Antibody Development Kinetics of *Plasmodium falciparum* Histidine Rich Protein-II (HRP-II)

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Abstract

Introduction The human malarial parasite *Plasmodium falciparum* secretes various intra and extra cellular proteins during its asexual life cycle in human RBC. Histidine Rich Protein-II *(HRP-II)* is one of the most prominent proteins, found to be secreted by *P. falciparum* throughout the asexual cycle with the peak during mature schizont stage of the parasite development in human IRBC. The high histidine content (35% of the total amino acids in protein) of this protein suggested the potential to bind divalent metal ions.

Objective Purification of extra cellular *PfHRP-II* antigen from *in-vitro* culture soup.

Development of anti *PfHRP-II* antibody and evaluation of antibody development kinetics in immunized rabbit

Methods Extra cellular *Pf HRP-II* has been purified from the in-vitro culture supernatant using metal chelate chromatography. Purified *PfHRP-II* antigen was immunized in inbred New Zealand white rabbits. Anti-*PfHRP-II* antibody development kinetics in the rabbits was measured using indigenously developed ELISA.

Results We have demonstrated by metal chelate chromatography, an extraordinary capacity of *HRP-II* to bind Nickel ions (Ni^{++}) and employed this characteristic to purify the extra-cellular *HRP-II* protein secreted by *P. falciparum* from culture supernatant. The identity of the purified protein was, verified by the relative molecular weight on SDS-PAGE, by reacting with polyclonal antibodies directed against it. The purified *PfHRP-II* antigen was immunized and produced antibody in rabbits. Purified *PfHRP-II* is found to be a good antigen provoking stable and high antibody response in immunized rabbits for about 12 months after immunization.

Conclusion Ni-NTA attached affinity chromatography could be useful in purification of *PfHRP-II* antigen from in-vitro culture supernatant of *Plasmodium falciparum*. Thus purified antigen retains its antigenicity during and after purification. Thus developed antibodies may be of diagnostic use for *Plasmodium falciparum*.

Keywords *Plasmodium falciparum*, Histidine Rich Protein-II *(HRP-II)*, Chelate chromatography, Antibodies Development

Introduction

Malaria has probably had a greater impact on world history than any other infectious disease. It has been responsible for the outcome of wars, population movements, and the growth and development of various nations throughout the world. More than 300 million individuals throughout the world are infected with malaria, and more than 1 million people a year, most of whom are children, are being killed by the disease. It is still a very common disease in many parts of the world, particularly in tropical and subtropical areas. Of the four most common species that infect humans, *P. vivax* and *P. falciparum* account for 95% of infections. *P. vivax* has the widest distribution, extending throughout the tropics, subtropics and temperate zones. *P. falciparum* is generally confined to the tropics. There has been a definite increase in the number of cases of *P. falciparum* malaria reported, which may be related to increased resistance to chloroquine.

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Malaria prevention is difficult, and no drug is universally effective. Vaccine development studies are ongoing, but malarial vaccines are not yet in general use (Garcia *et al.* 1997).

In many developing countries, resources for malaria diagnosis are sparse or unavailable. Small numbers of trained microscopists and microscopes often limits local clinics examining blood smears from a large number of patients suspected of having malaria. Consequently, malaria diagnosis is often made only on the basis of clinical symptoms although this is, at best, 50% accurate (WHO, 1994).

Specific diagnosis of malaria is, usually made by microscopic examination. However, microscopic examination even by expert microscopists is time consuming, labor-intensive, and it is difficult to diagnose mixed *P. falciparum* and *P. vivax* infections, later being present predominantly in ring stages. Microscopic examination will not be cost-effective in malaria control in an area with very low but persistent transmission, because the cost to diagnose one positive slide will be high. There is therefore a need to develop alternative diagnostic methods to detect cases especially those with low-grade parasitemia to supplement & perhaps to replace microscopy in malaria control programs. (Savant *et. al.*, 1990)

A key feature of the new WHO Global Malaria Control Strategy (WHO, 1992) is the rapid diagnosis of malaria at the village and district level, so that effective treatment can be administered quickly to reduce morbidity and mortality. The need for rapid diagnosis of *P. falciparum* is most acute because of the severe nature of this infection and its non-specific symptomatology.

