Tropical Biomedicine 37(4): 1050–1061 (2020)

Evaluation of anti-histidine-rich protein 2 monoclonal antibodies, developed by using poly (N-isopropylacrylamide) as an adjuvant for malarial diagnostic application

Vishalkumar, P.[1], Jayaprakash, N.S.[2*], Desai, P.K.[1], Krishnan, V.[2] and Vijayalakshmi, M.A.[2]
[1]Arkray Healthcare Pvt Ltd, Plot No. 336, 338, 340, Rd Number 30, Sachin 394230, Surat, India
[2]Centre for Bioseparation Technology (CBST), Vellore Institute of Technology (VIT), Vellore 632014, Tamil Nadu, India
[*]Corresponding author e-mail: nsjayaprakash@yahoo.com
Received 9 January 2020; received in revised form 19 July 2020; accepted 20 July 2020

Abstract: *Objective:* To evaluate the sensitivity and the stability of the monoclonal antibodies (Aa3c10, b10c1), against truncated Histidine-rich protein 2 (PfHRP2), developed using smart polymer, poly N-isopropylacrylamide, as adjuvant for malarial diagnostic applications in comparison with the available commercial antibodies. *Methods:* Two hybridoma clones (Aa3c10, b10c1) were used for the production of ascites in BALB/c mice. Purification of monoclonal antibodies from the ascites was carried out using affinity columns. The thermal stability study of monoclonal antibodies was done by storing it at 37°C and 45°C for thirty days. The stored antibodies were analyzed using SDS-PAGE and flow-through device where the antigen-antibody interaction was visualized by Protein A colloidal gold solution. Sensitivity was determined by endpoint dilution ELISA and the dissociation constant by competitive ELISA. Sensitive pair optimization was done by sandwich ELISA using biotinylated antibodies. Prototype preparation for lateral flow assay had a colloidal gold-based detection system. *Results:* Thermal stability experiments showed that both mAbs (Aa3c10; b10c1) are stable up to thirty days at 45°C while the commercially available mAbs were stable up to fifteen days only. Compared to commercial antibodies, the mAb Aa3c10, showed the highest sensitivity in end-point titre. In sensitive pair optimization, it was observed that the mAb, b10c1, as a detector and the mAb, Aa3c10, as a capture antibody showed the highest absorbance to detect 50pg/ml PfHRP2 antigen. The prototype formulation of lateral flow assay using the mAbs (Aa3c10; b10c1) showed good reactivity with WHO panel and no false-positive results were observed with twenty clinically negative samples and five *P. vivax* positive samples. *Conclusions:* The novel monoclonal antibodies (Aa3c10, b10c1) against truncated PfHRP2, could be a strong potential candidates that can be included in making RDTs with better sensitivity and stability.

INTRODUCTION

Malaria is one of the most important infectious diseases to cause significant mortality and morbidity worldwide (Baker *et al*., 2010). Early-stage diagnosis is critical for case management, treatment of the disease and in differentiating them from non-malarial fevers so as to select an appropriate treatment. Symptom dependent diagnosis is inaccurate, and results in poor management of febrile illness, over-treatment of malaria, and may induce drug resistance to current anti-malarials. Rapid diagnostic tests (RDTs) for malaria have a significant role to improve case management and thereby reduce morbidity and mortality, especially in remote areas, facilitating the timely delivery of appropriate treatment. Indeed, many RDTs today can achieve good sensitivity and specificity for *Plasmodium falciparum* at a parasitaemia more than 200

parasites/microlitre but at lower parasitaemia, variability in sensitivity is observed (Rakotonirina *et al.*, 2008; Wongsrichanalai *et al.*, 2007).

More than 200 different RDTs are available worldwide for the detection of *P. falciparum* which detects histidine-rich protein 2 (HRP2). The gene for PfHRP2, is a single copy subtelomeric gene located on chromosome 7 encoding an amino acid sequence containing 34% histidine, 37% alanine and 10% aspartic acid (Panton *et al.*, 1989; Rock *et al.*, 1987). HRP2 is a 60-105 kDa water-soluble protein specific to *P. falciparum*, synthesized and present throughout the asexual life cycle, identified as a surface-exposed protein in infected erythrocytes (Lopez *et al.*, 2000; Hayward *et al.*, 2000; Sharma *et al.*, 1988). The protein is found in the blood circulation of infected individuals. These features make PfHRP2 a prime target for diagnosis of *P. falciparum* infection.

As per the World Health Organization (WHO) and Foundation for Innovative and New Diagnostics (FIND), under the Malaria RDT Quality Assurance Programme, the levels of diversity for PfHRP2 targeted by malaria RDTs have been systematically investigated and it was found that PfHRP2 is highly variable. In the primary analysis of 74 isolates from mostly Southwest Pacific and Asian countries, a significant sequence variation in PfHRP2 was observed in isolates within the same country and between different countries (Baker *et al.*, 2005). This raised a critical point that the sequence variation could result in a significant difference in the presence and frequency of epitopes recognized by monoclonal antibodies (mAbs). Hence, this will have an impact on the detection sensitivities of RDTs for parasites from different regions. Since the introduction of lateral flow immunochromatographic assays for the detection of malaria about 20 years ago (Butler *et al.*, 2004; Shiff *et al.*, 1993), RDTs have played an increasing role in improving the quality of case management for malaria and assist the reduction of unnecessary anti-malarial drug usage (Albertini *et al.*, 2012; Moody *et al.*, 2002). The specificity, sensitivity, affinity, and heat stability of antibodies are important determinants of the quality and reliability of an RDT. Current malaria RDTs are developed in formats such as dipstick, strip, card, pad, well, or cassette. Three types of antibodies, including signal, capture, and control antibodies are generally incorporated in the device to render an indication of a complex antibody-antigen interaction (WHO guideline – How to use RDT, 2010). However, the variation of sensitivity has been reported in many current commercially available RDTs (Jorgensen *et al.*, 2006). The degradation of antibody performance with long exposure to high temperatures has led to a decrease in operational performance (Lee *et al.*, 2012). Due to commercial consideration, details such as mAbs isotype, subclasses, epitopes targeted, and laboratory of origin are either rarely published or incompletely documented by manufacturers (Baker *et al.*, 2005). Herein, the performance of the two newly developed mAbs (Reena *et al.*, 2017) was compared with the commercially available mAbs which are currently in use as a coating material in RDT. To address this problem, a selection of quality mAbs with high affinity, specificity and heat stability to the target antigen is important to improve malaria diagnosis in tropical regions.

