



Expression of Recombinant PfCelTOS Antigen in the Chloroplast of *Chlamydomonas reinhardtii* and its Potential Use in Detection of Malaria

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Abstract

Malaria is a serious but preventable and treatable infectious disease that is found in over 100 countries around the world. Correct and rapid diagnosis of malaria infection can rescue the patient of getting sicker and reduces the risk of disease spreading among humans. *Chlamydomonas reinhardtii* chloroplast is an attractive platform for expressing malaria antigens because it is capable of folding complex proteins, including those requiring disulfide bond formation, while lack the ability to glycosylate proteins; a valuable quality of any malaria protein expression system, since the *Plasmodium* parasite lacks *N*-linked glycosylation machinery. In this study, Cell-traversal protein for ookinetes and sporozoites (CelTOS) antigen from *Plasmodium falciparum* was expressed in the chloroplast of *C. reinhardtii* and a highly sensitive and specific indirect ELISA test was developed using *C. reinhardtii* expressed PfCelTOS to detect malaria. Results obtained demonstrated that expressed recombinant PfCelTOS accumulates as a soluble, properly folded and functional protein within *C. reinhardtii* chloroplast and indirect ELISA using sera from malaria-positive donors suggested the potential use of expressed PfCelTOS as a malaria antigen for diagnosis tests.

Keywords Malaria · Recombinant protein · *Chlamydomonas reinhardtii*

Introduction

Recombinant protein production has widespread uses in industries and medicine. The most widely used protein expression hosts are bacteria, yeast, insect or mammalian systems, each with its own strengths and weakness. Predominantly, *E. coli* is the first host of choice due to extensive knowledge about its genetics, short doubling time, low-cost media, easy genetic manipulation, and high yield of recombinant production. However, *E. coli* system is not capable of performing most post translational modifications and glycosylation found in eukaryotic cells and its cell wall contains toxic compounds [23]. Yeast has proven to be an alternative to bacteria and higher eukaryotic hosts. Yeast expression system has strengths of *E. coli* while offering

some of the advantages of higher eukaryotic hosts. Yeast expression system can perform post-translational modifications; however in most cases recombinant products are highly glycosylated [6]. Insect cell expression system is frequently used in producing recombinant proteins. Insect cell expression systems can produce recombinant proteins at high levels and they also perform significant post-translational modifications; however, protein expression in insect cells is time-consuming and difficult to scale-up [7, 18]. Mammalian systems result in high yield and provide all the post-translational modifications found in complex proteins. Despite of being expensive and difficult to scale-up, most of protein drugs are produced using mammalian cell expression systems [2, 9]. During the past decades, plants have been increasingly considered as alternative recombinant protein expression systems offering several advantages including being considered as Generally Recognized as Safe (GRAS) organisms, low and economic cost of production, recombinant proteins are free from human pathogens, facilities for genetic modification, planting and harvesting are available and finally correct folding of proteins and most post-translational modifications are possible. There are however

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some drawbacks of using plants to produce recombinant proteins such as slow growth and gene flow possibility [12, 25]. Microalgae are an attractive alternative to plant recombinant expression system. A variety of tools and techniques have been developed for genetic engineering of microalgae. Most progress in the field has been made with *Chlamydomonas reinhardtii*, biflagellate unicellular green algae found in fresh waters [8]. *C. reinhardtii* offers several advantages as recombinant expression platform including being considered as GRAS by the FDA, high growth rate, low-cost media, ease of cultivation, the possibility of post-translational modifications, the availability of promoters and markers for genetic modification, no concern of gene flow and contamination with human pathogens and the possibility of engineering its nuclear, chloroplast and mitochondrial genome [8, 13]. Despite the possibility of post translational modifications and glycosylation, nuclear engineering leads to random integration of target gene, positional and epigenetic effects and transgene silencing and yield of recombinant production is generally low [19]. In contrast, chloroplast expression has beneficial attributes, including targeted gene integration, lack of gene silencing, high yield of final product due to robust promoters, and the possibility to perform post translational modifications such as disulfide-bond formation; however, desirable glycosylation is avoided; therefore being ideal for producing complex proteins which exhibit little or no glycosylation [1, 21]. The purpose of this study was to exploit the potential of *C. reinhardtii* chloroplast to express one of the antigens of malaria infection agent, *Plasmodium* parasite, since the *Plasmodium* parasite lacks the N-linked glycosylation machinery. Malaria infection is still widespread in some parts of the world and threatens the lives of millions of people annually. Correct and rapid diagnosis of malaria infection can rescue the patient of getting sicker and reduces the risk of disease spreading among humans. Generally, malaria is diagnosed by the visual examination of Giemsa stained blood slides. Although it is a widely used and recommended technique for malaria diagnosis, this kind of diagnosis is time-consuming and error-prone and is only reliable when performed by skilled personnel. As a result, the remote and poor areas of the world would face major obstacles in precise detection as well as identification of malaria species. Over the last decades, innovative solutions and techniques have been proposed for solving these obstacles. These novel techniques are capable of accurate diagnosis while can be performed by minimally skilled personnel. One of the significant solutions developed by scientists is Malaria diagnosis kits made of malaria antigens or antibodies to plasmodia-specific proteins. For instance, NovaLisa test kit is an ELISA-based new rapid diagnosis test developed for Malaria Diagnosis in an Endemic Area of Thailand. Malaria Pf/Pv Ab Rapid Diagnostic using a Differential Diagnostic Marker is taken as the second example.