Human malarial parasite *Plasmodium falciparum* is the most pathogenic. *Plasmodium falciparum* infected erythrocytes (IRBCs) synthesize several histidine rich proteins (HRPs) that accumulate high levels of histidine but very low levels of amino acids such as Isoleucine or Methionine (Howard RJ, 1986). Three such proteins, designated *P. falciparum* histidine-rich proteins (HRP) I, II & III, are synthesized by asexual parasites within red blood cells. *PfHRP-I* is found associated with the cytoskeleton of IRBC and is localized under knob-like protrusions of the infected cell membranes (Howard RJ, 1986; Parra ME, 1993; Rock EP, 1987; Panton LJ, 1989; 1978; Sharma YD, 1988). *PfHRP-II* is expressed by both, knob-positive (k+) and knob-negative (K-) IRBC's. HRP-II is synthesized throughout the asexual cycle, transported in concentrated "packets" through the red cell cytoplasm and released from intact infected cells into the culture medium (Howard RJ, 1986). Extracellular *HRP-II* is water soluble (Howard RJ, 1986). Sequencing of the genomic DNA has shown that *HRP-II* contains 34% Histidine, as well as relatively high contents of alanine and aspartic acid (37% and 10% respectively). *HRP-II* contains many tandem repeats of the sequences AHH and AHHAAD[3]. *HRP-II* is very similar in sequence to *HRP-III* and both *HRP-II* and *HRP-III* are expressed simultaneously by some asexual *P. falciparum* parsites (Rock EP, 1987).

HRP-II is of particular interest because half of this protein is rapidly exported from infected cells into the extra-cellular medium[1]. Previous studies on the endogenous histidine rich protein of human serum and rabbit serum, called histidine rich glycoprotein, show that this protein binds some divalent metal ions but not others[4-11]. Previous study on the metal binding properties, especially capacity of Zinc affinity chromatography to be used for the purification of parasite protein *PfHRP-II* show that this protein binds to Zn ions. Zn chelate affinity chromatography could be used for purification of *PfHRP-II* from culture supernatants/extracts of parasitized cells.

It is therefore of particular interest to determine whether *PfHRP-II* released in culture supernatant has similar binding properties to Nickel ion in Ni-NTA agarose as that of other 6X-His tagged proteins, which could be useful for further study of *PfHRP-II* after purification using Ni-column. We utilized the extraordinary affinity of *HRP-II* for Ni-immobilized on metal chelate chromatography columns, and utilize this property to isolate and partially purify this malarial protein from culture supernatant.

Here we describe the ability of *Pf HRP-II* protein purified from in-vitro culture to provoke the antibody development and anti *Pf HRP-II* antibody development kinetics in experimental animal Rabbit.

Methodology

The culture adapted strain of *P. falciparum*, obtained with kind courtesy of Malaria Research Centre, New Delhi, were cultured in RPMI-1640 with 10% AB positive human serum using the method of Trager and Johnson, with minor modifications (Trager W, Jensen JB, 1978). The culture vials were monitored for parasitemia, after staining at every 24 hours interval. The media was changed and added the blood cells whenever necessary.

Preparation & purification of *PfHRP-II* antigen from culture supernatant

Collection of PfHRP-II antigen from P. falciparum culture supernatant:

The culture supernatant from the vials with 0.1-10% parasitemia, were collected daily in different

screw capped tubes and centrifuged at 500 g for 10 minutes. The supernatant was collected in screw-capped vials and stored at -20°C before further processing and was used for purification of *PfHRP-II* antigen.

Purification of PfHRP-II antigen from culture supernatant

200ml batch of culture supernatant was thawed quickly and centrifuged at 1500 rpm for 10 minutes. 50mM of Imidazole was added in each batch of 200 ml of centrifuged culture supernatant and incubated at 4°C in continuous stirring condition at 100 rpm for 12 hrs. The supernatant was centrifuged at 1500 rpm for 10 min. and processed for purification of *PfHRP-II* antigen using affinity chromatography through Ni-NTA matrix (Qiagen, USA) packed column.

