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Title:

Development of monoclonal antibodies against *Plasmodium falciparum* thioredoxin peroxidase 1 and its possible application for malaria diagnosis

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Abstract

Rapid diagnostic tests (RDTs) have been considered as an ideal alternative for light microscopy to detect malaria parasites especially in remote areas. The development and improvement of RDTs is an area of intensive research in the last decade. To date, few parasite proteins have been targeted in RDTs which are known to have certain deficiencies and made the researchers to look for other promising candidates to address this problem. *Plasmodium falciparum* thioredoxin peroxidase 1 (PfTPx-1) is abundantly expressed in the cytoplasm of the parasite and well conserved across *Plasmodium* species making this antigen a promising target for malaria diagnosis. Several monoclonal antibodies (mAbs) were produced against PfTPx-1. The binding affinities of mAbs were measured. Several immunochromatographic tests (ICTs) were developed using different combination of mAbs. All mAbs showed promising affinities to be used for diagnosis. The sensitivities of ICTs were evaluated using recombinant PfTPx-1 whose results lead us to the preparation of 4 different ICTs. These tests showed positive reaction with *P. falciparum* in vitro culture supernatant indicating the release of PfTPx-1 during schizont rupture. Altogether, these findings suggest that PfTPx-1 is a promising biomarker to diagnose *P. falciparum* infection. However, the diagnostic performance of this antigen should be further validated using clinical samples.
39  **Keywords:** *Plasmodium falciparum*; malaria diagnosis; rapid diagnostic test;

40  thioredoxin peroxidase 1
1. Background

Despite being preventable and treatable, malaria still remains as a major public health concern in the world with 1,238,000 global deaths in 2010 [1]. Emergence of drug resistance in the malaria parasite, insecticide resistance among the mosquito vectors and the unavailability of an efficient vaccine against malaria are important obstacles for controlling this parasitic disease.

Although parasite/parasite antigen-based diagnosis is increasing, most suspected cases in endemic areas are treated based on presumptive diagnosis [2]. Prompt and accurate diagnosis and treatment of the patients with appropriate antimalarial is an essential component of malaria control and elimination strategies. Therefore, since early 2010, WHO has recommended prompt parasitological confirmation by microscopy or rapid diagnostic test (RDT) for all suspected malaria patients before starting the treatment [2]. Malaria RDTs were introduced in early 1990s and recently, they have greatly enhanced the quality of malaria diagnosis in endemic areas [3]. These RDTs which are lateral flow immune-chromatographic tests (ICT), detect parasite antigens by specific monoclonal antibodies (mAbs). Commercial RDTs target one of three antigens namely histidine rich protein 2 (HRP-2), plasmodial lactate dehydrogenase (pLDH) and aldolase. Despite high sensitivity and specificity for Plasmodium falciparum infections,
commercial RDTs have known deficiencies such as variable detection thresholds especially in low transmission areas [4, 5]. There is a need, therefore, to improve current diagnostic techniques and to develop RDTs targeting additional antigens to address the current deficiencies as well as new challenges in malaria control.

Peroxiredoxin (Prx) is a ubiquitous family of antioxidant enzymes with molecular size of 20-30 kDa that are present in organisms from all kingdoms [6]. In different parasites, it is shown that Prxs may be potentially valuable candidates for drugs and vaccines targets [reviewed by 7]. In addition, Prxs may have diagnostic value for the detection of *Leishmania* spp., *Echinococcus granulosus*, *Fasciola gigantica*, *Taenia* spp. [reviewed by 7] and *Schistosoma japonicum* [8, 9]. The cytoplasmic Prxs from *P. falciparum*, *P. vivax* and *P. knowlesi* have been characterized by our group [10, 11, 12]. It was shown that *P. falciparum* thioredoxin peroxidase 1 (PfTPx-1) is constitutively and highly expressed through the erythrocytic cycle [13, 14] making it a promising candidate as a diagnostic antigen for malaria diagnosis. In this study, we produced several mAbs against PfTPx-1 and evaluated their potential to be used in RDTs.

### 2. Materials and Methods

2.1. Production and selection of mAbs
Recombinant PfTPx-1, *P. vivax* TPx-1 (PvTPx-1) and *P. knowlesi* TPx-1 (PkTPx-1) proteins were expressed as a fusion protein with N-terminal histidine-tag and purified [11]. mAbs were produced as previously described [15]. Briefly, BALB/c mice were immunized by rPfTPx-1 and hybridomas were developed by fusion of harvested splenocytes to SP2/0 myeloma cells. Single step hypoxanthine-aminopterin-thymidine (HAT) selection using methylcellulose and cloning of hybridoma was performed as previously described with some modifications [16]. Hybridoma cloning medium consisted of GIT medium (Nihon Pharmaceutical Co., Tokyo, Japan) containing 5% fetal bovine serum, 5% BriClone (NICB, Dublin, Ireland), HAT and 1.75% methylcellulose. Following 7-10 days incubation, hybridoma clones were picked and grown in wells of 96 well tissue culture microplates and screened using enzyme linked immunosorbent assay (ELISA) and Western blot. The animal experiments in this study were carried out in compliance with the Obihiro University of Agriculture and Veterinary Medicine Guidelines for Animal Experimentation (25-74).