Keeping the above observations as a limitation, new truncated PfHRP2 was designed in such a way that it covers almost all the possible major epitopes which are found in C terminus of the PfHRP2 protein (Verma *et al.*, 2015) and importantly, it is present in most isolates of the world (Baker *et al.*, 2005). The possible logic behind this idea could be the frequency and the abundance of target epitopes which in turn will define the binding affinity of mAbs, where a higher frequency and complete presence of the target epitope may result in greater sensitivity and higher binding affinity of the mAbs.

Chemical polymers have a specific advantage over conventional adjuvants, like most of the polymer is synthesized from a parent compound, and provides an opportunity to eliminate the additional reactive allergic epitopes that may be present

in the antigen. Complete Freund's adjuvant contains heat-killed *Mycobacteria*, which is an important component for stimulating antibody production. But, it is also associated with a number of undesirable side effects due to oil-coated *Mycobacteria*. This adjuvant sometimes deviate from the actual immune responses, and they are also found to be associated with local and systemic toxicities (Stills *et al.*, 2005) so to eliminate local and systemic toxicities of CFA, people looked for an alternative adjuvant which is both mild and have biodegradable properties, to develop antibodies against various antigens. The mAbs can be developed by different technology by using different adjuvant. These adjuvants sometimes deviates the actual immune responses, and they are also found to be associated with local and systemic toxicities. A careful consideration of antigen conformation as well as in-depth knowledge of adjuvant effects on antigens are required to ensure the preservation of antigen epitopes. An adjuvant that is biocompatible can preserve antigen epitopes from degradation upon delivery into animals and is capable of inducing a long-lasting antibody response as well as cell-mediated immunity. Poly(*N*-isopropylacrylamide) (PNiPAAm) as an adjuvant has the property to act as a reservoir of antigen with efficient delivery and protection of the antigen (Kumar *et al.*, 2007). This polymer is biocompatible and does not show toxicities under *in vivo* conditions. Thus, it acts as an antigen reservoir that not only provides a protective environment but also aids in the efficient antigen release. Moreover, PNiPAAm does not alter the susceptibility of the antigen to the immune responses, and at the same time, the polymer is immunologically inert (Schild, *et al.*, 1992). In the earlier study, the mAbs developed against truncated PfHRP2 with smart polymer were found to be highly specific to PfHRP2 (Verma *et al.*, 2015). In this work, two hybridomas (b10c1 and Aa3c10) were developed against truncated PfHRP2 (Verma *et al.*, 2015) to produce mAbs in mouse ascites. Further, the mAbs were characterized for its yield, reactivity, affinity, stability, specificity and sensitivity in comparison with commercially available antibodies (A and B) which were developed using complete Freund's adjuvant (CFA).

MATERIALS AND METHOD

Materials

IMDM (Iscove's Modified Dulbecco's Medium) media and all other analytical chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). ELISA plates were purchased from corning costar, USA. Consumable plastic wares were from Tarson, India. Secondary antibody for ELISA was purchased from Sigma-Aldrich, USA. rHRP2 antigen was obtained from Arkray healthcare, India. Purification resin was purchased from GE healthcare, Sweden. Electrophoresis materials were purchased from Biorad, USA. All filters were purchased from Millipore, USA.

The hybridomas producing mAbs [Aa3c10, b10c1 (developed against truncated recombinant PfHRP2 with smart polymer as adjuvant)] was obtained from the Centre for Bioseparation Technology (CBST), VIT, Vellore, India and the monoclonal antibodies A and B (developed against full length recombinant PfHRP2 with Freund's adjuvant) were obtained from Arkray Healthcare Pvt. Ltd., Surat, India.

Generation of ascites in BALB/c mice

Hybridoma cells were cultured in IMDM media under controlled aseptic environment. Five BALB/c female mice were primed with 0.2 ml of Freund's Incomplete Adjuvant and sterile 10mM PBS (1:1) in the peritoneal cavity. The grown hybridoma cells were harvested at the stationary phase and enumerated by the trypan blue method. The primed mice were inoculated with 3×10^6 cells and kept under observation. The ascitic fluid was collected after 9 days and filtered with pre-filter followed by 0.45 µm filtration and stored at -20°C for further use. Indirect ELISA was performed to test the specific reactivity of the ascites and also to find the concentration of anti-HRP2 IgG in ascites. All the animals used in the development of antibodies were approved by the Institutional Animal Ethics Committee (IAEC).

Purification of ascites

The collected ascites were processed and purified by affinity chromatography. Two different methods were compared using Protein G and Protein A affinity purification (Protein G sepharose, GE healthcare). Two millilitres of each resin was packed in separate tricorn columns (GE). For protein G, two different buffer systems (20mM Phosphate buffer, pH 7, with and without salt in binding condition) were tried to achieve maximum yield and purity. Ascites was diluted 10 times with binding buffer and loaded to the column with a flow rate of 1.5ml/min. The antibody was eluted with 100mM glycine pH 3 and neutralized with 1M tris pH 9. The purification process was carried out using Biologic LP purification system. Similarly, another run was performed with salt in the binding buffer. The column was equilibrated with 10mM Phosphate buffered saline (PBS), pH 7 and the sample was prepared and purified as mentioned above.