Another efficient strategy is diagnosis systems which are based on mathematical morphology. Loddo and colleagues have recently published a comprehensive review of mathematical morphology-based methods which are widely used for malaria detection and identification in images taken from stained blood smears.

Here we used *C. reinhardtii* chloroplast expression system to expressed CelTOS antigen from *P. falciparum* (PfCelTOS) to develop an ELISA test for detection of malaria infection. CelTOS is a required antigen for successful malaria infection with conserved sequence among the five species of the plasmodium that infect human [17]. Thus, a kit composed of this antigen enables detection of the infection with any species of the five plasmodia. PfCelTOS gene fused to FLAG tag sequence was cloned into pASapI vector and transformed to the chloroplast of *C. reinhardtii* by DNA-coated gold particle bombardment and integrated to the plastome through homologous recombination. Transformants were propagated to obtain homoplasmic cells and recombinant protein accumulation was then confirmed by western blotting. The biological conformation of expressed PfCelTOS and its potential as antigen for malaria diagnosis was assayed by indirect ELISA test using immune sera from malaria-positive donors.

Materials and Methods

Synthesis and Cloning of Target Gene

The mature amino acid sequence of PfCelTOS was retrieved from UniProt (PfCelTOS id: Q53UB8). The nucleotide sequence of target fragment with the addition of a C-terminal Flag sequence (5'-GATTATAAAGATGATGATGAC AAA-3') was optimized for expression in *C. reinhardtii* chloroplast and synthesized by Biomatik (Canada). A variety of parameters that are critical to the efficiency of gene expression were included such as *C. reinhardtii* chloroplast codon usage bias, GC content, CpG dinucleotides content, mRNA secondary structure, cryptic splicing sites, premature PolyA sites, internal chi sites and ribosomal binding sites, negative CpG islands, RNA instability motif (AU-rich elements; AREs), repeat sequences (direct repeat, reverse repeat, and Dyad repeat), and finally restriction sites that may interfere with cloning. *SapI* and *HindIII* sites were designed at the 5' and 3'-ends, respectively. The synthesized target gene was inserted in to *SapI/HindIII* sites of pASapI vector (Chlamydomonas Resource Center (<http://www.chlamy.org>), Minnesota University) under the control of *atpA* 5' promoter and UTR and *rbcL* 3' UTR sequences. The pASapI vector uses the rescue of prototrophy in *C. reinhardtii* non-photosynthetic *psbH* mutant strain, instead of bacterial antibiotic resistance gene, as a selectable marker [10]. Recombinant vector was

transformed into chemically competent *E. coli* DH5 α cells (Novagen Inc., Madison, WI) by heat-shock transformation method and verified by colony PCR, restriction endonuclease digestion and DNA sequencing [24].

***Chlamydomonas* Strain, Transformation and Growth Conditions**

C. reinhardtii CC-4388 (*psbH* :: *aadA*, mt+) strain (Chlamydomonas Resource Center (<http://www.chlamy.org>), Minnesota University) was used for transformation. *C. reinhardtii* CC-4388 is a non-photosynthetic *psbH* mutant strain in which homologous recombination results in the replacement of disrupted *psbH* with both a functional *psbH* and gene of interest.

