



RESEARCH ARTICLE

Antibody Responses Against *Plasmodium falciparum* MSP3 Protein During Natural Malaria Infection in Individuals Living in Malaria-Endemic Regions of India

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Abstract The humoral immune responses to blood-stage malaria proteins are requisite for the inhibition of parasite invasion. *Plasmodium falciparum* merozoite surface protein 3 (MSP3) is a secretory, expressed abundantly, merozoite surface protein that is important for the parasite invasion process. It has been shown to induce antibody responses during natural infections and is, therefore, considered to be the potential vaccine candidates against *Plasmodium*. Elucidating the immunogenicity and

prevalence of anti-parasite antibodies is important in identifying potential targets as candidates for malarial diagnosis and anti-malarial vaccine. The present study concerns the presence of antibodies against the MSP3 proteins of human malaria parasite- *P. falciparum* in infected individuals from endemic regions of India. Seventy-one anonymized *P. falciparum* infected serum samples were procured from the malaria fever clinic of ICMR-National Institute of Malaria Research (NIMR), New Delhi to detect the presence of antibodies against MSP3 protein by ELISA. The IgM antibody response against recombinant MSP3 was detected at significantly higher levels during acute malaria. The protein was found to be immunogenic and did not demonstrate any cross-reactivity with the serum of uninfected individuals or individuals infected with other *Plasmodium* species. The protein has hydrophilic regions in its N- and C-terminus which may contain immunogenic linear and conformational B-cell epitopes. The results from this study suggest that the MSP3 is immunogenic and likely a potential candidate for antibody-based diagnosis or vaccine development against the blood-stage of *P. falciparum*.

Significance statement Malaria is a major health concern in tropical and sub-tropical regions of world. For the management and control of malaria, vaccine development and effective diagnostic methods are essential. The understanding of humoral immune responses to malarial antigens are important for inhibition of parasite invasion. Elucidating the immunogenicity and prevalence of anti-parasite antibodies is important for developing potential targets as candidates for malarial diagnosis and as anti-malarial vaccine. The present study concerns the presence of antibodies against the *Plasmodium falciparum* MSP3 protein in infected individuals from endemic regions of India. The present study identifies MSP3 as an immunogenic molecule and likely a potential candidate for antibody-based diagnosis or vaccine development against the blood-stage of *P. falciparum*. The identification and characterization of immunogenic parasite antigens is important for the development of subunit vaccine development, diagnostic molecules, and transmission monitoring tools. The present study evaluates the natural antibody response profile in infected humans against *Plasmodium falciparum* MSP-3 that has relevance for the development of disease diagnostic and monitoring tools.

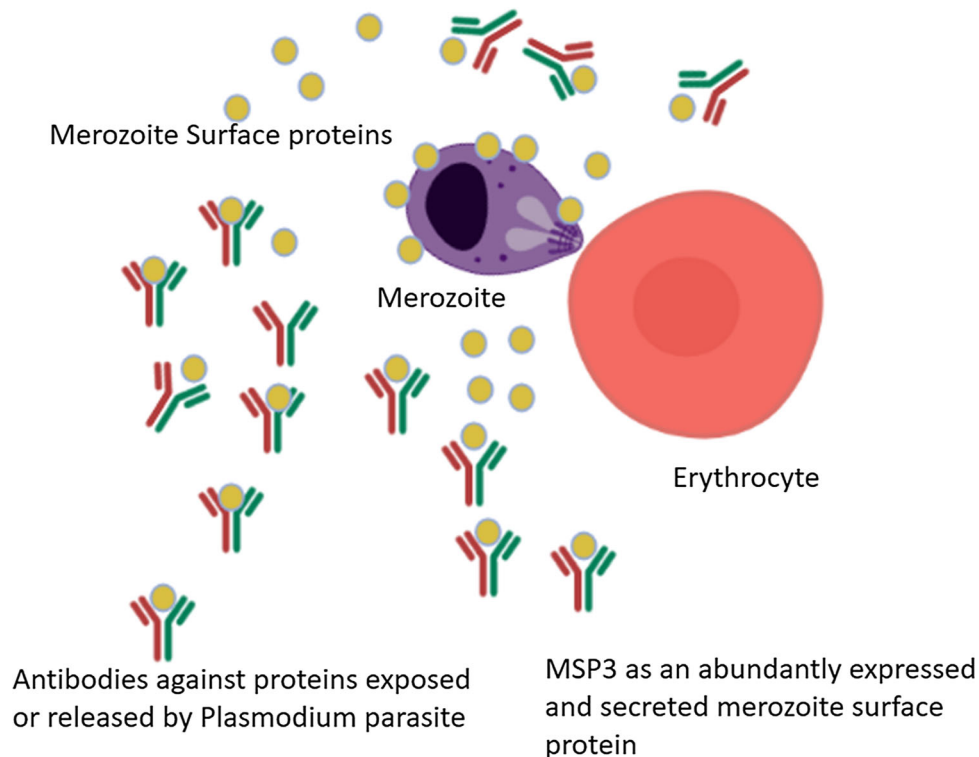
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Graphical abstract



