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Thermostable lamprey variable lymphocyte receptor antibody for detection of *Plasmodium falciparum* histidine rich protein-2

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Lampreys use variable lymphocyte receptors (VLR) comprising leucine-rich-repeat (LRR) segments for antigen recognition, distinct from immunoglobulin-based receptors of jawed vertebrates. Lamprey VLRs are as diverse and antigen-specific as immunoglobulin-based antibodies, with unique advantages including high avidity, pH stability, and recognition of novel antigen epitopes. Here we describe the generation of VLR monoclonal antibody against histidine rich protein-2 (HRP-2) of *Plasmodium falciparum*, a causative agent of malaria. HRP-2, expressed by all parasite stages and secreted into plasma, serves as an effective biomarker of infection. Lamprey larvae immunized with purified HRP-2 protein produced specific VLRB antibodies with relatively high serum titers. Using white blood cells from immunized lampreys, we constructed VLR cDNA libraries expressed on yeast surface. Through yeast display screening, we selected recombinant VLRB antibody 5A10 with high affinity and specificity for HRP-2, recognizing both recombinant and native proteins from *P. falciparum* culture supernatants and infected patient samples. The antibody retains its binding capacity at temperatures up to 70 °C, significantly outperforming a commercial mouse IgG-based anti-HRP-2 antibody. This HRP-2-specific VLR antibody shows promise for improved malaria diagnostics, particularly in tropical regions requiring heat-stable tests.

The adaptive immune response in jawless vertebrates, lampreys and hagfishes, provides an alternate antibody system in which variable lymphocyte receptors (VLR), composed of leucine-rich-repeat (LRR) segments, are used for antigen recognition in contrast to the immunoglobulin (Ig)-based receptors used by jawed vertebrates.[1–5] The lamprey genome encodes genes for five types of distinct VLR lymphocytes: VLRA, VLRB, VLRC, VLRD and VLRE.[6–11] Upon antigenic stimulation, VLRA and VLRC lymphocytes behave like T cells by differentiating into proinflammatory cytokine producing effector cells while maintaining their transmembrane antigen receptors.[8,10,12] By contrast, VLRB lymphocytes behave like B cells by responding to antigenic stimulation, undergo lymphoblastoid transformation, proliferation and differentiation into plasmacytes and plasma cells that secrete multivalent VLRB antibodies.[13,14] These antibodies are capable of agglutination and neutralization activity in response to immunization with particulate antigens, such as *Brucella abortus, Bacillus anthracis* exosporium and human red blood cells (RBCs).[1,13,15–19]

The germline VLRB gene is incomplete; it encodes portions of the N and C terminal regions that are separated by a non-coding intervening region. The VLRB gene is flanked by an extensive array of LRR cassettes[9,13,20,21] that can be incorporated as donor cassettes in a piecewise and stepwise fashion by a gene conversion like process.[7,9,13,20,22] The assembled mature VLRB genes are comprised of an N-terminal cap (LRRNT), LRR1, multiple LRRV modules, a connecting peptide, a C-terminal cap (LRRCT) and an invariant stalk region rich in threonine and proline residues.[13] Computational analysis of VLRB repertoire diversity suggests a potential repertoire of 10^{14} distinct VLRB receptors, which is comparable to immunoglobulin based receptor diversity.[7,13]

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VLRB monoclonal antibodies are highly specific for the antigen they are raised against as initially shown by their ability to antigenically differentiate spores from closely related *B. anthracis* with *B. cereus* species.[13,16] VLRBs can also recognize a wide variety of glycan epitopes, including terminal sulfated galactose residues on mammalian glycoproteins, making them valuable tools for studying glycan expression and developing novel biomarkers.[23,24] The VLRB antibodies are secreted as tetramers or pentamers of dimers.[16] Because of their multivalency, they display remarkable avidity to antigens with repetitive epitopes; for example, an anti-BclA VLRB monoclonal antibody agglutinates *B. antracis* spores at a 1000-fold greater dilution than a mouse anti-BclA IgG antibody. VLRB antibodies are stable at wide pH range between pH < 1.2 and pH > 11 while retaining their antigen binding specificity.[16] In view of their unique properties, we isolated VLRB monoclonal antibodies targeted against histidine rich protein-2 (HRP-2) from *Plasmodium falciparum* in order to explore their potential use in a diagnostic assay for *P. falciparum* detection.

Malaria in humans is caused by five different species of *Plasmodium* viz: *vivax, falciparum, ovale, malariae and knowlesi.* According to the World Health Organization, there were approximately 249 million malaria cases and 608,000 deaths reported worldwide in 2022.[25] *P. falciparum* is the most dangerous infection amongst all five; since, if untreated during early stages of infection, it can progress to severe clinical complications such as cerebral malaria, acute renal failure, severe anemia, and acute respiratory distress syndrome and death. A rapid diagnosis and treatment is therefore critical for preventing the morbidity and mortality associated with this infection. Thus, the World Health Organization recommends using rapid diagnostic tests (RDTs) in the settings where timely microscopic diagnosis is not feasible for early diagnosis of malaria.[26] Among three biomarkers used in malaria RDTs, histidine rich protein-2 (HRP-2) is the most commonly used target for *P. falciparum* diagnosis as it is more sensitively and specifically detected than other RDTs targets. This protein is a water-soluble protein secreted during sexual and asexual stages of the *P. falciparum* parasite.[27,28] This protein of 277 amino acids is composed mostly of histidine, alanine and aspartic acid (34% H, 37% A, and 10% D);[29,30] HRP-2 is secreted exclusively by *P. falciparum* and is found in the serum, blood and urine of infected patients, making it a valuable marker for diagnosis of *P. falciparum* infection.[31–33]

Currently, anti-HRP-2 monoclonal antibodies are used in RDTs for capturing HRP-2 and its homologue HRP-3 for diagnosis of *P. falciparum* infection. Although RDTs are highly sensitive and specific, they can be unstable at temperatures > 30 ℃ for a prolonged period of time.[34] The present studies examine the possibility that lamprey VLRs may offer specificity, avidity and thermostability advantages over traditional IgG based antibodies and could be considered for incorporation in RDTs for the diagnosis of malaria.