Protein A affinity purification was done with high salt containing buffer system. The column was equilibrated with the buffer containing 100mM tris, 3M NaCl, 1.5M glycine pH 8.8. The sample was diluted 10 times with the binding buffer and loaded on to the column. The antibody was eluted with 100mM citric acid and neutralized with 1M tris pH 9. The pooled antibodies purified using Protein G and Protein A were dialysed against 10mM PBS pH 7.3 and filtered by 0.22 µm syringe filter and stored at -20°C. The isotyping of the purified antibody was done by ELISA assay according to the manufacturer protocols (Sigma).

Thermal stability analysis of mAbs

The purified mAbs Aa3c10, b10c1 developed against truncated PfHRP2 with smart polymer and A, B developed against full-length recombinant PfHRP2 with Freund's adjuvant were analyzed for their thermal stability at two different temperatures. The mAbs (2mg/ml) were stored at 37°C and 45°C for thirty days and analyzed at an interval of five days. SDS-PAGE was done to test the structural alteration. The samples were analyzed by PAGE with 5µg/well under reduced and non-reduced conditions. Protein estimation was done to test the effect of temperature on protein concentration and reactivity analysis was done using immunodiagnostic flow-through device. Briefly, 40ng of PfHRP2 antigen was spotted on nitrocellulose membrane (NCM) of the flow-through device. Flow-through device is a kind of immuno-diagnostic device with 0.45 µm NCM for spotting of protein and supported by absorbent pad to absorb the buffer and access sample after specific interaction. Stack of NCM and absorbent pad is fixed between two halves of a plastic cassette having a test window. Spotted devices were kept at 37°C for overnight drying and stored at 2-8°C for analysis of mAbs. The purified mAbs (Aa3c10, b10c1, A and B), 500ng each were added to the device. The Ag-Ab interaction was visualized by Protein A colloidal gold solution.

Antibody binding sensitivity assay by endpoint dilution ELISA

The full length rHRP2 antigen (50ng/well) was coated on highbinding microtiter plate and incubated overnight at room temperature. The plate was then blocked with 2% BSA for 120 min at 37°C. Four different anti HRP2 monoclonal antibodies (Aa3c10; b10c1); commercial mAbs A and B were taken for this assay for comparison. All the mAbs were added in serial triple dilution (concentration of antibody dilution: 5, 1.67, 0.56, 0.19, 0.06, 0.02 ng/well). The plate was then incubated for 60min at 37°C and washed thrice with PBST (PBS with 0.1% v/v tween 20). Anti Mouse HRP conjugated antibody with 1:10000 dilution was added as a secondary antibody and incubated for 60 min at 37°C. After incubation plate was washed thrice with PBST. Plate was developed with TMB/H_2O_2 substrate and the reaction was stopped with 2N sulphuric acid. Plate was read at 450:620nm (Thermo multiskan ELISA reader) and results were plotted to observe statistical significance. All dilutions were examined in duplicate.

Determination of dissociation constant

Dissociation constant was measured in a solution of antigen-antibody complex by ELISA. Coating concentration need to

optimized prior to perform ELISA. Primary antibody of 1st plate need to transfer as a primary antibody of 2nd plate and likewise free unbound concentration of antibody need to identify and Antigen – antibody complex need to screen by ELISA. ELISA was optimized and performed as per the protocol reported previously (Bertrand *et al.*, 1985).

Biotinylation of antibody and Sandwich ELISA for optimization of highly specific antibody pair

Biotinylation of all four antibodies (Aa3c10, b10c1, A, B) were done as per the manufacturer's protocol (Thermo, US) and the extra biotin was removed by desalting using sephadex G25. The peak fractions were pooled together and stored in 40% glycerol at -20°C. ELISA plate was coated with 150 ng/well of mAbs (Aa3c10, b10C1, A and B) in 10mM PBS at room temperature for overnight. Then the plate was washed and blocked with 2% BSA at 37°C for 160min. The plate was washed thrice with PBST (PBS with 0.1% v/v tween 20) and the PfHRP2 antigen was added in serial double dilution (20pg/well to 0.63pg/well). The plate was incubated for 30 min at 37°C. After incubation, the plate was washed and biotinylated antibody was added at a dilution of 1:5000 and incubated for 30 min at 37°C. After incubation, the plate was washed and streptavidine-horse radish peroxidase is added (1:20000 dilution) and incubated for 30 min at 37°C. After incubation, the plate was washed and developed with TMB/H_2O_2 and the reaction was stopped by sulphuric acid. The plate was read at 450:620nm and results were plotted and analyzed to screen the best pair of antibodies.

Prototype preparation of lateral flow assay

Lateral flow assay was developed with different combinations of four different antibodies (Aa3c10,b10c1, A and B). Two different stock of coating solutions (3 mg/ml and 2.5 mg/ml in PBS) were prepared with all four antibodies and coated (Antibody coating volume: 2.5µl/mm) on NCM. Then the NCM was kept for drying at 37°C and blocked with BSA and again placed for drying in dehumidifying chamber for 24 hr.

The lateral flow assay had a colloidal gold-based detection system. The antigen will bind with NCM antibody and that Ag-Ab complex will be detected by binding with colloidal gold conjugated antibody. All antibodies were conjugated with 40 nm gold nanoparticles and sprayed on a conjugate pad and kept for drying at 37°C. Then it was assembled with different combinations (each of four antibodies as a capture antibody and as a detector antibody with each other) to test its analytical sensitivity against recombinant HRP2 protein spiked in normal blood (Spike concentration: 5 ng/ml to 0.3 ng/ml, serial double dilution) and also test its sensitivity-specificity against confirmed positive sample(Pf positive), negative sample(Normal human blood), cross-species infection sample(Pv Positive) as well as WHO panel (200 parasite/µl).

