Isolated DNA was quantified by Nanodrop at wavelength of 260nm and it is between the range of 7 - 100 µg/ml.

Nested PCR

Nested PCR was use for the parasitic strain identification. For the detection and identification of *Plasmodium* species we performed and optimized the nested-PCR method described as NP-2013 protocol, an update for the widely used NP-1993 to NP-2005 (SSU rRNA) protocols for all human malaria parasites molecular detection. For the *Plasmodium* genus detection a first PCR was done with specific primers (rPLU1 and rPLU5) followed by a second reaction using the primers rPLU3 and rPLU4. All genus specific nested PCR-positive results were analysed to species level using the second nested-PCR in 2 separate reactions as reported previously: rFAL1/rFAL2 (*P. falciparum*); rVIV1/rVIV2 (*P. vivax*) using the PCR amplification of the first reaction (rPLU1 and rPLU5) as a template.

All PCR was performed in 25 µl total volume, each containing 1X Q solution and 1X Coral Load PCR buffer including 1.5 mM MgCl2, 125 µM of each deoxynucleoside triphosphate and 0.5 U HotStar Taq Plus DNA Polymerase (Qiagen, Hilden, Germany). The additionally added MgCl2 and primers varied as detailed in Table. The first PCR round used 5 µl of extracted DNA from the blood samples. The second PCR reactions used 2 µl of the PCR product obtained for the first PCR. The amplification conditions for all PCR reactions are shown in Table.

Thermal cycling was performed in a Eppendorf thermal cycler. Each amplification run included two negative controls (ultrapure water and a negative control of DNA extraction). For nested-PCR reactions, an additional negative control was added, consisting of 2 µl of the negative control reaction of the first run of PCR. To visualize the products of the nested-PCR, analysis on a 2% agarose gel in 0.5 X Tris-EDTA. and stained with ethidium bromide (0.5 µg/ml) was used to verify the amplified product size and to check for non-specific

amplification. PCRs were considered positive if the specific amplicon of the expected base pair (bp) size was observed.

Table:1 Nested PCR :- Primers and Conditions

Species/ genus	Primer name	PCR primers (5′-3′)	Size(base Pair, bp)	Amplification Conditions
Genus - nested 1	rPLU1 rPLU5	TCAAAGATTAAGCCATGCAAGTGA CCTGTTGTTGCCTTAAACTTC	~1,670	Initial denaturation, 95ºC for 5 min; 25 cycles of 94ºC for 1 min, 58ºC for 1 min, and 72ºC for 2 min; final extension, 72ºC for 5 min
Genus - nested 2	rPLU3 rPLU4	TTTTTATAAGGATAACTACGGAAAAGC TACCCGTCATAGCCATGTTAGGCCAATACC	240	Initial denaturation, 95ºC for 5 min; 30 cycles of 94ºC for 1 min, 64ºC for 1 min, and 72ºC for 2 min; final extension, 72ºC for 5 min
P. falciparum	rFAl1 rFAl2	TTAAACTGGTTTGGGAAAACCAAATATATT ACACAATGAACTCAATCATGACTACCCGT C	206	Initial denaturation, 95ºC for 5 min; 30 cycles of 94ºC for 1 min, 58ºC for 1 min, and 72ºC for 2 min; final extension, 72ºC for 5 min
P. vivax	rVIV1 rVIV2	CGCTTCTAGCTT AATCCACATAAC TGATAC ACTTCCAAGCCG AAGCAAAGAAAG TCCTTA	121	Initial denaturation, 95ºC for 5 min; 30 cycles of 94ºC for 1 min, 58ºC for 1 min, and 72ºC for 2 min; final extension, 72ºC for 5

Ref.: Snounou G, Singh B. Nested PCR analysis of *Plasmodium* parasites.Methods Mol Med. 2002; 72: 189-203.

COX III Single Direct PCR

The concentration of extracted DNA was determined by Nanodrop. Three different dilutions were made (50ng, 10ng, 2ng). Then COXII single direct PCR was carried out to estimate the parasitic load. For the single direct PCR reactions, the primers short COX-III F/R were used in 20µl final volume. To visualize the products of the PCR, analysis on a 1.5% agarose gel in 0.5 X Tris-EDTA and stained with ethidium bromide (0.5 µg/ml).

Table 2: COX III PCR primer and Condition.

Primer name	PCR primer (5′-3′)	Size(base Pair, bp)	Amplification Conditions
COX III	AGCGGTTAACCTTTCTTTTTCCTTACG AGTGCATCATGTATGACAGCATGTTTACA	506 bp	Initial denaturation, 95ºC for 5 min; 25 cycles of 94ºC for 1 min, 65ºC for 1 min, and 72ºC for 2 min; final extension, 72ºC for 5 min

Ref: Echeverry et al. (2016)

Cloning

Plasmodial DNA was extracted from a blood using the DNA isolation kit (QIAamp DNA mini kit, QIAGEN, Germany) according to the recommendations of the manufacturer. A reaction mix with a final volume of 25 µL was obtained: P. vivax DNA, master mix, NFH_2O The reaction was performed in a mastercycler (MasterCycler, Eppendorf, Germany) as

follows: an initial cycle of 94°C for 15 min, followed by 30 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 5 min. As a positive control, one plasmids were used, containing a gene insert of the *P.vivax*. The digestion reaction was performed in a final volume of 10 µl of sample and EcoRI (Invitrogen), 2 µL of the enzyme reaction buffer, 10 µL of the PCR product and 7 µL of sterile DNAse-free water. The reactions were performed at 37°C 1 hour.

