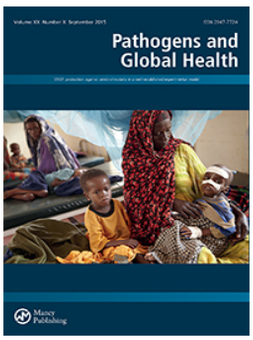

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Diagnostic potential of monoclonal antibodies developed against C-terminal polypeptide of *P. falciparum* Histidine Rich Protein2 (*Pf*HRP2) in malaria infected patients from India

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ABSTRACT

Malaria, caused by *Plasmodium falciparum* has become a major health burden in most tropical and developing countries. *P. falciparum* Histidine Rich Protein2 (*Pf*HRP2), which exhibits polymorphism, is being widely used as a diagnostic marker. Recently, we reported the development of monoclonal antibodies against conserved C-terminal 105 amino acids of *Pf*HRP2 for malaria diagnosis. Now, in this study, the diagnostic performance of two anti-C-terminal *Pf*HRP2 mAbs (b10c1 and Aa3c10) were evaluated with 100 blood samples from clinically identified malaria patients from seven different geographical centers in India. Sandwich ELISA, polymerase chain reaction (PCR) and statistical tools were used for the evaluation of the performance of the anti-C-terminal *Pf*HRP2 mAb. These mAbs detected *P. falciparum* (mean OD value 1.525 ± 0.56) malaria with great accuracy with no cross reactivity with *P. Plasmodium vivax* (mean OD value 0.285 ± 0.051) and normal healthy control samples (mean OD value 0.185 ± 0.06) in Sandwich ELISA assay. The samples which were RDT negative for *P. falciparum* were also reactive in Sandwich ELISA with mean OD value of (1.303 ± 0.532). The amount of *Pf*HRP2 antigen in the patients' blood sample was quantified and categorized into three distinct groups having the HRP2 antigen in high, intermediate and low amounts. The presence of *Pfhrp2* gene was also confirmed by PCR analysis. The sensitivity and specificity of the mAb were found to be 95 and 96% respectively. These data strongly suggest that the anti-C-terminal *Pf*HRP2 mAbs b10c1 and Aa3c10 have merits for improvising the existing malarial diagnostics.

KEYWORDS

Anti-C-terminal *Pf*HRP2 mAb; *Plasmodium falciparum*; *Plasmodium vivax*; Histidine Rich Protein2 (*Pf*HRP2); polymorphism; malaria diagnosis; malaria immunodiagnosis; malaria rapid diagnostic test

Introduction

Malaria is a major public health problem in tropical countries and approximately 24 million new cases of infection are reported every year in South East Asian countries alone [1]. Of the five species causing malaria in humans, *Plasmodium falciparum* is the most lethal and one of the major contributors of infant and child mortality [2]. With the emergence and spread of anti-malarial resistant *P. falciparum* parasite strains, early and accurate diagnosis of malaria is one of the significant factors in preventing complications and effective disease management [3].

Historically, microscopic detection of malarial parasites were done using thick and thin peripheral blood smears that still remains as a standard for laboratory diagnosis. Despite being cost effective, microscopy is time consuming and requires expertise. *P. falciparum* unique protein *Pf*HRP2 could be utilized as a diagnostic marker [4]. This protein is stable, persistent and is produced by both asexual and early sexual stages of the parasite and thereafter it is transported through the erythrocyte cytoplasm and secreted into the extracellular plasma [5]. *Pf*HRP2 is being considered as an ideal biomarker for the quantitative assessment of the magnitude of malarial infection caused due to *P.falciparum* parasite [6].

A number of anti-*Pf*HRP2 antibodies have been developed and used in Rapid Diagnostic Tests (RDTs), but due to genetic polymorphism in *pfhrp2*, there is variation in sensitivity and specificity of RDTs [7,8]. Variation in sensitivity and specificity amongst the anti-*Pf*HRP2 monoclonal antibodies (mAbs) is an important issue that needs to be addressed before they are employed for diagnostic purposes [9,10]. W.H.O guidelines recommend a mandatory parasitological confirmation of diagnosis in all patients suspected of malaria before initiation of treatment, particularly for children. Thus, an accurate laboratory diagnosis will prevent unnecessary/inappropriate treatment and restrict the use of anti-malarial drugs [11].

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The antigen C-terminal polypeptide of *Pf*HRP2 (rec*Pf*HRP2-T3) used in this study was developed based on unique regions of *Pf*HRP2 compared to its closest orthologue *Pf*HRP3 and the frequency of the peptide repeats present in the primary amino acid sequence using bioinformatics approaches. Importantly, this uniqueness is believed to be conserved in different *P. falciparum* isolates originating from various parts of the world. So far no attempts have been made to develop mAbs against this unique region comprising of various peptide repeats of *Pf*HRP2 [12,13].

