



Immune Responses to Defined *Plasmodium falciparum* Antigens and Disease Susceptibility in Two Subpopulations of Northern India

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Abstract: The aim of this study was to investigate the prevalence of naturally acquired immune response to malaria in individuals of different age groups belonging to areas of northern India, Loni PHC (LN) and Dhaulana PHC (SD) of district Ghaziabad. *Plasmodium falciparum*-infected erythrocyte lysate and six synthetic peptides from different stages of *P. falciparum* (CSP, MSP1, AMA1, RAP1, EBA175 and PfG27) were used to determine both humoral and cellular immune responses. Plasma of individual subject was also analyzed for IL-4, IL-10, IFN- γ and TNF- α level. We observed an age-wise increasing trend of immunity in these two populations. There was a significant association between the number of antibody responders and recognition of stage-specific epitopes by antibodies. Peripheral blood mononuclear cells of more than 75% of individuals proliferated in response to stimulation by all the antigens in LN area. IL-4 and IL-10 responses were significantly higher in individuals of LN Area; whereas IFN- γ and TNF- α responses were higher in individuals of SD Area. It was also noticed that the frequency of responders to stage-specific antigens was higher in individuals from the LN area where the frequency of malaria was lower. The naturally acquired immune responses to *P. falciparum* antigens reflected the reduced risk of malaria in the study groups. The results demonstrated immunogenicity of the epitopes to *P. falciparum* in population of this endemic zone.

Keywords: Malaria, *P. falciparum*, antigen, peptide, ELISA, Immune response.

1. Introduction

In areas where malaria is endemic, the acquisition of antimalarial immune protection is progressive, as observed by age-wise decrease of morbidity and mortality to malarial infection [1, 2]. Acquired immunity to malaria is achieved in adults as a cumulative effect after repeated exposure [3]. The humoral response to various antigens of Plasmodia is considered to be one of the important mechanisms mediating this immune state [4]. Malaria caused by *Plasmodium falciparum* remains a serious disease in the world endangering the life and development of infants and young children [5]. It is established from earlier studies that some of the important antigens, which induce protective responses were shared by *P. falciparum* parasites of different geographic regions [6].

Panels of stage specific antigens of *P. falciparum* have been characterized; they were taken to evaluate their immunogenicity for their inclusion in a malaria vaccine [5, 7, 8]. Several antigens of *P. falciparum* erythrocytic merozoite stage are under investigation for their inclusion in a subunit vaccine, among them merozoite surface proteins 1 and 2 (MSP1 and MSP2), the apical membrane antigen-1 (AMA1), rhoptry associated protein-1 (RAP1) and erythrocyte binding antigen-175 (EBA175) showed high immunogenicity producing partial or total protection in simian malaria [9]. Sera of the inhabitants living in malaria endemic areas showed marked immunoglobulin-G reactivities to these antigens [8, 10, 11, 12, 13, 14, 15]. The circumsporozoite protein (CSP) and thrombospondin related adhesive protein (TRAP), as well as liver stage antigens 1 and 3 (LSA-1 and LSA-3), are the most

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promising of these components. It was demonstrated that irradiated sporozoites were excellent material for a malaria vaccine, and a vaccine was assembled using the circumsporozoite (CS) protein [16, 17]. Recently, a switch from the original alum adjuvant to a more recent adjuvant and immune enhancer, along with the addition of an immunogenic fusion partner, namely the S antigen of hepatitis B (HBsAg), has caused the CS protein to be re-evaluated as a component in a vaccine against malaria. In fact, as RTS,S the CS protein is now showing great promise in trials in Africa [18, 19]. Study of the mosquito stage, where the idea is to reduce the chances of contact between the vector and the human host, has produced many potential targets that aim at the parasite's sexual stages. The most promising of these vaccine candidates are Pfs25 and Pfg27 [20, 21]. They have successfully shown the ability to block parasite infectivity to the mosquito vector, thus blocking the spread of the parasite between humans.