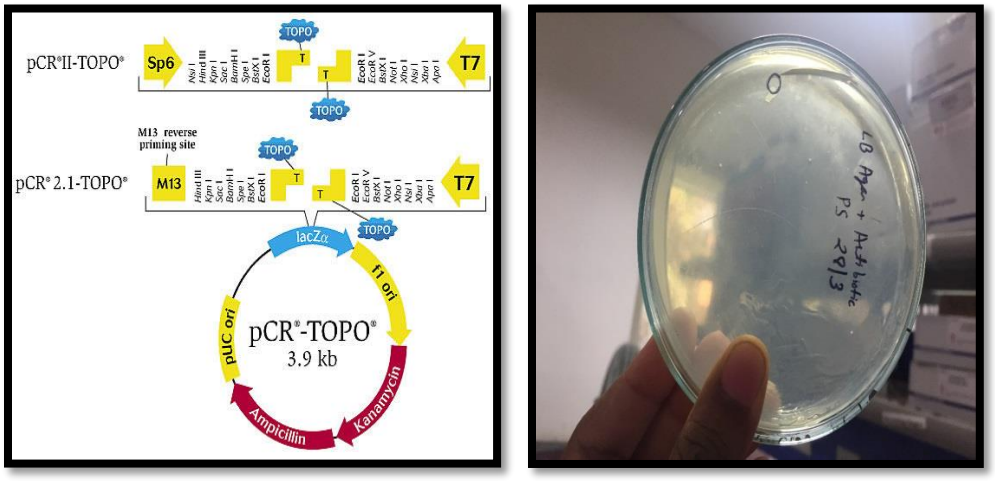


Fig 3: TOPO TA vector and transformed plate

Real Time PCR

Real-time PCR based on SYBR Green I was applied for accurate quantification of the target sequence in the current study. Primers were found to match with those used in the quantification study of *P. falciparum* / *P.vivax* DNA in blood samples by a real-time PCR assay using SYBR Green dye. A standard curve was constructed using 100-fold serially diluted

P. falciparum / *P.vivax* parasite DNA corresponding to 10^6 to 0.1 parasite per reaction. Amplification and detection were performed using QuantStudio detection system (Thermofisherscientific). Standards, samples, and negative controls were analyzed in duplicate for each run. A 20 µl of the PCR reaction was performed, consisting of 16 SYBR Green I PCR Master mix (Applied Biosystems, USA), 5 pmol forward primer, 5 pmol reverse primer, and different volume of DNA. Cycling parameters were 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15s and 60°C for 1 min. A threshold cycle value (Ct) was calculated for each sample by determining the point at which the fluorescence exceeded the threshold limit. A standard curve was obtained by plotting the Ct values against each standard of known concentration parasite DNA. Each real time PCR reaction was carried out in duplicate.

Total Protein Estimation

With the help of Bradford's regent total protein was estimated. For standard graph 1%BSA was use in different concentration. 0.2, , , , ,1.0µl 1% BSA is use and Diluted in 1x PBS. Then add 1 ml reagent and take OD at 650nm in colorimeter.

Plasma cytokine estimation

Sandwich ELISA was performed for the estimation of plasma cytokine levels in infected patients, control and healthy groups. Plasma TNF-α and IL-10 levels were determined using capture monoclonal anti-human antibody (PeproTech) and a paired biotinylated, anti-human antibody (PeproTech). Briefly, Microtitre plates were coated with 100 µl of capture antibody and incubated overnight at Room Temperature [18]. Plates were washed four times (0.1% tween-20 in 1X PBS) and was blocked with 300 µl blocking buffer (1% BSA in 1X PBS) for 1 h at R.T, after which plates were washed thoroughly four times. 100 µl of plasma sample or standards were added, in duplicates in each well and plates were incubated for 2h after which the plates were washed four times. Biotin-labelled antibody was added and the plates were

incubated at R.T for 2h after which the plates were washed four times and then incubated for 30 min. with Avidin-HRP enzyme. Plates were washed four times and 100 µl ABTS substrate solution was added. The reactions were allowed to develop for about 5 min. and then O.D was measured at a wavelength of 405 nm on a Microplate reader upto 30 min. at 5 min. intervals. Standards were included in duplicates in each assay plate and were used for the calculation of cytokine levels in the samples.

Results

Samples from malaria parasite infection were collected from 17 subjects aged (14-65yrs) participating in our current study. Five samples were collected from Nadiad, Gujarat. While 12 samples were collected from the Ahmedabad region of Gujarat. The samples collected were checked for the presence of parasite by preparing thin blood smear by the physician belong to the corresponding hospital. Then further confirmation of parasite was done by Nested PCR as described in materials and methods. Only two positive samples showed *P.falciparum* infection, rest all were *P.vivax* infected and one sample showed mix infection. The subjects suffering from recurrent fever but malaria parasite negative on both microscopy and real-time PCR were considered as the non-infected control group and three healthy individuals, who never had encountered malaria were also considered as healthy control. Such samples were also collected from both Ahmedabad and Demographic and clinical characteristics of the study subjects are given in Table 1. This study was approved by the Research Ethics Review Board of the Civil hospital, Sola and also permission for malaria patients' sample collection was taken from NIMR, Nadiad. Written informed consent was obtained from all study participants or their parents/guardians.

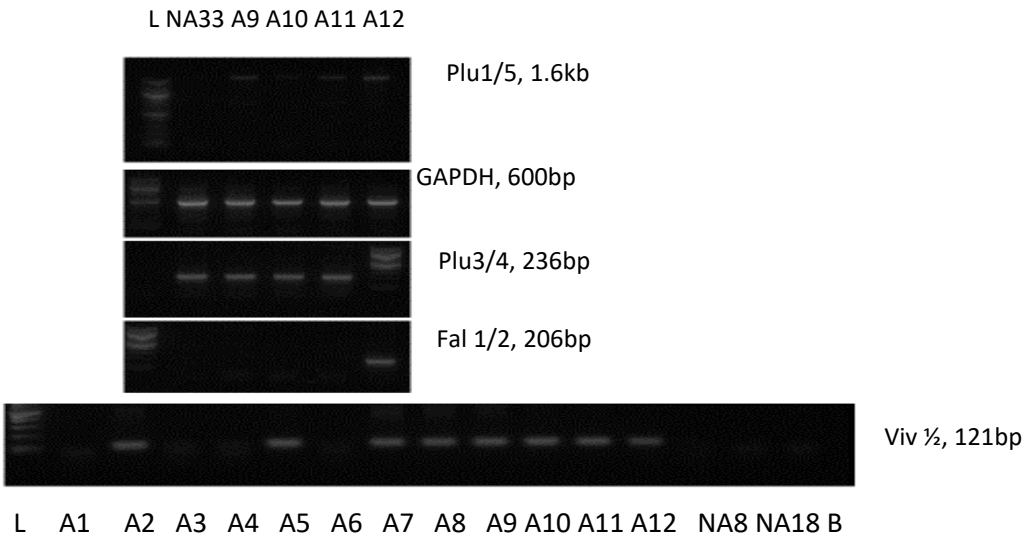


Fig4:- Identification of Parasite strain by Nested PCR. Agarose (2%) gel electrophoresis analysis of nested PCR. A1-A12 positive sample, NA: negative sample, B: blank, L: Ladder

Sensitivity and linearity of the Real-time PCR assay

Parasitic load was first checked by amplifying the COX-III parasitic gene as shown in Fig.2. The dilutions of 50, 10 and 2 ng were prepared and amplified the fragment via PCR. 2 samples were found to be PCR negative, which were rendered positive microscopically. The probable reason for this is such low parasite density cannot be detected by PCR. The load was then checked by real-time PCR analysis, in which the dilutions were prepared from 10^{10} – 0.1 copy number *of P. vivax* specific gene cloned plasmid and a standard graph was prepared as shown in Fig.No.3. 50 ng of the total DNA of the respective samples were then analyzed for the parasitic load identification. The parasitic load of the positive samples was calculated by plotting the Ct values on the standard graph. The mean standard curve was linear over 10 log range of copy number with correlation coefficient (r^2) of 0.9466 (Fig 2). A negative control (water instead of template DNA) with each PCR assay was included for stringent measures to control contamination.