C. reinhardtii CC-4388 strain was grown in TAP (Tris-Acetate-Phosphate) medium at 25 °C in low light (~ 5 $\mu\text{E}/\text{m}^2/\text{s}$) on a rotary shaker to a density of between 8.0×10^5 and 2.0×10^6 cells/ml (to mid log phase) and harvested by centrifugation at 3000 rpm for 5 min at room temperature. Approximately 1.5×10^7 cells were plated on HSM (high salt minimum) agar medium (selective medium) and transformed by particle bombardment [3]. Approximately 3 mg of 0.6- μm gold particles coated with 10–14 μg of recombinant vector was shot with the PDS-1000He particle delivery system (Bio-Rad, Hercules, CA, USA) under chamber vacuum of 28 inches Hg, plate distance of 9 cm, helium pressure of 1750 psi and 1350 psi rupture disc. Following transformation, plates were incubated at 25 °C in low light (~ 1–5 $\mu\text{E}/\text{m}^2/\text{s}$) overnight then moved to high constant illumination (~ 50 $\mu\text{E}/\text{m}^2/\text{s}$) and left for approximately 2–3 weeks to allow the transformed green colonies appeared.

PCR Screening of Transformants

Transformants were subcultured on HSM agar and screened for the presence of gene of interest using gene-specific primers. Cells were resuspended in 10 \times TE (Tris-EDTA) buffer and heated to 95 °C for 10 min. The cell lysate was then used as a template for PCR under standard conditions [22]. *C. reinhardtii* CC-4388 strain was used as untransformed control.

Homoplasmy of phototrophic transformants was assessed by three-primer method, as described by [10]. Briefly, gene-positive colonies were picked and restreaked to single colonies on HSM agar. This step was repeated for at least three times. To prepare cell lysates for PCR, single colonies were picked and resuspended in 25 μl of 10 \times TE buffer. Untransformed colony was used as negative control. Cell suspensions were heated to 95 °C for 10 min and then cooled to 4 °C. A standard PCR reaction using 2 μl of prepared lysates as templates and F/R1 and F/R2 primers (Table 1) was set up. Using this method, homoplasmy

Table 1 Primers which were used in three-primer method

Primer F	5'-GTCATTGCGAAAATACTGGTGC-3'
Primer R1	5'-ACGTCCACAGGCGTCGTAAGC-3'
Primer R2	5'-GATGACGTTTCTATGAGTTGGG-3'

transformants produce a 1.2 kb product only, but heteroplasmic transformants produce both the 1.2 kb product and a 1.0 kb product derived from the *C. reinhardtii* CC-4388 genome. In this way, integration of target gene into the chloroplast genome and homoplasmy is both confirmed. Figure 1 represents a scheme of the designed construct and the insertion site.

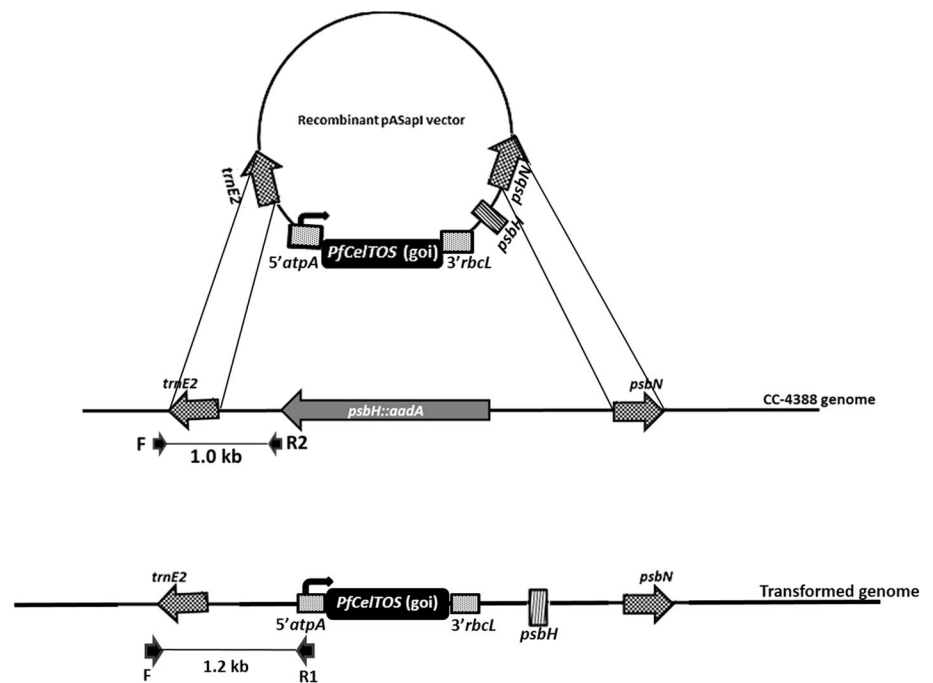
Protein Expression and Western Blot Analysis

