

Short communication

Development of Monoclonal Antibodies That Target 1-Cys Peroxiredoxin and Differentiate *Plasmodium falciparum* from *P. vivax* and *P. knowlesi*

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Abstract: Prompt and accurate diagnosis of malarial patients is a crucial factor in controlling the morbidity and mortality of the disease. Effective treatment decisions require a correct diagnosis among mixed-species malarial patients. Differential diagnosis is particularly important in cases of *Plasmodium vivax*, a species that shares endemicity with *P. falciparum* in most endemic areas. Moreover, it is difficult to identify *P. knowlesi* on the basis of morphology alone, and rapid diagnostic tests are still not available for this malaria species. Therefore, the development of diagnostic tests applicable to the field is urgently needed. 1-Cys peroxiredoxin (1-Cys-Prx) in *P. falciparum* is abundantly expressed in the mature asexual stages, making it a promising candidate as a diagnostic antigen. In this study, we produced five monoclonal antibodies (mAbs) against *P. falciparum* 1-Cys-Prx (Pf1-Cys-Prx) by immunizing BALB/c mice with recombinant Pf1-Cys-Prx and subsequent hybridoma production. Cross reactivity of established mAbs with the orthologous molecule of Pf1-Cys-Prx in *P. vivax* (Pv1-Cys-Prx) and *P. knowlesi* (Pk1-Cys-Prx) was examined. Western blot analyses showed that three mAbs reacted with Pv1-Cys-Prx and Pk1-Cys-Prx but two mAbs did not. These results indicate that the two mAbs were effective in differentiating *P. falciparum* from *P. vivax* and *P. knowlesi* and could be used in differential diagnosis as well as comparative molecular studies of human *Plasmodium* species.

Key words: malaria diagnosis, monoclonal antibody, peroxiredoxin, *Plasmodium knowlesi*, *P. vivax*

INTRODUCTION

Malaria is still considered to be one the most serious infectious diseases in the world. There were about 216 million cases of clinical malaria in 2010, 655,000 of which led to malaria related deaths [1]. Prompt and accurate diagnosis of malarial patients is fundamental in reducing the morbidity and mortality of the disease, avoiding unnecessary use of antimalarial agents and minimizing the spread of resistance to antimalarial drugs. Malaria elimination has been considered in countries with declining human malaria cases, and 32 countries have been categorized as malaria-eliminating countries in the summary report of Feachem *et al.* [2]. There is a need, therefore, to improve current diagnostic techniques as well as to address the new challenges in malaria control, including the presence of asymptomatic

Plasmodium vivax carriers [3].

P. knowlesi, “the fifth human malaria parasite,” is originally a parasite of Old World monkeys, and naturally acquired human infections were believed to be very rare and sporadic until a few years ago [4]. Several studies conducted in various countries in Southeast Asia showed that *P. knowlesi* human malaria was underestimated and misdiagnosed because of morphological similarities with *P. falciparum* and *P. malariae* [5, 6], underlining the difficulty of identifying knowlesi malaria on the basis of morphology alone [6]. The lack of any available rapid diagnostic test to detect *P. knowlesi* further indicates the urgent need to develop a diagnostic test applicable to the field.

Peroxiredoxins (Prxs) are cysteine dependent antioxidant enzymes which are ubiquitous and frequently present in abundance from prokaryotes to eukaryotes [7]. The criti-

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cal role and abundance of Prxs in parasites makes them potentially valuable candidates for drugs and vaccines as well as diagnostic targets [8]. It has been shown that Prx may have diagnostic value for the detection of *Leishmania* spp., *Echinococcus granulosus*, *Fasciola gigantica*, *Taenia* spp. [8] and *Schistosoma japonicum* [9]. 1-Cys peroxiredoxin (1-Cys-Prx) in *P. falciparum*, the orthologous molecule of human Prx VI, is abundantly expressed in the trophozoite stage of *P. falciparum* [10] making it a promising candidate as a diagnostic antigen. 1-Cys-Prx in *P. falciparum*, *P. vivax* and *P. knowlesi* has already been characterized by our group [11, 12]. In this study, we produced several monoclonal antibodies (mAbs) against *P. falciparum* 1-Cys-Prx (Pfl-Cys-Prx) and checked their cross reactivity with the orthologous molecule in *P. vivax* and *P. knowlesi*.