In Plasmodial infections, the balances between pro- and anti-inflammatory cytokines determine the level of anaemia, parasite load, degree of pyrexia, clinical severity and outcome of infection [22, 23, 24]. The role of pro- and anti-inflammatory cytokines in immune response to *P. falciparum* infection is well documented [25, 26, 27]. Proinflammatory cytokines, such as tumour necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) play a dominant role in the destruction of pathogens and confer protection, whereas cytokines like IL-4 and IL-10 are thought to play antagonistic roles and thus lowers the effect of TNF- α and IFN- γ [28, 29, 30].

Among Southeast Asian countries, India alone contributes more than 80% malaria cases and *P. falciparum* accounts for around 50% cases [31]. Large areas of India are under low to moderate intensity of malaria transmission. In these areas, immunity to

malaria is generally low; adults attain immunity after repeated exposures. The present study was conducted in two subpopulations living more or less closely in two village areas of district Ghaziabad, Uttar Pradesh. Individuals of the two areas were studied for their parasitologic and immunologic profiles. In the present report, we determined the immunogenicity of *P. falciparum*-infected erythrocyte lysate (Pf-crude antigen) and six *P. falciparum* stage-specific peptides, CSP, MSP1, AMA1, RAP1, EBA175 and Pfg27 by evaluating both humoral and cellular responses in a population where malaria is seasonal and the inhabitants experience with the natural course of *P. falciparum* infection.

2. Materials and Methods

2.1 Study Areas & Subjects

A cross-sectional survey was conducted from July to December 2007 in Ghaziabad district of Uttar Pradesh, in northern India, which is endemic for both *P. vivax* and *P. falciparum* malaria. The inhabitants of villages under Loni PHC (Area LN) and Dhaulana PHC (Area SD) participated in the study (Fig. 1). In these areas, malaria is seasonal; early and prolonged monsoons are responsible for intensive transmission of both the species. Among the anopheline population, *Anopheles culicifacies* is a most abundant vector followed by *Anopheles annularis* and *Anopheles subpictus* [32]. Blood samples were collected by active survey of fever cases for malaria. Axillary temperature was measured. Giemsa stained thick and thin blood smears were examined. Patients diagnosed with malaria were treated with recommended doses of antimalarials as per National Drug Policy. This study was approved by the Institutional Ethics Committee. Informed consent was obtained from all study subjects.

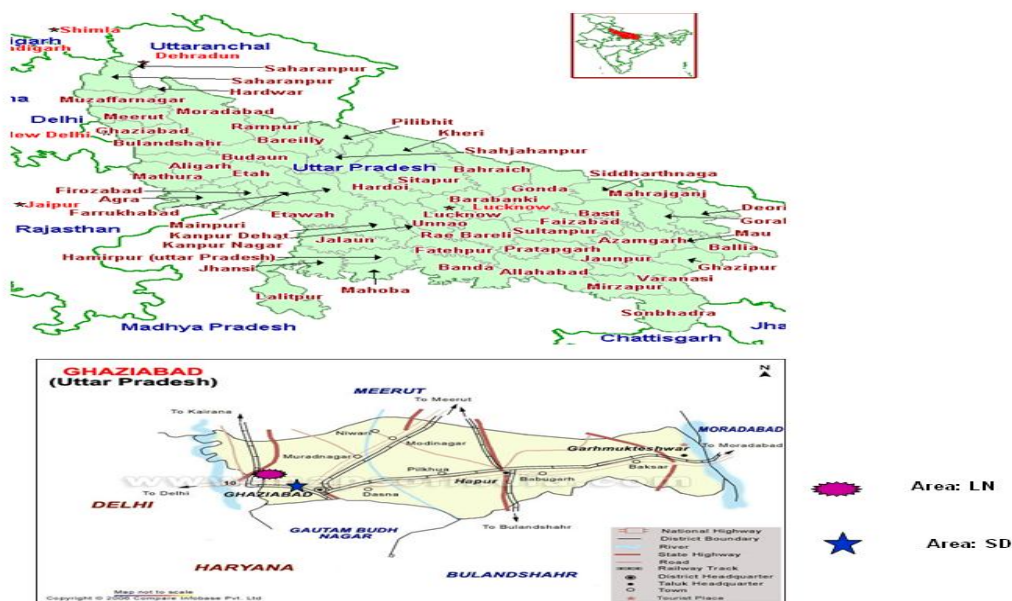


Fig. 1. Study site.