Homoplasmy transformants were grown in HSM medium at 25 °C in high constant light (~ 50 $\mu\text{E}/\text{m}^2/\text{s}$) on a rotary shaker. *C. reinhardtii* CC-4388 strain was grown in TAP medium at 25 °C in low light (~ 5 $\mu\text{E}/\text{m}^2/\text{s}$) on a rotary shaker as control. Algal cells were centrifuged to pellet and resuspended in 1 ml lysis buffer per 0.1 g wet algal paste (50 mM Tris pH 8.0, 400 mM NaCl, 0.1% Tween 20, protease inhibitor cocktail (Roche, Germany)) [14]. Cells were then frozen in liquid nitrogen and crushed using a mortar and pestle. The soluble protein fraction was isolated by centrifugation at 13,000 rpm for 15 min and total soluble protein was quantified using a Bradford assay. Equivalent amounts of TSP were then analyzed using western blotting. Proteins were separated on 12.5% SDS-PAGE gel and transferred to nitrocellulose membrane. The membrane was blocked with Skim milk 5% / TBS-T (Tris-Buffered Saline containing 0.5% v/v Tween 20) for 1 h at room temperature and overnight at 4 °C and then washed three times with TBS-T. The nitrocellulose membrane was incubated with anti-FLAG antibody at a concentration of 1:500 for 2 h at room temperature and overnight at 4 °C. The membrane was washed with TBS-T three times and treated using H₂O₂ and DAB solution (10 mM NiCl₂ was also added to enhance the diaminobenzidine colorimetric signal [20]) and placed in darkness to allow the protein bands appear.

Indirect Enzyme-Linked Immunosorbent Assay

To confirm the conformationally correct expression of recombinant PfCeITOS, indirect ELISA was performed using human sera obtained from patients suffering from malaria infection.

Fig. 1 A scheme of the designed construct and the insertion site



Serum Collection

64 positive serum samples were obtained from patients who were residents of malaria-endemic areas in south of Iran. Apart from showing *P. vivax* malaria symptoms, the disease was microscopically confirmed by detecting parasites in Giemsa stained blood films. 64 negative controls were obtained from healthy individuals who had never been exposed to malaria. To check cross reaction, nine samples were collected from patients suffering from kala-azar and Hydatidosis diseases. To define cut-off value, 30 normal sera were collected from healthy residents of malaria endemic areas. All serum samples were collected after receiving individuals' informed consent and frozen at -70°C until used.

Checkerboard Titration

A checkerboard titration experiment was performed to optimize two ELISA parameters; antigen concentration and antibody (sera) dilution. To make different concentrations of antigen, lyophilized TSP extracted from transformant was suspended in sodium carbonate buffer, pH 9.6 (coating buffer) and 50, 100, 150 and 200 $\mu\text{g}/\text{ml}$ stocks of TSP were prepared. Lyophilized TSP extracted from *C. reinhardtii* CC-4388 strain, untransformed parental strain, was used as control. 96-well flat bottom plate was coated with 100 μl of prepared stocks per well and incubated in a humid chamber at 4°C overnight. The plate was washed three times with PBS-Tween 20 (PBS-T). Microscopically positive and negative sera were then diluted to 1/10, 1/40,

1/80, and 1/160 in PBS-T and added to coated wells. Plate was incubated in a humid chamber at 37°C for 1 h and then washed three times with PBS-T. To assess the binding of antibody to recombinant PfCelTOS antigen, 100 μl of 1/1000 dilution of polyclonal anti-human IgG alkaline phosphatase conjugated (DakoCytomation, Denmark) was added to wells. Plate was again incubated for 1 h at 37°C and then washed three times with PBS-T. 100 μl of 1 mg/ml PNP substrate solution (Sigma, St. Louis, Illinois, USA) in Di-Ethanolamine was added to each well and allowed the color to develop at 37°C for 20 min in dark. 50 μl of 3N NaOH solution was finally added to stop the reaction. The optical density (OD) of the samples was measured at 405 nm using ELISA plate reader (Helsinki, Finland). All assays were tested in duplicate. Finally, the best protein concentration and antibody dilution were determined. The desired outcome is usually a dilution where the OD of positive sera has a more significant difference with the OD of negative sera and shows the changes with a more direct ratio.