Analysis of Parasite load in blood of malaria patients

Parasitic load in the Ahmedabad region was comparatively high than the Nadiad samples. One mixed infection was observed in the Ahmedabad region. The parasitic load in the Ahmedabad samples was in the range of less than 1 parasite to more than 10,000 parasites/50 ng of the total DNA as shown in Fig No.3. Highest parasitic load was found to be $10^{3.86}$ parasites. Among the Nadiad samples, the parasitic load was ranging from 10 – 100 parasites/50 ng of the total DNA as shown in Fig. No. 4.

Table3**:- Parasitic load in the samples determined by Q-PCR.**

Sample	Microscopically	PCR	Region	Ct-value	Parasitic load/50 ng of whole DNA	Fold change
A1	P	N	Ahmedabad	25.648	< 1	0.1912
A4	P	N	Ahmedabad	26.653	< 1	0.1434
A5	P	P	Ahmedabad	23.561	< 10	2.33
A8	P	P	Ahmedabad	18.181	$< 10^4$	327.647
A9	P	P	Ahmedabad	20.352	$< 10^3$	8.5
A10	P	P	Ahmedabad	22.571	< 100	14.5
A11	P	P	Ahmedabad	22.66	< 100	3.95
A12*	P	P	Ahmedabad	24.88	< 1	0.655
A12*	P	P	Ahmedabad	21.034	------	99.6
N1	P	P	Nadiad	23.045	< 10	4.983
N2	P	P	Nadiad	21.147	100	6.84
NH1	N	N	Ahmedabad	25.37	< 1	0.105
NH3	N	N	Ahmedabad	25.055	< 1	0.448
NA17	N	N	Ahmedabad	24.921	< 1	1
NA18	N	N	Ahmedabad	26.457	< 1	1
NTC	------	------	------	25.232	------	-------

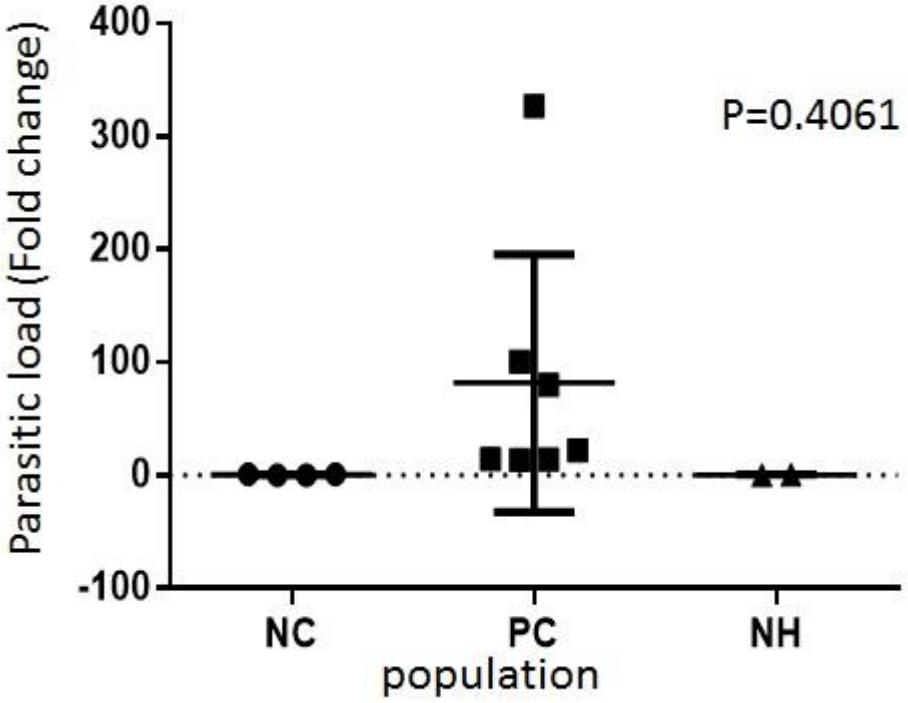


Fig 5: Parasitic load in Ahmedabad population.

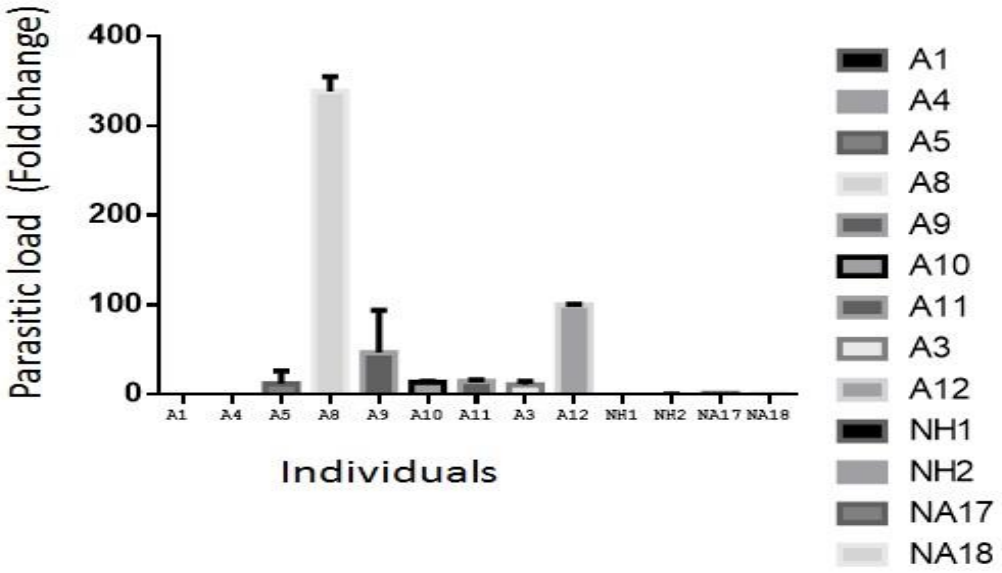


Fig 6: Parasitic load in individuls.

