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Research Article

DEVELOPMENT AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST ERYTHROCYTIC STAGES OF *PLASMODIUM VIVAX* AND USE IN DETECTION OF MALARIA

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ABSTRACT
Hybridomas producing monoclonal antibodies (MAbs) directed against *P. vivax* erythrocytic stage antigens were isolated. Here, we describe the production of six MAbs. Culture supernatant from growing hybridomas was tested for their reactivity with *P. vivax* erythrocytic stages by ELISA against *P. vivax* crude (PvC) antigen and by immunofluorescence assay on *P. vivax* infected erythrocyte smears. *P. vivax* positive hybridomas were checked for immunoglobulin isotyping by ELISA. Seroreactivity of MAbs was also determined against PvC antigen by inhibition ELISA and competitive binding assay. On Western blot, the MAbs identified proteins of different molecular weights. Mouse-specific IgG concentrations were determined in the culture supernatant of six hybridomas. Dot-blot assay showed specific reaction of monoclonal antibodies with the blood samples taken from *P. vivax* infected patients. The MAbs directed against parasite proteins would be helpful in the development of diagnostic test.

Key Words: *Malaria, Monoclonal Antibody, Hybridoma, P. vivax, Antigen, ELISA*

INTRODUCTION
Monoclonal antibodies (MAbs) are important reagents used in biomedical and microbiological research in diagnosis of various diseases including malaria. MAbs have an important impact on current progress in the identification and characterization of immunogenic blood stage antigens of Plasmodia and gained particular importance as immunodiagnostic reagents. In past few years, efforts have been made to supplement or replace the traditional and tedious microscopic readings of blood smears by using MAbs in an immunoassay.

Since the advent of hybridoma technology, a large number of MAbs has been produced against various antigens from Plasmodial source (Banyal and Inselburg 1985 and Andrysiak *et al.,* 1986). The first attempt to produce MAbs against sporozoites involved rodent, simian and human malaria parasite (Yoshida *et al.,* 1980; Nardin *et al.,* 1982 and Cochrane *et al.,* 1985). These MAbs reacted with circumsporozoite proteins and were found to be protective. The first MAbs against sporozoites of *P. falciparum* and *P. vivax* was developed to identify sporozoite antigens and was used for their field application (Zavala *et al.*, 1982; Wirtz et al., 1985 and Udhayakumar *et al.,* 1994). MAb 2C6111 specific for *P. vivax* erythrocytic stage was used to detect parasitized erythrocytes in blood samples (Perez *et al.,* 1995). This MAb binds to the mature trophozoite, schizont and gametes of *P. vivax*. To search for *P.vivax* parasites in human blood a MAb-immunofluorescence test has been developed. Its sensitivity is close to that usually achieved with Giemsa stained blood films. The possible location of some of the asexual erythrocytic stage polypeptides of *P. vivax* were characterized by immunofluorescence assay and Western blot techniques using a panel of MAbs. A high level of antigenic polymorphism prevailed in *P. vivax* isolates. MAbs against variant epitopes of a highly polymorphic protein Pv200 in schizonts of *P. vivax* have been used to analyze the variety of genetically distinct population of parasites present in clinical

isolates (Udagama *et al.,* 1990). Polymorphism of target antigens of transmission blocking immunity was investigated based on the reactivity of MAbs. Majority of the target antigens of transmission blocking immunity were found polymorphic in size and epitope (Premawansa *et al.,* 1990). MAbs from nine hybridomas specific for *P. vivax* erythrocytic stages from southeast Mexico were used to identify *P. vivax* antigens and their sub cellular distribution (Sanchez *et al.,* 1994). The antigenic diversity of *P. vivax* and their distribution in endemic areas of Thailand were studied by laboratory produced MAbs (Khusmith *et al.,* 1998). Attempt has been made to develop MAbs against a 19 kDa recombinant PvMSP1 for detection of *P. vivax* infection in Turkey and for their future use in development of diagnosis (Ak *et al.,* 2004). Some studies were conducted towards detection of *P. vivax* blood stage antigens using monoclonal and polyclonal antibodies by enzyme immunoassay and immunofluorescence assay (Perez *et al.,* 1995; Bracho and Perez 1996 and Joshi *et al.,* 2004).