Material and methods

Yeast strain and reagents

Saccharomyces cerevisiae strain EBY100 was used for yeast surface display.[35] Yeast strains were cultured in YPD media, which consisted of 10 g/L yeast extract, 20 g/L peptone, and 20 g/L dextrose. Solid media for yeast growth were prepared using YPD plates, containing YPD media supplemented with 15 g/L agar. For selection and maintenance of plasmid-bearing yeast strains, SD-CAA media was used, composed of 10.4 g/L sodium citrate, 7.4 g/L citric acid monohydrate, 20 g/L dextrose, 6.7 g/L yeast nitrogen base, 5 g/L casamino acids (lacking adenine, uracil, and tryptophan), 100 kU/L penicillin, and 0.1 g/L streptomycin.[36] SD-CAA plates were prepared by adding 15 g/L agar to the SD-CAA media.[37]

Expression and purification of recombinant histidine rich protein-2 (rHRP-2)

Plasmid encoding the recombinant HRP-2 (pET3d) was kindly gifted by Dr. Daniel Goldberg, Washington University, St. Louise, MO. Expression and purification of rHRP-2 was done using *E. coli* Rosetta™ (DE3) competent cells (Novagen) as previously described.[38] Briefly, bacterial cultures (5 ml) from a colony of plated *E. coli* Rosetta™ (DE3) competent cells transformed with the plasmid encoding rHRP-2 were grown overnight at 37 ℃ on shaking incubator. Two ml from this starter culture were seeded into 1 L LB media (with antibiotics) on shaking incubator at 37 ℃ for 5–6 h until the culture reaches an optical density of 0.6 at A600 nm. Cultures were induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) at 1 mM for 3 h at 37 ℃. The bacteria were harvested using centrifugation and lysed in lysis buffer containing BugBuster Protein Extraction Reagent (Novagen, cat#70584), 1 mg/ml Lysozyme, 1 mM PMSF, 10ug/ml DNase. The lysate was centrifuged at 13,000 g for 10 min and supernatant passed over an activated Ni^{2+} column (4 ml, QIAGEN, cat#1018244) prewashed five times with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole). The column was washed again three times with wash buffer and eluted in elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole). For the GST-HRP-2 chimeric protein, the HRP-2 gene was inserted into the pGEX vector, which was transformed into *E. coli* Rosetta (DE3) cells. The chimeric protein was induced by adding IPTG and purified with a glutathione Sepharose 4B column. The eluent was concentrated to 1 ml final volume using Amicon ultra-4 50 k MWCO (cat# UFC805024), desalted using Zeba Desalt spin column (Thermo Scientific) and the concentration was measured using BCA Protein assay kit (Thermo Scientific).

Preparation of HRP-2 immunogen

Soluble antigens alone do not induce detectable immune responses in lampreys. Although lampreys may respond to antigens administered with complete Fruend's adjuvant (CFA), this adjuvant causes significant mortality. However, lampreys respond well to antigens coating the surface of bacteria (*Bacillus anthracis*), viruses and mammalian cells (human O type erythrocytes).[15,17,39] Taking advantage of this observation, soluble HRP-2 protein was coupled to human Jurkat T cells using amine-based chemistry to enhance the immunogenicity of the soluble HRP-2 protein. Briefly, paraformaldehyde fixed Jurkat T cells (1×10^8) were washed and suspended in 500ul of 20 mM MES/150 mM NaCl, pH 5.5). In order to activate Jurkat T cells for amine based reaction with the protein, 250ul each of EDC (75 mg/ml, 400 mM)/NHS (11.5 mg/ml, 100 mM) was added to Jurkat T

cells for 20 min at room temperature. Cells were pelleted at 300 g for 2 min and washed with PBS 1x. 1 mg of purified HRP-2 protein was added to the pelleted Jurkat T cells and incubated on a rotating mixer for 3 h at room temperature. 1 M Tris pH 8.0 was then added and incubated overnight at 4℃ on a rotating mixer. Cells were pelleted by centrifuging at 300 g for 2 min and washed three times with PBS 1 × and stored at 4℃ for future use.

Animals and immunization

Sea lamprey larvae (8–15 cm, 2–4 years old) were immunized intracoelomically with 45 µg of HRP-2 coupled to the surface of Jurkat T cells (1×10^7 in 0.67 × PBS). The larvae were boosted twice at 2 week intervals and blood samples harvested on day 45 after lethally anaesthetizing lampreys in 1 g/L of Tricaine methanesulfonate (MS222) (cat # NC0872873, Western Chemical) to collect the lymphocyte-like cells. All experiments were approved by the Institutional Animal Care and Use Committee at Emory University. The study is reported in accordance with ARRIVE guidelines. All methods were performed in accordance with the relevant guidelines and regulations.

Enzyme-linked Immunosorbent assay (ELISA)

96 well ELISA plates were coated with HRP-2 antigen in PBS at 5 µg/ml overnight at 4℃. Plates were blocked with 3% milk powder in PBS 1 × and incubated with plasma from immunized animals at serial dilutions ranging from 1:10 to 1:10,000 for 1 h at 37℃. Plates were then washed and incubated with anti-VLRB mouse monoclonal antibody (4C4)[13] at 1:500 dilution for 1 h at 37℃. Binding of the VLRB antibodies in plasma to HRP-2 antigen was detected by incubating with a goat anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP) for 1 h at room temperature. Color was developed using tetramethylbenzidine (TMB) substrate and read at 450 nM after adding the stop solution (0.16 M sulfuric acid).

Anti HRP-2 VLRB library cloning in yeast

Blood samples collected from HRP-2 immunized lampreys were layered on top of 55% percoll (cat # 17-5445-01, GE Healthcare) and centrifuged at 400 g for 5 min to pellet RBCs and the plasma and leucocytes collected. RNA was isolated from the total leucocytes using RNAeasy kit (Qiagen) and reverse transcribed into cDNA using SuperScriprt III reverse transcriptase (Invitrogen) and random hexamer priming. The first round of PCR was done using primers that amplified the 5′- and 3′-untranslated region of the VLRB, VLRB-1st-F and VLRB-1st-R (Supplementary Table 1), respectively. PCR conditions were 95℃, 2 min, 40 cycles at 95 °C, 30 s; 60 °C, 30 s; and 70 °C, 1.5 min. The second round of PCR was performed using primers for 5' N-terminal LRR, VLRB-2nd-F and for 3' C-terminal LRR, VLRB-2nd-F (Supplementary Table 1). The PCR conditions were 94℃, 2 min, 35 cycles at 94 °C, 30 s; 64 °C, 30 s; and 68 °C, 1.5 min. These primers introduced a 50 bp homology with the yeast surface display vector (pCT-ESO) for the cloning of VLRB gene in yeast by homologous recombination and present the VLRB on the surface of yeast by fusion to the yeast protein Aga2p, that is covalently attached to the yeast cell wall.[40] Expression of VLRB fused to Aga2P is under the control of galactose inducible promoter and checked by expression of c-Myc epitope on VLRB gene. The VLRB library was then transformed into yeast by co-electroporation of the linearized yeast surface display vector pCT-ESO (digested with NheI, BamHI and NcoI) and amplified VLRB DNA.[41]