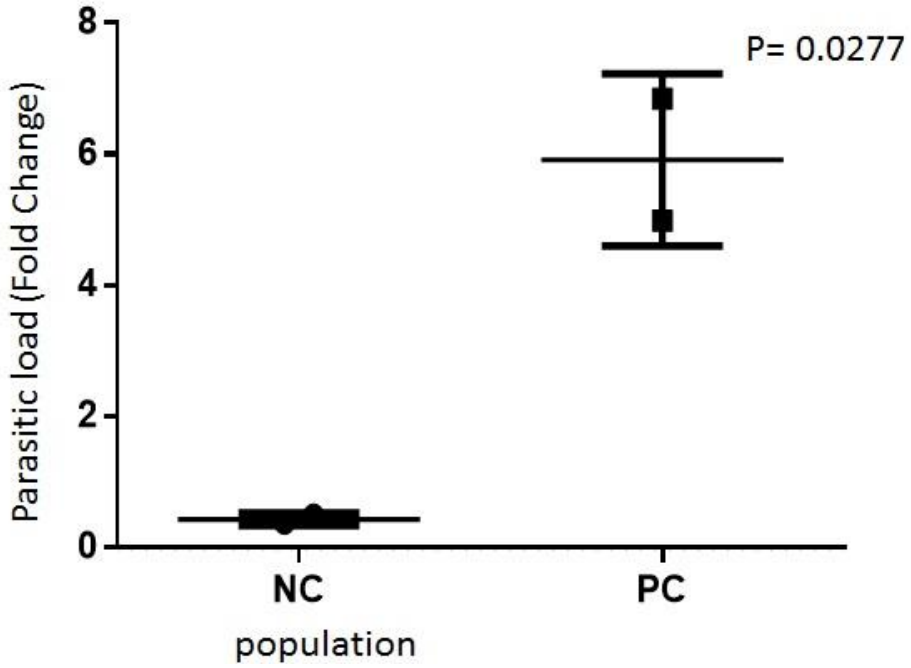


Fig 7: Parasitic load in Nadiad population.

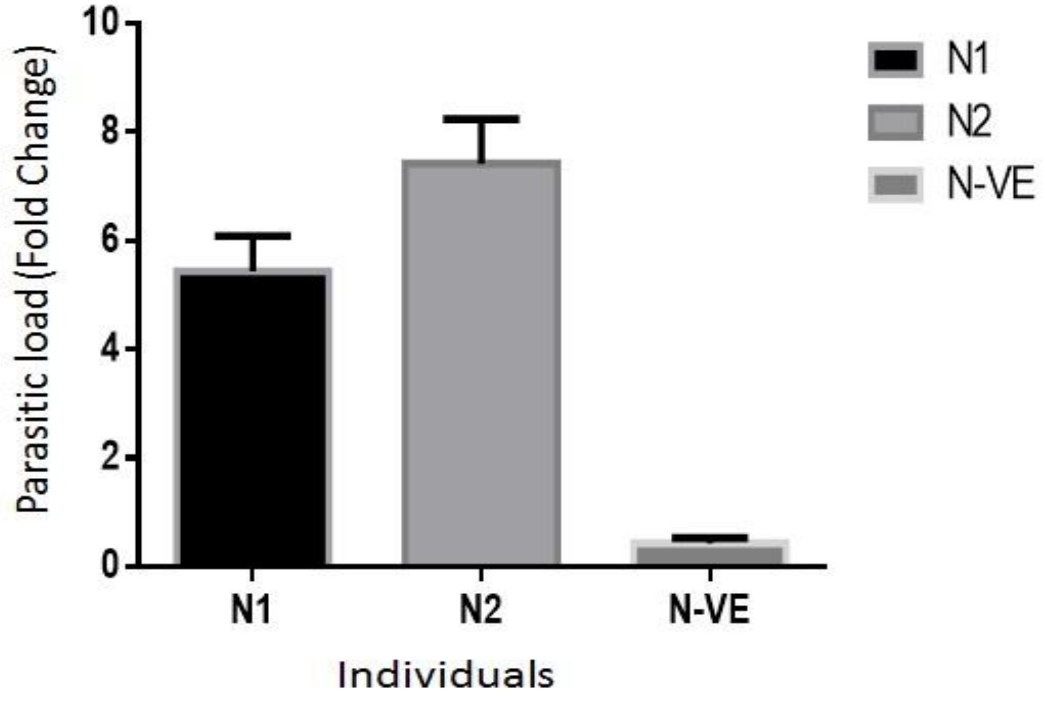


Fig 8: Parasitic load in individual samples.

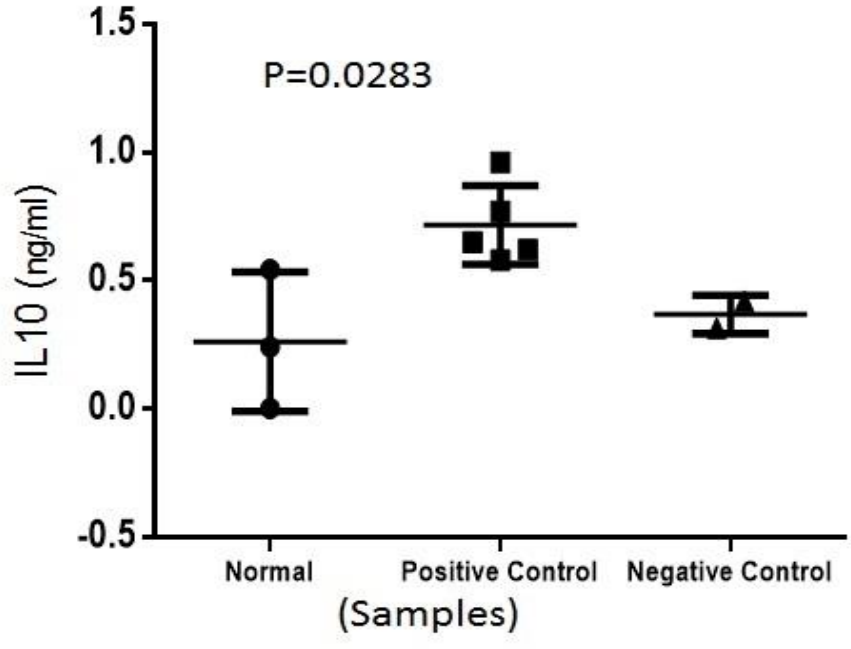
Plasma cytokine estimation

Sandwich ELISA was performed for the estimation of plasma cytokine levels in infected patients, control and healthy samples. Plasma TNF-α and IL-10 levels were determined using capture monoclonal anti-human antibody and a paired biotinylated, anti-human antibody (PeproTech).