Keywords MSP3 · Merozoite Surface Protein 3 · Malaria · Antibody response · Seroprevalence · *P. falciparum*

Introduction

Malaria is a fatal mosquito-borne disease, with an overall estimation of 220 million people infected per year worldwide [1]. The infection results in a wide variety of symptoms which include fever, malaise, and dehydration. The disease can be categorized into severe (complicated) and uncomplicated. Severe malaria can lead to anemia, multiple organs failure, coma, and even death. A substantial reduction in malaria mortality rates has been detected in the last 20 years; nonetheless, the burden of the disease still exists.

Humoral immune responses against *Plasmodium* species are critical to protective immunity and to develop naturally acquired immunity to malaria [2–4]. The latter prevents people from developing severe malarial symptoms. Studies

have shown that the passive transfer of antibody preparations or serum from clinically protected or partially immune subjects to non-immune individuals had anti-malarial potential [5, 6]. Asexual parasites were significantly decreased demonstrating those anti-malarial antibodies as generated in infected individuals are associated with partial protection against clinical malaria [7].

The merozoite membrane is comprised of the group of surface proteins that form an integral part of the merozoite membrane called as merozoite surface proteins (MSPs). MSPs that are attached directly to the merozoite membrane comprise of MSP1, MSP4, MSP5, and MSP10, while MSP6, MSP7, and MSP9, are joined via protein–protein interactions [8]. Many of these MSPs interact with the erythrocyte surface and play an important role in the invasion of erythrocytes. *P. falciparum* Merozoite Surface Protein 3 (MSP3) is a 43 kDa soluble protein situated on the surface of merozoites in association with other surface molecules. It undergoes proteolytic processing upon being secreted into the parasitophorous vacuole [9]. The protein was earlier recognized as secreted polymorphic antigen associated with merozoites [SPAM]). MSP3 was initially

identified when the purified antibodies obtained from clinically protected subjects were found to be effective in antibody-dependent cellular inhibition (ADCI), while antibodies directed against MSP3 were largely cytophilic [10–13]. The amino acid sequence of MSP3 consists of N-terminal and C-terminal regions. The N-terminal of MSP3 is polymorphic and has amino acid substitutions and multiple indels while the C-terminal domain of the protein has been found to be relatively conserved [14, 15].

The choice of MSP3 to study its seroprevalence was based on immuno-clinical analysis of the molecule which demonstrated MSP3 is immunogenic and associated with protection against clinical malaria [9, 16]. In addition, the MSP3 C-terminal has demonstrated complete sequence conservation in > 100 *P.falciparum* field isolates obtained from different geographical regions [16]. In the present study, we assessed the antigenicity of MSP3 molecule by analyzing the immune prevalence of anti-MSP3 antibodies using serum collected from infected individuals from different regions in India where malaria is endemic with a non-infected sample as a control.

Material and Methods

Ethics Statement

The Institutional Human Ethics Committee (ECR/NIMR/EC/2017/64) approved the use of anonymized *P. falciparum* infected sera samples preserved at ICMR-National Institute of Malaria Research (ICMR-NIMR), New Delhi, India. These samples were previously confirmed by microscopy, RDT and PCR for *P. falciparum* infection.

