Experimental Parasitology 150 (2015) 56–66

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Full length article

Novel monoclonal antibody against truncated C terminal region of Histidine Rich Protein2 (*Pf*HRP2) and its utility for the specific diagnosis of malaria caused by *Plasmodium falciparum*

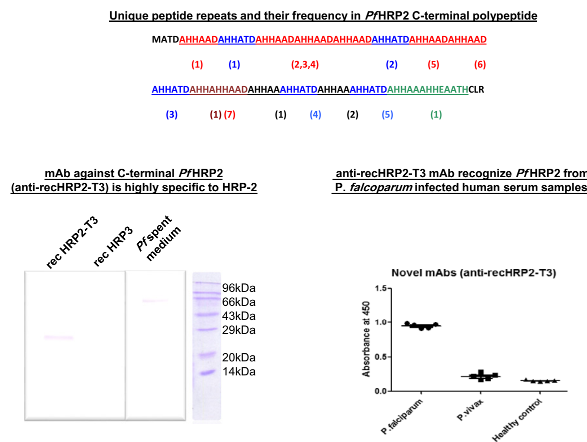
Reena Verma, N.S. Jayaprakash, M.A. Vijayalakshmi, Krishnan Venkataraman *

Centre for Bio Separation Technology (CBST), VIT University, Vellore 632 014, India

HIGHLIGHTS

- Highly polymorphic *Pf* HRP2 is used in malaria Rapid Diagnostic Tests.
- C-terminus of *Pf*HRP2 has unique and conserved peptide repeats.
- Recombinant C-terminal 105 amino acids of *Pf*HRP2 was used as an antigen.
- Selected *Pf*HRP2 mAbs were highly specific to *Pf*HRP2 but not to *Pf*HRP3.
- mAbs efficiently distinguished *P. falciparum vs P. vivax* with human serum samples.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 15 May 2014
Received in revised form 15 December 2014
Accepted 4 January 2015
Available online 12 January 2015

Keywords:
Plasmodium falciparum
Plasmodium vivax
Plasmodium falciparum Histidine Rich Protein2 (*Pf*HRP2)
Histidine Rich Protein3 (*Pf*HRP3)
Anti-*Pf*HRP2 monoclonal antibody
Malaria diagnosis

ABSTRACT

An accurate diagnosis of malarial infection is an important element in combating this deadly disease. Malaria diagnostic test including, microscopy and other molecular tests are highly sensitive but too complex for field conditions. Rapid detection tests for *P. falciparum* infection using monoclonal antibodies (mAbs) against highly polymorphic *Pf*HRP2 (Histidine Rich Protein2) are still most preferred test in field conditions, but with limitations such as specificity, and sensitivity leading to false positive and false negative results. To overcome these limitations, we carried out bioinformatics analysis *Pf*HRP2 and *Pf*HRP3 and found that the C-terminal region of *Pf*HRP2 (~105 amino acids) displayed relatively lower sequence identity with *Pf*HRP3. This C-terminal region of *Pf*HRP2 contained unique peptide repeats and was found to be conserved in various isolates of *P. falciparum*. Moreover, this region was also found to be highly antigenic as predicted by antigenicity propensity scores. Thus we constructed a cDNA clone of the truncated *Pf*HRP2 (rec*Pf*HRP2-T3) coding for C-terminal 105 amino acids and expressed it in *E. coli* and purified the polypeptide to homogeneity. The purified rec*Pf*HRP2-T3 was used as an antigen for development of both polyclonal and monoclonal antibody (mAb). The mAbs b10c1 and Aa3c10 developed against rec*Pf*HRP2-T3 was found to efficiently recognize recombinant *Pf*HRP2 but not *Pf*HRP3. In addition, the above mAbs

Abbreviations: rec*Pf*HRP2, recombinant *Pf*HRP2 protein; mAbs, monoclonal antibody; RDTs, rapid diagnostic tests; *Pf*HRP, *P.falciparum* Histidine Rich Protein; ELISA, enzyme Linked ImmunoAssay; *P.f*, *Plasmodium falciparum*; *P.v*, *Plasmodium vivax*.

* Corresponding author. Fax: +914162243092.
E-mail address: krishnan.v@vit.ac.in; bmkrishna1@yahoo.com (K. Venkataraman).

http://dx.doi.org/10.1016/j.exppara.2015.01.001
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reacted positively with spent media and serum sample of *P. falciparum* infection recognizing the native *Pf*HRP2. The affinity constant of both the clones were found to be 10^9 M^{-1}. Quantitatively, both these clones showed ~4.4 fold higher reactivity with *P. falciparum* infected serum compared to serum from healthy volunteers or *P. vivax* infected patient samples. Thus these anti-C-terminal *Pf*HRP2 mAbs (Aa3c10 and b10c1) display a very high potential for improvising the existing malarial diagnostic tools for detection of *P. falciparum* infection especially in areas where *Pf*HRP2 polymorphism is highly prevalent.

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1. Introduction

Malaria is one of the most important parasitic infections that affect mankind, and is associated with huge burden of morbidity and mortality. (http://whqlibdoc.who.int/publications/2010/9789241547925_eng.pdf). Four species of *Plasmodium* causes malaria in humans and among which *P. falciparum* is the most lethal (World Health Organization, 2011). In addition, it also causes cerebral malaria especially in children. Hence the early diagnosis of malaria is critical for the treatment. Histidine Rich Protein (*Pf*HRP2) which is believed to be expressed only by *P. falciparum* is considered to be one of the unique markers for diagnosis of malaria in humans (Rock et al., 1987). This abundant protein is water soluble with unique arrangement of amino acids that it contains multiple amino acid repeats that are rich in alanine, histidine, and aspartic acid (Howard et al., 1986; Rock et al., 1987). Because of the above described features, *Pf*HRP2 is considered as a potential biomarker in immunodetection and it is being widely used in RDTs (Baker et al., 2010; Mariette et al., 2008).