The proteins adsorbed in Ni-NTA agarose packed in column, was eluted using a gradient of 0-750 mM of Imidazole containing wash buffer. Elutes were collected in 15 ml screw capped tubes and stored at -20°C until further testing.

The collected fractions of elute, wash W_1, wash W_2 and Flow-through were tested for the presence of *PfHRP-II* antigen (Parasight-f test, B & D, USA).

The collected fractions of elute, wash W_1, wash W_2 and Flow-through were also tested for purity and molecular weight of the antigen present in different fractions using SDS-PAGE and western blotting (Ed Harlow & David Lane, 1989).

Fractions containing the purified antigen (*PfHRP-II*) was pooled, dialyzed and concentrated using Amicon apparatus.

The concentration of the antigen in solution was determined using standard Micro BCA Assay method and was stored it in small aliquots at -20°C containing 0.1% PMSF as a protease inhibitor.

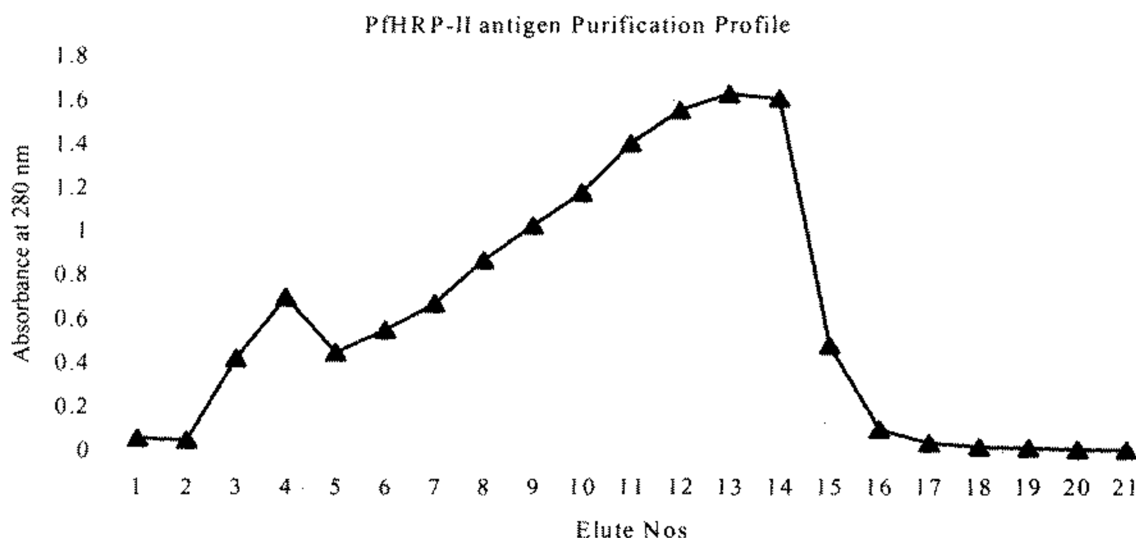
Generation of *anti-PfHRP-II* antibody in rabbits

Healthy New Zealand white rabbits were immunized with purified *PfHRP-II* antigen emulsified in CFA / IFA, as per standard protocol (Brian Law, 1998). The antibody titer in immunized rabbits was monitored by bleeding the immune rabbits at an interval of 4 weeks for 12 months post immunization. The antibody development kinetics was studied and noted as in result section. The sera with best antibody titer were pooled and purified the IgG fraction using Protein-G affinity chromatography (Harris *et al*, 1995). Thus produced antibody was analyzed by SDS-PAGE and western blot. Anti *PfHRP-II* antibody (IgG) was stored at -20°C after proper labeling. A part of the polyclonal antibody was conjugated with HRPO as per the standard protocol (John E. Coligan *et al.* 1992).