RESULTS

Production of ascites and antibody purification using Protein G and Protein A affinity chromatography

Two hybridoma clones (Aa3c10, b10C1) were injected separately into the peritoneal cavity of BALB/c mice and kept under observation. Mice were sacrificed after 10 days and ascites were collected and stored at -20°C after centrifugation and filtration through 0.45 µm filter. Indirect ELISA was performed to check the functional reactivity of the mAbs against full length rHRP2 antigen. Both the mAbs from the ascites showed specific reactivity against full length rHRP2 antigen. It was observed that, mAb Aa3c10 had higher reactivity (1.703 OD) than mAb b10c1 (1.3 OD) at 1:320000 dilutions. All the samples' OD was normalized with negative control (BSA and HRP2 -ve ascites) taken as 0.1 OD (Fig. 1 A).

To get a high yield of mAbs from ascites, purification was carried out using Protein G and Protein A sepharose resins separately. After purification, the eluted fractions were estimated by absorbance at 280 nm, using extinction coefficient (EC) 1.35 to check

the concentration of IgG. Purification with protein G sepharose resin gave a yield of 4 mg/ml whereas; protein A sepharose gave a yield of 10 mg/ml. The purity of the antibody was analyzed by reducing and non-reducing SDS-PAGE. It was found that a clear 150kDA band in non-reducing gel and 50kDa heavy chain and 25 kDa light chain bands in reducing gel were obtained with no other band of impurity (Fig. 1 B).

Thermal stability analysis of mAbs

The thermal stability study of mAbs (Aa3c10, b10c1, A, and B) was done using 2mg/ml of each as an initial concentration and stored it in aliquots at two different temperatures 37°C and 45°C. It was observed that the reactivity of mAbs, Aa3c10 & b10c1, were better when compared to commercially available mAbs, A & B, which reduced significantly after one month (Fig. 2A). To check the effect of storage temperature on antibody concentration, the samples were analyzed by absorbance at 280nm using EC 1.35. The results showed that the concentration of both A and B mAbs decreased to 1.70 mg/ml. However, the concentrations of mAbs (Aa3c10 and b10c1) reduced slightly to 1.99 mg/ml and 1.95 mg/ml respectively. To study the degradation of mAbs, the samples were analyzed by SDS-PAGE. It was found that both the mAbs (Aa3c10 and b10c1) have clear bands in non-reducing as well as reducing condition up to 30 days. In the case of mAbs (A and B), hazy bands of the heavy chain and light

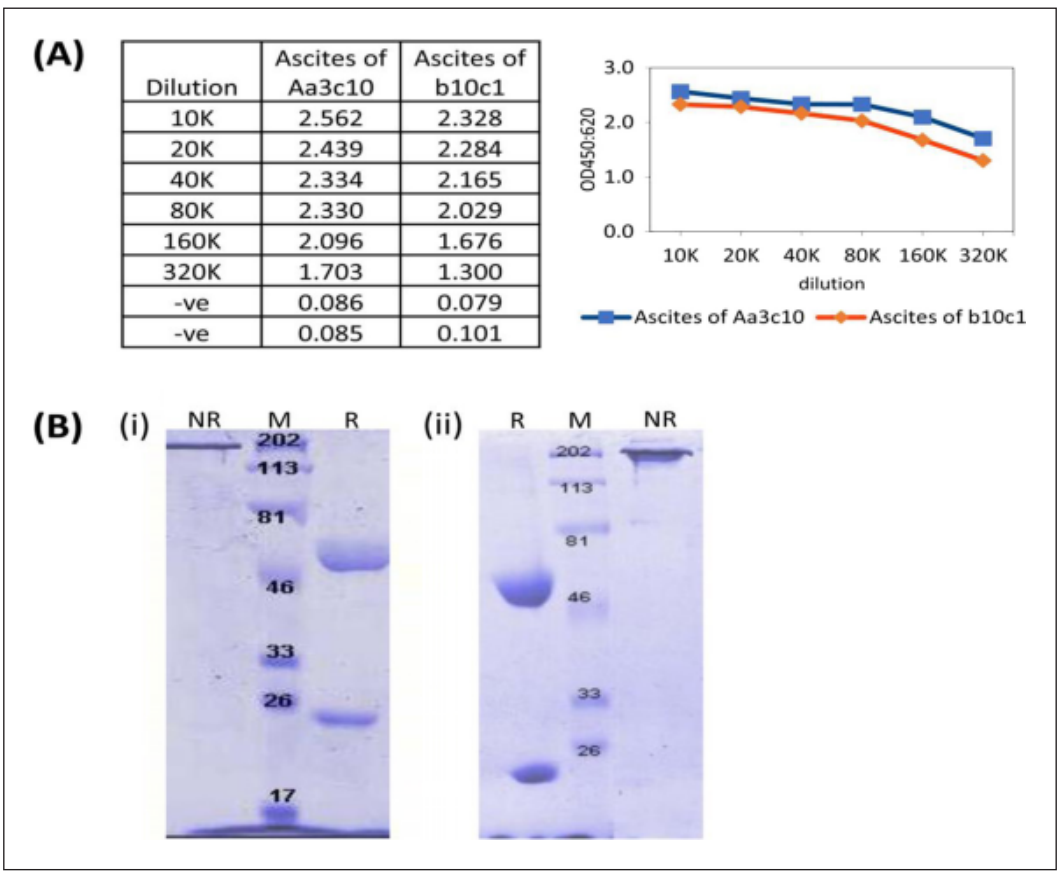


Figure 1. (A) Reactivity of mAbs, Aa3c10 and b10c1, against rHRP2 by indirect ELISA. ELISA plate was coated with rPfHRP2 followed by incubation with the serially double diluted purified mAbs. The assay data plotted are mean values of triplicates. (B) Coomassie blue stained 10% SDS-PAGE under non-reducing and reducing consitions of purified mAbs, (i) Aa3c10 and (ii) b10c1. Lane M, molecular weight marker; Lane NR, non-reduced; Lane R, reduced.

chain in reducing conditions were observed starting from 15th day of incubation along with multiple bands in both reducing as well as non-reducing conditions (Fig. 2B).