Recent reports have indicated that there is presence of definitive variations in the amino acid sequence of *Pf*HRP2 from different geographical locations (Baker et al., 2010; Mariette et al., 2008). In addition, there are also variations in frequency of the peptide repeats of *Pf*HRP2 which may serve as potential epitopes (Baker et al., 2005; Maltha et al., 2013). Several factors influence the binding of mAbs to the target epitope. First is the presence of highly repetitive nature of the peptide sequences in *Pf*HRP2 polypeptide which may serve as potential epitopes that are one or several amino acid shorter than a major epitope (a stretch of 10–15 amino acids) or minor epitope (a stretch of 3–6 amino acids) to be recognized by the mAbs. Second is the frequency of these targeted epitopes of *Pf*HRP2 protein and third is the geographical distribution of these *Pf*HRP2 epitopes in malarial parasites (Baker et al., 2005; Lee et al., 2006). So an ideal epitope for RDTs, which uses two antibody (capture and the detector), would be one that is present in most isolates of *P. falciparum* (100% prevalence) and has high copy number in each isolates (high frequency) so that the diagnosis would be effective and accurate.

So far a number of anti-*Pf*HRP2 antibodies have been developed and are used in RDTs. These antibodies are either made against complete *Pf*HRP2 polypeptide or with synthetic peptides of *Pf*HRP2 with the repeats (Tomar et al., 2006). Many of these antibodies are not sensitive and in addition cross reacts with *Pf*HRP3 which is very closely related to *Pf*HRP2 whose contribution to diagnosis is not well characterized. Moreover, the antibodies made against synthetic peptides may miss one or other amino acid which is substituted or absent in the target epitopes.

Keeping above described parameters as guidelines and its limitations, we have generated a truncated *Pf*HRP2 in such a way that it covers almost all the possible major epitopes which is found in C terminus of the *Pf*HRP2 protein. Importantly, it is present in most isolates of the world (Baker et al., 2005). The possible logic behind this idea could be the frequency and the abundance of target epitopes which in turn will define the binding affinity of mAb, where a higher frequency and complete presence of the target epitope may result in greater sensitivity and higher binding affinity of the mAb.

In this work we have successfully expressed C-terminal 105 amino acids of *Pf*HRP2 by recombinant DNA-technology in *E. coli* and purified the recombinant protein to homogeneity. It is further used as an antigen to generate both polyclonal and mAbs. The mAbs were developed by hybridoma technology by using an alternative adjuvant polyN-isopropylacrylamide (PNiPAAm) against rec*Pf*HRP2-T3 with the aim to preserve the conformation of epitopes. In addition, PNiPAAm has the property to act as a reservoir of antigen with efficient delivery and protection of the antigen (Kumar et al., 2007; Shakya et al., 2011; Zerpa et al., 2006). The mAbs developed against truncated *Pf*HRP2 were found to be highly specific to *Pf*HRP2. The two mAbs b10c1 and Aa3c10 were characterized for its affinity, specificity and sensitivity. Finally we have shown that the mAb developed against truncated polypeptide rec*Pf*HRP2-T3 is effective in recognizing native *Pf*HRP2 from spent medium of *P. falciparum* culture. With the successful development of highly specific antibody we demonstrated the utility of these mAbs in the specific detection of malaria caused by *P. falciparum* upon evaluation of malaria infected serum samples.

2. Material and methods

2.1. Materials

pET20b (+) expression vector, Competent *Escherichia coli, BL21 (DE3)* strains were obtained from Novagen (Madison, WI), Ampicillin (HiMedia Lab. Mumbai, INDIA). Genomic DNA isolation Kit (QIAGEN Venlo, Netherland), restriction digestion enzymes, (*Nco1, Xho1* and *Not1*) polymerases and ligases are from NEB (New England Biolabs Boston, MA). Monoclonal antibody against *Pf*HRP2 was a gift from SPAN Diagnostic Surat India). All analytical reagents and cell culture reagents were from Sigma (St.Louis,Mo, USA). Non-fat milk was procured from Hi Media (Mumbai, India). Tetra methyl benzidine (TMB)/H_2O_2 was purchased from Genei (Bangalore, Karnataka, India). ELISA plates were purchased from Nunc (Roskilde, Denmark) and all other plastic wares were from Cellstar, Greiner Bio-one (Frickenhausen, Germany). PNiPAAm adjuvant was a gift from Dr.Ashok Kumar, Department of Biological Sciences and Bioengineering, Indian Institute of Technology, Kanpur, 208016, India. *P. falciparum* culture and serum/plasma samples of malaria infected patients were obtained from the National Institute of Malaria Research (NIMR), New Delhi India.

2.1.1. Laboratory animals

All animals used in the development of antibodies were approved by the Institutional Animal Ethical Committee (IAEC).

2.2. Methods

2.2.1. Bioinformatics analysis

The *Pf*HRP2 and *Pf*HRP3 protein sequences used for alignment (tcoffee.crg.cat/) had Genbank Accession number XM002808697.1 and protein id = “XP-002808743.1” for *Pf*HRP2; Genbank accession number U69552.1 and protein id = “AAC47454.1” were for *Pf*HRP3 respectively. The selection of truncated *Pf*HRP2 comprising of C-terminal 105 amino acids named as *Pf*HRP2-T3 (see below)

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