Plasma levels of TNF-α and IL-10 in plasmodium infected, control and healthy groups from Ahmedabad region

The levels of TNF-α were found to be very high as compared to IL-10. The mean concentration of TNF-α was 1.826 ng/ml in patients with malaria and 1.24 ng/ml and 1.25 ng/ml in the negative (infected) control group and healthy group respectively We found significant correlation of TNF-α concentration with parasite load (r^2 =-0.7402 and P value 0.09). The concentrations of TNF-α were highly variable in the malaria patients ranging from (1.55 – 2.26 ng/ml). The mean concentration of IL-10 was 0.716 ng/ml in patients with malaria and 0.418 ng/ml and 0.391 ng/ml in negative control group and healthy group respectively. The level of IL-10 was significantly higher ($P<0.0022$) in cases with high parasite load with correlation coefficient $r^2 = 0.7004$ (Fig. 9a). The concentrations of IL-10 varied, ranging from 0.58 – 0.96 ng/ml. TNF-α levels were higher in *P. falciparum* infected samples as compared to *P.vivax* infected samples. While the levels of IL-10 were much higher in the *P. vivax* infected samples as shown in Fig.9a

Fig.9a : IL-10 concentration in the Ahmedabad and Nadiad samples



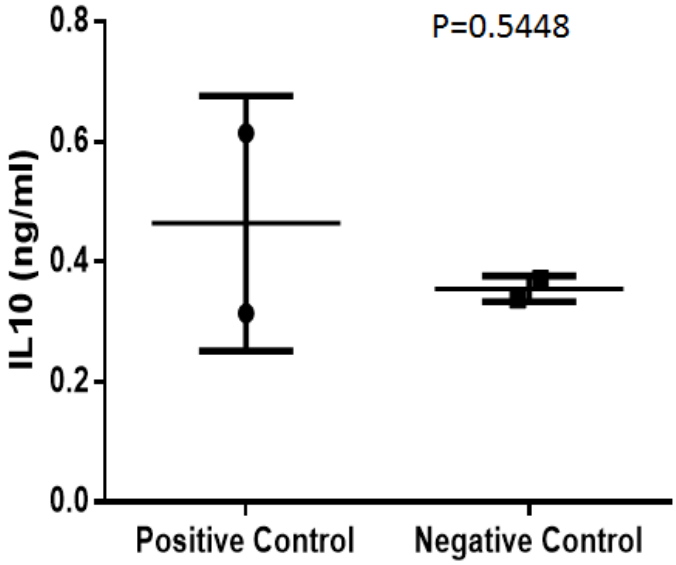


Fig.9b : TNF-α concentration in the Ahmedabad and Nadiad samples.

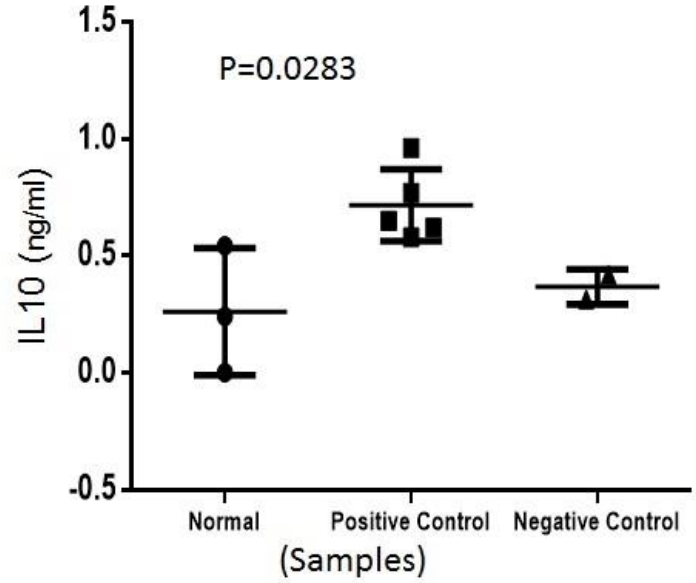


Fig.10a : TNF-α concentrations in the individual samples acquired from Ahmedabad and Nadiad