We produced recombinant C-terminal *Pf*HRP2 antigen comprising of 105 amino acids, successfully developed highly specific mAbs and evaluated its diagnostic potential by a sandwich ELISA method in clinical samples which originated from seven different geographical regions in India, which are considered as hotspots of malaria infection. Initially, these samples were analysed by PCR using species specific primers and confirmed whether infected samples harbour either *P. falciparum* or *Plasmodium vivax* or both (mixed infection) [14]. Further evaluation of these blood samples by anti-C-terminal (referred as anti-Ctp) *Pf*HRP2 mAb from hybridoma clones b10C1 and Aa3c10, demonstrated that these mAbs are highly efficient in the specific diagnosis of *P. falciparum* infections.

Materials and methods

Ethical statement

This study was approved by the Institutional Ethical Committee and was conducted in collaboration with the Christian Medical College and Hospital, (CMC), Vellore Tamil Nadu, India. Prior consent of the patients and healthy volunteers were taken before collection of blood.

Sample detail

Blood samples ($n = 100$) used for in study were part of an Acute Undifferentiated Fever (AUF). This work was a multicentre study coordinated by the Benjamin Pulimood Laboratory for Infection, Inflammation and Immunity (BMPLIII), Department of Medicine-1, Christian Medical Collage (CMC), Vellore. The seven different centres in India from where samples were collected along with the number of samples from each centre are: Ambur (Tamil Nadu)-16, R-Anantpur (Andhra Pradesh)-21, Mungelli (Chattisgarh)-10, Oddanchatram (Tamil Nadu)-4, Ratanagiri (Maharashtra)-27, Raxual (Bihar)-9, and Tezpur (Assam)-13. The samples were from patients tested for malaria infection by a genus and species specific PCR for *P. falciparum*, *P. vivax* and mixed infection as reported [14]. The sample details are summarized in Tables 1A–1C.

Rapid diagnostic test

All patient blood samples were initially tested with the RDT ParaHIT-Total Ver.1.0 Device 551C204-10 (Span Diagnostics Ltd., Surat, India) at the reference laboratory following manufacturer's instructions. This RDT simultaneously detects two antigens viz., pan antigen aldolase from Plasmodium and species specific HRP2 from *P. falciparum*. The RDT detects whether the observed infection is due to malaria caused by any of the *Plasmodium* species (*P. falciparum*, *P. malariae*, *P. vivax*, and *P. ovale*) by detecting the aldolase antigen and more specifically *P. falciparum* infection by *Pf*HRP2.

The RDT kits were quality checked using known positive and negative controls

Samples were collected from patients diagnosed with *P. falciparum* infection ($n = 69$), *P. vivax* infection ($n = 25$) and mixed infection ($n = 6$). Among the *P. falciparum* infected samples, 27 were RDT positive and 42 were RDT negative for *P. falciparum* antigen HRP2 (Table 1A). Twenty-five samples were positive for *P. vivax* malaria by RDT (pan positive for pLDH) and species PCR (Table 1B). Altogether 6 mixed infection samples were positive for both *P. falciparum* and *P. vivax* by RDT (Table 1C).

Blood samples from healthy individuals ($n = 9$) were used as healthy controls. In addition, blood samples ($n = 6$) from AUF patients, negative for malaria by PCR, were also used as negative control.

Polyclonal and monoclonal antibody

A cDNA clone of truncated rec*Pf*HRP2 (rec*Pf*HRP2-T3 comprising of C-terminal amino acids of *Pf*HRP2) was

Table 1A. P. falciparum infected human malaria sample details.

Name of the malaria centre	Number of patients	Genus PCR for *Plasmodium*	Species PCR for *Falciparum*	*Pf*HRP-2 RDT results	*Sandwich ELISA anti-Ctp *Pf*HRP-2 mAb with cut-off value 0.305	**PfHRP-2 specific PCR
Ambur	7	Positive	Positive	2+ve & 5−ve	7 positive	N/D
R-Anantpur	20	Positive	Positive	5+ve & 15−ve	20 positive	2+ve & 18 ND
Mungelli	10	Positive	Positive	5+ve & 5−ve	5+ve & 5−ve	3+ve, 5−ve & 2 ND
Oddanchatram	1	Positive	Positive	1+ve /	1 positive	ND
Ratanagiri	17	Positive	Positive	7+ve & 10−ve	17 positive	4+ve/ 1−ve/ 12 ND
Raxual	7	Positive	Positive	2+ve /5−ve	7 positive	N/D
Tezpur	7	Positive	Positive	5+ve/2−ve	7 positive	N/D

Note: ND refers to not determined due to non-availability of sample.
*Sandwich ELISA cut-off value was fixed at 0.305; the values above 0.305 were considered as positive for *P. falciparum* infection.
**PfHRP2 gene specific PCR.

Table 1B. P. vivax infected human malaria sample details.

Name of the malaria centre	Number of patients	Genus PCR for *Plasmodium*	Species PCR for *vivax*	RDT results	*Sandwich ELISA with anti-Ctp *Pf*HRP-2 mAb with cut-off value 0.305
Ambur	8	Positive	Positive	Pan positive	8–ve
Oddanchatram	2	Positive	Positive	Pan positive	2–ve
Ratanagiri	8	Positive	Positive	Pan positive	7–ve & 1+ve
Raxual	2	Positive	Positive	Pan positive	2–ve
Tezpur	5	Positive	Positive	Pan positive	5–ve

*Sandwich ELISA cut-off value was fixed at 0.305; the values above 0.305 were considered as positive for *P. falciparum* infection.