Using monoclonal antibodies against the histidine rich protein-2 of *P. falciparum,* two diagnostics kits Parasight-F (Becton Dickinson) and Malaquick test (ICT Diagnostics, Sydney) have been developed, which had the sensitivity greater than 95% and showed results in 5-10 min (Makler *et al.,* 1998). The first report on MAb based diagnostic approach using *P. knowlesi* lactate dehydrogenase (LDH) demonstrated biochemical and immunochemical distinction of parasite enzyme from the equivalent host enzyme (Kaushal *et al.,* 1995). A breakthrough in immunodiagnosis of *P. vivax* was introduced by OptiMAL test (Flow laboratories, Portland, Oregon) based on MAb raised against the intracellular metabolic enzyme: parasite LDH. It has sensitivities of 94% and 88% and specificities of 100% and 99% respectively, when compared to traditional microscopy (Palmer *et al.,* 1998). Further advancement was made in ParaSight-F test format by incorporating a MAb directed against a proprietary *P. vivax*-specific antigen and a composite paraSight F+V assay was developed by Becton Dickinson. The modified test kit was used to screen a total 4,894 symptomatic patients over a 2-year period in malaria endemic regions of Thailand and Peru. The overall sensitivity for *P. vivax* was 87% and the specificity for exclusion of *P. vivax* was also 87% (Forney *et al.,* 2003). These rapid diagnostic tests offer the chances to move the malaria diagnosis away from laboratory to patient and have the potential to enhance diagnostic capabilities in situations where skilled microscopy is not readily available (Moody 2002).

Main goal behind raising MAbs against various stages and enzymes of *Plasmodia* has potent application in diagnostics. In this study we have characterized MAbs reacting with *P. vivax* proteins for their future use as immunodiagnostic reagents.

MATERIALS AND METHODS

Preparation of Crude Antigen

Subsets of adult subjects having *P. vivax* infection confirmed by microscopy were bled through venipuncture for 5 to 6 ml blood in heparinized tube for antigen preparation. The patients diagnosed with malaria were treated with recommended antimalarials as per National Drug Policy. This study was approved by the ethics committee of the National Institute of Malaria Research. *P. vivax* infected blood containing ring, trophozoite, schizont and gametocyte stages; with about 1-1.5% parasitaemia was lysed (after removal of leucocytes and platelets through glass bead-cellulose column) with 2 volumes of 0.15% saponin at 37°C for 30 min. The lysed blood was sediment at 10,000 rpm for 20 minutes at 4°C. After three times washings with chilled PBS the pellet was sonicated at 14 µA for 90 seconds in the presence of protease arrest. The prepared lysate was termed as *P. vivax* crude (PvC) antigen. Similarly *P. falciparum* infected erythrocytes from in vitro culture and uninfected (normal) human erythrocytes were also processed for preparation of *P. falciparum* crude (PfC) antigen and normal human erythrocyte (HNRBC) antigen.

Development of Hybridoma

Four to six weeks old inbred BALB/c mice were used in this study. Pre-immunized blood was collected from each mouse by tail bleeding. Mice were immunized intraperitoneally with 100µg of PvC antigen (1mg/ml) in an emulsion with Freund's Complete Adjuvant (FCA). Subsequent boosters were given with

the same amount of antigen in an emulsion with Freund's Incomplete Adjuvant (FIA). Mice were bled by puncturing the tail vein at different times during the course of immunization. Sera were used to assess antibody titre by ELISA against crude antigens. The mice were allowed to rest for 1 month. Final booster with PvC was administered without adjuvant just before 4 days of fusion experiment.

Fusion

The method was followed with partial modification as described elsewhere (Kohler and Milstein 1975). Briefly, myeloma cells (SP_2O) and spleen cells were mixed at 1:4 ratios. Cells were pellet down by centrifugation at 1500 rpm for 5 min at RT. Pellet was dislodged by tapping and 1 ml pre-warmed (37°C) 50% (w/v) suspension of polyethylene glycol-1500 solution was added drop-wise to the cell pellet with gentle mixing over a period of 50 sec. Hypoxanthine Aminopterin Thymidine (HAT) medium was added drop by drop within 5 min with gentle mixing. The tube was left for another 5 min at RT followed by centrifugation at 1500 rpm for 8 min. Cell pellet was resuspended in 10 ml of HAT medium containing 10% FCS and 1 ml of Hybridoma Cloning Factor (HCF, Origen^{TM}, Fisher) maintaining the final concentration at 1 x 10^6 cells per ml. Culture was initiated in 96-well flat bottom tissue culture plates by placing 200 µL of cell suspension in each well. In every plate, spleen cells and SP_2O cells were added in two wells as control. The plates were kept in a humidified incubator with 5% CO_2 at 37°C. On fourth day after fusion, half of the supernatant was aspirated from each well and replaced with 100 µL of freshly prepared HAT medium. This step was repeated on day '7' and '10'. After the 10th day, the hybridomas were fed with HT medium on a regular basis every 3-5 days. From 14th day onwards plates were checked under an Inverted microscope using 10 x and 20 x objectives for appearance of clones and growth of the hybrids.

