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.

Keywords: *Plasmodium falciparum*; malaria diagnosis; rapid diagnostic test; 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,

59 commercial RDTs have known deficiencies such as variable detection thresholds
60 especially in low transmission areas [4, 5]. There is a need, therefore, to improve current
61 diagnostic techniques and to develop RDTs targeting additional antigens to address the
62 current deficiencies as well as new challenges in malaria control.

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

74

75 **2. Materials and Methods**

76 2.1. Production and selection of mAbs

77 Recombinant PfTPx-1, *P. vivax* TPx-1 (PvTPx-1) and *P. knowlesi* TPx-1
78 (PkTPx-1) proteins were expressed as a fusion protein with N-terminal histidine-tag and
79 purified [11]. mAbs were produced as previously described [15]. Briefly, BALB/c mice
80 were immunized by rPfTPx-1 and hybridomas were developed by fusion of harvested
81 splenocytes to SP2/0 myeloma cells. Single step hypoxanthine-aminopterin-thymidine
82 (HAT) selection using methylcellulose and cloning of hybridoma was performed as
83 previously described with some modifications [16]. Hybridoma cloning medium
84 consisted of GIT medium (Nihon Pharmaceutical Co., Tokyo, Japan) containing 5%
85 fetal bovine serum, 5% BriClone (NICB, Dublin, Ireland), HAT and 1.75%
86 methylcellulose. Following 7-10 days incubation, hybridoma clones were picked and
87 grown in wells of 96 well tissue culture microplates and screened using enzyme linked
88 immunosorbent assay (ELISA) and Western blot. The animal experiments in this study
89 were carried out in compliance with the Obihiro University of Agriculture and
90 Veterinary Medicine Guidelines for Animal Experimentation (25-74).
91
92 2.2. Purification of mAbs
93 Isotyping of mAbs was performed using IsoStrip mouse monoclonal antibody
94 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

113 block the non-coated gold nanoparticles' surfaces. After the immobilization and
114 blocking procedures, gold nanoparticle-conjugated mAb was separated by
115 centrifugation. The gold nanoparticle-conjugated mAb was pulse-sonicated for a few
116 seconds and was washed with 1 ml of PBS containing 0.05% (w/v) PEG and 0.5% (w/v)
117 BSA. After mixing, gold nanoparticle-conjugated mAb was collected by the same
118 process as described above. After pulse sonication, the gold nanoparticle-conjugated
119 mAb solution was diluted with the dilution buffer containing 20 mM Tris-HCl (pH: 8.2),
120 0.05% PEG and 3.5% sucrose to OD_{520}=6. The colloidal gold-conjugated mAb was
121 dried on a glass fiber and used as conjugate pad. 0.5 mg/ml of goat anti-mouse IgG
122 (ThermoFisher Scientific, IL, USA) and 1 mg/ml of mAb A1, E4 or D5 (Table 1) were
123 immobilized on the control and test line, respectively. The running buffer for ICTs was
124 100 mM Na borate (pH: 8), 1% triton X-100, 1% lactose, 1% casein [18].
125
126 2.5. Western blot
127 The *in vitro* culture supernatant *P. falciparum* 3D7 strain was centrifuged to
128 remove cell debris and then concentrated with saturated ammonium sulfate. The culture
129 supernatant proteins were electrophoretically separated using SDS-PAGE and
130 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).

149 3.2 . Binding affinities of mAbs

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

156

157 3.3 . The evaluation of ICTs targeting PfTPx-1

158 Using different combination of produced mAbs as colloidal gold-conjugated
159 and/or test line, several ICTs were developed and their sensitivities were evaluated
160 using rPfTPx-1. As a result, all IgM mAbs and mAb A4 did not show good sensitivities
161 (data not shown). Four ICTs showed the highest sensitivities (Fig. 1) using mAb A1 or
162 E4 as colloidal gold-conjugated and mAb A1, E4 or D5 as test line. These ICTs were
163 able to detect 0.2-0.5 ng of rPfTPx-1 and were further evaluated by *in vitro* culture
164 supernatant. All of them showed positive result when *P. falciparum in vitro* culture
165 supernatant was used as sample, indicating the presence of PfTPx-1 in the culture
166 supernatant. In order to confirm this, Western blot analysis was done by transferring the

167 culture supernatant proteins onto nitrocellulose membrane and PfTPx-1 was detected by
168 specific antibodies. As shown in Figure 2, an approximate 22 kDa band corresponding
169 to PfTPx-1 was appeared, confirming the presence of PfTPx-1 in the culture supernatant.
170 Moreover, no reaction was seen with uninfected human erythrocytes lysate.
171
172 **4. Discussion**
173 A prompt and reliable diagnostic system remains to be a challenge for malaria
174 control. Most of malaria death could be prevented if the patients diagnosed and treated
175 promptly and accurately. Since the traditional microscopy is cumbersome and requires
176 experienced technicians, RDTs have been introduced as an ideal alternative for
177 microcopy. The current commercial RDTs for malaria have known pitfalls such as
178 genetic diversity and persistence of HRP-2 which made WHO to evaluate the
179 performance of RDTs [21]. To address this problem, improvement of current RDTs and
180 evaluation of new target antigens are necessary. A number of alternative diagnostic
181 targets have been introduced namely dihydrofolate reductase–thymidylate synthase,
182 heme-detoxification protein, glutamate-rich protein, heat-shock protein 70,
183 hypoxanthine phosphoribosyl transferase and 1-Cys peroxiredoxin [15, 19, 22, 23].
184 In this study, we introduced TPx-1 as a new promising candidate for malaria