Protein Expression and Purification

MSP3 was expressed as two separate recombinant proteins or polypeptides representing the N-terminal and C-terminal regions to allow detection of possible antibodies against variable and conserved regions. The constructs were designated as MSP3N and MSP3C representing the variable N-terminal and significantly conserved C-terminal conserved regions [15]. *P. falciparum* MSP3N and MSP3C were amplified from 3D7 strain genomic DNA, cloned in DH5 alpha (NEB) cells, and expressed in BL21 (DE3) cells. The recombinant proteins were affinity purified as C-terminally His-tagged proteins using 5 ml HisTrap HP-column (GE healthcare) followed by 5 mL HiTrap QHP anion exchanged chromatography column (GE healthcare).

Dot-Blot

This assay was done using recombinant MSP3N and MSP3C proteins on nitrocellulose membrane strips. For this, 0.5–2 µg of the recombinant protein in 10 µL of buffer was put on the membrane using a vacuum manifold. The membrane was blocked with 3%BSA and *P. falciparum* infected human sera diluted at 1:50 in 1X PBS was applied to the nitrocellulose membrane (BioRad). Uninfected sera and sera from other *Plasmodium* species were used as control. The blots were processed for antibody signal detection in the same way as western blotting.

Enzyme-Linked Immunosorbent Assay (ELISA)

The presence of anti-MSP3 antibodies in Clinical samples was done by ELISA. For this, 1 µg of purified protein prepared in 1X coating buffer was coated on flat bottom ELISA plate in triplicates and incubated overnight at 4 °C. Next day, the plate was washed and blocked with 3% BSA for 1 h. The patient sera in 1:100–1:10,000 dilutions was added to the plate and incubated for 2 h at 37 °C. The plate was washed again with 1xPBST thrice. Following washing, the secondary antibody, i.e., goat-anti-human IgM-HRP antibodies were added to the wells in 1:10,000 dilution and incubated for 1 h at 37 °C. After washing, 90 µL of TMB substrate was added to the plate. The color was allowed to develop for 10–15 min in dark. 50 µL of 0.1 M H₂SO₄ was used to stop the reaction. The results were analyzed at 450 nm using an ELISA plate reader (Spectrostar). The serum antibody titres were also determined using ELISA.

Results and Discussion

Expression of PfMSP3N and PfMSP3C as Recombinant Polypeptide Fragments

The gene fragment clones were designed from N- and C-terminal regions of MSP3 protein. These were designated as MSP3N and MSP3C, respectively (Fig 1A). Parkers Hydrophilicity prediction was employed to identify hydrophilic and hydrophobic regions of the protein. We observed that both N and C-terminal regions had hydrophilic regions which likely contain immunogenic linear and conformational B-cell epitopes (Fig 1B). The individual gene fragments were amplified and cloned in the bacterial expression vector. The cloned MSP3 gene sequences (cloned as MSP3N and MSP3C) were expressed as C-terminal His-tagged proteins. The His-tagged recombinant