Briefly, tryptophan-auxotroph *S. cerevisiae* strain EBY100 was grown to the log phase in YPD media at 30 °C until it reached an optical density of 1.0 at 600 nM. Yeast cells were pelleted at 1000 g for 3 min at 4℃, washed with ice cold water, and resuspended in 10 mM Tris–HCl, 10 mM DTT, 100 mM lithium acetate, pH 7.6 and incubated at 225 rpm for 20 min at 30 °C. After incubation, yeast cells were pelleted at 1000 g for 3 min at 4℃, washed with ice cold water and resuspended in ice cold 1 M sorbitol at a concentration of 10^9 cell per ml. 1 µg of linearized pCT-ESO-BDNF vector, 2 µg of the prechilled, purified VLRB DNA and 300µL of yeast cells were electroporated at 2.5 kV using a Micropulser™ electroporator (Bio-Rad). Immediately, 1 ml of 1:1 mixture of sorbitol: YPD media was added, incubated at 30 °C and 225 rpm for 1 h. Yeast cells were maintained in SDCAA media and aliquots of the transformed yeast library were stored at − 80 °C in 15% glycerol. The total number of transformants from electroporation were estimated to be 1×10^6 VLRB transformed yeast cells.

Magnetic- activated cell sorting for HRP-2 specific VLRB library enrichment

Lamprey VLRB clones specific for HRP-2 expressed on the surface of yeast were enriched by two rounds of magnetic activated cell sorting (MACS) as per manufacturer's instruction using Miltneyi LS columns (Miltneyi Biotech). Briefly, recombinant HRP-2 was biotinylated using EZ-Link Sulfo-NHS-LC-Biotinylation Kit (Thermo Fisher Scientific). Anti HRP-2 non-sorted yeast library preincubated with biotinylated HRP-2 was then incubated with Streptavidin microbeads (Miltneyi Biotech) and passed over a magnetic column. Yeast library obtained from this round of magnetic sorting was subjected to another round of MACS for further enrichment of anti-HRP-2 specific VLRB clones in the yeast library followed by flow sorting using anti-Myc Alexa Fluor 488 (clone 4A6, 16-224, EMD Millipore) and streptavidin R-phycoerythrin (R-PE) conjugate (cat# 7100-09, SouthernBiotech). Eluted cells were resuspended in SD-CAA medium and plated on SD-CAA plates. Individual colonies of plated yeast cells were grown overnight at 30℃ in SDCAA medium, centrifuged and resuspended in SGCAA medium for inducing the expression of VLRB. 50 µl of yeast cells from induced culture were incubated with biotinylated HRP-2 and stained with mouse α-Myc Alexa Fluor 488 and streptavidin R-phycoerythrin (R-PE) conjugate. HRP-2 binding was checked on BD Accuri™ C6 flow cytometer (BD BioSciences). Dual positivity for fluorochrome Alexa-488 and PE represented the HRP-2 binding positive clone.[41]

Individual colonies of yeast expressing VLRB were cultured and sequenced as described below.

Sequence characterization of anti-HRP-2 VLRB clones

HRP-2 reactive clones displayed on yeast cell surface were amplified using KOD hot start DNA polymerase (Novagen) and Zymolase (Zymo Research) and sequenced. Briefly, 2 µl of yeast culture was digested with Zymolase

for 30 min at 37℃. Sequencing PCR was done using primers VLRB-Seq-F and VLRB-Seq-R (Supplementary Table 1). The PCR conditions were: 94℃, 2 min, 40 cycles at 94 °C, 30 s; 58 °C, 30 s; and 70 °C, 1.0 min.

Sequence alignment, phylogenetic analysis and 3D structure prediction

Sequence alignments were performed using the ClustalW algorithm,[42] with additional manual inspection to ensure accuracy. Phylogenetic trees were generated using the Maximum Likelihood method implemented in MEGA software (version 12), utilizing up to 7 parallel computing threads.[43,44] The robustness of the phylogenetic tree was evaluated through bootstrap analysis with 1000 replications, providing statistical support for branching patterns. Three-dimensional structural predictions were performed using AlphaFold2.[45,46]

Lentiviral constructs for lamprey antibodies and virus infection (and antibody purification)

Lamprey anti-HRP-2 monoclonal antibody 5A10 was fused with the Fc portion of mouse IgG2a and inserted into the lentivirus vector (pCDH-EF1-IRES-GFP, System Biosciences). The lentiviral expression construct 5A10-mFcIgG2a was co-transfected into 293FT cells (Invitrogen) with pCMVR8.74 (Addgene plasmid 22036) and pMD2G (Addgene plasmid 22036) using polyethylenimine (PEI). The virus-derived constructs (pCMVR8.74 for packaging and pMD2G for envelop protein) were provided by D. Trono (EPFL, Switzerland). Culture medium containing lentivirus was harvested at 48 h after transfection and removed cell debris by 0.45 µm filters. The recombinant lentiviruses were mixed with polybrene (5 ug/ml) and infected into HEK293T cells. Infected cells were cultured in serum-free medium (Sigma-Aldrich); the culture supernatant was collected for antibody purification on a protein A column as per manufacturer's instructions (Affi gel Protein A MAPS II kit, BioRad cat no.1536159). VLRB 5A10 antibody yield was about 30 mg/L without aggregation or precipitation.

Western and dot blot analysis

Reactivity of anti HRP-2 VLRB clone 5A10 to recombinant and native HRP-2 was measured by western blot analysis. Briefly, HRP-2 (recombinant and native) electrophoresed under reducing conditions on 4–20% gradient gel (SDS-PAGE precast gel, Lonza).[47] For Western blots, the proteins were transferred onto the nitrocellulose membranes after electrophoresis. The membranes were then incubated with lamprey monoclonal antibody 5A10 and detected using anti-mouse IgG conjugated to Alkaline phosphatase (Life Tech, cat no: G21060). Color was developed using Western Blue Substrate (Promega, cat no: 53841). Dot blot analysis was carried out by using nitrocellulose membrane. Samples were added in serial dilutions on the membrane and were left to filter by gravity. The membrane was blocked, reacted with anti-HRP-2 VLRB 5A10 and color visualized by Western Blue Substrate. The original scanned images from two or three experimental repeats were used for all blots and a gel.