2.2 Antigens

2.2.1 *P. falciparum* crude antigen (PfC)

PfC was prepared from a well adapted, established *P. falciparum* culture line by the published method [33]. Briefly, the parasite culture was maintained *in vitro* using human O+ RBC and AB+ serum by candle-jar technique [34]. Cultures enriched with late trophozoites and schizont stages were taken in antigen preparation. Parasites were freed from host erythrocytes by saponin lysis and disrupted by sonication at 14μA for 90 seconds in a sonicator (MSE Soniprep, UK). The soluble extract was taken as antigen after centrifugation and purification by removing host contamination by adsorbing with rabbit antisera raised against human erythrocytes [35].

2.2.2 Peptides

The immunodominant parts of *P. falciparum* CSP, MSP1, AMA1, RAP1, EBA175 and Pfg27 were used (Table 1). They were synthesized by Fmoc-chemistry followed by purification through gel filtration chromatography [36, 37]. The purity of each peptide was assessed by HPLC and amino acid analysis. All the peptides were found to be >90% pure. They were used at a final concentration of 10μg/ml for ELISA and LTT. The details of the peptides used are given below:

Circumsporozoite Protein (CSP): This is a major surface protein of sporozoites. It is nearly a 67kDa protein. All CSPs have a central region of tandemly repeated amino acids.

Merozoite Surface Protein 1 (MSP1): It is synthesized by the intracellular schizont as a 185-205kDa precursor protein. Following the proteolytic processing of the precursor, only carboxy-terminal 19kDa remains on the surface. This fragment is a conserved region of MSP1.

Apical Membrane Antigen 1 (AMA1): This protein is involved in the invasion of RBC. It is a 66kDa protein located in the apical membrane of the merozoite.

Rhoptry Associated Protein 1 (RAP1): This protein is also involved in the invasion of RBC. It is an 80kDa protein located in rhoptry organelles of the merozoite.

Erythrocyte Binding Antigen 175 (EBA175): The erythrocyte binding protein is located in the micronemes of *P. falciparum*. The Pf-EBA 175kDa is a sialic acid-binding protein. Antibodies to region II of EBA175 block erythrocyte binding and also inhibit merozoite invasion.

Pf-Gametocyte Antigen 27 (Pfg27): This is a more potent immunogen of gametocytes of 25-27kDa. It is synthesized at the early stage of gametocytes when they are not even morphologically distinguishable.

Table 1. Synthetic peptides from *Plasmodium falciparum* stage-specific antigens.

Antigens	Sequences	References
CSP	(NANP) ₃	[7]
MSP1	NSGCFRHLDEREACKLL	[13]
AMA1	DGNCEDIPHVNEFSAIDL	[8]
RAP1	LTPLEELY	[10]
EBA175	NEREDERTLTKEYEDIVLK	[12]
Pfg27	KPLDKFGNIYDYHYEH	[20]

2.3 Antibody estimation

An indirect ELISA was performed following published protocol [1]. In brief, the 96-well round bottom polystyrene plates (Iwaki, Japan) were coated with individual peptides at a protein concentration of 10μg/ml and with PfC antigen at 20μg/ml by incubating for 1 h at 37°C, then overnight at 4°C. Sera were tested at 1:200 dilutions in duplicate for each antigen. After addition of sera at a respective dilution onto the first layer, they were allowed to react for 1 h at 37°C and 1 hr more at room temperature followed by addition of antihuman IgG conjugated with horseradish peroxidase (Dakopatts, Denmark) at an optimum dilution to trap the antigen-antibody complexes. After incubation for 1 h at 37°C and 1 hr more at room temperature, the enzyme-specific substrate, ortho-phenylenediamine/hydrogen peroxide (Sigma, Aldrich) was added. The reaction was terminated with 8N sulfuric acid and absorbance was read at 490nm in an ELISA reader (Microscan, ECIL, India) and results expressed as O.D. values. In each plate, reference antibody positive and negative samples were tested as controls.

