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Research paper

A chemiluminescent-western blot assay for quantitative detection of *Plasmodium falciparum* circumsporozoite protein

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ABSTRACT

Highly sensitive and reliable assays based on the quantitation of immunologically relevant component(s) in recombinant or whole parasite-based vaccines would facilitate pre-clinical and clinical phases and the monitoring of malaria vaccine deployment. Here we report a laboratory-grade Western Blot assay for quantitative detection of *Plasmodium falciparum* circumsporozoite protein (*PfCSP*) in *P. falciparum* sporozoite (*PfSPZ*) and in recombinant (*rPfCSP*) product. This assay is based on the immuno-reactivity of an anti-*P. falciparum* CSP monoclonal antibody (mAb 2A10) with the NANP-repeat units on *PfCSP*. The antigen–antibody complex is detected by reaction with a commercially obtained chemiluminescence-linked Immunodetection system. The linear range for detecting the recombinant *P. falciparum* CSP (*rPfCSP*) in this assay is 3–12 pg ($R^2 = 0.9399$). The range for detecting the day 15 salivary-gland *PfSPZ* is between 0.0625 and 1 parasite ($R^2 = 0.9448$) and approximately 10.0 pg of *PfCSP* was detected on each sporozoite. The assay was highly reproducible in measuring the *PfCSP* on *PfSPZ*. The inter-assay Coefficient of Variation (CV%) was 10.31% while the intra-assay CV% on three different days was 6.05%, 2.03% and 1.42% respectively. These results suggest that this ECL-WB assay is highly sensitive and robust with a low degree of inter-assay and intra-assay variations. To our knowledge, this is the most sensitive immunoassay for the detection of a recombinant or native malarial protein and may have a wider range of applications including the quantification of immunological component(s) in a vaccine formulation, determination of the antigenic integrity in adjuvanted-vaccine and in stability studies. In addition, this assay can be applied to measure the mosquito infectivity in malaria transmission areas and to determine the effects of intervention measures on malaria transmission.
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1. Introduction

A quantitative method for sensitive and reproducible detection of immunologically active vaccine component(s) based on reactivity with immuno-dominant, protective epitope(s) would support the pre-clinical and clinical development of candidate malaria vaccines. Vaccines against pre-erythrocytic stage malaria rely on blocking the release of merozoites, the infectious liver form parasites from entering into the blood stream and thus preventing the pathogenesis-causing blood-stage life cycle. Currently the leading anti-pre-erythrocytic stage vaccine candidates for *Plasmodium falciparum* malaria incorporate the circumsporozoite protein (CSP) as the major protective antigenic component (Hill, 2006). CSP constitutes approximately 90% of the malaria sporozoite surface, and antibodies to the central repeat region on CSP are

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known to neutralize the virulence of malaria sporozoites *in vitro* and *in vivo* (Potocnjak et al., 1980; Ballou et al., 1985; Young et al., 1985; Zavala et al., 1985b; Egan et al., 1987). To date, more than a dozen recombinant or synthetically constructed CSP-based vaccines have undergone clinical testing, with RTS, S, currently in Phase III trials in infants in seven countries in Africa being the most advanced (Agnandji et al., 2011).

Across all *Plasmodium* species, CSP shares a similar structure and can be divided into three distinct domains of equal length. Of these, the central domain is comprised of highly immunogenic repeating amino acid units. The amino acid sequence length and number of repeat units are unique for each *Plasmodium* species (McCutchan et al., 1996). In *P. falciparum* the repeat is comprised of 37 to 41 copies of the major repeat sequence NANP and four copies of a minor repeat NVDP. A salient feature of the *PfCSP* repeats is that while the number of repeat units may vary, their amino acid sequence is conserved in all *P. falciparum* isolates sequenced (Weber and Hockmeyer, 1985; Zavala et al., 1985a; Rich and Ayala, 2000; Zeeshan et al., 2012).

Assays that measure the biologically relevant and non-variant epitopes in malaria vaccine constructs are urgently needed. From that perspective, based on its immunodominant and conserved nature, *P. falciparum* CSP repeats are an excellent target to measure the amount of *PfCSP* in recombinantly expressed products and malaria sporozoites. Several high-affinity and protective monoclonal antibodies that recognize the *PfCSP* repeat units are available (Wirtz et al., 1987; Burkot et al., 1991).