Positive wells were cloned and sub-cloned by limiting dilution 96-well flat bottom tissue culture plates to obtain stable hybrids. When cell growth in the wells was 25% confluent, hybridoma culture supernatants were assayed for specific antibodies by ELISA. The specific hybrid cells were expanded by seeding in 24 well tissue culture plates followed by 6 well tissue culture plate and then in 25 cm^2 culture flask. One half of the cells were cryopreserved at every expansion in liquid nitrogen.

Enzyme Linked Immunosorbent Assay (ELISA)

The supernatant from wells showing clones was screened for antibody activity by indirect ELISA in 96-well high protein binding U bottom polystyrene ELISA plates (Iwaki, Japan). Briefly, plates were coated with PvC antigen at a concentration of 5 µg/mL in 0.1 M carbonate-bicarbonate buffer, pH-9.6 and incubated over night at 4°C. Plates were washed thrice with PBS-Tween 20 (PBS-T) and incubated with culture supernatant from wells of selected hybrids for 1 h at 37°C and 1 h at RT. Plates were then washed thrice with PBS-T and incubated with 1:2000 dilution of HRP conjugated anti-mouse antibodies (polyvalent) for 1h at 37°C and 1h at RT. Plates were washed and orthophenylenediamine/hydrogen peroxide (OPD/H_2O_2) as substrate was added to each well. The reaction was terminated by addition of 8N sulphuric acid and the absorbance was read at 492 nm in an ELISA reader, results were expressed as O.D. values. The 100µL reaction volume was maintained throughout the steps. Pre immune sera and SP_2O culture supernatant were used as negative controls. Each test samples and reagent blank (devoid of primary antibody) were assayed in duplicates.

Immunofluorescent Antibody Test (IFAT)

The specificity of the monoclonal antibodies was determined by localization of antigens in *P. vivax* erythrocytic stages by IFA test. Basically the IFA procedure described previously was followed (Collins and Skinner 1972). Briefly, blood samples were collected in heparinized tube from microscopically positive *P. vivax* patients having all the blood stages viz. ring, trophozoite, schizont and gametocytes. Samples were centrifuged at 1500 rpm at 4-6°C for 10 min and plasma was aspirated. RBC pellet was washed thrice with chilled PBS and pellet was resuspended in PBS and parasitaemia was adjusted to 0.1%. Thick smears of the parasite suspension (10µL) were prepared on multi test glass slide. Smears were air dried and stored at -20°C till further use. Similar way smears of *P. falciparum* infected erythrocytes from in vitro culture were prepared on multi test slides for determining cross-reactivity.

Before use the slides were brought at RT. Smears were washed with PBS and blocked with 1% BSA for 30 min at 37°C, washed thrice and then air dried. Culture supernatant from growing hybridomas was allowed to react for 1 hr at 37°C with erythrocytic stages of *P. vivax* and *P. falciparum*. The supernatant of SP_2O culture was taken as negative control. After four washings with PBS, smears were treated for 1 hr at 37°C with fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG (1:50) followed by counter staining of parasite DNA with ethidium bromide (50µg/ml) for 2 min at RT. After four washing the spots were air dried and then mounted with 60% glycerol in PBS with a rectangular glass cover slip. Smears were examined under UV microscope using 100X oil immersion objective.

Immunglobulin Isotype and Immunoglobulin G Subclass Analysis

Immunoglobulin isotype specificity and Immunoglobulin G (IgG) subclass was determined followed by standard ELISA procedure as mentioned with a little modification (Ferreira *et al.*, 1998). Briefly, high protein binding polystyrene ELISA plates were coated with PvC antigen. After washing, wells were blocked with 1% BSA for 1 h at 37°C. Neat hybridoma supernatant was added in each well after washings and then kept for overnight at 4°C. Plates were washed as earlier and then secondary antibody (goat antimouse IgG and IgM) conjugated with HRP was added. Plates were incubated for 1h at 37°C followed by 1h at RT. Plates were washed thrice before addition of OPD/H_2O_2 substrate solution and incubated for 10 min at RT in dark. Reaction was stopped with 50 µL of 8N H_2SO_4 and absorbance was read at 492nm.

For determination of IgG subclass, neat hybridoma supernatant was added in another PvC antigen coated plate, 2 wells each for IgG1, IgG2a, IgG2b and IgG3. After overnight incubation at 4°C, anti mouse IgG1, IgG2a, IgG2b and IgG3 monoclonals were added in respective wells. Then plates were incubated for 1h at 37°C followed by 1h at RT. Plates were washed as earlier and secondary antibody conjugated with HRP (goat antimouse anti-IgG) was added. ELISA plates were incubated for 1h at 37°C followed by 1h at RT. Plates were washed thrice and then OPD/H_2O_2 substrate solution was added to each well and incubated for 10 min at RT in dark. After stopping reaction absorbance was read at 492nm. The 100µL reaction volume was maintained throughout the steps.