MATERIALS AND METHODS

Recombinant protein production and purification

Recombinant proteins were produced as described previously [12, 13]. Briefly, the PCR products were digested with restriction enzymes (Supplementary Table S1) and then inserted into pET-28a expression vector (EMD Biosciences, San Diego, CA) by overnight incubation with ligation mixture (Takara, Otsu, Japan) at 15°C. The recombinant plasmids containing the coding sequence of Pfl-Cys-Prx, *P. vivax* 1-Cys-Prx (Pv1-Cys-Prx) and *P. knowlesi* 1-Cys-Prx (Pk1-Cys-Prx) were transfected into *Escherichia coli* (strain BL21). Expression of the recombinant proteins (rPfl-Cys-Prx, rPv1-Cys-Prx and rPk1-Cys-Prx) as a fusion protein with N-terminal histidine-tag was induced by adding 1 mM isopropyl thio- β -D-galactoside (IPTG) and purified using Ni-NTA agarose beads (Qiagen Inc., Valencia, CA). The expression and purification of the recombinant proteins were evaluated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) under re-

ducing conditions and subsequent Coomassie Brilliant Blue R-250 (CBB) staining. The concentration of each expressed protein was measured using bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL).

Preparation of monoclonal antibodies

In order to produce mAbs, rPfl-Cys-Prx was used to immunize 8–10 week-old BALB/c mice. rPfl-Cys-Prx was mixed with an equal volume of complete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO) for initial immunization and with incomplete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO) for two subsequent immunizations. The antibody titer was measured by indirect enzyme-linked immunosorbent assay (ELISA). Prefusion boost was conducted three days before harvesting the spleen by injecting 0.2 ml of rPfl-Cys-Prx into the tail vein. Hybridomas were developed by fusion of harvested splenocytes to Sp2/0 myeloma cells and screened using ELISA and Western blot. Isotyping of mAbs was performed using IsoStrip mouse monoclonal antibody isotyping kit (Roche Diagnostics, Indianapolis, IN). The animal experiments in this study were carried out in compliance with the Obihiro University of Agriculture and Veterinary Medicine Guidelines for Animal Experimentation (permission numbers: 23–155 and 24–43).

Epitope mapping

The full length of the Pfl-Cys-Prx was split into four equal parts (Pfl-Cys-Prx (amino acid nos. 1–55), Pfl-Cys-Prx (56–110), Pfl-Cys-Prx (111–165) and Pfl-Cys-Prx (166–220)) with 55 amino acids each and primers were designed to amplify these DNA fragments (Supplementary Table S1). The amplified DNA fragments were digested with restriction enzymes and inserted into pGEX4T-1 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) by overnight incubation with ligation mixture (Takara, Otsu, Japan) at 15°C. The recombinant plasmids were transfected into

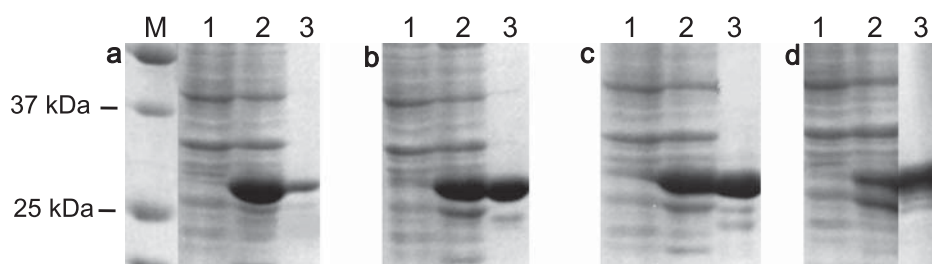


Fig. 1. SDS/PAGE image of production of recombinant proteins for epitope mapping. The full length of Pfl-Cys-Prx was split into four parts (Pfl-Cys-Prx (amino acid nos. 1–55), Pfl-Cys-Prx (56–110), Pfl-Cys-Prx (111–165) and Pfl-Cys-Prx (166–220)). Recombinant proteins were expressed in *E. coli*. M, Marker. Lane 1: *E. coli* culture before adding IPTG; lane 2: 4h after adding IPTG; and lane 3: purified recombinant proteins. Panel a: Pfl-Cys-Prx (1–55); panel b: Pfl-Cys-Prx (56–110); panel c: Pfl-Cys-Prx (111–165); and panel d: Pfl-Cys-Prx (166–220).

E. coli (strain BL21). The transformed colony was incubated in LB broth (Sigma-Aldrich, St. Louis, MO) for expression of the glutathione S-transferase (GST)-tagged protein. The recombinant protein was purified with Glutathione Sepharose 3B following the manufacturer's protocol (GE Healthcare Bio-Sciences AB). The purity of the recombinant protein was evaluated by 12% SDS/PAGE under reducing conditions and subsequent CBB staining (Fig. 1).