2.4 Lymphocyte proliferation assay

This assay was done in a subset of 15 and 18 older children of Both LN and SD areas. Within 24 hr after bleeding, peripheral blood mononuclear cells (PBMC) were isolated from venous blood by density gradient. The proliferation assay was performed following published method with slight modification [13]. A total of 0.5x10⁶ PBMCs was used in a volume of 200μl cultures per well in 96-well flat bottom tissue culture plates (Greiner, GmBH). All 6 peptides (CSP, MSP1, AMA1, RAP1, EBA175 and Pfg27) of *P. falciparum* were used at 1μg/ml; PfC and NRBC antigens at 10μg/ml and phytohemagglutinin (SIGMA-Aldrich, USA) as positive control at 5μg/ml concentrations. After incubation for 120 h at 37°C in 5%, CO₂ atmosphere cultures were pulsed with 1μCi of ³H-thymidine per well and incubated for 18 h. After incubation, the cultures were harvested and radioactivity measured in β-scintillation counter (Beckman, USA).

2.5 Cytokines Assay

Plasma of each subject was kept at -70°C until use. Cytokines as IFN-γ, IL-4, IL-10, TNF-α were

determined by two-site sandwich ELISA using commercially developed two-site ELISA assay kits (Source: Peptotech Asia, Rocky hills, New Jersey, USA) following published method with slight modification [24]. Mean absorbance values were determined against known concentrations of IL-4, IFN- γ , IL-10 and TNF- α by Sandwich ELISA to estimate cytokine concentrations in plasma.

2.6 Data Analysis

Parasite density in blood smears of study subjects was estimated by counting the number of parasites per 200 leucocytes and the counts were converted to a number of parasites/ μ l blood taking 8000 leucocytes/ μ l as a standard mean [38]. For the purpose of analysis, the subjects of each area were divided into three age groups with corresponding parasitologic data. Each ELISA plate coated with individual antigen contained blank, reference negative and positive controls. Assaying 20 sera of non-malarial, non-endemic young adult's determined cutoff values for negative sera. The thresholds for positivity for each antigen were set above mean OD+3 SD values of negative serum. ELISA OD values for each antigen were compared and differences between intra-group and inter-group were tested by one-way ANOVA. The ELISA OD values of sera from two areas tested against 7 antigens were compared to see correlation of seroresponses between antigens by Pearson's coefficient of correlation. *In vitro* lymphocyte proliferation in the presence of various antigens was calculated by geometric mean counts per minute for each set of triplicate wells. The stimulation index (SI) was determined as the geometric mean counts per minute of antigen/mitogen stimulated culture divided by the geometric mean counts per minute of unstimulated culture. Cytokine estimation data were presented as mean \pm SD/mean \pm SEM/median (IQR). Box diagrams were plotted taking median values with 25th and 75th percentiles, and bars for 10th and 90th percentiles of cytokine concentrations and values

outside the 10th and 90th percentiles were plotted as points. For all tests, P values <0.05 were considered significant.

3. Results and Discussion

In surveying a population of 1223, malaria slides positive cases were 318 (26%), which include 214 *P. falciparum* and 104 *P. vivax* cases. Fig. 2 shows the epidemiological profile and parasite episodes of individuals belonging to LN and SD areas. Area LN and Area SD were distinguished on clinical and parasitologic data. In LN Area, children presented with one febrile episode associated with parasitaemia <5000/ μ l; a less number of children found parasitaemic compared to SD Area. In SD Area, children presented with more than one parasite episodes associated with >5000/ μ l. Based on this, from LN and SD areas, 81 and 84 children of 1-<5 yr; 45 and 46 school children of 5-15 yr age groups and a batch of 34 and 32 adults were selected for the immunologic study, respectively.

In this study, we have monitored a population living in an endemic area of malaria, where the occurrence of seasonal fluctuations of transmission was envisaged. The *P. falciparum* parasitaemia among different age groups could not be correlated statistically as observed by other workers also [38]. In our study population, 32 and 78 children below 5 years; 26 and 51 of 5-15 year; 11 and 16 above 15 years from LN and SD areas were found *P. falciparum* positive and mean parasitaemia was lower in LN area compared to SD (Table 2). The data on parasitaemia of two areas could suggest that they differ in malaria prevalence based on the number of *P. falciparum* positive cases. The *P. falciparum* parasitaemia among different age groups could not be correlated statistically. Both younger and older children were equally prone to *P. falciparum* infection.