Cut-off Point Calculation

To define a border between positive and negative sera in the study population, cut-off value was calculated using indirect ELISA with microscopically negative human sera collected from residents of malaria-endemic areas in south of Iran. The cut-off value was calculated as the $\text{mean} + 2\text{SD}$ of OD value.

Assay of Clinical Parameters of Designed Indirect ELISA

To evaluate the sensitivity and specificity of the test, ELISA was carried out using microscopically positive human sera collected from residents of malaria-endemic areas in south of Iran and microscopically negative human sera collected from residents of non-endemic areas in Iran. OD values higher than cut-off were considered positive. The cross reaction with sera collected from other diseases (kala-azar and Hydatidosis diseases) was then investigated.

Statistical Analysis

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and diagnostic efficiency of ELISA compared to microscopy test were calculated as follows:

Sensitivity	$TP/(TP + FN) \times 100\%$
Specificity	$TN/(TN + FP) \times 100\%$
Positive predictive value (PPV)	$TP/(TP + FP) \times 100\%$
Negative predictive value (NPV)	$TN/(TN + FN) \times 100\%$
Efficiency	$(TN + TP)/total \times 100\%$
Validity	$(Sensitivity + specificity)/2 \times 100\%$

TP true positive, *TN* true negative, *FN* false negative, *FP* false positive

The degree of agreement between ELISA and microscopy test was determined by calculating kappa (κ) values with 95% confidence intervals, using the Epi-Info software.

Results

Construction of Transgenic Algal Chloroplasts Expressing PfCelTOS

PfCelTOS sequence flanked by a FLAG affinity tag on the carboxy end of protein was optimized for expression in *C. reinhardtii* chloroplast and synthesized de novo. The synthesized target gene was cloned into pASapI vector, a chloroplast expression vector containing *atpA* 5' promoter and UTR and *rbcl* 3' UTR sequences and using the endogenous photosynthesis gene, *psbH*, as a selectable marker. *C. reinhardtii* CC-4388 strain was transformed with recombinant vector by particle bombardment. The integration of gene of interest and chloroplast homoplasmy was confirmed by PCR as described in material and methods (Figs. 2, 3). TSP was quantified using Bradford



Fig. 2 PCR analysis of *C. reinhardtii* transformants. M: DNA marker; NC: PCR negative control; PC: PCR positive control (vector containing synthesized target gene was used as positive template in PCR). CC-4388 was used as untransformed control. C-TC1/C-TC2/C-TC3: PfCelTOS-FLAG tag—transformed cells. Sharp bands detected in transformants and positive control verified the integration of gene of interest

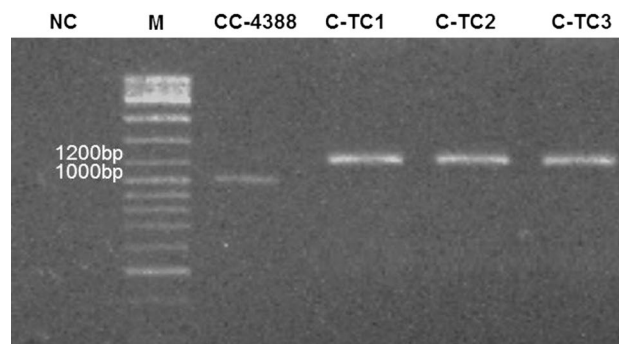


Fig. 3 Chloroplast homoplasmy confirmation by PCR using a mix of three primers. Homoplasmic transformants produced a 1.2 kb product, where CC-4388 produced 1.0 kb product. M: DNA marker; NC: PCR negative control. 1200 bp band detected in transformants verified the chloroplast homoplasmy

assay and protein accumulation was confirmed by Western blotting (Fig. 4). TSP extracted from 100 ml culture in Mid logarithmic phase is reported in Table 2. Furthermore, TSP was monitored over 9 months and the results showed that *C. reinhardtii* chloroplast was an appropriate environment for protein storage leading to stable accumulation of proteins since there was no significant changes in TSP over a 9-month period.

Checkerboard Titration and Cut-off Point Calculation

According to the results of checkerboard titration, 100 μ g TSP/ml of sodium carbonate buffer and 1/10 dilution of sera were determined as the best concentration of antigen and dilution of sera, respectively (Figs. 5, 6). The cut-off point for designed ELISA test was calculated as OD 0.667.