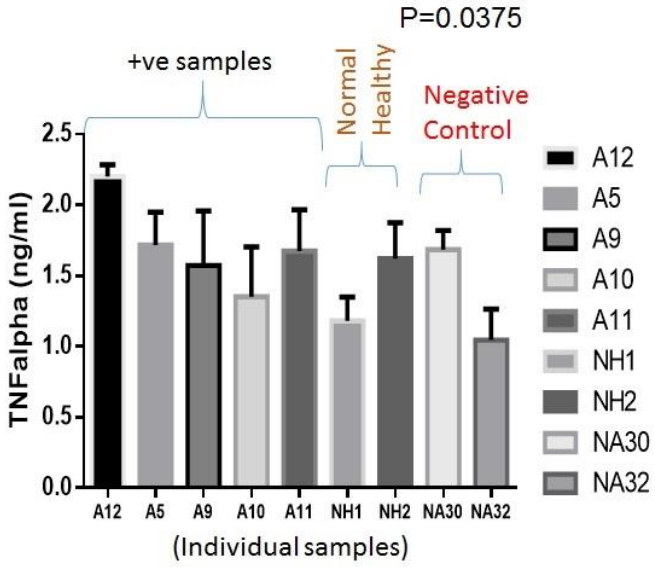


Fig.10b : IL-10 concentrations in the individual samples acquired from Ahmedabad

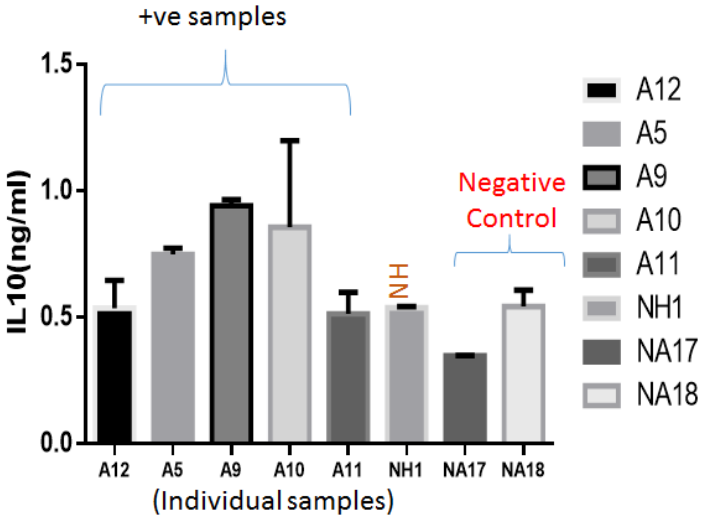
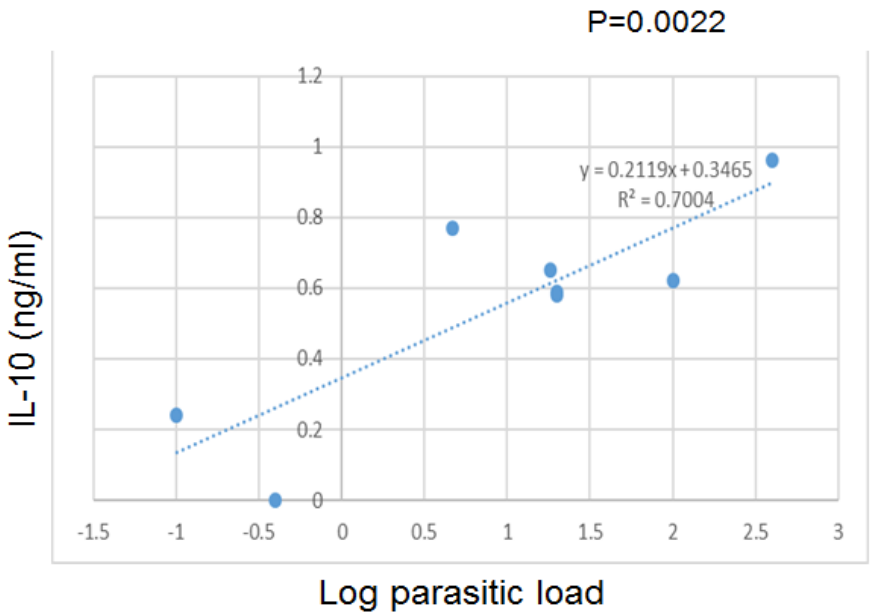


Fig.11a : Parasitic load vs IL-10 In Ahmedabad and Nadiad



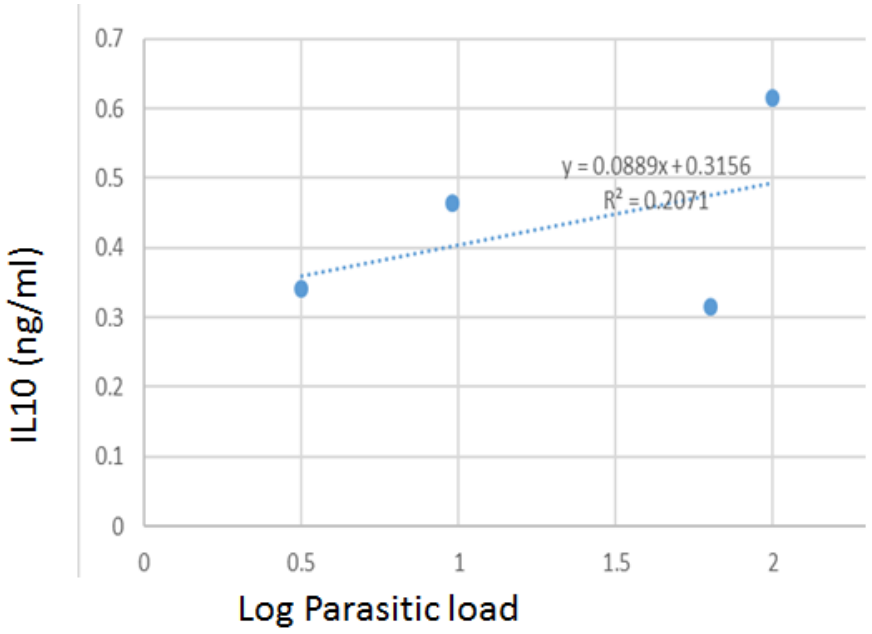
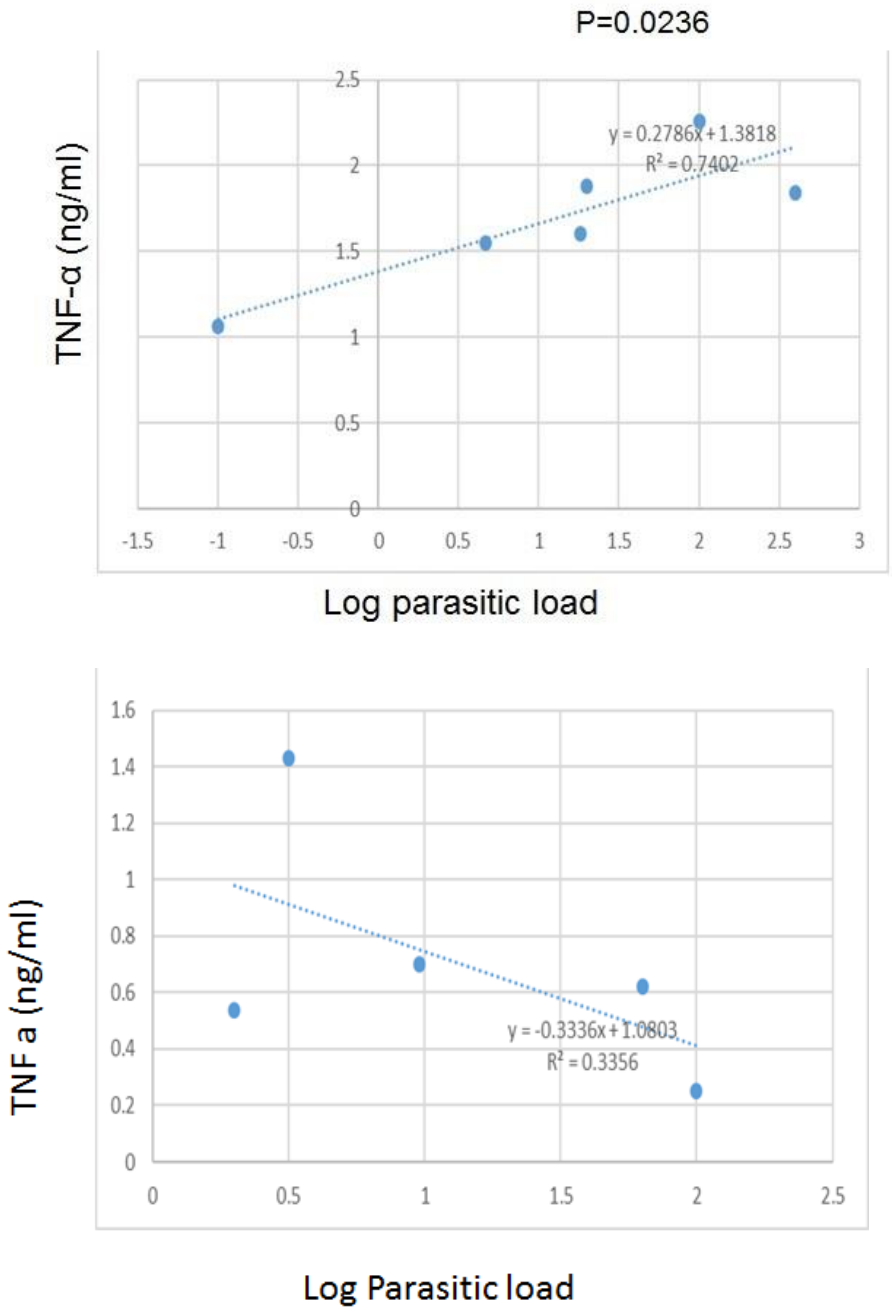