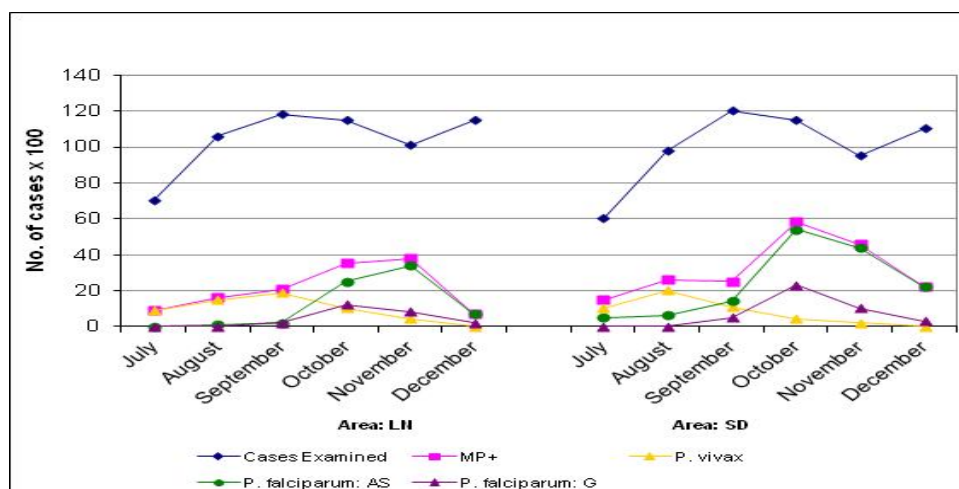


Fig. 2. Malaria profile in two areas (LN & SD) with seasonal transmission of malaria.

Table 2. Characteristics of study population.

Study site	Blood slide examined	Age group	Mean age (years)	Malaria slide+	Pf+ (%)	Pf count/ μ l
LN	625	1 to <5 yr	2.6	62	32 (25.4)	1550
		5 to 15 yr	10.6	45	26 (20.6)	8436
		>15 yr	32.3	19	11 (8.7)	7635
SD	598	1 to <5 yr	2.4	103	78 (40.6)	18900
		5 to 15 yr	10.2	72	51 (26.6)	15650
		>15 yr	31.5	17	16(8.3)	10645

For immunologic investigation, synthetic peptides were used that represented epitopes from conserved and semiconserved regions of *P. falciparum* asexual/sexual blood stages. All 160 and 162 sera obtained from LN and SD areas were tested for their reactivity to *P. falciparum* antigens CSP, MSP1, AMA1, RAP1, EBA175, Pfg27 and parasite lysate (PfC). Figs. 3 and 4 show the absorbance levels of antigen-specific IgG antibodies in various age groups indicating the intensity of immune responses and seroresponder frequency among younger and older children. Antibodies detected against the peptides and Pf crude antigen were lower in subjects from SD Area than LN ($P < 0.01$). The adults of both LN and SD areas showed similar seroreactivity ($P < 0.05$). In younger children, the antibody response rate to CSP, MSP1, EBA175, AMA1, RAP1, Pfg27 and Pf infected erythrocyte lysate was higher in children of both LN and SD areas ($P < 0.01$). In older children's groups, seropositivity to MSP1 and PFC was observed in almost all the subjects ($P < 0.05$). In adult groups, almost all the individuals showed seropositivity to these antigens.

The main objective of the study was to investigate whether naturally acquired immune responses to different stage-specific antigens of *P. falciparum* could reflect the less risk of infection in the study population. Evaluation of age-specific prevalence of antibodies to a

variety of malarial antigens may help in exploration of trends in malaria endemicity in two subpopulations. Although the antigens of *P. falciparum* used here are to some extent polymorphic, they contain conserved/semiconserved sequences that induce strong antibody responses. In the present study, all six antigens clearly vary in immunogenicity; MSP1 appears to be highly immunogenic at moderate malaria endemicity. Our findings revealed an overall higher ELISA IgG reactivity for samples from LN compared to SD against the tested antigens. Age-associated pattern of ELISA reactivity may suggest that antibodies to all these stage-specific antigens may represent serological markers of age-acquired immunity to distinguish between areas of different transmission intensity. We found that IgG responses to antigens were detectable in almost all donors. The highest prevalence of seropositivity occurred in subjects of more than 15 years age in both the areas as observed earlier also [4]. Among adults of both areas, seroprevalence was high, whereas parasite prevalence fell, which may be the expected outcome among individuals who acquired a significant degree of immunity [39]. Serological cohort studies are an efficient means of obtaining information on the force of infection in areas of very low transmission and single conversion events may be detected [40].