Table 1C. Mixed infected human malaria sample details.

Name of the malaria centre	Number of patients	Genus PCR for *Plasmodium*	Species PCR for *P. falciparum* & *P. vivax*	RDT results	*Sandwich ELISA with anti-Ctp *Pf*HRP-2 mAb with cut-off value 0.305
Ambur	1	Positive	N/D	N/D	1+ve
Ananthapur	1	Positive	*P. f* & *P. v*	*P. f* & Pan positive	1+ve
Oddanchatram	1	Positive	N/*D*	N/D	1+ve
Ratanagiri	2	Positive	*P. f* & *P. v*	*P. f* & Pan positive	1–ve & 1+ve
Tezpur	1	Positive	*P. f* & *P. v*	*P. f* & Pan positive	1+ve

*Sandwich ELISA cut-off value was fixed at 0.305; the values above 0.305 were considered as positive for *P. falciparum* infection.

constructed, expressed and recombinant protein was purified to near homogeneity by use of Immobilized Metal Affinity Chromatography (IMAC) [12]. The rec*Pf*HRP2-T3 protein was used as an antigen for the development of both polyclonal (in laboratory-bred female New Zealand rabbits) and monoclonal antibody (6–12 weeks old female BALB/c mice). Two clones from the mAbs produced (b10c1 and Aa3c10) having affinity constant of $10^9 M^{-1}$ (determined by Surface Plasma Resonance using Ni-NTA column) were very promising [12,13]. Purified mAbs and polyclonal antibodies, were evaluated with malaria infected patient samples.

Generation of recombinant PfHRP2 (recPfHRP2-T1) for screening purposes

P. falciparum (field isolate from Rourkela, India) deposited at National Institute of Malaria Research (ICMR) (Ministry of Health & Family Welfare), New Delhi, India was used as a source of genomic DNA and exon-2, which codes for the functional *Pf*HRP2 was cloned into bacterial expression system, successfully expressed in *E. coli* and the antigen was purified to homogeneity (rec*Pf*HRP2-T1) by IMAC for screening purposes.

Screening of malaria patient blood samples by sandwich ELISA with anti-Ctp PfHRP2 mAb

Blood samples were screened by an in-house sandwich ELISA. Microtiter plates were coated with polyclonal antibody (capture antibody, 1 µg/100µL/well) and incubated overnight at 4 °C. Plates were blocked by adding 200µL of PBS containing 5% skimmed milk and incubated at 37 °C for 2hr. The plates were then washed thrice with wash buffer (PBS with 0.1% Tween-20 [PBST]). Blood sample (50 µl) was diluted with equal volume of PBS and 100 µL of diluted sample was added in duplicates and incubated at 37 °C for 2hr. After incubation and wash, mAb against truncated *Pf*HRP2 (1:10000 dilution of anti-Ctp *Pf*HRP2 mAb) was added (detector antibody) and incubated at 37 °C for 2hr. After washing, the plates were incubated with secondary antibody conjugated to horseradish peroxidase. 100 µl tetramethylbenzidine (TMB)/H_2O_2 solution was used as a substrate for the enzyme with 50 µl 2 M sulphuric acid as stopping solution [15,16]. The optical density (OD) was measured at 450 nm with a multiwell plate reader (FLUOstar Optima, BMG Labtech, Ortenberg, Germany). Purified recombinant *Pf*HRP2 was used as a positive control. Both the mAbs (b10c1 and Aa3c10) were used separately in duplicates and the average OD values were used to plot the graphs as the standard deviations were less than 10% between the two mAbs. The secondary antibody used in the experiment was anti-mouse IgG raised in goat purchased from Sigma (Sigma Aldrich Corporation, St. Louis, MO).

Determination of limit of blank, limit of detection and limit of quantification

The limit of blank = mean of 10 blank samples + 1.64 (S.D of blank);

The limit of detection = mean value of healthy control + 2SD (cut-off value);

The limit of quantification = is the cut-off value from where the test can be defined as positive or negative [17].

The OD value above the cut off value was considered as positive for *P. falciparum* infection. An overall cut-off value (mean value of healthy control ± 2SD) for *Pf*HRP2 antigen was calculated as reported by Noedl H and coworkers [18].

Quantification of PfHRP2 antigen in patient blood samples by Sandwich ELISA

To quantify the *Pf*HRP2 antigen in the blood samples; sandwich ELISA was carried out first to detect the antigen in blood samples. Subsequently, the quantification of *Pf*HRP2 antigen in the samples was done using a standard graph using decreasing concentration (serial double dilution) of purified recombinant *Pf*HRP2-T1 (1000, 500, 250 … 0.498 ng/ml) and was spiked in healthy blood samples. Sandwich ELISA was performed as described. The results are represented as a mean ± S.D. from 3 independent assays for each concentration and best fit curve was obtained using the Graph Pad Prism 5.0. The limit of detection (LOD) was taken as the lowest concentration of the antigen at which the mean OD is distinguishable from the background. The lower limit of quantification (LOQ) was calculated as the point at which the response is no longer linear [15,16]. The *Pf*HRP2 concentration in the patients' blood sample was determined by extrapolating the ELISA values of *P. falciparum* infected blood sample with the standard graph. To ensure that LOD and LOQ were biologically significant, background values were derived by analysis of negative blood samples from the same geographical regions.