Results

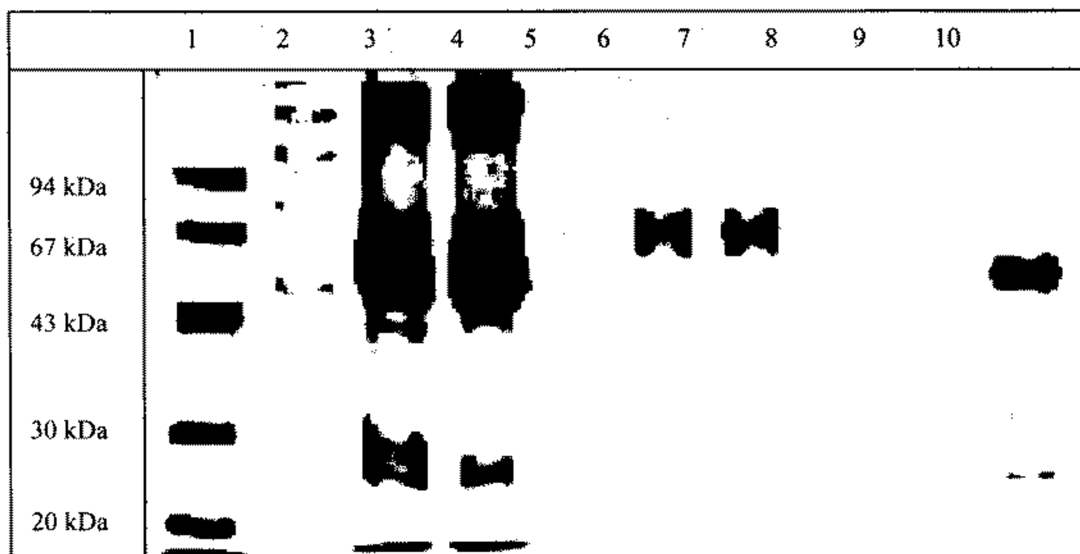
The metal binding character of *PfHRP-II* was examined by metal-chelate affinity chromatography of culture supernatants. The eluted solution was collected in fractions of 1 ml each in each tube and measured absorbance at 280 nm. Presence of *Pf HRP-II* in each fraction was tested using a commercial antigen capture assay (Parasight-f test). Figure-I, shows the elution profile of *PfHRP-II* from the culture supernatant. The profile shows two peaks of adsorption at 280 nm. The presence of *PfHRP-II* was detected in fractions 3, 4, 5 & 6.

Chromatogram showing purification profile of PfHRP-II antigen



Analysis of the antigen *PfHRP-II* by SDS-PAGE

The fractions which showed positive in commercial antigen testing system were pooled and analyzed in 10% SDS-PAGE. (Fig. II), which resolved as a 72 kDa band. This was, further confirmed by western blotting using commercially available antibody. In an average, 300 - 400 µg of *Pf HRP-II* could be purified from 200ml of culture supernatant.

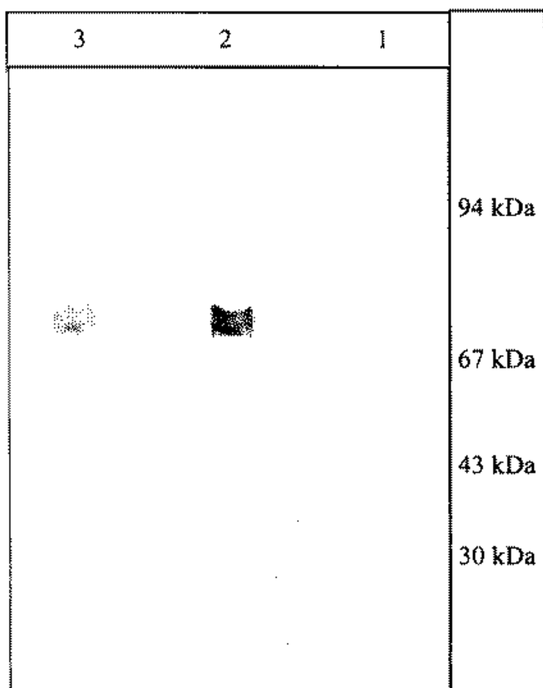


10 % SDS-PAGE Gel Stained with Coomassie Blue-R Stain

Lane 1: Mol Wt Marker (14 - 94 kDa)
Lane 2: Mol Wt Marker (55 - 245 kDa)
Lane 3: Load (*P. falciparum* Culture Soup)
Lane 4: Flow Through
Lane 5: Blank
Lane 6: Purified *Pf HRP-II* (Elute 4)
Lane 7: Purified *Pf HRP-II* (Elute 5)
Lane 8: Blank
Lane 9: Blank
Lane10: BSA (Fraction -V)