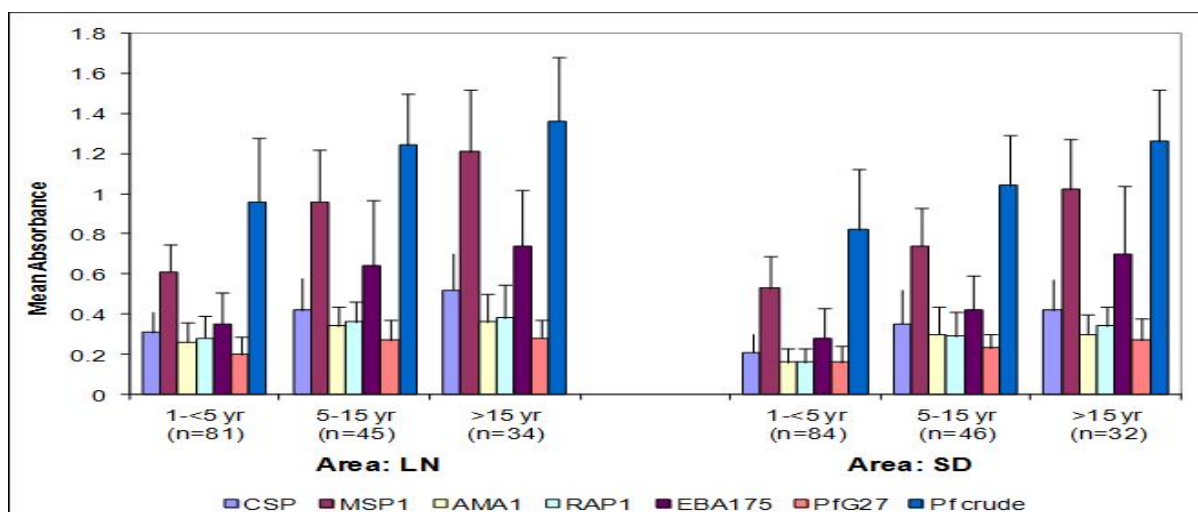


Fig. 3. Antibody profile of study subjects in different age groups. Sera were tested for antimalarial IgG antibody against 6 Pf stage-specific peptides (CSP, MSP1, EBA175, AMA1, RAP1 and Pfg27) and Pf infected erythrocyte lysate (Pf crude). Antibodies detected against the peptides and Pf crude antigen were lower in subjects from SD Area than LN ($P < 0.01$). The adults of both areas showed similar seroreactivity ($P < 0.05$).