Fig.11b Parasitic load vs TNF-α in Ahmedabad and Nadiad

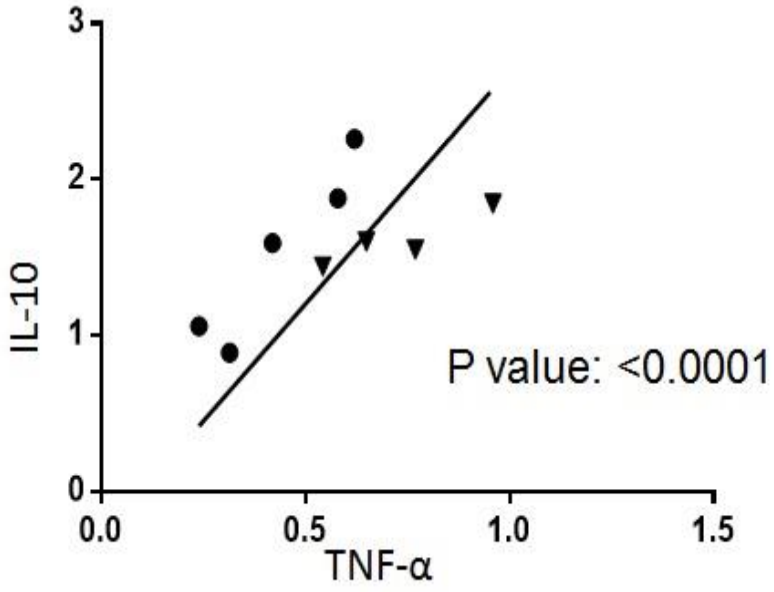


Plasma levels of TNF-α and IL-10 in plasmodium infected, control and healthy groups from Nadiad region

The mean concentration of TNF-α was found to be 0.56 ng/ml in the patients with malaria infection and 0.985 ng/ml in the control group, for which the significance value was P = 0.5810, r^2 = -2.019. as shown in Fig. No.10. The levels of TNF-α hence dropped drastically during infection when compared to the control subjects. Surprisingly, opposite was the case for IL-10, the IL-10 levels increased in the infected samples as compared to the control population. P=0.1762, r^2 =-16.96 as shown in Fig. No.10

Correlation of IL-10 vs TNF-α in Plasmodium infected population.

In this study we found a positive correlation between IL10 concentration vs TNF-α in the above said subjects from Ahmedabad. The r^2 value was 0.4256 and the correlation was significant P<0.0001 as shown in Fig. No.11. In case of Nadiad sample although the correlation was significant P= 0.0458 but r value is -0.005 as shown in Fig. No.11.



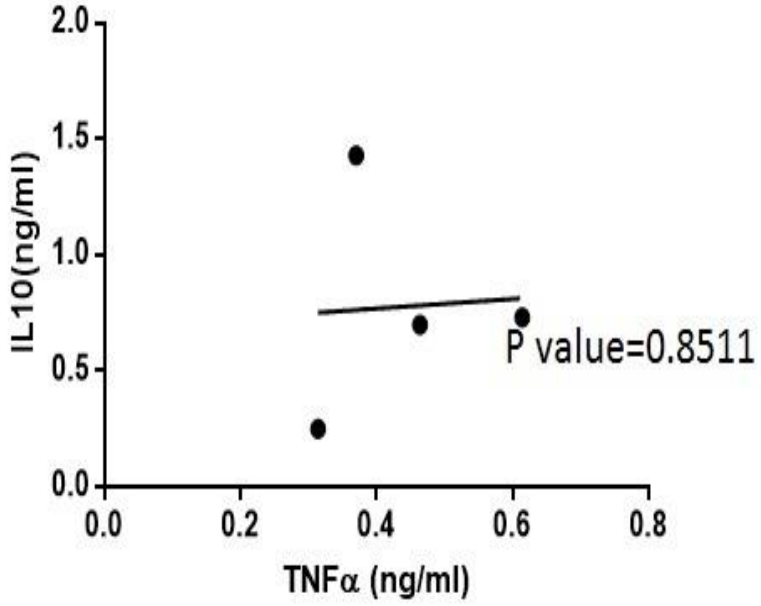


Fig.12 : IL-10 V/s TNF-α

Relative levels of TNF-α and IL-10 in Ahmedabad and Nadiad regions

Baseline plasma levels of individual cytokines were first compared between control, healthy and infected groups of both the regions.P<0.0001, r=0.4256. In Ahmedabad region, the levels of TNF-α in plasma was found to be high (1.826 ng/ml) in positive samples as compared to the positive samples from Nadiad (0.56 ng/ml). The control population from Nadiad region exhibited higher levels (0.985 ng/ml) of TNF-α when compared to the infected group (0.56 ng/ml) of the same region. In contradiction, the control population exhibited the lower concentrations (0.35 ng/ml) of TNF-α as compared to infected individuals (0.464 ng/ml). P value=0.1482, r^2 value=-0.005

Correlation of IL-10 with the parasitic load

Expression levels of IL-10 were analyzed in the plasma of malaria infected individuals by sandwich ELISA. Levels of IL-10 were significantly higher in cases with high parasitic load (P =). No such correlation of TNF-α was found with parasitic load.