To ensure that each of the samples gave a signal across the several points within the previously determined linear range of the assay, some of the patient's blood samples were serially diluted as 1:2, 1:4 … 1:2048 in PBST for quantification of *Pf*HRP2 antigen.

Detection of Pfhrp2 gene in RDT+ve and RDT–ve of P. falciparum patient's sample by PCR

Genomic DNA from patients infected with *P. falciparum* ($n = 20$), *P. vivax* ($n = 5$) and healthy control ($n = 2$) were used for PCR analysis of *Pf*hrp2 gene. *Pf*HRP-2 exon-2 specific primers as reported earlier by Baker et al., [19] were used for amplification with incorporation of the restriction site *NcoI* and *XhoI* in the forward and reverse primers respectively. The primers for Exon-2 of *Pfhrp2* gene were 5′ CCATGGCAAAAGGACTTAATTTAAATAAGAG 3′- forward primer and 5′ CTCGAGAATAAATTTAATGGCGTAGGCA 3′- reverse primer.

The PCR was carried out in an autoclaved PCR tube in a thermal cycler by denaturing the genomic DNA at 94 °C for 10 min. Subsequently 35 cycles of amplification were carried out where in each cycle consisted of denaturation at 94 °C for 1 min, annealing at 55 °C for 50sec and extension at 70 °C for 1 min followed by a final extension at 72 °C for 10 min. Each reaction mixture consisted of 0.2 mM dNTPs, thermo stable polymerase 5 units (Sigma Aldrich) and 100 ng template DNA (patients' genomic DNA). Expression plasmid coding for the *Pfhrp2* gene was used as a positive control. Genomic DNA from patients infected with *P. vivax* and healthy volunteers were used as negative controls for *Pfhrp2* gene.

Statistical analysis

Statistical analysis of the patient's ELISA data was done using the Graph Pad Prism 5 and 6. Statistical data are presented in the tabular form. Using the Graph Pad, we evaluated the tests through Receiver Operating Characteristic (ROC) analysis, in which sensitivity and specificity were calculated as a function of cut-off value, (specificity was plotted against sensitivity) and the Area Under the ROC curves (AUCs) was calculated [20].

Further, with the sandwich ELISA data (with 100 samples), the sensitivity and specificity of generated mAb was calculated using the formula 1 – (sensitivity) and 2 – (specificity), where the terms are defined as, True positive (patient has the disease and test is positive), False positive (patient does not have the disease but the test is positive), True negative (patient does not have disease and the test is negative) and False negative (patients has the disease but the test is negative) [21].

$$\text{Sensitivity} = \text{True positive}/\text{True positive} + \text{False negatives}(\text{Formula } -1)$$

$$\text{Specificity} = \text{True negative}/\text{True negative} + \text{False positive } (\text{Formula } -2)$$

Results

Sample detail

The sample details are summarized in Table 1. The content in Tables 1A, 1B and 1C consists of the outcome of genus and species PCR, RDTs and the results obtained with Sandwich ELISA, *Pf*HRP2 specific PCR.

Screening of malaria patients' blood samples by sandwich ELISA

The result of sandwich ELISA of blood samples with *P. falciparum*, *P. vivax*, mixed infection and healthy control is depicted graphically in Figure 1(A). The limit of blank was found to be 0.283 (mean of healthy control + 1.64 SD) and the limit of quantification was found to be 0.305 (cut-off value), which was calculated using OD values of healthy controls (mean + 2SD). Out of 69 samples of falciparum malaria, 64 were found to be positive with above mentioned cut-off value. Among the 25 vivax malaria samples, 24 were found to be negative and among the 6 mixed infections samples, 5 were found to be positive. The mean OD for the *P. falciparum* samples was 1.525 ± 0.671, *P. vivax* 0.295 ± 0.055 and mixed infection samples 0.766 ± 0.29. The mean OD value of healthy controls was 0.185 ± 0.06. Other statistical comparison of Sandwich ELISA OD values of patients' sample is summarized in Table 2.