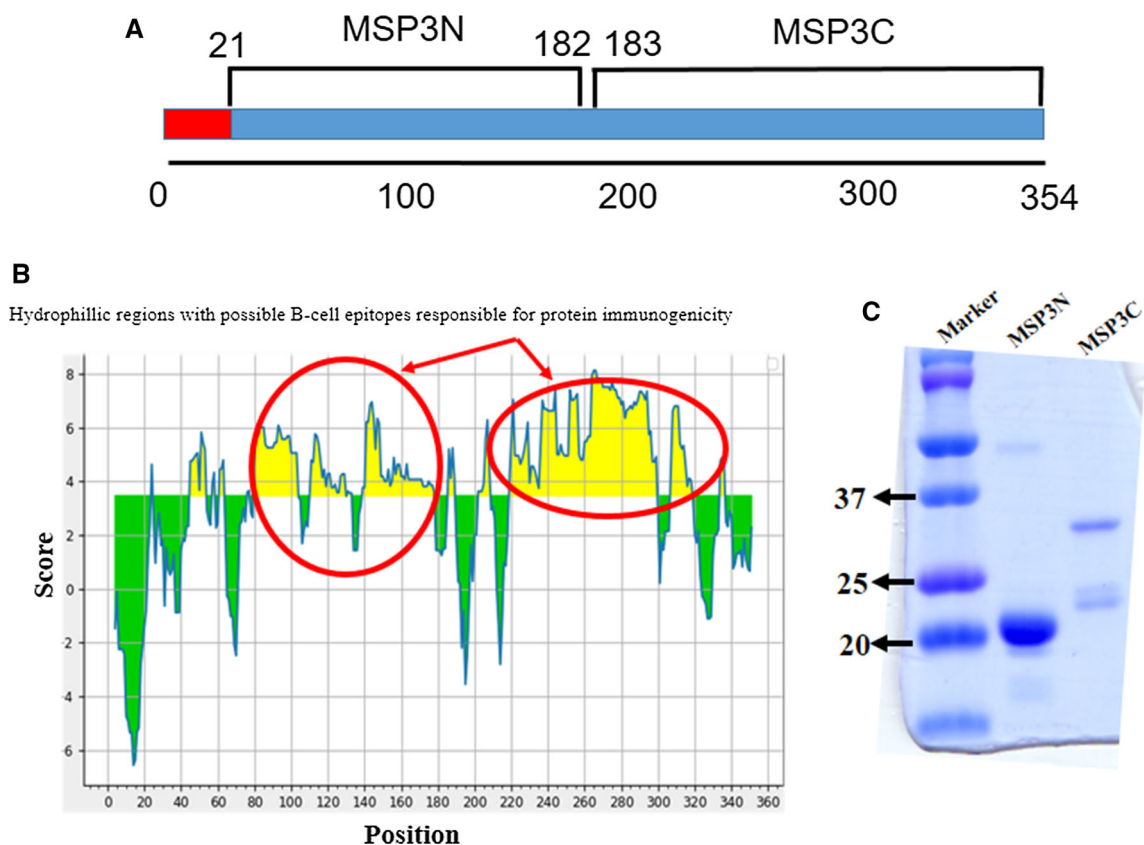


Fig. 1 Expression of rMSP3 protein. Schematic diagram showing the organization of PfMSP3 expressed as MSP3N and MSP3C recombinant polypeptides (A). Parkers hydrophilicity prediction showing hydrophilic (yellow) regions of the protein (B) Coomassie-stained

SDS-PAGE gel (C) shows migration and recognition of purified rMSP3N and rMSP3C in reduced dye. Lane M, molecular mass marker

protein, MSP3N, encompassed amino acid residues 21 to 182 while MSP3C encompassed amino acid residues 183 to 354 (Fig 1A). Both MSP3N and MSP3C polypeptides were expressed as soluble proteins and were purified by metal affinity chromatography. The elution fractions that contained the recombinant proteins were further subjected to anion-exchange chromatography to remove any contaminating host-cell proteins. The apparent mobilities of MSP3N and MSP3C corresponded to ~20 kDa and ~30 kDa, respectively, on SDS-PAGE gel (Fig 1C). The SDS-PAGE gel analysis suggests that the proteins were >98.0% pure and minimal contamination of the host proteins was observed in the purified protein samples.

Prevalence of Anti-MSP3 Antibodies in Malaria Infected Individuals

The antigenicity of MSP3N and MSP3C was evaluated using serum collected from malaria-infected individuals from endemic regions of India. For this, the ELISA-based analysis was setup. The 96-well plates were coated with

recombinant MSP3N and MSP3C proteins separately, followed by the addition of serum from infected patients (Fig 2A, B). The infected samples were found to be seropositive for anti-MSP3N and anti-MSP3C antibodies. The recognition of MSP3N and MSP3C by sera of infected individuals suggests that antibodies are produced against MSP3N and MSP3C during *P. falciparum* infection. These results illustrate the generation of humoral responses against MSP3 during natural *P. falciparum* infections.