Thermo-stability under different storage conditions

To assess the stability of the anti HRP-2 VLRB 5A10 at different storage conditions, anti HRP-2 VLRB 5A10 was incubated at following temperatures: 4℃ for 1 month; room temperature for 1 month; 37℃ for one month; 42℃ for 24 h, 36 h, one month; 56℃ for 1 h, 2 h, 36 h; 70℃ for 1 h, 2 h. 96 well ELISA plates were coated with native HRP-2 antigen at 10 µg/ml overnight at 4℃. Plates were blocked with 3% milk powder in PBS 1 × and incubated with VLRB 5A10 (preincubated at various stated conditions) for 1 h at 37℃. Plates were washed and incubated with goat anti- mouse IgG (H + L) \enzyme conjugated secondary monoclonal antibody (ThermoFisher Scientific, cat no: 31430) at 1:500 dilution for 1 h at 37℃. After washing three times, TMB substrate was added (KPL, cat no: 5120–0053) for 20- 30 min at 37℃ for color development. Color developed was read at 450 nM. Commercially available monoclonal IgG anti-*Plasmodium falciparum* antibody (Immunology Consultants Laboratory, Inc cat no: MPFG-55A) specific for HRP-2 was also used in the ELISA for comparison.

Sandwich enzyme-linked immunosorbent assay (ELISA)

96-well plates were coated overnight at 4℃ with 50ul of 1ug/ml unlabeled lamprey 5A10 antibodies. After removing the unbound antibodies using washing steps, 50ul of 3% BSA in TBS was added and incubated at room temperature for 1 h. Following one wash with PBS containing 0.1% Tween 20 (PBST), human plasma samples were diluted 1:250 with PBS, applied 50 ul to the well and kept for 3 h at room temperature. After three washes with PBST, biotin-labeled 5A10 were added to the well and incubated at room temperature for 1 h. After three additional washes with PBST, peroxidase-labelled streptavidin (at 1:1000 dilution) was added and incubated for 1 h at room temperature. Then, three final washes with PBST were performed followed by the addition of 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate for 5–10 min. Hydrochloric acid (HCL) was added to stop the reaction. The optical density was measured at 450 nm (OD450).

Plasma samples from Kenya donors

Plasma samples from 60 microscopically diagnosed malaria positive residents in the Asembo Bay area of western Kenya next to Lake Victoria who participated in a birth cohort study were used to test reactivity of the VLRB antibody. A detailed description of the Asembo Bay study site and the epidemiology of malaria in this area has been reported elsewhere.[48–50] The original cohort study was approved by the Institutional Review Board of Centers for Disease Control and Prevention and the Ethical Review Committee of Kenya Medical Research Institute; informed consent was obtained from all study participants for performing various assays including for immunological studies using stored specimens. Plasma from a subset of participants were tested for determining if lamprey antibody 5A10 can recognize HRP-2 antigens present in the plasma of individuals previously exposed to malaria. All studies were performed in accordance with the relevant guidelines and regulations.

Results

The most consistent antibody responses are obtainable by immunizing lampreys with mammalian cells (mouse or human erythrocytes) and bacteria (*B. anthracis*) that have repetitive protein and carbohydrate epitopes on their surface (Alder MN, 2008). Taking advantage of the immunogenicity of mammalian cells as a carrier, Ni^{2+} column-purified HRP-2 protein (Fig. 1A) was covalently linked to the surface of Jurkat T cells prior to immunization in lampreys. Lampreys generated strong humoral immune responses against HRP-2 with high anti-HRP-2 serum titers of ≥ 1:2500 in all three animals immunized (Fig. 1B).

Total RNA from the lymphocytes of these hyperimmunized animals was reverse transcribed into cDNA and cloned into a yeast surface display vector by in vivo homologous recombination in transformed yeast cells. The VLRB cDNA library was expressed on the surface of yeast cell in fusion with the yeast agglutinin protein Aga2p that gets attached to the yeast cell wall in covalent association by disulfide linkage with another yeast protein Aga1p.[40] The yeast surface displayed VLRB cDNA library was enriched for VLRB clones that bind to HRP-2 by two subsequent rounds of magnetic activated cell sorting (MACS) using biotinylated HRP-2. VLRB cDNA expressed with a c-Myc tag under the control of a galactose inducible promoter, which allowed evaluation of the expression of the VLRB library after a media change from glucose- to galactose-rich to induce the expression of VLRB on the yeast cell surface. After the first round of MACS sorting using biotinylated rHRP-2, the enrichment of VLRB clones binding to HRP-2 increased from 0.1% (non-sorted) to 0.5%, and a subsequent round of MACS sorting enriched the HRP-2 specific VLRB clones to 6% (Fig. 2A). The VLRB library was then sorted using anti-Myc Alexa Fluor 488 and streptavidin R-PE and plated on SDCAA plates. We have isolated fifteen clones that contain five unique sequences and can bond to the HRP-2 protein. The binding specificity of representative four clones was verified by flow cytomerty analysis (Fig. 2B). Sequence analysis of the HRP-2 specific VLRB clones revealed that the clones (clones II and 5A10 and clones 17 and A3) have their identical amino acid sequence of the LRR N-terminal (LRRNT), LRR1, LRRV1, LRRV2, LRRV3, LRRVe, connecting peptide (CP) and LRR C-terminal domains (LRRCT) (Fig. 3A). The different sequence clones have only a few amino-acid differences in the N-terminal regions, LRRNT and LRR1 (Fig. 3A). Structural analysis indicated that the different amino acids are located in and close to the first and second β-strands (Fig. 3A,B,C). These implied that all of the selected VLRB clones bind the same epitope on HRP-2 due to similar VLRB gene sequence and one of these, monoclonal antibody VLRB 5A10, was selected for subsequent assays. Of note, the insert loop of the clones in the LRRCT domain has the same amino acid sequence as those of the VLRB clones, anti-H-trisaccharide Tn4-22 and O13[51] and antigen-unknown VLR2913[52] (Supplementary Fig. 1).