Fig. 4 Western blot detection of recombinant PfCelTOS from total soluble protein extracted from transformants. NC: CC-4388 as negative control. Related band was not detected in C-TC2

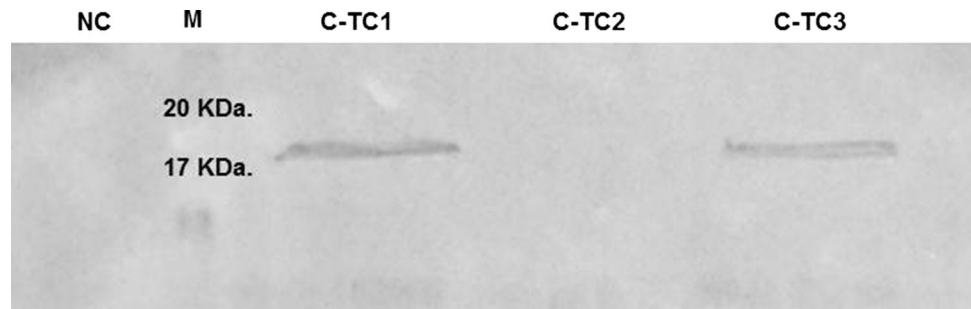


Table 2 TSP extracted from 100 ml culture in Mid logarithmic phase

Transformant	C-TC1	C-TC2	C-TC3
mg/100 ml culture after verifying transformation	67	64.8	62
mg/100 ml culture after 9 months of transformation	62.5	57	58

Assay of Clinical Parameters of ELISA

Comparison between designed ELISA and microscopy results, as a gold standard to detect malaria infection, is shown in Table 3. Diagnostic values including sensitivity, specificity, PPV, NPV, efficiency, and validity are presented in Table 4. It is also necessary to mention that no cross reaction was observed with sera collected from other diseases.

Discussion

Malaria is a serious infection caused by parasites of the *Plasmodium* species and transmitted among humans by the bite of infected female mosquitoes of the genus *Anopheles*. Malaria disease has a large negative impact on economic growth due to the remarkable costs of health care, decreased productivity, loss of investment and tourism and so on [15]. In this regard, malaria control is increasingly an important element to reduce poverty in malaria-endemic countries. Correct and rapid diagnosis can rescue the patient of getting sicker and also can reduce the risk of disease spreading among humans. ELISA could be considered as a reliable and cost-effective technique for the diagnosis of malaria infection in a short time. Nowadays, there are some malaria diagnostic kits based on recombinant proteins which have been developed, distributed and tested in the field. In 2006, Noedl and colleagues tested the sensitivity and specificity of

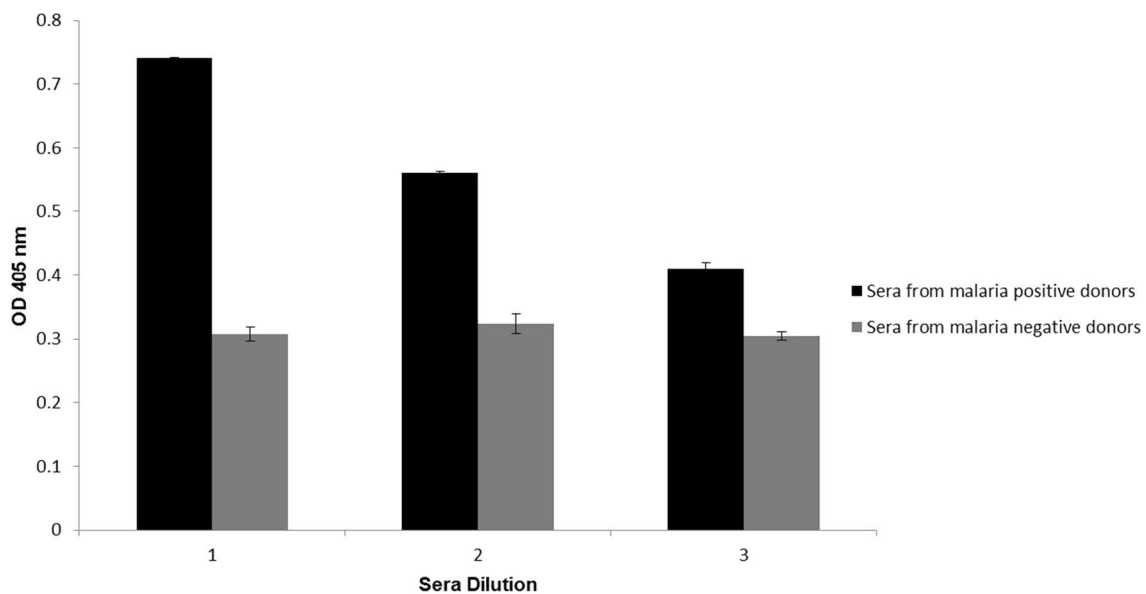


Fig. 5 Immunoreactivity of *C. reinhardtii* CC-4388 extract containing recombinant PfCelTOS with sera obtained from malaria-positive and -negative donors. 100 µg TSP/ml of sodium carbonate buffer and