Interestingly, many of the RDT–ve samples were reactive in this ELISA with mean OD (Figure 1(B)) value

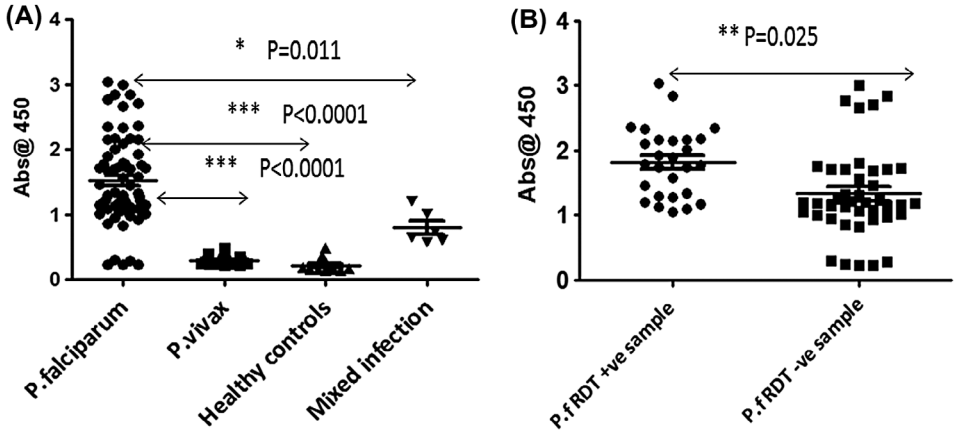


Figure 1. (A) Differential analysis of *P. falciparum*, *P. vivax* and mixed infection samples by Sandwich ELISA with anti-Ctp *Pf*HRP-2 mAb. Malaria infected patient blood samples from different malaria field stations within India were obtained. Samples were classified based on PCR analysis using genus and species sequence specific primers. These samples were further probed with mAb developed against C-terminal polypeptide of *Pf*HRP2 {*P. falciparum* (*n*) = 69; *P. vivax* (*n*) = 25; Mixed infection (*n*) = 6; Healthy volunteers (*n*) = 10}. (B) Analysis of Sandwich ELISA results of *P. falciparum* infected patient's samples. The samples were tested first with commercially available RDT kit. The results of *P. falciparum* infected samples from RDT Kit was (*n* = 27 RDT+ve and *n* = 42 RDT−ve).

Table 2. Statistical analysis of sandwich ELISA value of P. falciparum and P. vivax infected human blood samples.

Malaria patient's blood sample	Number of patient's sample	Average sandwich ELISA Value	High sandwich ELISA value	Low sandwich ELISA value	*P* value by *t*-test
P. *falciparum*	69	1.523 ± 0.671	3.045	0.300	*P* value between *P. falciparum* and *P. vivax* $P < 0.0001$ SE ± 0.081
P. Vivax	25	0.285 ± 0.055	0.462	0.211	*P* value between *P. falciparum* and *P. vivax* $P < 0.0001$ SE ± 0.015
Mixed malaria infection	6	0.761 ± 0.3025	1.12	0.35	*P* value between *P. falciparum* and mixed infection $P = 0.011$
Healthy control	9	0.1856 ± 0.061 Cut-off = 0.305 (Mean of healthy control ± 2SD)	0.364	0.142	*P* value between healthy control and *P. falciparum* $P < 0.0001$
Malaria Negative	6	0.240 ± 0.047	0.286	0.163	$P < 0.0001$

of 1.303 ± 0.532. The 6 malaria negative samples mentioned in sample detail had a mean OD of 0.240 ± 0.047 (Table 2).

Five samples, which were positive for *P. falciparum* infection by genus and species, based on PCR analysis, but negative to *Pf*HRP2-PCR, showed very poor reactivity with our anti-Ctp *Pf*HRP2- mAb with mean OD of 0.258 ± 0.034.

Quantification of PfHRP2 antigen in P. falciparum infected patients by Sandwich ELISA

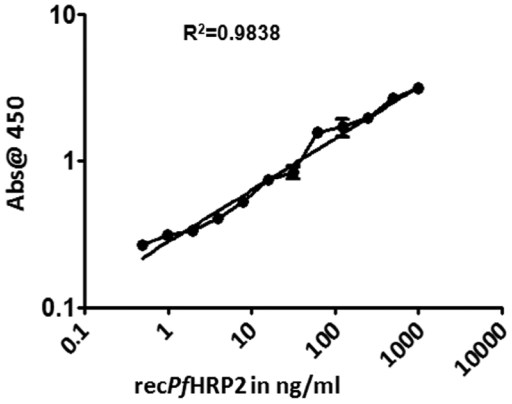
Established along the scores obtained in a sandwich ELISA for *P. falciparum* infected patient samples, the *Pf*HRP2 antigen amount was estimated from the standard graph (Figure 2(A)). Depending on the antigen concentrations, these samples could be categorized into 3 groups, viz. high (>1000 ng/ml mainly RDT+ve samples), intermediate (150 ng/ml-1000 ng/ml more of RDT+ve) and low (0.5 ng/ml to 150 ng/ml of RDT−ve samples). From Figure 2(B), according to the cut-off value of 0.305, the lowest amounts of *Pf*HRP2 antigen quantified from the patient's samples were found to be 0.956 ng/ml. The assay had working linear range corresponding to 1.0 ng/ml to 1000 ng/ml, which corresponds to 1 pg/µl of the antigen. Lower limit of detection for *Pf*HRP2 protein was 50 pg/test with 50 µl of blood sample.