The anti-HRP-2 VLRB monoclonal antibody 5A10 was expressed as a fusion protein with Fc portion of mouse IgG2a and cloned into the lentivirus vector (pCDH-EF1-IRES-GFP) for its secretion and purification on a protein A column. The purified lamprey VLRB monoclonal antibody 5A10 was of the expected size (Fig. 4A) and bound to recombinant HRP-2 proteins with and without GST on Western blot analysis (Fig. 4B). VLRB 5A10 monoclonal antibody also recognized native HRP-2 from different isolates of *P. falciparum (PfW2 and PfHB3)* (Fig. 4C). *Plasmodium falciparum* isolate *Pf W2* secretes HRP-2 and another related histidine rich protein-3 (*Pf*HRP-3), while the *Pf*HB3 strain only secretes HRP-2 in its culture supernatant. Culture supernatant from another strain of *Plasmodium falciparum, Pf* Dd2, that only secretes HRP-3 was also reactive with VLRB 5A10 (Supplementary Fig. 2). *Pf*HRP-2 shares around 80–90% sequence homology to *Pf*HRP-3[30], thereby

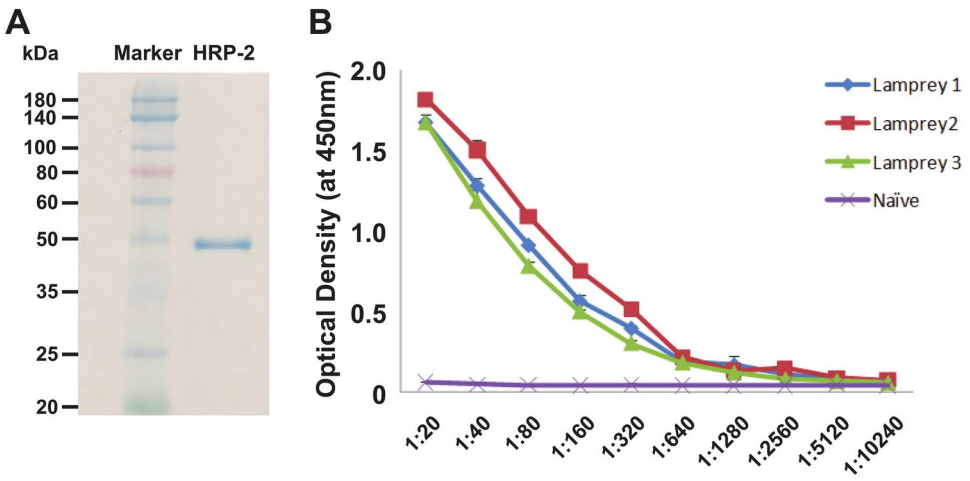

Fig. 1. Generating anti-HRP-2 VLRB monoclonal antibodies in sea lampreys. (**A**) Plasmid containing the gene in pET3d vector was transformed in *E. coli* strain DE3 and HRP-2 expression was induced by addition of 1 mM IPTG. HRP-2 was purified from culture supernatants by metal chelate chromatography on Ni^{2+} NTA-Sepharose column. The purified protein was separated by SDS-PAGE and stained with Coomassie Blue. The representative gel from two repeats was shown. (**B**) HRP-2 specific VLRB antibodies were generated by immunizing lamprey larvae three times with purified HRP-2 protein conjugated to Jurkat T cells at two-week intervals, and cells were harvested 14 days after 3rd immunization. HRP-2 specific plasma VLRB reactivity titers were measured by ELISA. The cutoff value is determined by the mean of the naïve plasma O.D. ± 2 sd.

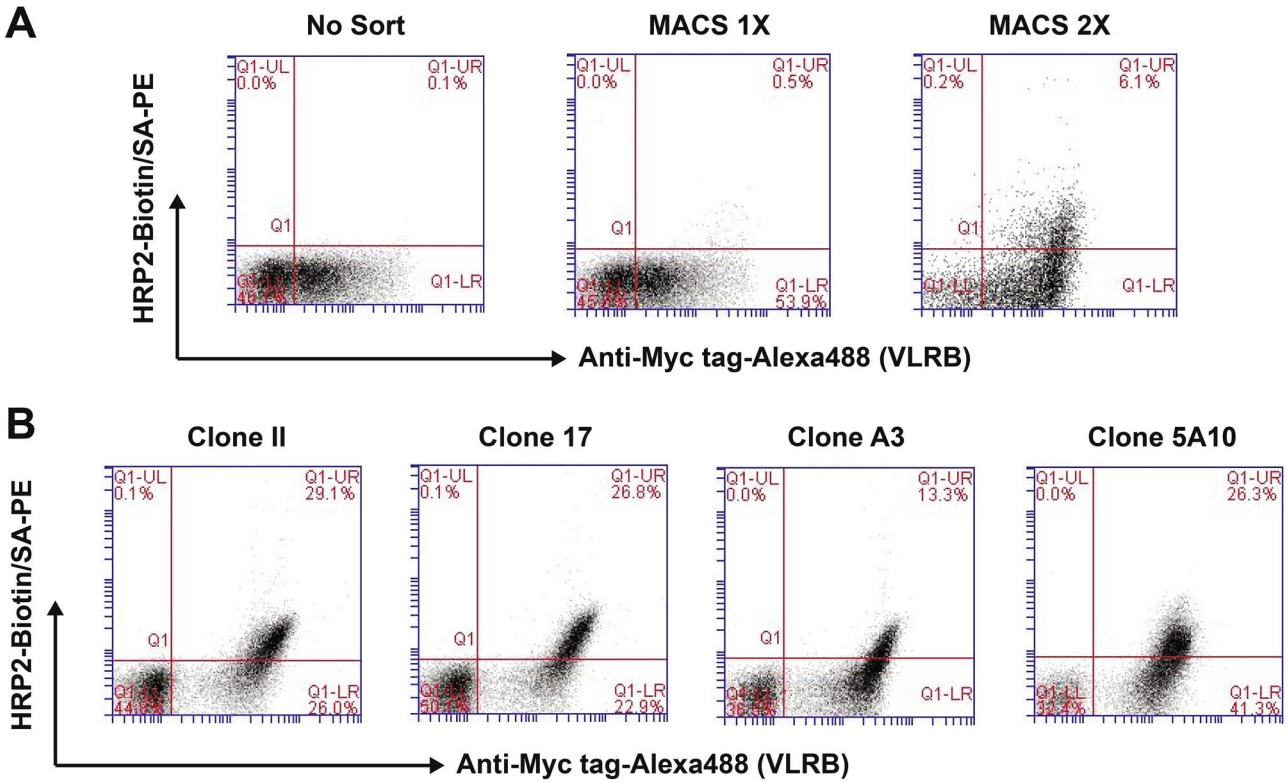

Fig. 2. Selection of HRP-2 specific lamprey VLRB monoclonal antibodies using yeast surface display. (**A**) VLRB cDNA libraries from the lymphocytes of HRP-2 immunized lampreys were expressed on the yeast surface by fusion to the Aga2p yeast protein and tagged with a myc epitope to enable detection of VLRB surface expression. VLRB cDNA yeast library was enriched for HRP-2 binding VLRB clones by two rounds of magnetic- activated cell sorting (MACS) using biotinylated HRP-2 followed by florescence activated cell sorting (FACS). HRP-2 specific VLRB clones were selected by incubating the transformed yeast cells with biotinylated HRP-2 antigen and staining with streptavidin-PE and anti-Myc Alexa 488. (**B**) Reactivity of anti HRP-2 VLRB monoclonal antibodies expressed on yeast surface to recombinant HRP-2.