Sera Obtained from Other *Plasmodium* Species Infected Individuals Showed No Cross-Reactivity for PfMSP3 Protein

The amount of antigenic relatedness between the MSP3 proteins of different *Plasmodium* species was measured by analyzing the cross-reactivity of MSP3 to sera against other *Plasmodium* species. For this, ELISA assays were performed using sera of *Plasmodium*-infected individuals at 1:100 dilution (Fig 2C). The sera of *P. vivax* and *P. malariae* infected individuals showed no significant cross-reactivity

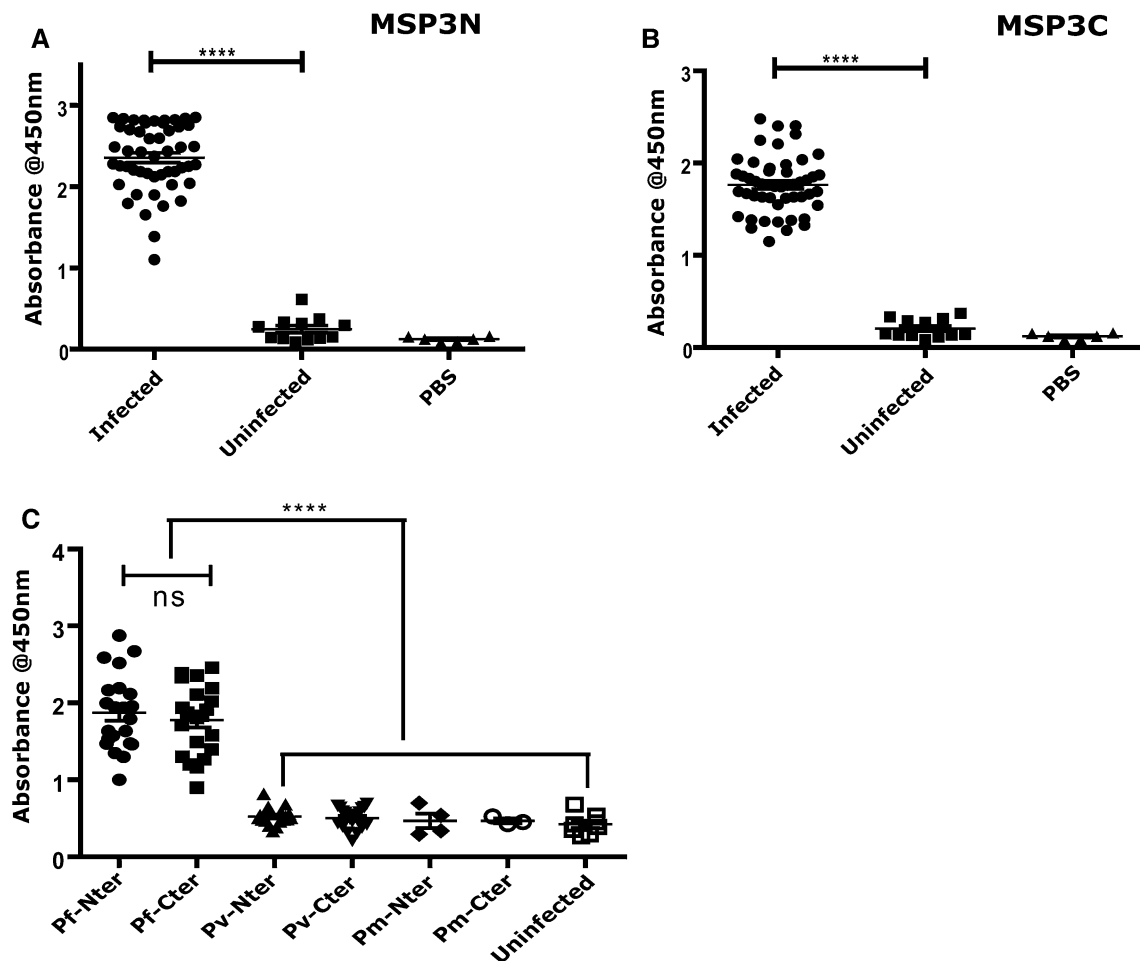


Fig. 2 Antibody responses against PfMSP-3 antigen in endemic regions of India The figure shows the antibody responses generated in *Plasmodium falciparum*-infected individuals ($n = 71$) against PfMSP-3 antigen (MSP3N and MSP3C) as determined by optical density (OD) following ELISA (A, B). The sera that were obtained from

individuals infected with *Plasmodium vivax* ($n = 10$) or *Plasmodium malariae* ($n = 3$) showed no cross-reactivity for PfMSP3 protein (C). Data have been presented as mean \pm SEM. **** $P < 0.0001$; ns, non-significant; by unpaired T-test

for PfMSP3 protein (Fig 2C). The samples used were confirmed positive for *P.vivax* and *P. malariae* by microscopy and PCR.