IgG quantification in hybridoma culture supernatants

Estimation of IgG in hybridoma culture supernatant was done by using Easy-titre® mouse IgG Assay kit. The procedure was followed as instructed by manufacturer. Briefly, 20µL of the sensitized beads were transferred to each well of 96-well microplate. Then 20µL of samples or standards were added into the appropriate number of wells containing beads, mixed and kept for 5 min followed by addition of 100µL of blocking buffer in each well and kept for another 5 min. The absorbance was read at 405 nm. Standard curve was plotted and sample concentrations were determined from the standard curve. Starting IgG concentration was calculated using dilution factor of each sample.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis Using MAbs

The components of crude antigens of PvC, PfC and HNRBC of different molecular weights were analyzed on denatured, non reducing SDS-PAGE. Briefly, 10% polyacrylamide gel was prepared and samples were boiled for 10 min in the presence of sample buffer containing 1% SDS before electrophoresis. Electrophoresis was carried out at 70V for 30 min then 100V for remaining time of completion of the run. After completion of electrophoresis, one gel was stained with Coomassie brilliant blue R-250 to check the banding patterns resolved on the gel. Molecular weight of each protein band was determined by comparing with standard molecular weight marker.

Another unstained gel was used for western blot analysis. Briefly, proteins from the unstained SDS-PAGE slab gel were transferred electrophoretically to polyvinylidene difluoride (PVDF) membrane using transfer buffer (150mM Glycine, 20mM TrisHCl, 0.01% SDS, 20% methanol) at 50mA for overnight. Membrane was excised according to the lane of SDS-PAGE. Each strip was washed with PBS-T and then blocked with 3% non-fat milk containing 0.5% BSA for an hour at 37°C. After blocking, the PVDF strips were incubated 2 h at room temperature with hybridoma culture supernatant. The strips were washed

thrice with PBS-T for 10 min then incubated with anti-mouse IgG-HRP conjugate (1:500) for 1h at 37°C followed by 1h at room temperature. This was followed by washing and treatment with enzyme specific substrate solution (amino ethyl carbazole, AEC & H_2O_2) to identify the protein bands reacted with MAbs.

Purification of IgG from Culture Supernatant

Culture supernatants from six hybridoma lines that contained varying concentrations of IgG were fractionated by 40% ammonium sulphate precipitation and further centrifuging at 10,000 rpm for 20 min at 4°C. The pellets obtained after salt precipitation was dissolved in 1 ml PBS and put for dialysis overnight in refrigerator against large volume (2 L each time with 3 intermittent changing) of PBS. After dialysis, each IgG preparation was passed through Sephadex G-50 column equilibrated with PBS and fractions were collected. Each fraction was read at 280 nm for protein concentration. The fractions with high amount of protein were pooled and concentrated by Amicon ultra centrifugal filtration by using 10 kDa cut-off membranes. Concentrated antibody solutions were stored at -20°C for further use.

Inhibition ELISA

P. vivax antigen-specific reactions of the MAbs were determined by Inhibition ELISA against PvC antigen. At first step, purified IgG (10 µg/mL) of individual MAbs were treated separately with antigen, then both pretreated and untreated MAbs were tested for reactivity by Indirect ELISA. The O.D. values of untreated and treated samples were measured and results were expressed as percent inhibition.

Competitive Binding Assay between MAbs with P. Vivax Crude Antigen

Seroreactivity of the MAbs was determined against PvC antigen by indirect ELISA. Assay was done in two steps. In the first step, PvC antigen coated wells were incubated with optimum concentration of purified IgG (10 µg/mL) of each MAbs followed by incubation with second MAb. The control wells were filled with SP_2O culture supernatant. Results were expressed in percentage O.D. increase in ELISA following incubation with the second MAb and represent the means of duplicates.

Dot-blot Immnoassay

The highly reactive MAbs detected by ELISA and IFA were tested for antigen detection in malaria patients' blood by dot-blot ELISA. Finger-prick blood from *P. vivax*, *P. falciparum* infected patients and healthy normal were used in this assay. Approximately, 5 µL lysed blood samples containing *P. vivax* or *P. falciparum* infected erythrocytes; also parasite negative blood was blotted on nitrocellulose membrane. The blots were dried at RT, and then blocked with 3% BSA keeping 30 min at 37°C. The blotted strips were treated with 3% H_2O_2 for inactivating endogenous peroxidase. Culture supernatants containing MAbs were poured onto the treated membrane strips and were allowed to react for 2 hr at RT. After washing with PBS-T, antigens captured by MAbs were detected by anti-mouse IgG-HRP conjugate (1:500) for 1 hr at RT. This was followed by washing and treatment with enzyme specific substrate solution (aminoethyl carbazole, AEC & H_2O_2) to develop colour.