suggesting that lamprey VLRB 5A10 cross reacts with *Pf*HRP-3 due to high degree of sequence similarity with *Pf*HRP-2. Nevertheless, the lamprey monoclonal VLRB 5A10 was not cross reactive with any other protein from *plasmodium* species (*Pv* 25, *Pf* 25, *Pf* 230 and *Pf* 480) or proteins from other unrelated non-malarial species Crypto27 and Giardia intestinalis protein (Supplementary Fig. 2). The detection limit for both VLRB 5A10 and mouse anti-HRP-2 was 40 ng/ml (Supplementary Fig. 2).

Stability and biological activity of VLRB 5A10 at different storage temperatures was tested by incubating the purified lamprey monoclonal antibody at different temperatures: room temperature for 1 month; 37℃ for one month; 42℃ for 24 h, 36 h, one month; 56℃ for 36 h; and 70℃ for 2 h. VLRB 5A10 monoclonal antibody retains its biological activity and binds to native HRP-2 at all of these storage conditions (Fig. 5). A commercially available anti-HRP-2 mouse monoclonal antibody used for comparison also binds to the native HRP-2 at all conditions except for 70℃ (Fig. 5).

To test if 5A10 can be used for detection of HRP-2 in clinically *P. falciparum* positive patient samples by sandwich Enzyme-Linked Immunosorbent Assay (ELISA), we biotinylated the 5A10 mAb and used it for the secondary antibody. All 60 of the patient samples were recognized by 5A10 as well as by mouse anti-HRP-2 antibody, whereas normal human plasma samples were not (Fig. 6).

Discussion

We have developed a system to produce lamprey VLRB antibodies specific for detection of *Plasmodium falciparum's* HRP-2 and characterized its specificity and other biological properties. Previous studies indicate that it is exceedingly difficult to generate an immune response to soluble antigens like BSA, KLH or bovine −γ in lampreys.[1,14,18] However, consistent immune responses were induced in lampreys to repetitive epitopes presented on the surface of particulates antigens like *B. anthracis* exosporium, bacteriophage, *Brucella abortus* and human red blood cells.[15,17,39] We used the ability of lampreys to mount an immune response to the repetitive HRP-2 particulate antigen coupled to the surface of Jurkat T cells to consistently elicit a high titer (1 ≥ 2500) humoral response against the HRP-2 antigen. A VLRB cDNA yeast surface display library was then constructed from the lymphocytes of lampreys immunized with this immunogen. After two rounds of magnetic activated cell sorting (MACS) using biotinylated rHRP-2 followed by flow cytometry sorting, the VLRB antibodies selected for binding to rHRP-2 were identical in sequence—except for one or two amino acid differences at the