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

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

202 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 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 *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

239 release of PfTPx-1 in the culture supernatant was further confirmed by Western blot
240 studies. Taken together, these findings suggest that TPx-1 is a promising candidate for
241 malaria diagnosis.
242
243 **Abbreviations**
244 PfTPx-1: *Plasmodium falciparum* thioredoxin peroxidase 1; mAb: monoclonal
245 antibody; ICT: Immunochromatographic test; RDT: Rapid diagnostic test; HRP-2:
246 Histidine rich protein 2; pLDH: Plasmodial lactate dehydrogenase; Prx: Peroxiredoxin;
247 PvTPx-1: *P. vivax* TPx-1; PkTPx-1: *P. knowlesi* TPx-1; HAT:
248 Hypoxanthine-aminopterin-thymidine; ELISA: Enzyme linked immunosorbent assay;
249 SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis;
250
251 **Acknowledgments**
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369 Table 1. Dissociation constants of monoclonal antibodies determined by ELISA

mAb*	Isotype	K_d (nM)#	SD¶
A1	IgG1	1.21	0.08
A4	IgG3	0.61	0.11
E4	IgG1	1.295	0.66
D5	IgG1	1.225	0.80
C6	IgG1	0.615	0.09
F1	IgM	0.96	0.49
B3	IgM	0.67	0.03
C4	IgM	0.975	0.69
A5	IgM	0.89	0.08
D6	IgM	0.79	0.04
F8	IgM	0.78	0.08

370 * Monoclonal antibodies tested.

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

372 ¶ Standard deviation.

373 **Figure captions**

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

380

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

Supplementary information

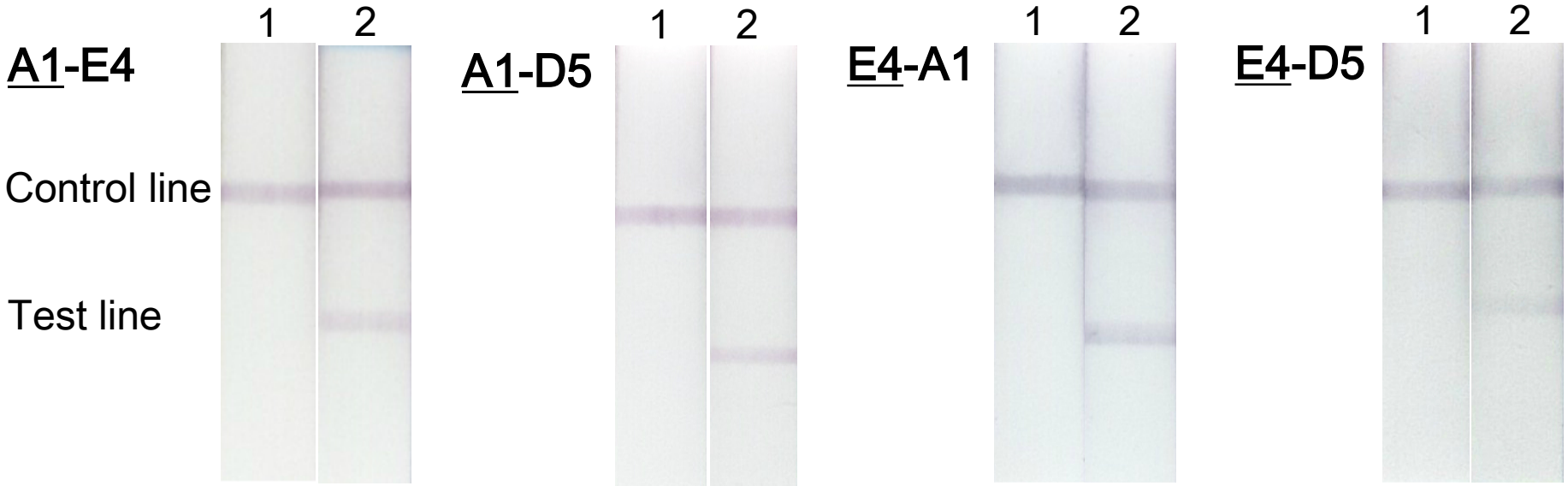
Table S1. Primers for amplification of *P. falciparum* TPx-1 (PfTPx-1), *P. vivax* TPx-1 (PvTPx-1) and *P. knowlesi* TPx-1 (PkTPx-1). Start and stop codons are underlined and restriction sites are italicized.

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



Hakimi et al. Figure 2