In this paper, we report a highly sensitive enhanced chemiluminescent-based, quantitative Western Blot for the detection of *PfCSP* on day 15 *P. falciparum* salivary gland sporozoites and in *Escherichia coli* expressed protein. This assay is based on the immuno-reactivity of mAb 2A10 that recognizes the NANP repeat on *PfCSP* followed by protein band visualization by incubation with a chemiluminescent detection system. The band intensity was measured as integrated optical density (IOD). IOD values obtained from recombinant or sporozoite-produced, native *PfCSP* were fitted by using a non-linear regression to generate a standard curve which was then used to convert the IOD values from a test sample into the quantitative measure of *PfCSP*. We also present data on assay reproducibility measured as inter-assay and intra-assay variations.

2. Materials and methods

2.1. Recombinant P. falciparum CSP

A recombinant plasmid encoding the *P. falciparum* CSP amino acid sequence 27–123[NANPNVDP]$_3$[NANP]$_{21}$300–411 was transformed into *E. coli* BL21 (λDE3) (Life Technologies, C6000-03; Grand Island, NY) that allowed periplasmic protein expression under the control of lac promoter. The recombinant CSP was purified on a heparin sepharose affinity column and fractions were pooled and further purified to homogeneity on a gel filtration column. The purified protein gave a single band on SDS-PAGE and the NH_2-terminal amino acid sequencing confirmed that the purified protein was *PfCSP* (data not shown).

2.2. Production of P. falciparum sporozoites

Three to five day old female *Anopheles stephensi* mosquitoes were membrane fed with *P. falciparum* (NF54) gametocytes cultured using human erythrocytes and serum. Cages containing gametocyte fed mosquitoes were maintained at 26 °C, 80% relative humidity. Eight to nine days after feeding, eight mosquitoes from each cage were taken out and their midguts were dissected in order to count the oocysts. Remaining mosquitoes in each cage were maintained until day 15 and the infected mosquitoes were dissected to harvest the sporozoites (Mlambo et al., 2010).

On day 15 after the gametocyte feed, mosquitoes from all cages were pooled and distributed in roughly equal numbers to four different cages, then transferred to −20 °C freezer for 20 min. When the mosquitoes were found to be inactivated completely, they were dipped in 70% ethanol and transferred to a Petri dish containing RPMI-1640 medium (Thermo Scientific — HyClone Classical Liquid Media — RPMI 1640, SH30255 01 — MA; Rockford, IL) with 5% human serum and 1× penicillin — streptomycin (Life Technologies, 15140122; Grand Island, NY). The thorax of each mosquito was cut anterior to the wings and the anterior part was suspended in 50 μL of RPMI-1640 and centrifuged through a sterile column of glass wool in a microcentrifuge tube for 1 min at 13,000 rpm twice. The column was rinsed with 50 μL of RPMI-1640.

PfSPZs were further purified on a discontinuous Renografin-60 (Bracco Diagnostics, NDC 0270-0707-50; Irvine, CA) gradient following centrifugation for 20 min at 10,000 rpm at 4 °C (Pacheco et al., 1979). The inter-phase was removed and mixed with 25 ml of complete RPMI-1640 medium and centrifuged for 10 min at 10,000 rpm and 4 °C. The pellet containing *P. falciparum (PfSPZ)* was suspended in complete RPMI-1640 medium. *PfSPZ* count was determined in a hemocytometer with light-microscopy. *PfSPZ* counts were determined by two independent operators for accuracy.

2.3. Preparation of P. falciparum sporozoites and recombinant PfCSP for the enhanced chemiluminescence-Western Blot

A stock solution of 1000 *PfSPZ*/μL in protein gel loading solution was prepared and heated at 100 °C for 5 min. The *PfSPZ* lysate was centrifuged at 13,000 rpm for 2 min and supernatant was collected. A 10-fold serial dilution of the *PfSPZ* lysate was made to maintain the equivalence of *PfSPZ* counts between 1 and 10,000 sporozoites per 10 μL of sample volume. *E. coli* produced recombinant *P. falciparum* CS protein (*rPfCSP*) was diluted to a stock concentration of 1 ng/10μL using 2× SDS-PAGE gel loading dye (Quality Biological INC, 351-082-011; Gaithersburg, MD) and then further two-fold serial dilutions were prepared. The protein concentration of the stock *rPfCSP* was determined by using a Bradford Protein Assay (Thermo Scientific, PI-23200; Rockford, IL).

2.4. Anti-P. falciparum CSP mAb 2A10

This mouse monoclonal antibody recognizes the repeat NANP sequences within the *P. falciparum* CSP and was used for the Western blot studies. The hybridoma expressing 2A10 monoclonal antibody was procured from MR4/ATCC, Virginia (Nardin et al., 1982). The antibody was generated as ascitic