RESULTS AND DISCUSSION

Before fusion, immunized sera from 2 mice were checked for antibody titre by ELISA against PvC and HNRBC antigens. On the day of fusion antibody titre with PvC antigen was 1:32,000 and with human normal RBC lysate was 1:1,600. A total of 257 colonies were observed after 14 days from two sets of culture in four 96-well plates. The initial screening for antibody activity was carried out as soon as growth of hybrid cells under inverted microscope and change in pH of growth medium was observed. Once stable lines were established by seeing growth and antibody activity after a few passage, cloning was done by limiting dilution method. For cloning, a change was made by using Hybridoma Cloning Factor (HCF, Origen^{TM}, Fisher) over conventional feeder cell layer.

In this study, MAbs were produced by conventional techniques through the fusion of spleen cells isolated from mouse immunized with *P. vivax* infected erythrocytes with mouse myeloma cells to produce MAb secreting hybridomas lines. Freshly isolated hybridoma cultures often grow slowly and are less tolerant of low cell densities than their plasmacytoma parent cell growth. The screening for antibody activity was carried out as soon as growth of hybrid cells was observed in microscopic monitoring. Though the cells

for fusion were diluted to limit the number of independent hybrid cells per well, it was observed that growth rate of the hybrids was variable producing their own clones. Since the hybridoma cell line is immortal, an unlimited production of any specific antibodies could be achieved (Kohler *et al.,* 1976). Some parasite antigen positive clones were detected soon after fusion but then might be lost due to overgrowth of negative or other positive clones. The use of impure or crude antigen in mouse immunization is tolerated since the detection of MAb of interest is based on the selection strategy. Therefore, antibodies with selected properties or reactivity for specific antigenic structures could be selected.

Hybridomas producing MAbs directed against PvC antigens were isolated as described under Materials and Methods. The first screening was performed by ELISA to determine immunoreactivity followed by IFA in order to demonstrate the antigenic localization. The presence of antimalarial antibody in hybridoma culture medium was determined by indirect ELISA. Supernatant collected from each well was tested for PvC and NRBC antigen specific antibodies by ELISA. A total of 182 antibody secreting hybridoma clones were produced. Of the 182, ELISA positive clones were 109, 42 and 31 with PvC, PvC & NRBC and NRBC antigens, respectively. Immunoglobulin isotyping demonstrated 67 IgG, 10 IgG & IgM and 3 IgM positive, respectively. A total number of 27 stable, well-grown, *P. vivax* positive hybridoma culture lines were obtained. Out of 27 lines, 15 clones showed consistent growth after a series of passages. Culture supernatant collected from these lines reacted with different preparations of PvC antigens by indirect ELISA. Of the 15 hybridomas, 13 produced IgG isotype, 1 showed both IgG and IgM responses and 1 produced IgM isotype, but titre was low as observed from ELISA O.D. values. Of the 13 IgG producing hybridomas, 11 showed stable growth after several passages. Of the 11 stable, well grown, *P. vivax* antibody positive hybridomas, 6 clones (MAb-1, MAb-2, MAb-3, MAb-4, MAb-6 and MAb-8) were taken for further characterization.