2.2. Purification of mAbs

Isotyping of mAbs was performed using IsoStrip mouse monoclonal antibody isotyping kit (Roche Diagnostics, Indianapolis, IN). IgG and IgM mAbs from
hybridoma culture supernatant were purified using protein G (GenScript, NJ, USA) and protein L (GenScript, NJ, USA), respectively, according to manufacturer’s instructions. The purity of the mAbs was evaluated by SDS-PAGE under reducing conditions.

2.3. Measurement of binding affinities of mAbs by ELISA

The binding affinities of mAbs were determined by measuring the dissociation constant ($K_d$) as described before [17]. Briefly, constant amounts of mAbs were incubated with various concentrations of rPfTPx-1 until the equilibrium was reached. The mixture of antigen-antibody was then transferred to micro-titer plates previously coated with rPfTPx-1 and the remaining unsaturated mAbs were measured by indirect ELISA. $K_d$ was determined using Klotz plot [17].

2.4. Preparation of immunochromatographic tests

Fifty µg/ml of mAb A1 or E4 (Table 1) was added gently to 1 ml of gold nanoparticles (BBI Solutions, Cardiff, UK), then mixed and kept for 10 min at room temperature for the immobilization of antibodies onto the gold nanoparticles’ surfaces by physical adsorption. After immobilization, 10 µl of 5% (w/v) polyethylene glycol (PEG) and 100 µl of 10% (w/v) bovine serum albumin (BSA) solution were added to
block the non-coated gold nanoparticles’ surfaces. After the immobilization and blocking procedures, gold nanoparticle-conjugated mAb was separated by centrifugation. The gold nanoparticle-conjugated mAb was pulse-sonicated for a few seconds and was washed with 1 ml of PBS containing 0.05% (w/v) PEG and 0.5% (w/v) BSA. After mixing, gold nanoparticle-conjugated mAb was collected by the same process as described above. After pulse sonication, the gold nanoparticle-conjugated mAb solution was diluted with the dilution buffer containing 20 mM Tris-HCl (pH: 8.2), 0.05% PEG and 3.5% sucrose to OD$_{520}$=6. The colloidal gold-conjugated mAb was dried on a glass fiber and used as conjugate pad. 0.5 mg/ml of goat anti-mouse IgG (ThermoFisher Scientific, IL, USA) and 1 mg/ml of mAb A1, E4 or D5 (Table 1) were immobilized on the control and test line, respectively. The running buffer for ICTs was 100 mM Na borate (pH: 8), 1% triton X-100, 1% lactose, 1% casein [18].

2.5. Western blot

The in vitro culture supernatant P. falciparum 3D7 strain was centrifuged to remove cell debris and then concentrated with saturated ammonium sulfate. The culture supernatant proteins were electrophoretically separated using SDS-PAGE and transferred onto nitrocellulose membrane. Uninfected human erythrocyte lysate was
used to check the cross reaction of anti-PfTPx-1 antibodies with human peroxiredoxins. The membrane was blocked by 5% skim milk and further incubated with mAb and anti-rPfTPx-1 polyclonal antibody. Bound antibodies were detected using an anti-mouse IgG horseradish peroxidase linked whole antibody (GE Healthcare, Buckinghamshire, UK).

3. Results

3.1. Production and screening of mAbs

In this study, 11 different mAbs were produced against PfTPx-1 by screening the hybridoma clones with ELISA and Western blot. Isotyping showed that 5 mAbs are IgG and 6 mAbs are IgM with kappa light chains (Table 1). Since TPx-1 is a well conserved enzyme in *Plasmodium* spp., we decided to clarify the cross reactivity of established mAbs with the orthologous molecule of PfTPx-1 in *P. vivax* (PvTPx-1) and *P. knowlesi* (PkTPx-1). Western blot analysis was conducted using recombinant PvTPx-1 (rPvTPx-1) and rPkTPx-1 proteins. As a result, all 6 IgM mAbs bind to rPvTPx-1 and rPkTPx-1 while IgG mAbs did not, indicating the different targeting epitopes (data not shown).
3.2. Binding affinities of mAbs

In order to evaluate the potential of mAbs to be used for diagnosis, the binding affinities of mAbs were determined by measuring the dissociation constant (K_d) using regression analysis and Klotz plot (Supplementary Fig. S1) [17]. As it could be seen in table 1, K_d of all mAbs was around 1 or less than 1 nM indicating high affinity for all mAbs which is comparable with commercially used mAbs for malaria RDTs as well as previous studies [19, 20].