Determination of sensitivity by endpoint dilution ELISA and dissociation constant

The ELISA plate was coated with different concentrations of recombinant PfHRP2 antigen and endpoint titre value was tested. The absorbance was normalized with control (wells coated with BSA) taken as 0.07. The result (Fig. 3) shows that the mAb, Aa3c10c, has the highest end-titre sensitivity at 0.02 ng/well whereas mAbs, b10c1, A and B showed nearly equal sensitivity but lesser than the mAb, Aa3c10. The dissociation constant was determined by competitive ELISA. It was observed that the dissociation constant (Kd) values of mAbs, Aa3c10, and b10c1, are 2.52 X 10^{-9} and 2.91 X 10^{-11} respectively. Dissociation constant of A and B were 2.51 X 10^{-8} and 2.98 X 10^{-10} as per data provided by manufacturer.

Sensitive pair optimization by sandwich ELISA

All four biotinylated mAbs were used to do sandwich ELISA in different combinations. The dilution, 1:5000, was found to be significant which contained an optimum concentration of biotin- conjugated mAbs to be used for sandwich ELISA. To determine the accuracy and linearity of recombinant PfHRP2 detection, both smart polymer mAbs Aa3c10 and b10c1 were designed to use as capture or detection antibodies in combination with CFA mAbs (A & B) for sandwich ELISA. The OD of each pair was plotted in a line graph (Fig. 4). The ODs of mean reactivity with recombinant PfHRP2 protein diluted serially from 200pg/ml to 6.25pg/mL and detected by mAbs are represented in tabular form (Fig. 4). The values given in the first three columns are the result of ELISA done to screen the best capture antibody and the values in the other three columns are for the best detector antibody. Highest ODs at 50pg/ml concen-

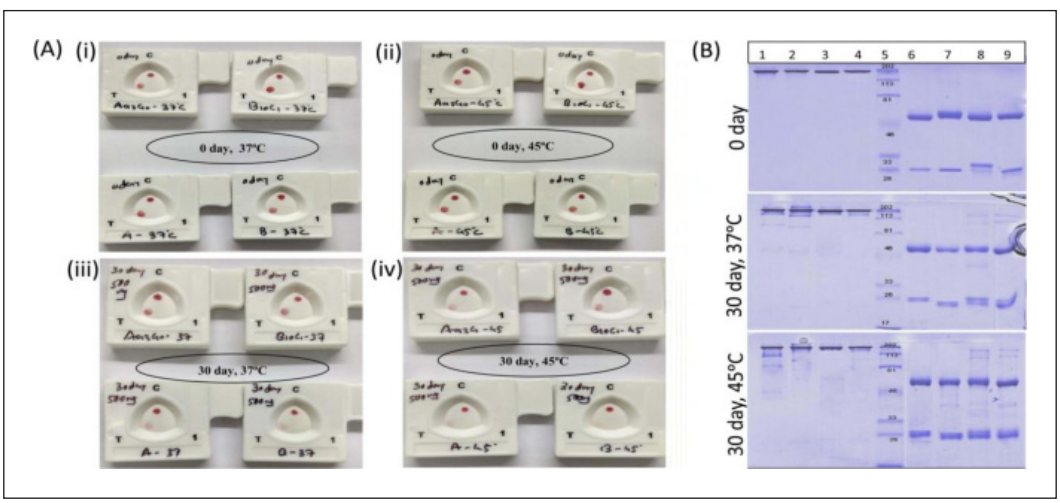


Figure 2. Thermal stability analysis of mAbs. (A) Reactivity of the mAbs (Aa3c10, b10c1, A and B) incubated at different temperatures (37°C and 45°C), against 40ng of HRP2 antigen coated on flow through device, on 0th day (i & ii), after 30 days (iii & iv). Row 1 and 3 contains mAbs, Aa3c10 and b10c1. Row 2 and 4 contains mAbs, A and B. (B). Coomassie blue stained 10% SDS-PAGE under non-reducing (NR) and reducing (R) conditions of the mAbs (Aa3c10, b10c1, A and B) stored at 37°C and 45°C for 0 and 30 days of incubation. Lane 1, A antibody (NR); 2, B antibody (NR); 3, b10c1 antibody (NR); 4, Aa3c10 antibody (NR); 5, molecular weight marker; 6, Aa3c10 antibody (R); 7, b10c1 antibody (R); 8, B antibody (R); 9, A antibody (R)

Conc in ng/100µl	Aa3c10	b10c1	A	B
5	1.988	1.252	1.844	1.484
1.67	1.207	0.628	1.079	0.740
0.56	0.610	0.305	0.556	0.349
0.19	0.303	0.189	0.276	0.194
0.06	0.196	0.145	0.172	0.150
0.02	0.162	0.137	0.136	0.133
-ve	0.071	0.073	0.078	0.075