Analyzing the Titer of Sera from Infected Individuals

The titer refers to a serum dilution that still recognizes the antigen and gives an end point color above the control. Malaria vaccine formulations identify antigens that generate high titers. Sera collected from infected patients displayed high titers against MSP3 protein. The ELISA gave end point measurements even up to 5000 times dilution although a significant difference was observed at 500 times dilution (Fig 3A, B). The ability of sera obtained from infected individuals to identify recombinant MSP3N and MSP3C was established by testing its reactivity against

these proteins employing dot blotting. The immunoblotting using recombinant proteins was performed using sera obtained from infected individuals. The results demonstrated that the sera from infected individuals show binding affinity towards the recombinant proteins indicating the presence of anti-MSP3 antibodies.

Conclusion

Malaria continues to be a serious health problem in several tropical regions. To combat it, continuous diagnostic improvements and an effective malaria vaccine have long been sought after [17, 18]. An approach for developing effective diagnostic molecules or vaccines includes the identification of proteins that are secreted into the host or those that can generate immune responses. The role of the

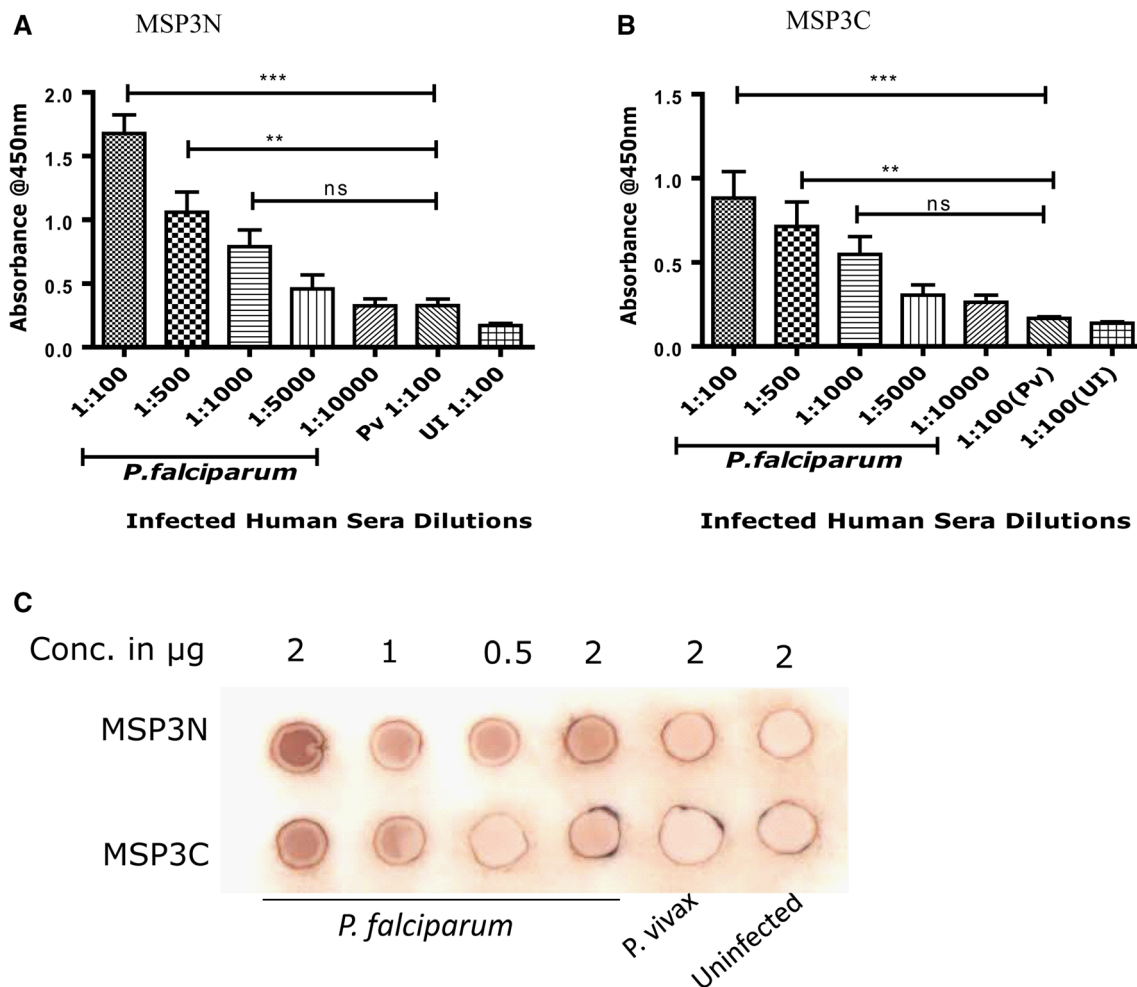


Fig. 3 In order to determine, the serum dilution which is insufficient for detecting recombinant protein, the serum titer was measured. The serum was serially diluted in the antibody dilution buffer and ELISA was conducted. The infected sera show significantly high absorbance even at 1:500 dilution (A, B). Dot-blot analysis of the recombinant

protein using *P.falciparum* infected sera was conducted. *P. vivax* and uninfected sera were used as control (C). Data have been presented as mean \pm SEM. *** $P < 0.001$; ** $P < 0.01$; ns, non-significant; by One way ANOVA using Tukeys Multiple comparison test

immune response against malaria has been increasingly highlighted in the past few years [19]. The seroprevalence and antibody responses against various malaria parasite antigens are being extensively studied for the development or understanding of acquired immunity and for vaccine development [2, 20]. The asexual erythrocytic stages of *Plasmodium* cause the symptoms and clinical manifestations of malaria. The proteins or antigens expressed during this stage are thus considered important targets for humoral responses [21]. Many of the blood-stage candidates have been characterized for their immunogenicity.

India is endemic to malaria and contributes 6% of all malaria cases in the world and 6% of the deaths [1]. The identification of anti-malarial antibodies by ELISA is considered to be a potentially useful epidemiological tool. For example, the antibodies against *Plasmodium* circumsporozoite antigens have been correlated with transmission