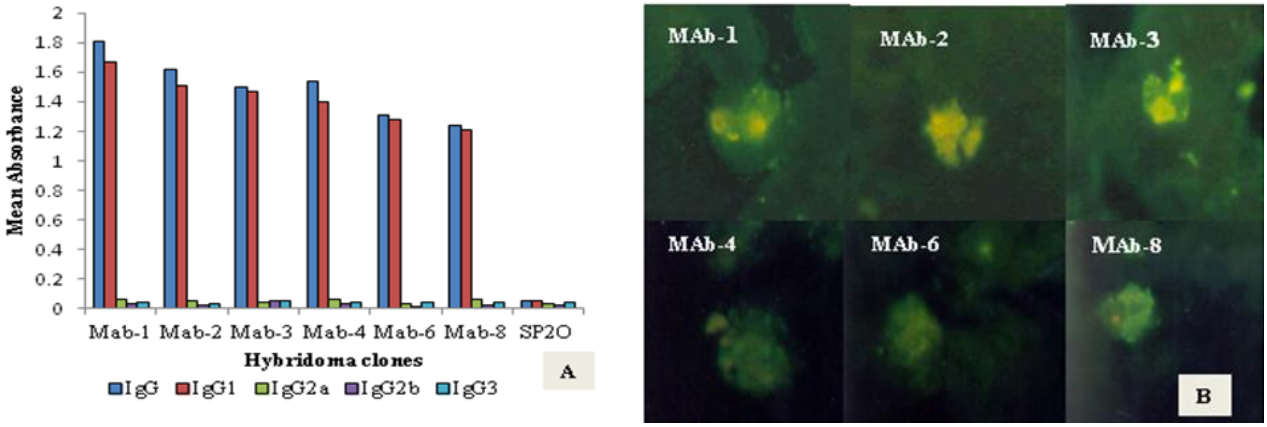

Figure 1: Immunoglobulin G subclass analysis and reactivity of monoclonal antibodies with *P. vivax* parasite by IFA test. A - Culture supernatants of 6 stable, well grown, *P. vivax* antibody positive hybridomas (MAb-1, MAb-2, MAb-3, MAb-4, MAb-6 and MAb-8) were tested for IgG subclass determination. Antibodies secreted by 6 hybridomas were IgG1 type. B - The stable, well grown, *P. vivax* antibody positive 6 hybridomas (MAb-1, MAb-2, MAb-3, MAb-4, MAb-6 and MAb-8) producing MAbs directed against various proteins of *P. vivax* erythrocytic stages were screened by IFA test to demonstrate the reactivity with antigenic determinants present in the native protein of the parasite. All 6 MAbs showed reactivity with blood stages of *P. vivax* with varying intensity on examination under UV light in a fluorescent microscope.

Supernatant was tested for IgG subclass determination. Antibodies secreted by 6 hybridomas were IgG1 type (Fig. 1A). They were grown in large volume, supernatant was collected from each line and cells were cryopreserved for future use. Hybridomas producing MAbs directed against various proteins of *P. vivax*

erythrocytic stages were also screened by IFA in order to demonstrate the reactivity of MAbs with antigenic determinants present in the native protein of the parasite. On examination under UV light in a fluorescent microscope, all MAbs showed reactivity with blood stages of *P. vivax* with varying intensity (Fig. 1B). None of the MAbs reacted with *P. falciparum* by IFA.

To investigate the species specificity of MAbs, we used Western blot analysis. The PvC, PfC and HNRBC antigens were subjected to separation in SDS-PAGE. The gels after staining were checked for the band patterns resolved on the polyacrylamide gel. These crude preparations demonstrated nearly 10 prominent bands of different molecular weights. On Western blot, the MAbs identified proteins with apparent molecular weights of 33 kDa, 42 kDa, 65 kDa and 66 kDa in *P. vivax* infected erythrocyte lysates. MAb-2 identified two proteins of apparent molecular weights of 42 kDa and 66 kDa. The panel of MAbs was taken for investigation allowed to pick up *P. vivax* proteins of different molecular weights. These MAbs did not recognize any *P. falciparum* proteins on Western blot (Fig 2).

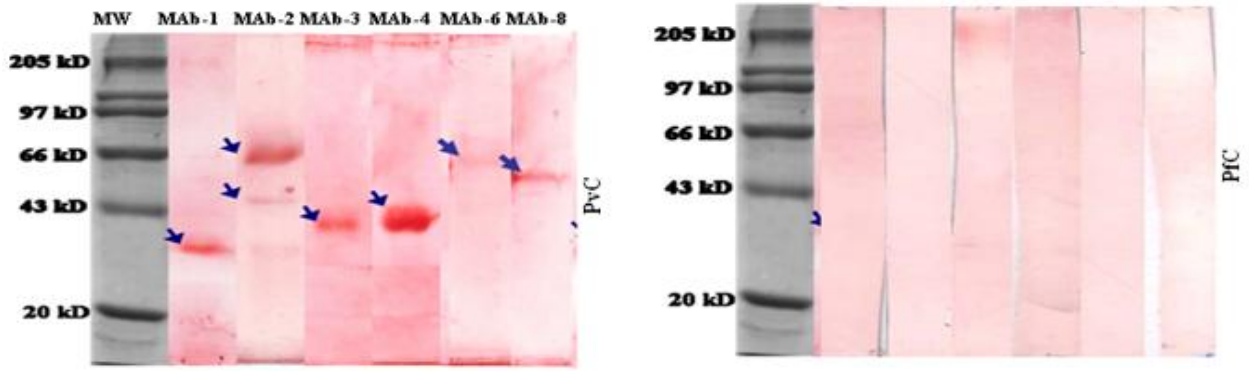

Figure 2: Western blot analyses of 6 MAbs with *P. vivax* (PvC) and *P. falciparum* (PfC) lysates. 10 % SDS-PAGE gel of PvC and PfC was transferred to PVDF membrane and lanes were separated into strips. They were treated with culture supernatants of each MAbs. On Western blot, the MAbs identified proteins (marked with arrows) with apparent molecular weights of 33 kDa, 42 kDa, 65 kDa and 66 kDa in *P. vivax* infected erythrocyte lysates; none of them recognized any *P. falciparum* proteins.