3.3. The evaluation of ICTs targeting PfTPx-1

Using different combination of produced mAbs as colloidal gold-conjugated and/or test line, several ICTs were developed and their sensitivities were evaluated using rPfTPx-1. As a result, all IgM mAbs and mAb A4 did not show good sensitivities (data not shown). Four ICTs showed the highest sensitivities (Fig. 1) using mAb A1 or E4 as colloidal gold-conjugated and mAb A1, E4 or D5 as test line. These ICTs were able to detect 0.2-0.5 ng of rPfTPx-1 and were further evaluated by in vitro culture supernatant. All of them showed positive result when P. falciparum in vitro culture supernatant was used as sample, indicating the presence of PfTPx-1 in the culture supernatant. In order to confirm this, Western blot analysis was done by transferring the
culture supernatant proteins onto nitrocellulose membrane and PfTPx-1 was detected by specific antibodies. As shown in Figure 2, an approximate 22 kDa band corresponding to PfTPx-1 was appeared, confirming the presence of PfTPx-1 in the culture supernatant. Moreover, no reaction was seen with uninfected human erythrocytes lysate.

4. Discussion

A prompt and reliable diagnostic system remains to be a challenge for malaria control. Most of malaria death could be prevented if the patients diagnosed and treated promptly and accurately. Since the traditional microscopy is cumbersome and requires experienced technicians, RDTs have been introduced as an ideal alternative for microscopy. The current commercial RDTs for malaria have known pitfalls such as genetic diversity and persistence of HRP-2 which made WHO to evaluate the performance of RDTs [21]. To address this problem, improvement of current RDTs and evaluation of new target antigens are necessary. A number of alternative diagnostic targets have been introduced namely dihydrofolate reductase–thymidylate synthase, heme-detoxification protein, glutamate-rich protein, heat-shock protein 70, hypoxanthine phosphoribosyl transferase and 1-Cys peroxiredoxin [15, 19, 22, 23].

In this study, we introduced TPx-1 as a new promising candidate for malaria
diagnosis. TPx-1 is well conserved across the genus *Plasmodium*, with 83% amino acid sequence identity among *P. falciparum*, *P. vivax* and *P. knowlesi* (supplementary Fig. S2) and PfTPx-1 shares 45% amino acid identity with human orthologue, Prx1. TPx-1 is constitutively expressed in asexual erythrocytic stages and gametocytes of *Plasmodium* suggesting a housekeeping role for this enzyme [14, 24]. PfTPx-1 is a cytoplasmic peroxiredoxin [13], which reduces and detoxifies hydrogen peroxides through the action of the redox-active cysteine [25]. Moreover, during the trophozoite stage, PfTPx-1 is one of the most abundantly expressed proteins in the parasite cytoplasm, accounts for 0.25 to 0.5% of the total cellular protein [13].

Eleven mAbs were produced against PfTPx-1 by immunizing mice with recombinant protein and subsequent hybridoma production. All mAbs showed high affinities (Kd of around 1nM) to be used for diagnostic purposes (Table 1). All IgM mAbs bind to rPfTPx-1, rPvTPx-1 and rPkTPx-1 while IgG mAbs reacted only with rPfTPx-1. This indicates that all IgM mAbs possibly target a common epitope in PfTPx-1, PvTPx-1 and PkTPx-1, while, IgG mAbs possibly target PfTPx-1-specific epitopes making these mAbs specific for *P. falciparum* and could be used for diagnosing this human malaria parasite.

In order to evaluate the combination of two mAbs for detection of PfTPx-1,
different ICTs were developed and evaluated using rPfTPx-1 (data not shown). IgG1 mAbs showed the highest sensitivities and showed positive results when *P. falciparum* \textit{in vitro} culture supernatant was applied on these tests (Fig. 1). The presence of PfTPx-1 in culture supernatant was further confirmed by Western blot studies (Fig. 2). Since PfTPx-1 is a cytoplasmic protein and is not associated with the parasite membrane [10], it might be released upon schizont rupture. Furuta \textit{et al}, (2008) reported that malarial TPx-1 is a ligand protein for Toll-like receptor 4 and induces IgE-mediated protection [26]. They suggested that TPx-1 might be released from the parasite when schizonts are ruptured or infected erythrocytes are destroyed in the spleen [26]. During asexual development of *P. falciparum* in the RBCs, late stages parasites are retained in the capillary system of various organs which is called sequestration [27]. Since PfTPx-1 might be released during schizont rupture into the circulation, targeting PfTPx-1 may improve the detection of sequestered parasites which cannot be seen by microscopy.