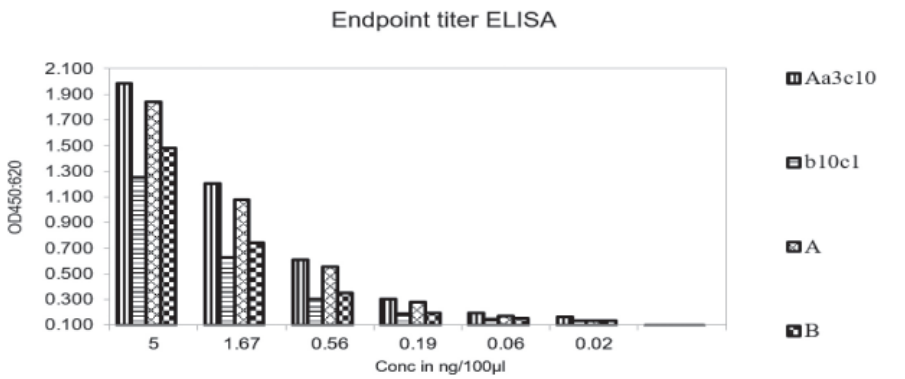
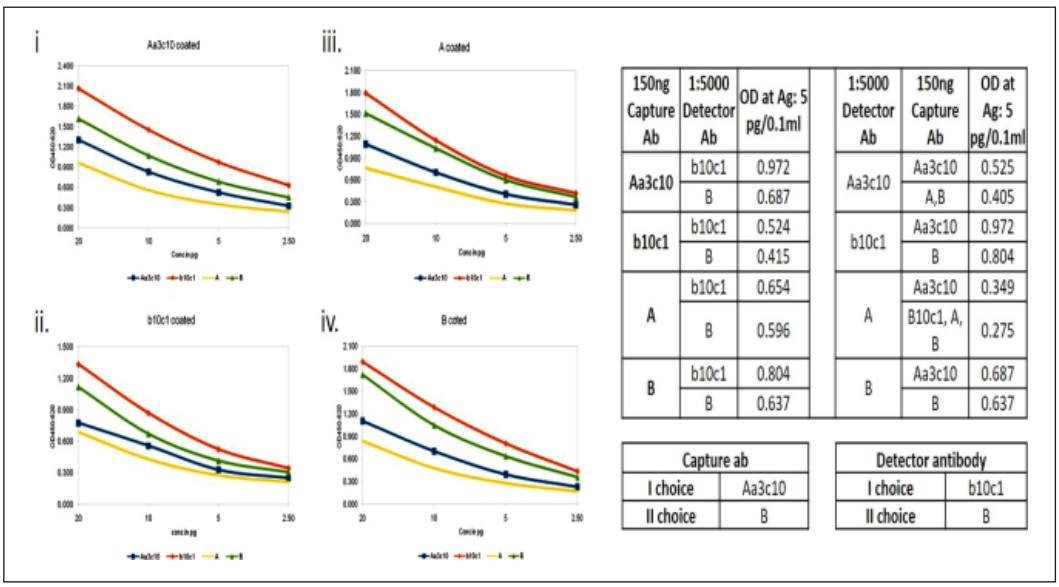


Figure 3. Sensitivity of mAbs against rHRP2 by indirect ELISA. Recombinant PfHRP2 (50ng/well) was coated in ELISA plate, followed by incubation with purified mAbs (in serial triple dilution, concentration range 5ng to 0.02ng/well). The OD values are represented in the tabular form and plotted in a graph.



150ng Capture Ab	1:5000 Detector Ab	OD at Ag: 5 pg/0.1ml
Aa3c10	b10c1	0.972
	B	0.687
b10c1	b10c1	0.524
	B	0.415
A	b10c1	0.654
	B	0.596
B	b10c1	0.804
	B	0.637

1:5000 Detector Ab	150ng Capture Ab	OD at Ag: 5 pg/0.1ml
Aa3c10	Aa3c10	0.525
	A,B	0.405
b10c1	Aa3c10	0.972
	B	0.804
A	Aa3c10	0.349
	B10c1, A, B	0.275
B	Aa3c10	0.687
	B	0.637

Capture ab	
I choice	Aa3c10
II choice	B

Detector antibody	
I choice	b10c1
II choice	B

Figure 4. Optimization of highly specific antibody pair by sandwich ELISA. Purified mAbs (150ng/well) was coated in ELISA plate followed by incubation with HRP2 at different concentrations. Biotinylated antibody was used as detection antibody followed by streptavidin-horse radish peroxidase incubation and developed with TMB/H_2O_2. The assay data was plotted as graph representing the mAbs, (i) Aa3c10; (ii) b10c1; (iii) A; (iv) B coated in ELISA plate.

Table 1. Lateral flow assay. Four different antibody (Aa3c10, b10c1, A and B) were sprayed on nitrocellulose membrane and lateral flow assay was prepared and tested with recombinant HRP2 protein spiked in normal blood and clinical positive sample

Sample	Dilution	Pair of Ab	Result (Reactivity)
Recombinant protein spike with parasite free blood	5 ng/ml	Aa3c10 & b10c1	+3
		A & B	+3
	2.5 ng/ml	Aa3c10 & b10c1	+3
		A & B	+3
	1.25 ng/ml	Aa3c10 & b10c1	+2
		A & B	+2
	0.62 ng/ml	Aa3c10 & b10c1	+1
		A & B	±1
	0.31 ng/ml	Aa3c10 & b10c1	±1
		A & B	0
Clinical Pf positive sample	2000 p/µl	Aa3c10 & b10c1	+3
		A & B	+3
	1000 p/µl	Aa3c10 & b10c1	+3
		A & B	+2
	200 p/ul	Aa3c10 & b10c1	+2
		A & B	±1
	100 p/µl	Aa3c10 & b10c1	+1
		A & B	±1

tration is mentioned in the table with respective combinations of antibodies. It was observed that the mAb, b10c1, as a detector and the mAb, Aa3c10, as a capture antibody showed the highest absorbance to detect 50pg/ml PfHRP2 antigen.

Prototype formulation for lateral flow assay

Two different concentrations of smart polymer mAbs and CFA mAbs were examined and the result is mentioned in Table 1. It was observed that prototype prepared from stock concentration of 3 mg/ml of mAbs, b10c1 and Aa3c10 detect 0.6 ng/ml recombinant PfHRP2 spiked with blood with +1 reactivity whereas both CFA mAbs gave ±1 reactivity (+3: strong reactivity, +2: Medium reactivity, +1: low reactivity, 0: No reactivity, ± : hazy appearance). Also, it was found that the smart polymer mAbs pair had +1 reactivity when checked with parasite blood dilution containing 100 parasites/µl. For further confirmation, all combinations (smart polymer mAbs and CFA mAbs) were tested using WHO panel with 200 parasites/µl dilution and it was found to be +1/+2 reactivity. For specificity, 20 clinically negative samples and five *P. vivax* positive samples were tested with all combination and no false-positive results were observed.

DISCUSSION

The aim of this study was to assess and compare the binding capability of four different anti HRP2 monoclonal antibodies (Aa3c10; b10c1; developed against truncated HRP2 antigen by using smart polymer as an adjuvant and commercial mAbs A and B which were developed against full length recombinant PfHRP2 antigen by using CFA/FIA as an adjuvant) against malaria PfHRP2 antigen. Most antibodies deployed currently in commercial malaria RDT devices were produced in the early 1990s.