Correlation of cytokines relative to parasitic strains

In *P.vivax* infected samples from Ahmedabad region, the TNF-α levels were found to be high (mean = 1.826 ng/ml). While IL-10 levels related negatively with TNF-α. The range of IL-10 was from 0.58 ng/ml – 0.96 ng/ml with the mean of 0.716 ng/ml in *P.vivax* infected samples acquired from Ahmedabad. Interestingly, there was much less difference in the concentrations of IL-10 and TNF-α of *P.vivax* infected samples from Nadiad. The mean concentration of TNF-α in *P.vivax* infected samples from Nadiad was 0.56 ng/ml having the range of 0.25 ng/ml – 0.73 ng/ml. Similarly IL-10 concentration ranged between 0.34 ng/ml – 0.37 ng/ml with the mean of 0.35 ng/ml.

Discussion

The ability of the immune system to establish a precise balance between Th1 and Th2 responses can be crucial to guarantee effective clearance of the pathogen without excessive damage to the tissue. In this study we measured the levels of TNF-α and IL-10 in the plasma of patients suffering from malaria. IL-10 levels were found to be high in the malaria infected individuals having high parasitic load exception was found in A12 sample (*P. falciparum* infected). It is noted earlier that circulating levels of IL-10 decreases markedly within 1 week after treatment, with anti-malarials [19]. Functionally IL-10 is well described as a suppressive or deactivating cytokine; in vitro, it has been shown to inhibit antigen presentation [35], antigen-specific T cell proliferation and type 1 cytokine production [36] and to render macrophages refractory to activation by IFN-c for intracellular killing [37]. The endogenous production of IL-10 has been shown to hinder the clearance of many other infectious organisms including Klebsiella pneumoniae, Brucella abortus, Candida albicans, Trypanosoma cruzi and Mycobacterium avium [38–42]. IL-10 has been shown to inhibit the production of TNF-α by monocytes [20] and there was a positive correlation between IL-10 and TNF-α levels in the plasma of malaria patients. TNF-α concentrations were also found to be high in the samples from Ahmedabad region (mean – 1.826 ng/ml). While there was reduction in TNF-α levels in the plasma of samples collected from Nadiad when compared to the control group of the same region. The presence of TNF-α with IL-10 in the early stage of the disease may suggest activation of monocytes. The desperate need of reliable and rapid diagnostic and prognostic tools can be fulfilled. IL-10 contributed towards the region specific difference in basal cytokine response. The balance between pro- and anti-inflammatory cytokines are thought to play a pivotal role in the pathogenesis and in regulation of immune responses in plasmodium malaria, but still their role is a mystery in the how it leads to pathogenesis and its relationship to host protection. An increase in IL-10 levels during uncomplicated acute malaria is thought to reflect effective early responses by Th1 cytokines [21]. But excessive production of IL-12 facilitates the class switching of IgG2a isotype. And excessive of Th1 responses leads to the damage of self tissues, which in turn is regulated by IL-10 by switching the response to Th2. The increased levels of IL-10 in samples having high parasitic load from Ahmedabad region and the samples from Nadiad depicts Th2 shift in the basal cytokine response. the positive correlation between IL-10 levels and P.vivax density [39] and the detection of low plasma concentrations of IL-10

in asymptomatic carriers of very low parasitemias (Table 2) allow for two competing interpretations. High IL-10 levels may be a consequence of high parasite density if a minimum density threshold is required to trigger substantial IL-10 production. Alternatively, increased IL-10 levels may favor parasitemultiplication by inhibiting parasite-killing effector mechanisms in humans [44] and mice [35]. IFN-γ is a critical mediator of clinical immunity to malaria and is associated with protective mechanisms [22, 23]. Pathogenic Th1-type responses have been suggested to be downregulated by certain factors such as IL-10 [6]. And IL-10 is also referred to be pleotropic immune-modulatory cytokine which is known to regulate Th1 and Th2 reactions in many instances [24, 25]. However the role of IL-10 in malaria remains controversial, as abnormally high levels have been shown to be associated with both severity of the disease and its protection [19, 26, 27],[28]. In our study IL-10 shows the best evidence of its direct relationship with parasitemia. It was also been demonstrated in children with parasitic density [4]. Our analysis showed that TNF-α did not significantly contribute to any group. Analysis that is compatible with the possibility that these cytokines follow a pattern that is rather determined by other factors like IL-10, without influencing the process of pathogenesis directly.Positive correlation between IL10 and TNFa in our study suggest with increase in parasite density IL10 expression go up in contrary it downregulate TNFa expression. Clinical outcome of the disease is a consequence of the complex interaction between the pathogen and the host and survival of pathogen largely depends on the type of cytokine (Th1 or Th2) being produced by host immune cells on encounter. Finally, our result suggest that the concentration of IL-10 is inversely proportional to TNF-α, and it is thought that IL-10 regulates the production of TNF-α by monocytes mediated by IFN-γ [20]. Still, each cytokine has their own unique function and most of them exhibit exceptionally wide range of bio-chemical effects that simultaneously acts on a particular cell typ, which creates a complex cytokine network, and meakes a precise correlation to be difficult with malaria severity. In conclusion, mechanisms that link cytokines with the bio-chemical aspects of cell regulation and expression of adhesion molecules in the microvasculature are seeming to be the potential modulators of severity of malaria. On the basis of these observations, measurement of these two cytokines can serve to be helpful for diagnostic purposes.

Summary

- The real–time PCR assay has immense application value as a rapid and accurate test for identification strain as well as parasitic load in the purpose of diagnosis and prognosis of Malaria.
- This study emphasizes that IL–10 production correlates with parasitic burden in active human Malaria, making it a biomarker of disease severity.
- IL-10 : Strongest predictor of the disease in the endemic region.
- *P. falciparum* elicits more inflammatory response than *P. vivax*
- Proposed study suggests immunsupression in malaria positive subjects due to low expression of MHC II gene.

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