PCR amplification of hrp2 gene

All 10 RDT+ve samples showed the presence of 855 base pair PCR amplified product as expected, which is in conformity with the amplification of *Pfhrp2* gene from cDNA construct containing *Pfhrp2* gene (Figure 3(A)). Among the 10 RDT−ve samples, four were positive by *Pfhrp*2-PCR (band at 855 base pair) and another five, which were having very low ELISA OD values (Figure 1(B)), were found to be negative by *Pfhrp*2-PCR (Figure 3(B)). There was no amplification of *Pfhrp2* gene as observed with respect to *P. vivax* samples (data not shown) indicating the absence of *Pfhrp2* in the genome of *P. vivax*. Additional bands were also observed and this could be due to the primers binding to more than one site in the *hrp*2 gene that code for some of the peptide repeats present in the *Pf*HRP-2 resulting in different sized PCR products [19].

(A)

r*Pf*HRP2 ng/ml	O.D at 450 nM Mean value	±S.D
1000	3.179	0.15347
500	2.709	0.08755
250	1.958667	0.005033
125	1.710333	0.238888
62.5	1.560667	0.007024
31.25	0.834667	0.078233
15.625	0.746333	0.042771
7.812	0.529333	0.02829
3.906	0.404	0.025534
1.953	0.337	0.025239
0.976	0.31333	0.020257
0.488	0. 262	0.012
Negative	0.136	0.0012
Blank	0.0356	0.0034



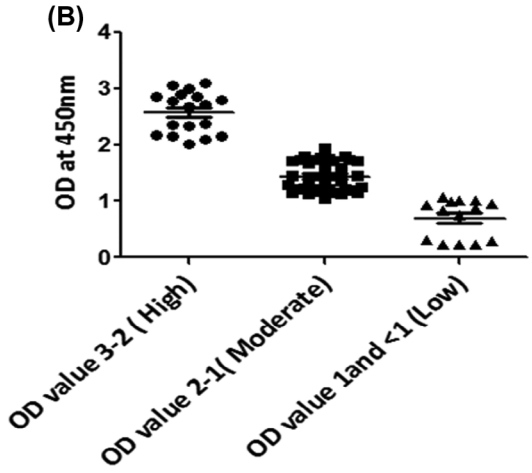


Figure 2. (A) Standard Graph for quantification of *Pf*HRP2. Known concentrations (1000–0.488 ng) of purified recombinant *Pf*HRP2 antigen was spiked in healthy blood samples. Subsequently Sandwich ELISA was carried out as described in methods. The OD values against concentration were plotted on log scale on both axis and the values are fitted to true line with standard deviation. The best fit curve was obtained. The minimal OD value = 0.262 corresponds to 0.488 ng/ml, while the highest OD value = 3.179 corresponds to 1000 ng/ml of the *Pf*HRP-2 antigen (Elisa values are mean of triplicates; ± SD). (B) Quantification of *Pf*HRP-2 antigen levels in *P. falciparum* patients' sample. From the standard graph the unknown concentration was extrapolated and the samples were categorized into 3 groups viz. high, moderate and low. The concentration of *Pf*HRP2 antigen in the blood that could be detected was in the range between 0.976 and 1000 ng/ml. This range is LOD in which the levels of native *Pf*HRP-2 antigen in blood samples become feasible for its determination.

Statistical analysis

A summary of *Pf*HRP2 antigen levels in malaria patient samples along with their mean, range and test of significance and *P* values are provided in Table 2. In *t*-test, the *P* value ($p < 0.001$) indicated that the differences in OD value of sandwich ELISA results between *P. falciparum* and *P. vivax* samples were highly significant. With unpaired t-test between RDT–ve and RDT+ve of *P. falciparum* samples, the *P* value was less than 0.05 and hence the difference in the mean of the OD value was significant. These data clearly indicate that the anti-Ctp *Pf*HRP2 mAb is highly specific and could efficiently differentiate falciparum vs vivax infection. From the data obtained with sandwich ELISA, the sensitivity was found to be 0.957 (64 true positive from *P. falciparum* + 4 positive from the mixed infection /68 true positive + 2 false negative from *P. falciparum* + 1 from mixed infection). Similarly, the specificity was found to be 0.964 (true negative 24 from *P. vivax* + 3 from *P. falciparum* as they were *P. vivax* positive by microscopy /27 true negative + 1 false positive from *P. vivax*).

ROC analysis

Figure 4 shows the ROC curves for sandwich ELISA using the comparative O.D. values of *P. falciparum* with healthy control and with *P. vivax*. First, we examined the behaviour of ROC curve between malaria patients and healthy control where we found AUC to be 0.984 with standard error of 0.017 and *P* value < 0.0001 (Figure 4(A)). The range of cut-off value of specificity and sensitivity determination are given along with the graph. According to the behaviour of ROC curve between *P. falciparum* and *P. vivax,* we found AUC to be 0.96 with standard error of 0.022 and a *P* value of < 0.0001. The cut-off range value is depicted in Figure 4(B).