1/10 dilution of sera were chosen as the best concentration of antigen and dilution of sera, respectively

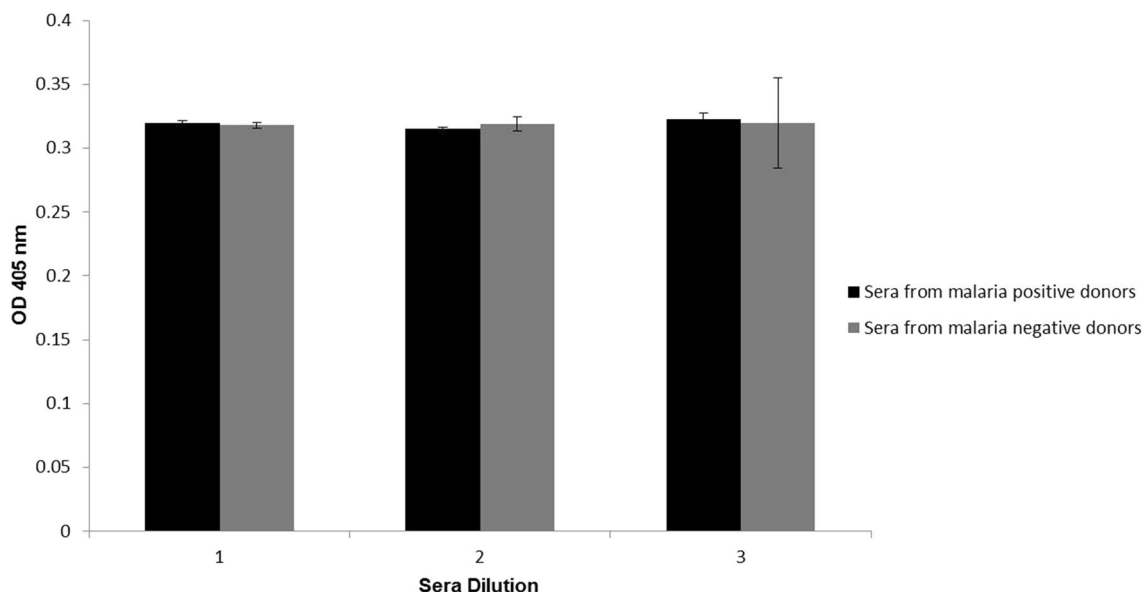


Fig. 6 Immunoreactivity of *C. reinhardtii* CC-4388 extract with sera obtained from malaria-positive and -negative donors. 100 µg TSP/ml of sodium carbonate buffer and 1/10 dilution of sera were chosen as the best concentration of antigen and dilution of sera, respectively

Table 3 Comparison between ELISA and microscopy results

	ELISA+	ELISA–	Total
Microscopy (positive)	78.12%	21.88%	100%
Microscopy (negative)	7.82%	92.18%	100%
Total	42.96%	57.04%	100%

Table 4 Assay of clinical parameters of designed ELISA

Sensitivity	Specificity	Positive predictive value	Negative predictive value	Efficiency	Validity
78%	92%	90%	80%	85%	85%

a commercially available HRP2-based ELISA antigen detection assay for the diagnosis of *P. falciparum*. Based on the results obtained, they reported the overall sensitivity and specificity as well as the positive and NPV of the kit for *P. falciparum* malaria 98.8%, 100%, 100%, and 99.8%, respectively. They concluded that in comparison to microscopy as the gold standard, the results for *P. falciparum* were considerably superior and remarkable and the kit could serve as an excellent alternative method for *P. falciparum* detection. Considering Indirect Fluorescence Antibody Test (IFAT) and ELISA as the most widely used methods for measuring malarial antibody titres, in 2007, Doderer and colleagues assessed the sensitivity and specificity of DiaMed, a new malaria antibody ELISA kit using a combination of crude soluble *P. falciparum* extract and recombinant *P. vivax*