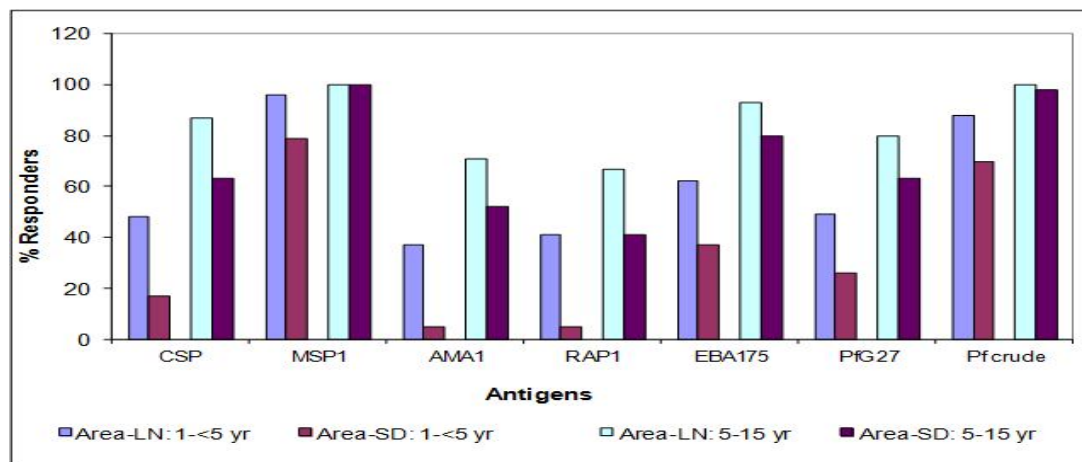


Fig. 4. Seroresponders frequency. In younger children, the antibody response rate to CSP, MSP1, EBA175, AMA1, RAP1, Pfg27 and Pf infected erythrocyte lysate was higher in children of both LN and SD Areas ($P < 0.01$). In older children's groups, seropositivity to MSP1 and Pf crude was observed in almost all the subjects ($P < 0.05$).

Lymphocyte proliferative response frequency in study cases of LN Area was higher (above 60%) compared to SD Area with antigens CSP, MSP1, AMA1, RAP1, EBA175 and Pf-C ($P < 0.001$), but in Pfg27 peptide, the response was almost alike in children of both areas (Fig. 5). Cytokines as IFN- γ , IL-4, IL-10, TNF- α were determined in plasma by two-site sandwich ELISA (Fig. 6). IL-4 and IL-10 responses were significantly higher in individuals of LN Area ($P < 0.01$), whereas IFN- γ and TNF- α responses to these antigens were higher in individuals of SD Area ($P < 0.05$).

For *P. falciparum* malaria in humans, the existence of functionally different CD4+ T cells has been established experimentally in naturally exposed individuals. These T cells respond to malaria antigen *in vitro* by proliferation and by secretion of cytokines, such as IL-4 and IFN- γ [41]. The cytokines as IL-4 and IFN- γ have strong counterregulatory activity, failure to mount a strong IFN- γ responses may be a tendency to

respond to malarial infection. Anti-inflammatory cytokines as IL-4 and IL-10 counteract the production of the pro-inflammatory cytokines. The cytokine, TNF- α has a central role for both protection and malaria pathogenesis. The levels of TNF- α and IFN- γ (both pro-inflammatory) are also increased with the disease. In comparison to healthy normal's (HC) both were elevated among the malaria cases ($P < 0.001$). TNF- α and IFN- γ both are also known to check parasitaemia thus are important at early stage of the infection [23, 27]. But at very higher concentration these cytokines are harmful for the host itself. The most effective T-cell responses against malaria are those that promote production of specific antibodies. The use of all these epitopes in the context of vaccine could be determined based on the specific antibody and T-cell responses. But as such antigenicity of these epitopes could be brought under use in determining immune response *in vitro*.

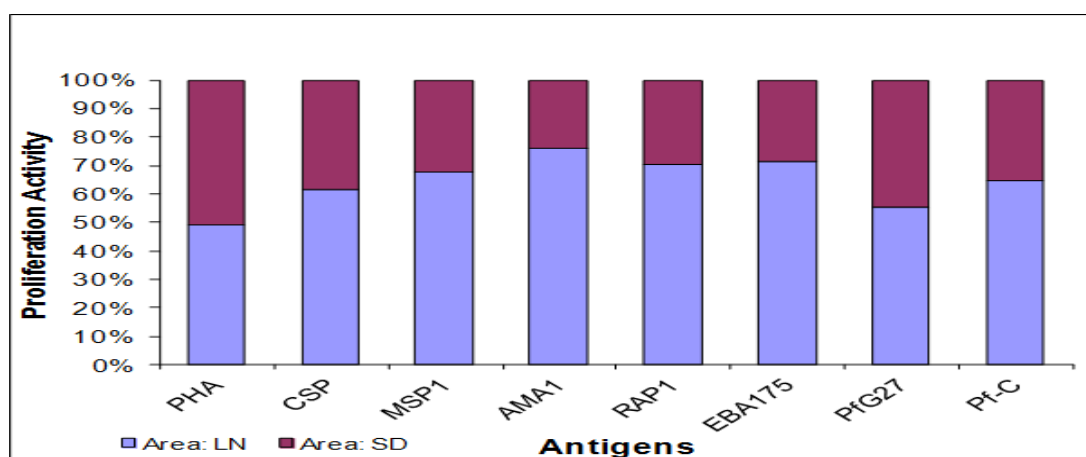


Fig. 5. Lymphocyte proliferation activity. Lymphocyte proliferative response frequency in study cases of LN Area was higher (above 60%) compared to SD Area with antigens CSP, MSP1, AMA1, RAP1, EBA175 and Pf-C ($P < 0.001$), but in Pfg27 peptide, the response was almost alike in children of both areas (NS).