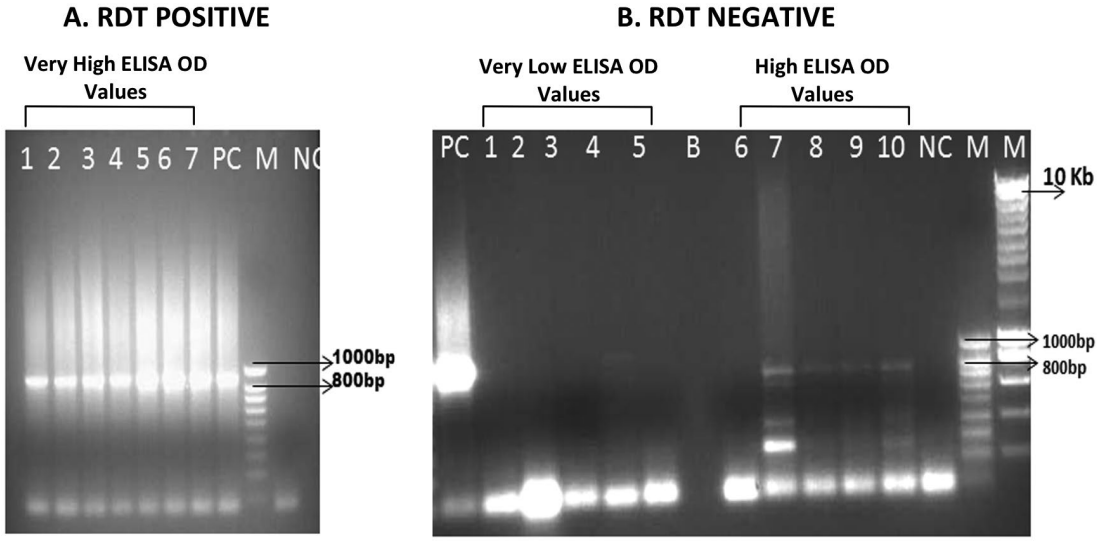
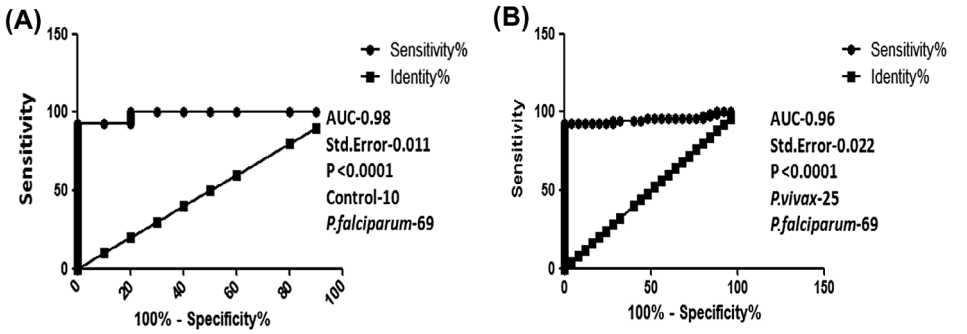


Figure 3. Analysis of *PfHrp2* genes in *P. falciparum* infected malaria patient samples. Genomic DNA was isolated from representative malaria patient samples (7 from RDT positive and 10 from RDT negative samples; PCR was carried out using *Pfhrp2* gene specific primers as described in methods. (A) Analysis of *Pfhrp*2 gene in *P. falciparum* infected patient samples which tested RDT positive (7 samples). All the RDT positive samples showed *Pfhrp*2 amplification. Lanes 1–7: RDT positive *P. falciparum* patient's genomic DNA, PC- Positive control, M- DNA marker, NC- Negative Control. (B) RDT negative (10 samples; 5 with high values and 5 with very low values in Sandwich ELISA) PC- Positive control, NC – Negative Control, M- DNA marker. 5 samples (Lanes 1–5) did not show *Pfhrp*2 amplification and these samples also exhibited very low OD values in Sandwich ELISA. Lanes 7–10 showed amplification of *Pfhrp*-2 gene and they exhibited high OD values in sandwich ELISA. Please note: 'for the last sample, not discernible *hrp*2 gene amplification was observed'.



Cut off	Sensitivity	95% CI	Specificity	Cut off	Sensitivity	95% CI	Specificity
>0.234	97.1%	89.2 to 99.65 %	80	>0.296	100	94.4% to 100%	68 %
>0.2975	95%	85.5 to 98.5 %	80	> 0.304	100	94.4% to 100%	80 %
> 0.315	94%	84.8 to 97.16 %	82	>0.313	100	94.4% to 100%	84 %
>0.424	93%	83.9 to 97.61 %	92	>0.441	100	94.4% to 100%	96%

Figure 4. ROC curves obtained by analyzing the patients' sandwich ELISA results. (A) ROC curve between *P. falciparum* and healthy control with AUC of 0.98. (B) ROC curve between *P. falciparum* and *P. vivax* with AUC of 0.96.

Discussion

Malaria presents a huge diagnostic challenge at both the clinical and laboratory levels. Among all the five species of *Plasmodium*, malaria caused by *P. falciparum* poses serious health concerns as it causes cerebral malaria and death in children [22–24]. In addition, pregnant women are often predisposed to placental malaria affecting the health of the growing child [25,26].