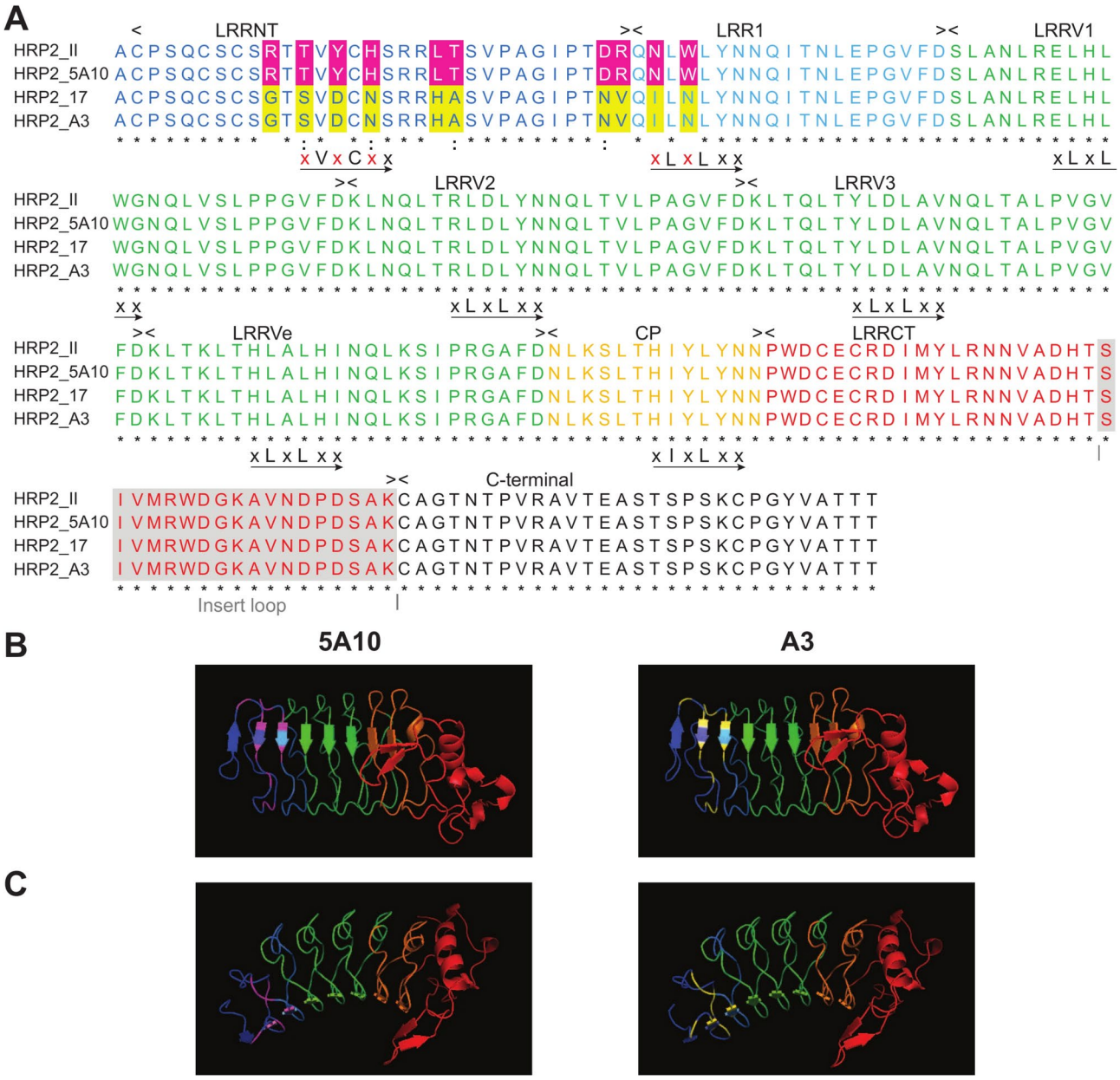

Fig. 3. (**A**) Amino acid sequence alignment of the selected HRP-2 specific VLRB monoclonal antibodies. HRP-2 specific VLRB monoclonal antibodies were isolated by yeast surface display. Amino acid alignments for the LRR N-Terminal (LRRNT), LRR1, LRRV1, LRRV2, LRRV3, LRRVe, connecting peptide (CP) and C-terminal LRR domains (LRRCT) are indicated. Insert loop, a key component for antigen recognition, and seven β-strands (xL/V/IxL/Cxx) from LRRNT to LRRCT regions are shown. Different amino acids in LRRNT and LRR1 regions between VLRB clones II and 5A10 and 17 and A3 are shown in magenta and yellow, respectively. * Same amino acids, : Similar amino acids. (**B**) Comparison of the predicted 3D structures of VLRB clones 5A10 and A3. The LRRNT region is shown in blue, the LRR1 region is in light blue, and the LRRVs are in green, whereas the CP region is presented in orange, and the LRRCT region is in red. Different amino acids in LRRNT and LRR1 regions are shown in magenta (5A10) and yellow (A3), respectively. (**C**) Side views of the predicted 3D structures of 5A10 and A3, showing a 90-degree rotation from B. Amino acid sequences of VLRB clones were deposited in the GenBank NCBI database (accession numbers PV433236-PV433237).

N-terminus—indicating binding to the same epitope on HRP-2. In view of the repetitive alanine and histidine sequences in *Pf*HRP-2 that present multiple repeats of the tripeptide (AHH), pentapeptide (AHHAA) and hexapeptide (AHHAAD), we therefore used the monoclonal VLRB 5A10 lamprey antibody as both capturing and detecting antibody by biotinylating the detection antibody reagent.

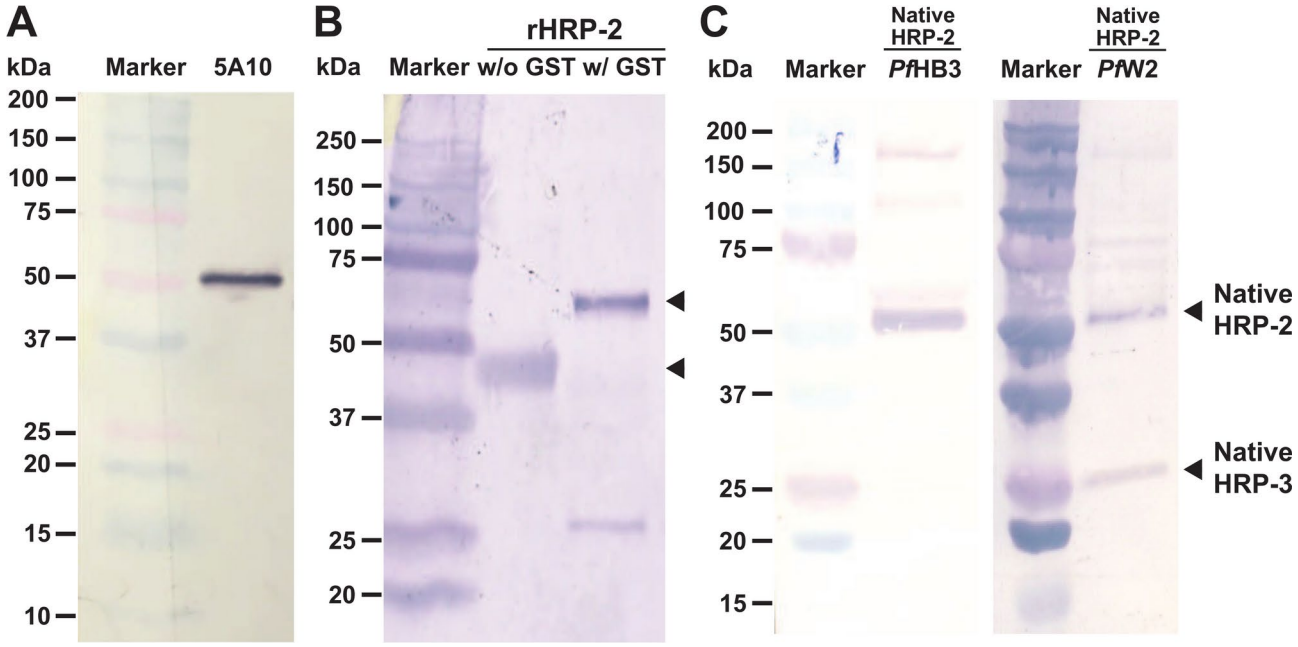

Fig. 4. Binding of anti-HRP-2 VLRB 5A10 with HRP-2. (**A**) Protein expression of anti-HRP-2 monoclonal VLRB 5A10 by HEK293T cells. The purified protein was separated by SDS-PAGE, transferred onto the membrane and detected anti-mouse IgG conjugated to alkaline phosphatase. (**B**) Western blot analysis shows the reactivity of anti-HRP-2 VLRB monoclonal antibody 5A10 to the recombinant HRP-2 with and without GST. (**C**) Reactivity of anti-HRP-2 VLRB monoclonal antibody 5A10 to the native HRP-2 purified from culture supernatants of different isolates of *Plasmodium falciparum* (*Pf*HB3, *Pf*W2). Different blots were separated by the white space. Representative blots from two or three repeats were shown.