RESULTS AND DISCUSSION

In this study, five different mAbs were produced against Pf1-Cys-Prx by screening the hybridoma clones with ELISA and Western blot. Isotyping showed that all of the mAbs are IgG1 with kappa light chains. In order to clarify the cross reactivity of established mAbs with the orthologous molecule of Pf1-Cys-Prx in *P. vivax* (Pv1-Cys-Prx) and *P. knowlesi* (Pk1-Cys-Prx), Western blot analysis was conducted using recombinant Pv1-Cys-Prx (rPv1-Cys-Prx) and rPk1-Cys-Prx protein (Fig. 2). As a result, mAbs 1, 2 and 3 reacted with rPv1-Cys-Prx and rPk1-Cys-Prx while mAbs 4 and 5 did not, indicating that mAbs 4 and 5 could differentiate *P. falciparum* from *P. vivax* and *P. knowlesi*.

Epitope mapping to determine the target epitope of each mAbs showed that mAbs 1, 2 and 3 reacted with Pf1-Cys-Prx (166–220), which is the C-terminal part of Pf1-

Cys-Prx, while mAbs 4 and 5 reacted with Pf1-Cys-Prx (111–165) (Fig. 3). Moreover, none of the mAbs reacted with GST. Deduced amino acid sequence analysis of Pf1-Cys-Prx (111–165) and Pf1-Cys-Prx (166–220) with the corresponding part in Pv1-Cys-Prx and Pk1-Cys-Prx showed 93% and 60% identity, respectively (Supplementary Fig. S1). Epitopes can be divided into linear and conformational residues wherein linear epitopes are a sequence of amino acids, while conformational epitopes result from a three-dimensional structure [14]. Although mAbs 4 and 5-targeting epitopes are located in Pf1-Cys-Prx (111–165),

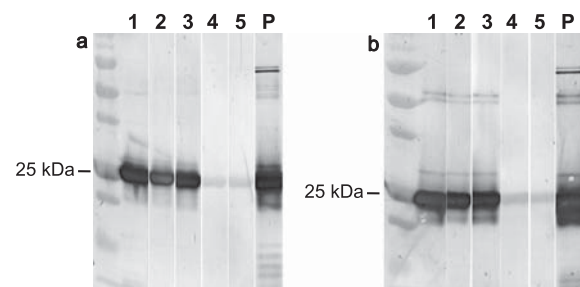


Fig. 2. Reactivity of produced mAbs against Pf1-Cys-Prx with Pv1-Cys-Prx and Pk1-Cys-Prx. Western blot analysis using mAbs (lanes 1–5) and specific polyclonal antibody against rPf1-Cys-Prx (lane P). Panel a: rPv1-Cys-Prx; and panel b: rPk1-Cys-Prx.

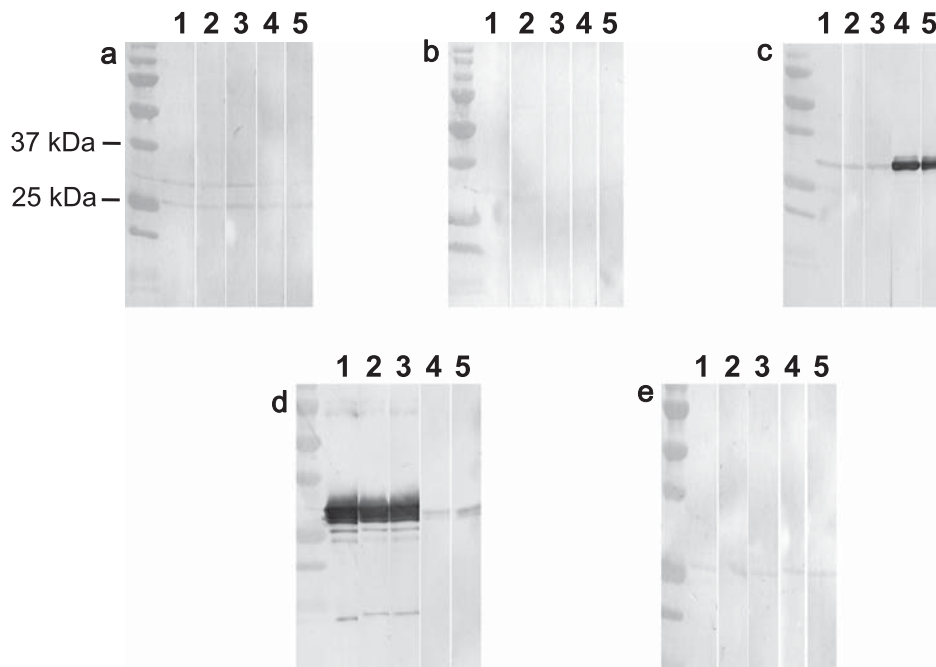


Fig. 3. Epitope mapping. Western blot analysis shows the reactivity of each mAbs (lanes 1–5) to recombinant proteins. Panel a: Pf1-Cys-Prx (1–55); panel b: Pf1-Cys-Prx (56–110); panel c: Pf1-Cys-Prx (111–165); panel d: Pf1-Cys-Prx (166–220); and panel e: GST.

which had a high identity with the corresponding part in Pv1-Cys-Prx and Pk1-Cys-Prx, their binding epitope residues may exist in the non-identical part, or possibly they may be targeting conformational epitopes.