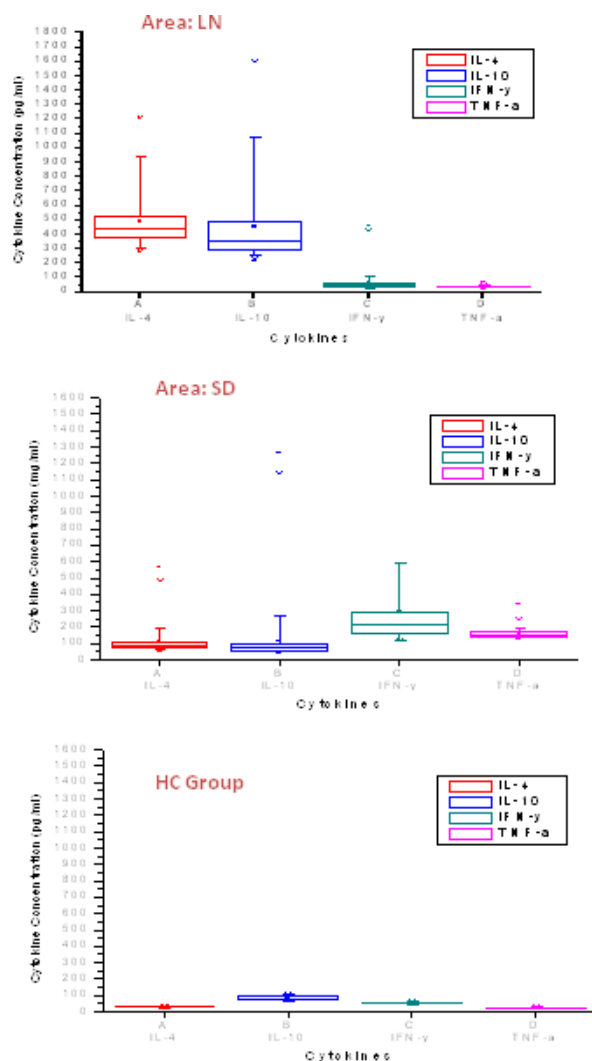


Fig. 6. Estimation of cytokines in plasma samples by two-site sandwich ELISA. Cytokines as IFN- γ , IL-4, IL-10, TNF- α were determined by two-site sandwich ELISA. IL-4 and IL-10 responses were significantly higher in individuals of LN Area to all the antigens ($P < 0.01$), whereas IFN- γ and TNF- α responses to these antigens were higher in individuals of SD Area ($P < 0.05$).

4. Conclusion

All synthetic peptides were able to detect antibodies and also induce cellular responses in some individuals. In areas with moderate endemicity, antibody responses were observed in 30-90% of the donors of LN Area and 5-80% of SD Area to any given peptide. Age-wise increasing trend of antibody has been observed in individuals of both areas, which showed correlation with reduced malaria morbidity. Clinical protection was achieved in some individuals showed higher proliferative responses and elevated levels of antibodies to MSP1, AMA1, RAPI and EBA175 peptides. The overall immune responses among individuals of two ecotypes highlight the immunogenicity of these molecules and their relation to clinical protection. Results obtained from this study

may be useful in monitoring natural immunity to *P. falciparum* malaria by using these epitopes as marker antigens, especially in younger age groups where low antibody responses and high parasite episodes could throw light in malaria prevention and control programme. Malaria epidemiology and transmission, in particular, is highly affected even by minor differences in environmental conditions. This type of study that take into account of microepidemiological approach and compares very close areas with slightly distinct characteristics are important to assess the patterns of establishment of malaria parasites in an area and response of human populations to them.

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