PNiPAAm, a synthetic polymer is being suggested as an alternative adjuvant for the development of mAb as it forms a complex with the antigen and slowly releases the antigen inside the host. This, in turn, helps in maintaining the antigen levels over a period of time, and additionally, it is believed to preserve the antigen's conformation inside the host system (Heredia *et al.*, 2005; Schild *et al.*, 1992). It may be one of the reasons for the high sensitivity of mAbs developed by using PNiPAAM polymer.

The study showed that mAbs, Aa3c10, and b10c1, generated using PNiPAAm polymer exhibited strong binding specificity and sensitivity against recombinant and native PfHRP2 (blood sample), as shown by ELISA and lateral flow assay (Fig. 2 and 4). Both the antibodies showed no significant loss of sensitivity of binding in the sandwich format when tested either as capture antibody or detection antibody by partnering with CFA mAbs (Fig. 5). The results suggest that the best combination to use for sandwich ELISA is the mAb, b10c1, as a detector and mAb, Aa3c10, as a capture antibody to achieve significant results. The detection sensitivity was best when the mAb, Aa3c10, was used as a capture antibody although different secondary antibodies used for detection may contribute to better detection sensitivity. As an extensive number of repeated epitopes exist in PfHRP2 (Baker *et al.*, 2010), epitope numbers are not likely contributing to the difference in binding of mAbs (Tischenko *et al.*, 1998). This further validated the utility of the generated antibodies using PNiPAAm as an adjuvant.

The Kd value of the mAb, b10c1, showed the highest binding affinity 2.91 X 10^{-11}, although it had a weaker sensitivity in the ELISA screening (Fig. 4) whereas Aa3c10 showed 2.52 X 10^{-9} and had stronger sensitivity in ELISA. The repetitive nature of the epitope contributes to the very slow dissociation rates of these mAbs, as the antigen is providing high avidity, and consequently an apparent strong affinity. Hence, the mAbs generated through PNiPAAm polymer may help in capturing the antigen to its lowest limiting value. This, in turn, will improve the potency of the mAb to be used in diagnostic purposes under field conditions. Moreover, this strong apparent affinity is ideal for RDTs as mAbs that have a faster rate of association and a slower rate of dissociation (a low Kd value) would be considered to give a greater binding ability to target biomarker. Thus, the kinetic properties of mAbs, Aa3c10, and b10c1, make them potential candidates for malaria RDTs.

Another aspect that is very much important for reagents used in RDTs in the tropical and subtropical world is their ability to withstand high ambient storage temperatures. The present study identified that both mAbs (Aa3c10; b10c1) are stable up to 30 days at 45°C while the commercially available mAbs (A & B) were stable up to 15 days only. Moreover, the multiple bands in SDS PAGE of mAbs (A & B) may correspond to different parts of the mAb (e.g. the Fab region and Fc region) unfolding and that could be possible reason for losing reactivity at high temperature. According to the literature, the CH2 domain of an antibody tends to unfold earlier, followed by the Fab and the CH3 domains. The thermal transition of the Fab domain commonly occurs between the CH2 and CH3 domains or overlapping with either one of these domains in a monoclonal antibody (Chiuan *et al.*, 2014).

The prototype formulation of lateral flow assay using the mAbs (Aa3c10; b10c1) were tried as per the existing formulation of Arkray healthcare Pvt. Ltd. (formerly Span Diagnostics Pvt. Ltd.) by replacing the commercial antibody and found to be reactive. Further confirmation using WHO panel with 200 parasites/µl showed +2 reactivity. When tested for specificity with 20 clinically negative samples and five *P. vivax* positive samples with all combinations, no false-positive results were observed. This again confirmed that the two new mAbs (Aa3c10; b10c1) could be strong potential candidates that can be included in making RDTs. These mAbs have to be validated with large blood/serum samples of malaria patients in field conditions using immunochromatography tests. This may eliminate the current issue of low sensitivity and stability with the existing systems.

Abbreviations

Complete Freund's Adjuvant (CFA), Enzyme Linked Immunosorbent Assay (ELISA); histidine rich protein (HRP), Monoclonal antibody (mAb), Optical Density (OD), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), poly(N-isopropylacrylamide) (PNiPAAm), Rapid Diagnostic Tests (RDTs), PBS (Phosphate buffered saline).

REFERENCES

Albertini, A., Lee, E., Coulibaly, S.O., Sleshi, M., Faye, B., Mationg, M.L., Ouedraogo, K., Tsadik, A.G., Feleke, S.M., Diallo, I., Gaye, O., Luchavez, J., Bennett, J., Bell, D. (2012). Malaria rapid diagnostic test transport and storage conditions in Burkina Faso, Senegal, Ethiopia and the Philippines. *Malaria Journal* **11**: 406.

Baker, J., Ho, M.F., Pelecanos, A., Gatton, M., Chen, N., Abdullah, S., Albertini, A., Ariey, F., Barnwell, J., Bell, D., Cunningham, J., Djalle, D., Echeverry, D.F., Gamboa, D., Hii, J., Kyaw, M.P., Luchavez, J., Membi, C., Menard, D., Murillo, C., Nhem, S., Ogutu, B., Onyor, P., Oyibo, W., Wang, S.Q., McCarthy, J. & Cheng, Q. (2010). Global sequence variation in the histidine-rich proteins 2 and 3 of *Plasmodium falciparum*: implications for the performance of malaria rapid diagnostic tests. *Malaria Journal* **9**: 129.

Baker, J., McCarthy, J., Gatton, M., Kyle, D.E., Belizario, V., Luchavez, J., Bell, D. & Cheng, Q. (2005). Genetic diversity of *Plasmodium falciparum* histidine rich protein 2 (PfHRP2) and its effect on the performance of PfHRP2-based rapid diagnostic Tests. *Journal of Infectious Diseases* **192**: 870-877.