intensity. In the present study, the antibody responses generated against PfMSP-3 antigen were analyzed using ELISA-based diagnostic assay in *Pf*-infected serum samples. We conducted the study in infected patients to assess antibody responses against the variable and conserved regions of MSP-3 protein during *P. falciparum* infections in the endemic Indian setting. The serum samples from *P. falciparum*-infected patients collected from endemic regions and deposited in the NIMR repository were analyzed for anti-MSP-3 antibody levels by ELISA. Our results demonstrated that the high titer antibodies were generated in patients against MSP3. Our data showed significantly higher levels of anti-MSP-3 antibody in *P. falciparum*-infected individuals. Our data is in agreement with earlier reports stating that MSP-3 is highly immunogenic and elicits a rapid humoral response during acute infections. In this study, we observed over 99.9% of patients to be seropositive against

recombinant PfMSP-3 antigen. Such a strong induction of antibody responses could be due to the presence of immunogenic epitopes in protein. The in silico analysis of the protein shows the presence of hydrophilic regions which likely score best for B-cell epitopes. We employed Parker Hydrophilicity Prediction to identify the hydrophilic regions in the protein. We also characterized the cross-reactivity of PfMSP3 with antibodies induced against other strains of *Plasmodium* parasite. The sera that were obtained from other *Plasmodium* species infected individuals showed no cross-reactivity for PfMSP3 protein.

Difficulties in developing effective anti-malarial vaccines are in part due to poor understanding of potential targets that can induce immunity. The identification and characterization of immunogenic parasite antigens is vital for the development of subunit vaccine development, diagnostic molecules, and transmission monitoring tools. The present study evaluated the natural antibody response profile in infected humans against *P. falciparum* MSP-3. The antibody levels were compared between *P. falciparum* infected and non-infected individuals. The serum from parasite-positive individuals had significantly higher anti-MSP3 levels. Serologically measured antibodies may be a biomarker of exposure and may reflect the acquisition of functional antibodies.

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Author Contribution AA, AQ and LK designed and conceived the idea. AQ and SK performed the experiments. AQ wrote the manuscript. All authors reviewed the manuscript.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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