Western blotting for confirmation of the purity of the antigen *PfHRP-II*

The protein revealed in the SDS-PAGE, was conformed by Western blotting, which blotted a single band around 72 kDa, in only the lane in which *PfHRP-II* was run but did not blot in the lane in which *rKAHRP* (recombinant *PfHRP-I*) was run, confirming its purity.



Western blotting with the commercial antibody for confirmation of purified *PfHRP-II*

Lane1:	Mol Wt. marker (14 - 94 kDa)
Lane2:	*Pf HRP-II*
Lane 3:	*Pf HRP-II*
Primary Antibody:	Rabbit anti *Pf HRP-II* IgG (1:1000) {Commercial}
Conjugate:	Goat anti rabbit IgG HRP (1:5000)
Substrate:	DAB + H_2O_2+ PBS

Generation of anti *Pf HRP-II* antibody in rabbits

Polyclonal antiserum was raised in in-bred New-Zealand white rabbits, by immunizing 150µg of purified *Pf HRP-II* antigen emulsified with CFA in primary and subsequently antigen emulsified with IFA during secondary immunizations, using standard protocol as mentioned in methodology.

Bleed the rabbits before immunization. After immunization of the rabbits with the purified *PfHRP-II* antigen, the bleeding of the rabbits was continued at 30 days interval. Antibody titers of the sera collected from the immune-rabbits were estimated by standardized ELISA and studied the kinetics of antibody development as mentioned below.

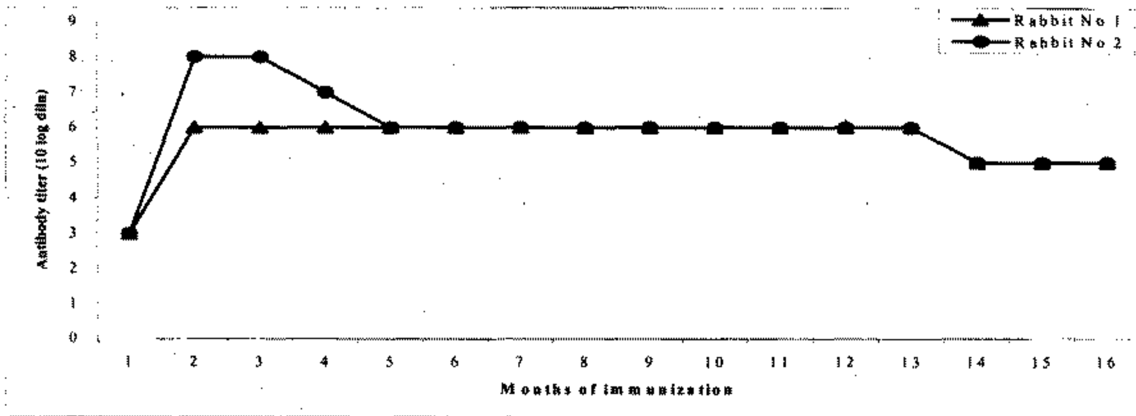
Purification of antibody from rabbit sera

The stored immune rabbit sera were pooled together and purified IgG fraction of antibody using Protein -G column. The chromatogram below shows polyclonal antibody purified from Protein-G column. Different fractions obtained during the Purification were used for protein estimation and are plotted in Table below. It was observed that majority of IgG could be purified in single step with protein - G column. In an average 2 - 3 mg/ml of *IgG* could be purified from immune rabbit sera.