The binding affinities of mAbs were determined by measuring the dissociation constant (K_d) as described before [15]. Briefly, constant amounts of mAbs were incubated with various concentrations of rPfl-Cys-Prx until the equilibrium was reached. The remaining unsaturated mAbs were measured by indirect ELISA. K_d was determined using Klotz plot [15]. As a result, mAbs 1, 2 and 3 showed the highest affinity with K_d , namely 0.39 ± 0.16 , 2.23 ± 0.83 and 1.64 ± 0.66 nM, respectively (Table 1). These findings are comparable with mAbs from previous studies showing a K_d of 12.03 ± 3.1 and 24.21 ± 2.07 nM against *Plasmodium* lactate dehydrogenase (pLDH) [16], and 0.16 ± 0.13 and 1.0 ± 0.049 nM against histidine-rich protein 2 (HRP-2) [17]. However, mAbs 4 and 5 showed a lower affinity (Table 1). These results suggest that mAbs 1, 2 and 3 are good candidates for use in the diagnosis of falciparum malaria, while mAbs 4 and 5 are useful for species differentiation in smear-confirmed malarial patients.

Diagnosis of mixed-species malarial patients is a crucial factor in decisions regarding treatment [16]. *P. knowlesi* co-exists with other human *Plasmodium* species in Southeast Asian countries [4]. Diagnosis of *P. knowlesi* through microscopy alone is difficult because of its morphological similarities with other *Plasmodium* species. The early trophozoites of *P. knowlesi* are similar to those of *P. falciparum* while the late trophozoites and schizonts morphologically resemble those of *P. malaria* [6]. Some researchers have investigated the use of commercial RDTs as a way to detect knowlesi infections. McCutchan *et al.* [18] showed that mAbs targeting pLDH of *P. falciparum* and *P. vivax* can also detect *P. knowlesi* and attributed this to the highly similar amino acid sequences among these malaria parasites. In another study using two different commercial RDTs,

P. knowlesi did not react with HRP-2-based RDT but was detected with pLDH-based RDT [19]. These results suggest that pLDH-based RDTs are not suitable for knowlesi malaria detection or differentiation. In the present study, however, mAbs 4 and 5 could differentiate falciparum from knowlesi malaria, indicating that they are good candidates for uses as differential diagnosis tools in *P. knowlesi* endemic Southeast Asian countries.

Since the diagnosis of malaria is based on direct detection of the parasites by microscopy or the parasites' antigens, mAbs may play an important role in the detection of malaria parasites. Commercial antigen detection ELISAs and RDTs use mAbs that target one of three antigens (HRP-2, pLDH and aldolase) [3] being used extensively for case management and prevalence surveys. However, the available RDTs are known to have certain deficiencies, such as the fact that HRP-2 is only expressed in *P. falciparum* and persists even after parasite clearance, thus causing false positive results [20]. The genetic variation of HRP-2 in different geographical regions has also been reported, a problem that affects the sensitivity of tests in the field [21, 22], and a large proportion of field isolates in the Peruvian Amazon lack this antigen [23]. On the other hand, pLDH-based tests were less efficient in detecting low parasite densities and yielded variable results in different studies [24], while aldolase targeting tests have been shown to lack sensitivity in recent comparative studies, possibly due to the low concentration of this antigen in the parasite [24]. The development of RDTs targeting other antigens may overcome some of the deficiencies of current RDTs and improve the identification of human malaria parasites [3]. Pfl-Cys-Prx is highly expressed in the asexual stage of *P. falciparum* and accounts for 0.5% of total cellular protein during the trophozoite stage [11]. Moreover, Pfl-Cys-Prx does not exhibit genetic diversity and therefore maybe a great potential candidate for diagnostic use.

In summary, we produced five different mAbs targeting Pfl-Cys-Prx, two of which were able to differentiate *P. falciparum* from *P. vivax* and *P. knowlesi*. These mAbs can therefore be used in differential diagnosis as well as comparative molecular biology studies.

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Table 1. Dissociation constants of monoclonal antibodies determined by ELISA

mAb*	Isotype	K_d (nM) [#]	SD [†]
1	IgG1	0.39	0.16
2	IgG1	2.23	0.83
3	IgG1	1.64	0.66
4	IgG1	8743	2263
5	IgG1	4873	851

* Monoclonal antibodies tested.

[#] The dissociation constants (K_d) are calculated by Klotz plot [15].

[†] Standard deviation.

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