Though selection of hybridomas secreting desired antibodies is quite tedious, yet the screening assays should be rapid, sensitive, reliable and easy to perform. The most commonly used assay is ELISA. Frequently, the isolation of a MAb has been the first step in the identification of a minor, but functionally important component in a mixture of antigens. In present study, MAbs produced against parasite proteins were found to be seroreactive with *P. vivax* infected RBC lysate by ELISA. They were characterized by isotyping to determine the subclass of immunoglobulin by ELISA. In IFA test, the MAbs did not react with uninfected erythrocytes as observed in thin smear preparations. The immunofluorescence data suggest that the antigenic materials recognized by the MAbs are synthesized in late trophozoite and in schizont stages. These antigens are present in merozoites and are also shed from the infected erythrocytes. Thus the MAbs with this characteristic may affect the invasion of merozoites to new RBCs. On immunoblotting, the MAbs mainly demonstrated protein bands of apparent molecular weights of 33 kDa, 42 kDa, 65 kDa and 66 kDa. Identification of two proteins by MAb-2 on Western blot could be the sharing of common epitopes in the proteins with shifts in molecular weights.

The culture supernatant of individual hybridoma was assayed for quantification of mouse-specific IgG by Pierce easy titre Mouse IgG assay kit. The cell culture fluid contained varying concentration of IgG (Table 1). Purification of Immunoglobulin-G from hybridoma supernatant was carried out by ammonium sulphate precipitation followed by Sephadex G-50 column chromatography.

Table 1: Estimation of mouse IgG in culture supernatant of hybridomas

Hybridoma lines	IgG concentration (ng/ml)
MAb-1	31.20
MAb-2	71.43
MAb-3	89.29
MAb-4	196.43
MAb-6	187.50
MAb-8	40.56

A simplified competitive ELISA was performed using *P. vivax* crude antigen. The results presented in Table 2 show an O.D. increase in presence of other MAbs and therefore indicate the additive effect in recognition of parasite specific epitopes on *P. vivax* proteins identified these MAbs. An inhibition ELISA was performed to determine inhibitory reactivity of pre-treated MAb by *P. vivax* antigen-specific reactions. The differences in mean absorbance of untreated and treated samples were expressed as percent inhibition. The reactivity was observed in all 6 MAbs and the highly reactive was MAb-4 (Fig. 3A).

Table 2: Competitive binding assay between antibodies with *P. vivax* crude antigen

Mean ELISA O.D. (% increase in binding)						
MAbs	**MAb-1**	**MAb-2**	**MAb-3**	**MAb-4**	**MAb-6**	**MAb-8**
Alone	0.21	0.20	0.22	0.56	0.23	0.22
MAb-1	-	0.36 (41.67%)	0.42 (50%)	0.65 (67.69%)	0.30 (30%)	0.32 (34.38%)
MAb-2	-	-	0.24 (16.67%)	0.60 (66.67%)	0.36 (44.44%)	0.40 (50%)
MAb-3	-	-	-	0.71 (69.01%)	0.34 (35.29%)	0.30 (26.67%)
MAb-4	-	-	-	-	0.58 (3.48%)	0.60 (6.67%)
MAb-6	-	-	-	-	-	0.38 (36.11%)

Specificity and cross-reactivity of MAbs were analyzed using the dot blot method. We found that all 6 MAbs recognized *P. vivax* antigens with varying intensities but no reaction was seen between any of these antibodies with *P. falciparum* infected and normal blood (Fig 3B).

The parasitic proteins were shown to be detected by the dot-blot immunoassay, inhibition ELISA and competitive binding assay. On dot-blot, culture supernatant of individual hybridoma lines showed the specificity of reactions with *P. vivax* infected blood at varying intensity. Inhibition ELISA showed varied degree of reaction as observed in untreated and treated MAbs with parasite proteins. For proper assessment of inhibiting or competitive binding, both antibodies should be under saturating conditions. The competitive binding should result in the antibody mixture having peak intensity equal to the peak of the two antibodies alone. Lack of binding to a test-cell population might mean that the epitope is absent or merely inaccessible. The prominent role of antibody-mediated immune protection in the asexual erythrocytic infection in *P. vivax* is well established and some of the protein antigens showed merits for further immunological studies as vaccine candidates (Dutta *et al.*, 2001; Valderrama-Aguirre *et al.*, 2005; Rodrigues *et al.*, 2005; Seth *et al.*, 2010). The biological evaluation on antimalarial activity by determining effect on parasite growth of the MAbs could not be done since in vitro culture of *P. vivax* is not yet established.