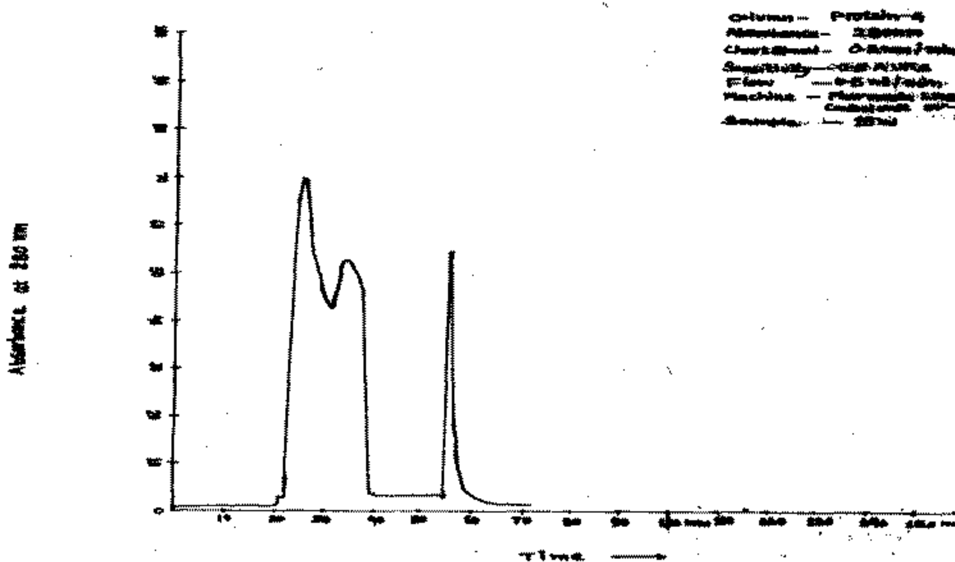
Kinetics of anti PfHRP-II polyclonal antibody production in Rabbits

The kinetics of the generation of polyclonal antibody in serum of the immunized rabbits were studied by using ELISA and was noted as below in table:

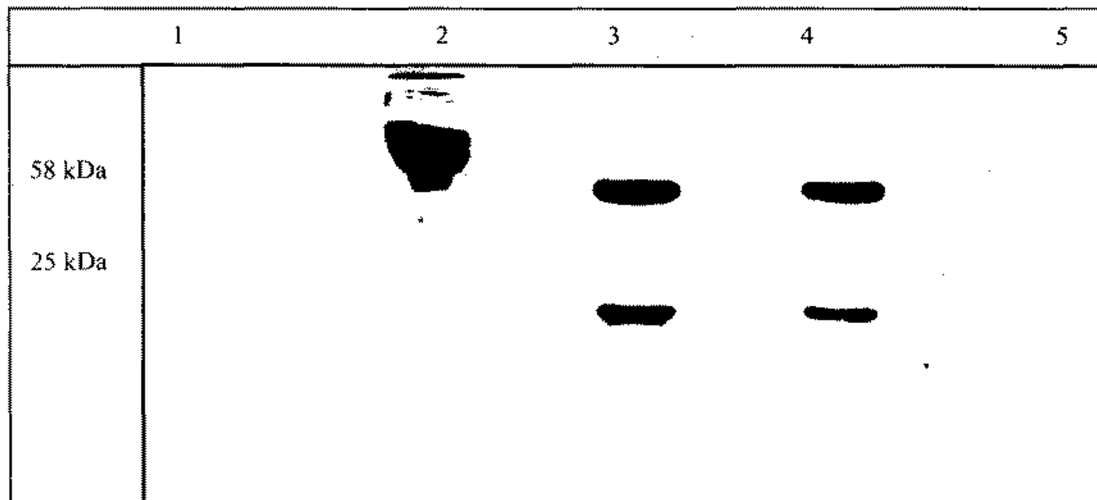
In both the rabbits, immunized with native *PfHRP-II* antigen, antibody titer started increasing after 2 weeks of primary immunization and reached at peak (6/7log dilution) on 3rd month of primary immunization. Antibody titer remained at a plateau / stable for more than 12 months and then slight fall occurred by 1 log dilution and remained stable till 16 months of Primary immunization, as shown in chart below.