A key concern regarding to sensitivity of HRP-2-based RDTs is the genetic variation of this antigen (Supplementary Table S2) in different geographical regions [28, 29, 30]. Besides the genetic diversity, lack of HRP-2 gene in *P. falciparum* isolates have been reported from various countries [31, 32, 33], which limits the application of HRP-2-based RDTs in these regions. PfTPx-1 does not show genetic variation
(Supplementary Table S2) which is another promising advantage of this antigen for diagnostic purposes.

Another major concern with RDTs targeting PfHRP-2 is the persistence of this antigen in the blood for long period after parasite clearance that not only produces false positive results but also decreases the usefulness of this antigen for drug-susceptibility testing and patients treatment follow up [reviewed by 34]. Therefore, a positive result of PfHRP-2-based test may indicate a previous infection and should be confirmed using other diagnostics such as microscopy, PCR or RDT targeting other antigens [35]. To further evaluate PfTPx-1 as a diagnostic antigen, it is worthy to determine the half life of this antigen in the patient’s blood.

5. Conclusions

Here, we introduced and evaluated PfTPx-1 as a promising candidate for malaria diagnosis. The abundance and consistent expression of PfTPx-1 in *P. falciparum* together with having no genetic diversity makes this antigen a promising target for malaria diagnosis. Moreover, TPx-1 is well conserved across *Plasmodium* species and different from human orthologue. Four different ICTs targeting PfTPx-1 were developed and were able to detect this antigen in *P. falciparum in vitro* culture supernatant. The
release of PfTPx-1 in the culture supernatant was further confirmed by Western blot studies. Taken together, these findings suggest that TPx-1 is a promising candidate for malaria diagnosis.

Abbreviations

PfTPx-1: *Plasmodium falciparum* thioredoxin peroxidase 1; mAb: monoclonal antibody; ICT: Immunochromatographic test; RDT: Rapid diagnostic test; HRP-2: Histidine rich protein 2; pLDH: Plasmodial lactate dehydrogenase; Prx: Peroxiredoxin; PvTPx-1: *P. vivax* TPx-1; PkTPx-1: *P. knowlesi* TPx-1; HAT: Hypoxanthine-aminopterin-thymidine; ELISA: Enzyme linked immunosorbent assay; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis;

Acknowledgments

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<th>Isotype</th>
<th>$K_d$ (nM)#</th>
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* Monoclonal antibodies tested.

# The dissociation constants ($K_d$) are calculated by Klotz plot [18].

¶ Standard deviation.
Figure captions

**Fig. 1.** Reactivity of developed immunochromatographic tests with *P. falciparum in vitro* culture supernatant. Monoclonal antibodies (mAbs) A1 or E4 was used as gold conjugate (underlined). mAbs A1, E4 or D5 was used in test line and goat anti-mouse IgG was used as control line. Lane 1: supernatant of non-infected culture was used as negative control; lane 2: 10 µl of *P. falciparum in vitro* culture supernatant was used.

**Fig. 2.** Western blot analysis. Western blot analysis of *P. falciparum in vitro* culture supernatant. Culture supernatant and uninfected human erythrocyte lysate proteins were electrophoretically separated using SDS-PAGE and transferred onto nitrocellulose membrane. M, Marker. Reactivity of mouse anti-PfTPx-1 polyclonal and monoclonal antibody A1 with culture supernatant (lane 1 and 3, respectively) and human erythrocyte (lane 2 and 4, respectively).
Supplementary information

Table S1. Primers for amplification of *P. falciparum* TPx-1 (PfTPx-1), *P. vivax* TPx-1 (PvTPx-1) and *P. knowlesi* TPx-1 (PkTPx-1). Start and stop codons are underlined and restriction sites are italicized.

Table S2. Total single nucleotide polymorphisms (SNPs) are from 143 *P. falciparum* strains. Source: PlasmoDB database.

Fig. S1. Klotz plot of the binding affinities of mAbs measured by indirect ELISA. mAbs incubated with different concentration of rPfTPx-1 until the equilibrium is reached and the free mAbs are measured by ELISA. A0: OD in the absence of antigen; A: OD in the presence of antigen; a0: antigen concentration (nM).

Fig. S2. Amino acid sequence alignment of PfTPx-1, PvTPx-1 and PkTPx-1. The identical residues among three sequences are boxed.
Hakimi et al. Figure 1

A1-E4  1  2  A1-D5  1  2  E4-A1  1  2  E4-D5  1  2

Control line

Test line