antigens and compared the ELISA kit with IFAT. The results showed that the DiaMed ELISA test had clinical sensitivity and sensitivity of 84.2% and 99.6%, respectively, which in comparison to IFAT method with 70.5% sensitivity and 99.6% specificity, the ELISA method seems more sensitive especially for *P. vivax* infections. In 2008, Kifude and colleagues evaluated the performance of a commercial ELISA kit in the detection of *P. falciparum* Histidine-Rich Protein 2 in Blood, Plasma, and Serum. They proved that PfHRP2 ELISA using whole-blood and serum samples seems to be an acceptable adjunct to microscopy for malaria detection. In a comprehensive study conducted in 2007, the potential of a RDT, an antibody ELISA, and a pLDH ELISA in detecting asymptomatic malaria parasitaemia in blood donors was evaluated and compared together. While the sensitivity (88.0%), specificity (99.1%), and NPV (99.0%) of the RDT were noticeably higher than the ELISAs, the pLDH ELISA had the highest PPV (91.6%) and demonstrated a generally comparable performance to the RDT. The lowest sensitivity (69.9%) as well as the highest false positive and false negative rates were related to the antibody ELISA.

In an attempt to develop a sensitive and inexpensive detection test, we decided to develop an ELISA detection test with algae-produced PfCelTOS antigen. The chloroplast of *C. reinhardtii* is an attractive platform for expressing malaria antigens because it is capable of folding complex proteins, including those requiring disulfide bond formations, while lack the ability to glycosylate proteins. Therefore, *C. reinhardtii* chloroplast is an ideal system for expressing complex proteins with little or no glycosylation such as proteins of *Plasmodium* parasite. For example, although Pfs25 and

Pfs28 are complex but non-glycosylated proteins of *P. falciparum* that their expression in eukaryotic systems has led to incorrect conformations and also glycosylation of them, they were successfully expressed in *C. reinhardtii* chloroplast [14]. *P. falciparum* surface protein Pfs48/45 C-terminal domain is another example which was also produced in *C. reinhardtii* chloroplast; however, it accumulated mostly in the insoluble fraction [16]. *Plasmodium* parasite has two hosts during its complex life cycle, a mosquito and a vertebrate. *Plasmodium* invasion into both hosts is mediated by a protein called Cell-traversal protein for ookinetes and sporozoites (CelTOS). CelTOS protein is required for successful malaria infection. Moreover, CelTOS is highly conserved among the *Plasmodium* species indicating its vital role in malaria infection [17]. The potential of CelTOS as malaria pre-erythrocytic vaccine candidate and its cross-species protection has been proved in previous studies [4, 5, 11]. Here, we successfully expressed PfCelTOS in the chloroplast of *C. reinhardtii* and investigated its potential as an antigen in malaria detection test. Conventionally, staining of peripheral blood and microscopy examination for malaria infection is adopted as the gold standard. A total of 100% microscopy positive sera, 78.12% showed positive and 21.8% showed negative responses in designed indirect ELISA test. The high percent of positive responses indicates the correct folding of recombinant PfCelTOS in *C. reinhardtii* chloroplast and also confirms its protected structure among *Plasmodium* species. The false negative responses can be due to the reason that the patients were probably in the early stages of infection and antibody titer for detection in ELISA test was low, however, microscopy test, as the gold standard method for malaria detection, was able to rapidly detect parasite in blood. Of 100% microscopy negative sera, 92.18% showed negative response in ELISA and only 7.82% percent showed false positive response which can be due to reaction with other factors of sera. The degree of agreement between ELISA and the microscopy was determined by calculating kappa (κ) value. Our results showed that there is a substantial agreement in the results between microscopy, as a standard method, and ELISA using *C. reinhardtii* extract containing recombinant PfCelTOS for malaria detection. Moreover, there was no cross reaction between *C. reinhardtii* CC-4388 extract containing recombinant PfCelTOS with sera collected from other diseases indicating specific immunoreactivity of recombinant PfCelTOS with sera from malaria patients. As compared to the sensitivity and specificity of some commercial kits and taking into account the remarkable benefits of PfCelTOS (cross-species protection with no cross reactivity with other diseases), the algae-produced PfCelTOS antigen seems to have the potential to be used for developing an ELISA detection test for malaria infection. It should be mentioned that in this study the recombinant antigen was not purified and the crude extract of algae cells

was used in ELISA test. By extracting the antigen, better results could be definitely obtained.

Finally, since PfCelTOS is proved as malaria pre-erythrocytic vaccine candidate and on the other hand *C. reinhardtii* is GRAS and can be used in oral vaccination, our project can continue to investigate the potential of transformants in malaria oral vaccination.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with animals performed by any of the authors.

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