Chromatogram: Antibody purification from immune rabbit sera



SDS-PAGE (12%) showing two bands of IgG



Lane 1: *Anti PfHRP-II IgG1 Pab,* ***Lane 2:*** *Flow Through,* ***Lane 3:*** *Anti PfHRP-II IgG1 Pab*
Lane 4: *Anti PfHRP-II IgG1 Pab,* ***Lane 5:*** *Mol. Wt. marker (14 - 94 kDa)*

Discussion & Conclusion

Malaria probably has a greater impact on world history than any other infectious disease. In many developing countries, resources for malaria diagnosis are sparse or unavailable. Small numbers of trained microscopists and microscopes often limits local clinics examining blood smears from a large number of patients suspected of having malaria. Consequently, malaria diagnosis is often made only on the basis of clinical symptoms although this is, at best, 50% accurate (WHO, 1996). A key feature of the new WHO Global Malaria Control Strategy (WHO, 1992) is the rapid diagnosis of malaria at the village and district level, so that effective treatment can be administered quickly to reduce morbidity and mortality. The need for rapid diagnosis of *P. falciparum* is most acute because of the severe nature of this infection and its non-specific symptomatology. Ugen and group evaluated a rapid manual test incorporating a dual antibody immunoassay against the *HRP-II* antigen of *P. falciparum* (Parra *et al.* 1991). A similar but different antibody has been incorporated in an ELISA to detect *P. falciparum* infection (Taylor & Voller, 1993) with titrated dilutions of infected blood to parasitemia as low as 0.001- 0.0002%. In another study, Christine Beadle and group, (Christine Beadle *et al.* 1994) used a dipstick antigen-capture assay for the detection of *PfHRP-II* antigen, in Kenya and an experimental challenge study in the USA, found that the assay was 11-100% sensitive in different groups.

Purification of *PfHRP-II* from Culture Supernatant

In this study, method for *in-vitro* culture of *P. falciparum*, and maintenance of culture adopted Indian wild strain of *P. falciparum in-vitro* were standardized. Parasite culture at 0.1-10 % parasitemia were maintained *in-vitro* using the standard methodology (Pillai *et al.* 1996) and spent culture supernatant *P. falciparum* were collected and stored at -20^{0}C, for the purification of *PfHRP-II* antigen.

In this study, we have standardized a novel method, and buffer system, for the purification of extra-cellular *PfHRP-II* antigen secreted by the parasites in culture soup during the asexual cycle of life cycle. We have purified the native extra-cellular *PfHRP-II* antigen from the culture soup, in required quantity and quality.

Lindsay J. Panton and his group have demonstrated by metal chelate chromatography, an extraordinary capacity of HRP-II to bind Zinc ions (ZN^{+2}) and employed this characteristic to isolate the extra-cellular protein. The identity of the purified protein was verified by relative molecular wt. on denaturing polyacrylamide gels, by reacting with monoclonal antibodies & mono-specific rabbit antiserum (L. J. Panton *et al.* 1989).

Lindsay J. Panton and his group have also found that *HRP-II* binds even more strongly to a column charged with Cu^{+2}, requiring a minimum of 450 mM Imidazole to release it (L.J.Panton *et al.* 1989).

In this study, we have employed the extraordinary capacity of *HRP-II* antigen of *P. falciparum*, to bind metal ions like, Zn^{+2}, Cu^{+2}, Ni^{+2}. We used a commercially available matrix charged with Ni^{+2}

ion, originally manufactured for the purification of recombinant protein tagged with six histidine residues, expressed in bacterial system (*E. coli)*. We designed and standardized our own buffer system and the protocol to purify native *PfHRP-II* protein (which contains only four histidine residues at one stretch), from *P. falciparum* culture supernatant.

Immobilized metal affinity chromatography (IMAC) was first used to purify proteins in 1975 (Porath *et al.* 1975), using the chelating ligand Iminodiacetic acid (IDA). IDA was charged with metal ions such as Zn^{+2}, Cu^{+2}, Ni^{+2} and then used to purify a variety of different proteins and peptides (Sulkowski, 1985).