For a long time, the *in vivo* production in mice by ascites induction has been preferred for its cost effectiveness and high concentration of MAbs produced. But the growing ethical concern about mice led to an increased emphasis on *in vitro* methods being parallel to the *in vivo* methods both in capacity and cost effectiveness (Trebak *et al.,* 1999). As observed, all six hybridoma clones secreted mouse IgG antibody though at low concentration of 31-196 ng/ml but their reactivity has been detected by ELISA and IFA. It is reported that conventional low cell density culture methods result *in vitro* production of MAbs which are released in culture medium at concentrations between 1 - 100 µg/ml (Falkenberg 1998). In the recent past, efforts have been made to design high density culture systems, leading to the development of various bioreactors. They can generate high yields of MAbs (100 mg/week on an average), but only allow the production of one MAb at a time and suffer the disadvantage of expense, complexity and proneness to contamination (Kreutz *et al.*, 1997).

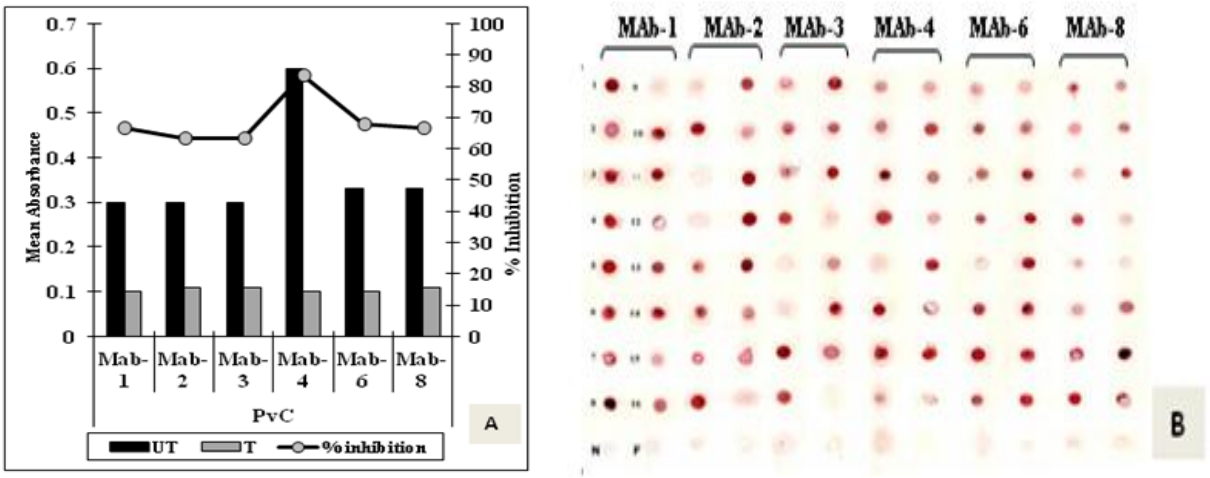

Figure 3: Inhibition ELISA and Dot-blot ELISA. A - An inhibition ELISA was performed to determine inhibitory reactivity of pre-treated MAb by *P. vivax* antigen-specific reactions. The *P. vivax* antigen-specific reactions were observed in all 6 MAbs and the highly reactive was MAb-4. B - Representative dot-blot assay results of *P. vivax* infected blood from 16 patients (1-16), 1 *P. falciparum* infected blood (F) and 1 normal blood (N) with six MAbs. Results show that of the 16 *P. vivax* infected blood, 15, 12, 12,15, 14 and 13 were detected by Mab-1 , Mab-2, Mab-3, Mab-4, Mab-6 and Mab-8., respectively. All 6 MAbs did not react with *P. falciparum* infected and normal blood.

There is a need in the field for antibodies specific for *P. vivax* blood stage proteins in a diagnostic assay. The detection of parasite antigens present in whole blood or plasma or erythrocytes can constitute a method for the diagnosis of acute *P. vivax* infection using MAbs, which react with almost all the stages available in patient's smears. Such antigens may contain epitopes unique to the *P. vivax* and also seem to be conserved within all or most of the isolates to allow species specific detection of malaria.

From this present study, it may be concluded that the isolation of a novel MAb is the initial step for identifying a minor but functionally important component presents in a mixture of antigens. By antigen-specific screening of culture supernatants and cloning of secreting cells, it becomes possible to produce immortal cell lines synthesizing homogeneous antibody. Monospecific antibodies derived from hybridomas could have applications as diagnostic or immunoassay reagents. Immunoassays using monoclonal antibodies are sufficiently sensitive to allow the diagnosis of infectious disease by the detection of antigen rather than antibody. There are monoclonals available in kit form against blood stage antigens which can detect up to 50-100 parasites/ul. The currently available diagnostic test kits are imported and they are not cost-effective. Keeping this in view, present study was aimed to characterize

the hybridoma lines/monoclonal antibodies for studying the antigenic characteristics of *P. vivax* clinical isolates and to develop an immunodiagnostic test. There is always a need for an indigenous kit.

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