Bertrand, F., Alain, F., Lisa, D.O. & Michel, E.G. (1985). Measurements of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay. *Journal of Immunological Methods* **77**: 305-319.

Butler, D. (2004). Global fund changes tack on malaria therapy. *Nature* **429**: 588.

Chiuan, H.L., Martina, J., Qin, C., Stephen, M. & James, M.C. (2014). Production and characterization of specific monoclonal antibodies binding the *Plasmodium falciparum* diagnostic biomarker, histidine-rich protein 2. *Malaria Journal* **13**: 277.

Hayward, R.E., Sullivan, D.J. & Day, K.P. (2000). *Plasmodium falciparum*: histidine rich protein II is expressed during gametocyte development. *Experimental Parasitology* **96**: 139-146.

Heredia, K.L., Bontempo, D., Ly, T., Bayers, J.T., Halstenberg, S. & Maynard, H.D. (2005). *In situ* preparation of protein-"smart" polymer conjugates with retention of bioactivity. *Journal of the American Chemical Society* **4**: 16955-60.

WHO guideline – How to use RDT, (2010).

Jorgensen, P., Chanthap, L., Rebueno, A., Tsuyuoka, R. & Bell, D. (2006). Malaria rapid diagnostic tests in tropical climates: the need for a cool chain. *The American Journal of Tropical Medicine and Hygiene* **74**: 750-754.

Kumar, A., Srivastava, A., Yugalaeu, I.Y. & Mattiasson, B. (2007). Smart polymer: physical forms and bioengineering application. *Progress in Polymer Science* **32**: 1205-1237.

Lee, N., Gatton, M.L., Pelecanos, A., Bubb, M., Gonzalez, I., Bell, D., Cheng, Q. & McCarthy, J.S. (2012). Identification of optimal epitopes for *Plasmodium falciparum* rapid diagnostic tests that target histidine-rich proteins 2 and 3. *Journal of Clinical Microbiology* **50**: 1397-1405.

Lopez, R., Urquiza, M., Curtidor, H., Eduardo, J.C., Mora, H., Puentes, A. & Patarroyo, M.E. (2000). *Plasmodium falciparum*: red blood cell binding studies of peptides derived from histidine rich KAHRP-I, HRP-II and HRP-III proteins. *Acta Tropica* **75**: 349-359.

Moody, A. (2002). Rapid diagnostic tests for malaria parasites. *Clinical Microbiology Reviews* **15**: 66-78.

Panton, L.J., McPhie, P., Maloy, W.L., Wellems, T.E., Taylor, D.W. & Howard, R.J. (1989). Purification and partial characterization of an unusual protein of *Plasmodium falciparum*: histidine rich protein II. *Molecular and Biochemical Parasitology* **35**: 149-60.

Rakotonirina, H., Barnadas, C., Raherijafy, R., Hery, H., Ratsimbasoa, A., Randrianasolo, L., Jahevitra, M., Andriantsoanirina, V. & Menard, D. (2008). Accuracy and reliability of malaria diagnostic techniques for guiding febrile outpatient treatment in malaria-endemic countries. *The American Journal of Tropical Medicine and Hygiene* **78**: 217-221.

Reena, V., Sara, C., Jayaprakash, N.S., Manoharan, A., Vijayalakshmi, M.A. & Krishanan, V. (2017). Diagnostic potential of monoclonal antibodies developed against C-terminal polypeptide of *P. falciparum* Histidine Rich Protein2 (P*f*HRP2) in malaria infected patients from India. *Pathogens and Global Health* **111**: 297-305.

Rock, E.P., Marsh, K., Saul, A.J., Wellems, T.E., Taylor, D.W., Maloy, W.L. & Howard, R.J. (1987). Comparative analysis of the *Plasmodium falciparum* histidine rich proteins HRP-I, HRP-II, and HRP-III in malaria parasites of diverse origin. *Parasitology* **95**: 209-227.

Schild, H.G. (1992). Poly(N-isopropylacrylamide): experiment, theory, and application. *Progress in Polymer Science* **19**: 163-249.

Sharma, Y.D. (1988). Genomic organization, structure and possible function of histidine rich proteins of malaria parasites. *International Journal of Biochemistry* **20**: 471-477.

Shiff, C.J., Premji, Z. & Minjas, J.N. (1993). The rapid manual ParaSight-F test. A new diagnostic tool for *Plasmodium falciparum* infection. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **87**: 646-648.

Stills, H.F. (2005). Adjuvants and antibody production: Dispelling the myths associated with Freund's complete and other adjuvants. *Journal of Institute for Laboratory Animal Research* **46**: 280-293.

Tischenko, V.M., Abramov, V.M. & Zav'yalov, V.P. (1998). Investigation of the cooperative structure of Fc fragments from myeloma immunoglobulin G. *Biochemistry* **37**: 5576-5581.

Reena, V., Jayaprakash, N.S., Vijayalakshmi, M.A. & Krishnan, V. (2015). Novel monoclonal against truncated C terminal region of Histidine Rich Protein2 (PfHRP2) and its utility for specific diagnosis of malaria caused by *Plasmodium falciparum*. *Experimental Parasitology* **150**: 56-66.

Reena, V., Ravichandran, R., Jayaprakash, N.S., Ashok, K., Vijayalakshmi, M.A. & Krishnan, V. (2015) Adjuvant PolyN-isopropylacrylamide generates more efficient monoclonal antibodies against truncated recombinant Histidine Rich Protein (HRP)-2 of *Plasmodium falciparum* for malaria diagnosis. *Biotechnology Journal* **10**: 772-782.

Wongsrichanalai, C., Barcus, M.J., Muth, S., Sutamihardja, A. & Wernsdorfer, W.H. (2007). Review of malaria diagnostic tools: microscopy and rapid diagnostic test (RDT). *The American Journal of Tropical Medicine and Hygiene* **77**: 119-127.