Nitriloacetic acid (NTA), available from QIAGEN, is a tetra-dentate chelating adsorbent developed at Hoffmann -La-Roche. NTA occupies four of six ligand binding sites in the coordination sphere of the nickel ion, leaving two sites free to interact with the 6X-His tag. NTA binds metal ions far more stable than other available chelating resins and retains the ions under a wide variety of conditions, especially under stringent wash conditions. (Hochuli *et.al.*1989). Ni-NTA available from QIAGEN is exclusively used for the purification of 6x His tagged proteins.

Lindsay J. Panton and his group (L. J. Panton et. al., 1989), took chelating sepaharose 6B (Pharmacia), equilibrated with equilibration buffer and charged with Zinc chloride or Copper sulphate. Parasites were bio-synthetically labeled with histidine [^{3}H]. Then purified *PfHRP-II* antigen from the *P. falciparum* culture soup. The adsorbed protein in the column matrix was eluted with a 200ml linear gradient of 0-750 mM Imidazole in PBS. Fractions containing peaks of radioactivity were pooled, dialyzed against 20mM Ammonium Bicarbonate with 3 mM octyl B-glucoside at 4^0C. The pools were lyophilized and resuspended in 500µl of water. An aliquot of each pool was analyzed using SDS-PAGE and flurography.

In our study, we took Ni-NTA agarose packed column and purified the *PfHRP-II* antigen from the *P. falciparum* culture supernatant with the methodology mentioned as in methodology section. The fractions of the elute were tested for the presence of *PfHRP-II* using a commercial test, Parasight-f test and fractions showing positive reaction were pooled together and dialyzed against PBS pH 7.2. An aliquot of each pool was analyzed using 10 % SDS-PAGE.

Washing the column with low molar concentration of Imidazole with detergent Triton X-100 (0.1%) was able to reduce the non-specific binding of protein from the culture supernatant into the Ni-NTA agarose column. Even-though the 0-750 mM Imidazole was used for elution of *PfHRP-II*, most of the *PfHRP-II* eluted at low concentration of Imidazole. This shows the weak binding of *PfHRP-II* into the Ni-NTA agarose column, in compare to many histidine tagged recombinant proteins. This may be due to presence of only four histidine at a stretch in the polypeptide chain of *PfHRP-II*.

The SDS-PAGE picture in our study is also similar to the study of L. J. Panton, i. e. *PfHRP-II* antigen we purified has a molecular Wt. of 70-72 kDa, which is similar to that of L. J. Panton's study.

Generation of polyclonal antibody

In this study, polyclonal antibody to *PfHRP-II* was raised in Newzealand white rabbits using the standard methodology (Brian Law, 1997; Ed. Harlow & W. B. Lane, 1989). We also studied the antibody development kinetics in rabbits. Antibody titer in rabbits increased gradually and reached a peak around 3rd month of immunization. Antibody titer remained constant for a long period from 4th to 12th month post immunization. This constant maintenance of the antibody titer in rabbit sera may be due to immunization of the native *PfHRP-II* antigen, but not a small fraction of the antigen. *This finding of maintenance of antibody titer at a constant level for long period is corroborating with others study.* Manmohan Singh's study (1997) found that after immunization of tetanus toxoid emulsified with alum in rats, antibody titre reached at peak (5 log dilution) on 10 weeks after immunization and it lasted till 20 weeks of immunization. Antibody titer dropped to 4-log dilution after 30 weeks and remained same till the end of the study (50 weeks after immunization). In a similar study by Ying Men et al., 1995 found that the antibody titer reached at a peak of 10^5 after 5 weeks of immunization of tetanus toxoid emulsified with alum in rats, and it remained at $10^{4.5}$ from 15 - 30 weeks post immunization. The antibody titer remained at 10^4 from 35 to 45 weeks. This indicates the high immunogenic nature of the purified *PfHRP-II* antigen.

The rabbit sera with high antibody titer were pooled together, and purified using Protein-G affinity chromatography. In 12% SDS-PAGE, it gave one band of heavy chain at 58 kDa and the other band of light chain at 25 kDa. The purified polyclonal antibody reacted only with purified *PfHRP-II*, and did not cross- reacted with *rKAHRP*, in western blotting. We could purify about 2-3 mg/ml of IgG from immune rabbit sera.

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