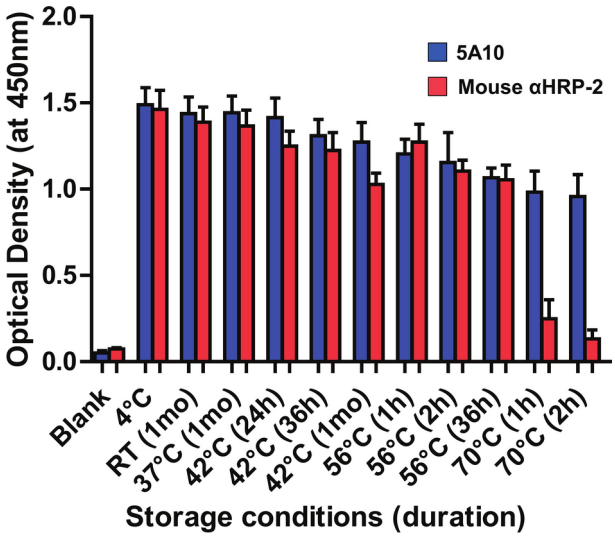

Fig. 5. Stability of the anti-HRP-2 VLRB 5A10 at different storage conditions. Anti-HRP-2 VLRB 5A10 was incubated at 4℃ for 1 month; room temperature for 1 month; 37℃ for one month; 42℃ for 24 h, 36 h, one month; 56℃ for 1 h, 2 h, 36 h; 70℃ for 1 h, 2 h. Anti-HRP-2 VLRB 5A10 reacted to native HRP-2 protein at temperatures up to 70℃ by ELISA. Commercially available mouse monoclonal IgG anti-*Plasmodium falciparum* antibody specific for HRP-2 binds very weakly with native HRP-2 as compared to lamprey VLRB 5A10 at 70℃.

VLRB antibodies offer several unique and potentially advantageous properties over traditional monoclonal antibodies that make them excellent candidates as diagnostic agents. Lamprey VLRB antibodies bind with high avidity and specificity to the immunized antigen and can differentiate between closely related proteins. For example, VLRB antibodies can discriminate between spores of *Bacillus anthracis* from *Bacillus cereus* despite 89

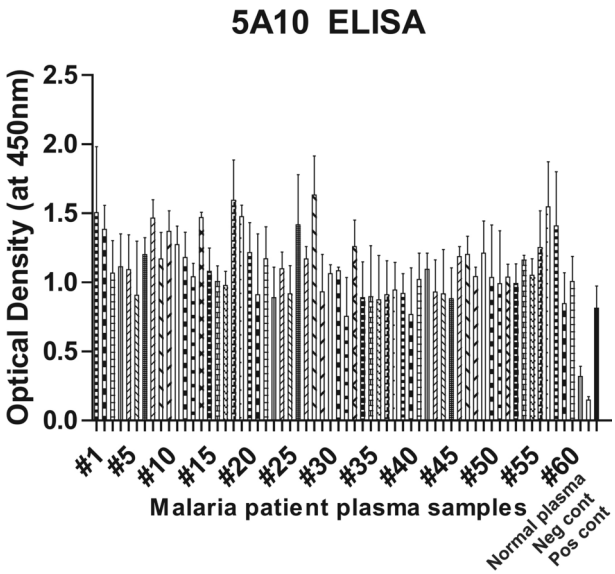

Fig. 6. VLRB 5A10 detection of *Plasmodium falciparum Pf*HRP-2 in 60 patients by sandwich ELISA. Non-labeled VLRB 5A10 was coated on a 96-well plate. Plasma samples were incubated on the plate and biotin-labeled 5A10 was added after washing. Streptavidin-labeled horseradish peroxidase (HRP) and its substrate TMB (3,3',5,5'-tetramethylbenzidine) were used to detect the binding between *Pf*HRP-2 and VLRB 5A10. Mouse anti-HRP-2 (IgG) was coated on the plate for positive control (pos cont) and a non-coated plate was used for negative control (neg cont). Patient plasma #60 was used for the controls. Optical density (OD) of 450 nm was measured using a microplate reader after adding a stop solution (sulphuric acid).

percent sequence homology between the two spores.[16] Lamprey VLRB monoclonal antibodies also agglutinate *B. anthracis* spores at a 1000-fold higher dilution (5 pg/ml) compared to a IgG2b monoclonal antibody (5 ng/ml) due to the avidity effect of the secreted multivalent VLRB antibodies for binding to antigens with repetitive epitopes.[16] Lamprey VLRB antibodies are stable at high salt conditions and at pH levels ranging from 2 to 11 without compromising the antigen-binding activity.[16] Because VLRB genes encode only a single polypeptide chain, it is easier to manipulate the DNA of these antibodies including mutagenesis to increase affinity of the VLRB antibodies for antigen binding.

Malaria is a global infectious disease that accounts for nearly 608,000 deaths annually worldwide.[25] Diagnosis of malaria using commercially available rapid diagnostic tests (RDTs) for the most part target the histidine rich protein-2 of *Plasmodium falciparum* (*Pf*HRP-2), which is a highly stable specific biomarker for *P. falciparum* infection. *Pf*HRP-2 has an abundance of histidine and alanine repeats that are shared by another antigenically similar protein, *Pf*HRP-3, which also contributes to the reactivity of *Pf*HRP-2 based RDTs.[30] Although *Pf*HRP-2 based RDTs have a high detection rate, their stability will be influenced by the local storage and temperature conditions in tropical weather conditions in endemic countries. In this context, the unique properties of lamprey VLRB monoclonal antibodies, including specificity and thermostability at high temperatures, could offer a favorable alternative source of antibodies than conventional antibodies for use in RDT products.[53–55] Therefore, we plan to collaborate with RDT manufacturers for validating potential advantages of employing lamprey antibodies for improving stability and performance of RDTs.

Data availability

The datasets used and/or analyzed in this study are available from the corresponding author upon request. Nucleotide and amino acid sequence data supporting the findings of this study have been deposited in GenBank/NCBI under accession numbers PV433236-PV433237 (https://submit.ncbi.nlm.nih.gov/subs/genbank/SUB15230038/overview).

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Author contributions

D.T., B.S., M.H., B.R.H. and U.V. conceived and designed the experiments. D.T., B.S., M.H., S.S., E.R., O.L. and B.R.H. performed the experiments. D.T., B.S., M.H., S.S., M.A., E.R., B.R.H. and U.V. analyzed the data. D.T., B.S., M.H., M.A., E.R. and U.V. wrote the paper.

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Declarations

Ethical Statement

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

Competing interests

The authors declare no competing interests.

Additional information

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