In our study, the values obtained with sandwich ELISA were found to be statistically significant and higher than those of vivax malaria and healthy control (Figure 1 and Table 2). Thus, we successfully differentiated the blood samples of *P. falciparum* infection from *P. vivax* and healthy control, which proved the high specificity of generated mAb for specific diagnosis of malaria caused by *P. falciparum*.

Mixed infections were observed from both *P. falciparum* and P. *vivax* parasites as determined by genus and species specific PCR based analysis. Among the 6 mixed infection samples, 4 were confirmed to be *P. falciparum* and *P. vivax* by microscopy, and our sandwich ELISA method further confirmed the *P. falciparum* infection. The other two samples were also confirmed to be of *P. falciparum* and *P. vivax* by PCR.

The generated anti-Ctp *Pf*HRP2 mAbs (b10c1 and Aa3c10) were showing sensitivity of 95 with 96% specificity and the data obtained in our study clearly strengthens the diagnostic potential of our mAb, as genetic polymorphism is reported to be one of the major reasons for variation in specificity and sensitivity for most of the mAbs that are being used in RDTs [26,27].

A recent study from our laboratory showed that the maximum number of unique peptides of *Pf*HRP2 protein are present in the C-terminal region. We identified the probable epitopes by the software programme developed by Kolaskar and Tongoankar to predict the antigenicity of the C-terminal polypeptide of *Pf*HRP2. In this work, we included samples which were confirmed as *P. falciparum* positive by PCR (genus and species) but negative by RDT. The reason for negativity in the RDTs is not clear. However, one cannot rule out the differences in the affinity of the mAbs when compared to that of antibodies used for preparing the commercial RDTs. At present, the epitopes of the antibodies employed in RDTs are not well defined. Indeed, if it is different from the epitopes recognized by our mAb (anti-Ctp *Pf*HRP2 mAb), it is plausible that some of the samples which tested PCR positive (genus and species) and showed positivity in our sandwich ELISA but displayed RDT negative (Figure 1(B)) may be due to lack of specific epitopes recognized by the antibody used in RDT kit [28]. Detection of such genotype will be of more importance; otherwise these patients may not be treated with antimalarials, which lead to undesirable delay in treatment [28], especially in remote centres where the tertiary health facility is lacking. In our study anti-Ctp *Pf*HRP-2mAb (b10c1 and Aa3c10) could detect *P. falciparum* RDT−ve samples with great accuracy, as these patients may not be treated with antimalarials which leads to delay in treatment.

*Pf*HRP2 has also been reported to be one of the indicators of severity of infection [6]. Therefore, it was important to quantify *Pf*HRP2 antigen in the patients' blood sample. In this study, we could attain this through the standard graph. The assay had working linear range corresponding to 1.0–1000 ng/ml which corresponds to 1 pg/µl of the antigen. Lower limit of detection of *Pf*HRP2 protein was 50 pg/test with 50 µl of blood sample in the ELISA based assays reported here. In RDT based assays, usually 5–10 µl of blood samples are used which implies that the detection of PfHRP2 antigen by the anti-Ctp *Pf*HRP2 mAb will be in the range of 5–10 pg/test which falls well within the lowest detection limit of antigen 1 pg/µl by these mAbs. This data has huge implications for developing the anti-Ctp *Pf*HRP2 mAbs viz., b10c1 and Aa3c10 in RDT based assay system.

Importantly, recent reports have revealed the deletion of *Pfhrp2* and *Pfhrp3* genes in some parasite isolates found in India and also in the Amazon region of Peru [29,30]. Our study with 10 RDT+ve samples showed the presence of 855 bp and the PCR product obtained was in accordance with the available literature [31]. With disparity of the sandwich ELISA results in the RDT−ve samples, the PCR results validated the potentiality of our mAb.

The statistical parameter of a diagnostic test can be greatly affected by the predetermined cut-off values; therefore, to meaningfully compare the different ELISA values with the generated mAb, we performed ROC analysis. The ROC curves distinguish true positive vs. false positive samples for all the cut off values with high sensitivity and specificity. The sample size, although limited, covered the major malaria dominating geographical areas in India where our future work will focus on large set of samples including drug resistant malaria strains.

Our anti-Ctp *Pf*HRP2 mAbs (b10c1 and Aa3c10) have very good potentials for the development of new RDT kits which might assist in bridging the gap where there are variations due to polymorphism of *Pf*HRP2 leading to differential sensitivity and also reducing false negative results.

Geolocation

India

Ethical approval

This study was approved by the Institutional Ethical Committee and was conducted in collaboration with Christian Medical College and Hospital, Vellore (CMC), Tamil Nadu, India. Prior consent of the patients and healthy volunteers were taken before collection of blood. All animals used in the development of antibodies were approved by the Institutional Animal Ethical Committee (IAEC). Animal Ethical Clearance number: 1333/C/10/CPCSEA, Dated 30/3/2010, New Delhi- Approval of Institutional Animal Ethics Committee.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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