Antibodies elicited during natural infection in a predominantly *Plasmodium falciparum* transmission area cross-react with sexual stage-specific antigen in *P. vivax*

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Abstract

Infections caused by *Plasmodium falciparum* and *P. vivax* account for more than 90% of global malaria burden. Exposure to malaria parasite elicits immune responses during natural infection and it is generally believed that the immunity is not only stage specific but also species specific. However, partial genomic similarity for various antigens in different *Plasmodium* spp. raises the possibility of immunological cross-reactivity at the level of specific antigens. Serum samples collected from children who were permanent residents of a *P. falciparum* transmission area in Zimbabwe were screened for antibody reactivity against Pfs48/45, a *P. falciparum* gametocyte antigen and Pvs48/45, a *P. vivax* homolog of Pfs48/45 using ELISA. Western blotting was used to further confirm identity of the specific antibody reactivity to the Pfs48/45 and Pvs48/45 proteins. Pan *Plasmodium* PCR and nested PCR were used to confirm infection with the *Plasmodium* species. Twenty-seven percent (49/181) of the participants were found to be sero-positive for Pfs48/45 and 73% (n=36) of these Pfs48/45 positive sera also showed reactivity with Pvs48/45. Immune cross-reactivity revealed by ELISA was also confirmed by Western blot analysis using a panel of randomly selected 23 Pfs48/45 and Pvs48/45 ELISA positive samples. Nested PCR analysis of 27 blood samples randomly selected from the 36 that showed positive ELISA reactivity to both Pfs48/45 and Pvs48/45 antigens confirmed infection with *P. falciparum* and generalized absence of *P. vivax* except for a single sample which revealed PCR positivity for both *P. vivax* and

P. falciparum. Our studies with sera samples from a predominantly *P. falciparum* transmission area in Zimbabwe suggest immunological cross-reactivity with Pvs48/45, thus raising the possibility of partial species cross-reactive immunity and possible cross-boosting of immunity during co-infection with *P. falciparum* and *P. vivax*.

Keywords

Cross reactivity; *Plasmodium falciparum*; *Plasmodium Vivax*; antibody responses

Introduction

Current estimates indicate 95 countries and territories as having ongoing malaria transmission (WHO, 2015) in spite of a world-wide reduction in malaria incidence among populations at risk. About 50% of the world's population is at risk globally among whom, young children, pregnant women and non-immune travellers are especially vulnerable. According to the WHO, out of an estimated 247 million malaria cases, about 88% of malaria cases and 90% of malaria deaths occur in sub-Saharan Africa.

Malaria is transmitted by female *Anopheles* mosquitoes and is caused by a protozoan of the genus *Plasmodium*. Four well-established *Plasmodium* species infect humans, *P. vivax*, *P. malariae*, *P. falciparum* and *P. ovale*; of these *P. falciparum* is most prevalent in sub-Saharan Africa while *P. vivax* dominates outside of sub-Saharan Africa. *P. falciparum* and *P. vivax* are endemic and coexist in many countries (Mueller et al., 2009) and account for more than 90% of global malaria burden (WHO, 2015) and in eastern and southern Africa, *P. vivax* represents around 10% of malaria cases. *P. falciparum* is responsible for most malaria-related deaths globally, and malaria caused by *P. vivax* is considered relatively benign, though sometimes causing life-threatening outcomes (Guerra et al., 2010; Price et al., 2007). Additionally, human infection with *P. knowlesi* have also been detected in some areas and it is believed to be due to zoonoses (Antinori et al., 2013). It is generally accepted that immune responses against the malaria parasite are by and large specific to the infecting species with little known cross reactivity at least between the major human parasites *P. falciparum* and *P. vivax* (Clyde, 1990; Nussenzweig and Chen, 1974; Nussenzweig et al., 1972). However, some studies have reported on cross-reactivity of few antigens specific to pre-erythrocytic and erythrocytic asexual stages of multiple *Plasmodium* species. This includes: (i) recognition of *P. falciparum* asexual stage antigens by antibodies in sera from people exposed to *P. vivax*, (Kumar et al., 1992), and suppression of *P. falciparum* by sera from a case of *P. vivax* (Nagao et al., 2008), (ii) recognition of circumsporozoite protein (CSP) of *P. falciparum* and *P. berghei* by antibodies elicited by VMP001, a *P. vivax* CSP-based vaccine (VMP00) (Yadava et al., 2012), (iii) induction of cross-reactive and cross-protective antibodies by PfCelTOS, a highly conserved cell-traversal protein expressed on the surface of ookinetes and sporozoites (Bergmann-Leitner et al., 2010), and (iv) reported cross reactivity between apical membrane antigen 1, cytoadherence-linked asexual gene 9 product and merozoite surface protein 5 of *P. falciparum* and *P. vivax* (Costa et al., 2013; Igonet et al., 2007; Woodberry et al., 2008).

A search of the genome sequences (www.plasmodb.org) indicates that there are several antigens in the sexual stages which share significant sequence similarity, however the question of possible immunological cross-reactivity has remained a matter of conjecture. The availability of recombinant proteins offers an opportunity to test it directly. More recently, we have shown cross reactive immunity of murine sera raised against Pfs48/45 and Pvs48/45, as well as cross boosting of immune responses against heterologous P48/45 (Cao et al., 2016). Pfs48/45, a protein produced during *P. falciparum* gametocyte development, is expressed on the surface of male and female gametes and zygotes and plays a key role in male gamete fertility and sexual development. Monoclonal antibodies and polyclonal antibodies against recombinant Pfs48/45 expressed in *E. coli* have been shown to prevent zygote development and reduced oocyst formation in the mosquito midgut (Chowdhury et al., 2009; Outchkourov et al., 2007; Outchkourov et al., 2008; Rener et al., 1983; van Dijk et al., 2001). Comparison of Pfs48/45 and Pvs48/45 reveals 61% and 55% identity at the level of DNA and protein sequences, respectively; arguing in favour of antigenic and possibly functional immune cross-reactivity between Pfs48/45 and Pvs48/45. The availability of *E. coli* – expressed recombinant Pfs48/45 and Pvs48/45 allowed us to investigate the immune cross reactivity using human sera from a primarily *P. falciparum* endemic region in Zimbabwe. In Zimbabwe *P. falciparum* is the major parasite species responsible for ~100% total malaria cases with no officially documented transmission of *P. vivax* (WHO, 2015). We reasoned that recognition of Pvs48/45 antigen by sera from individuals resident in *P. falciparum* endemic Zimbabwe will likely to be due to recognition of cross-reactive epitopes by antibodies elicited by Pfs48/45 during natural exposure to *P. falciparum*, and not due to a prior *P. vivax* infection. Demonstration of any immunological cross-reactivity at the level of key vaccine targets is further suggested to likely influence natural dynamics of malaria transmission in the areas co-endemic for *P. falciparum* and *P. vivax*.

Materials and Methods

Study area and participants

The study was first reviewed by University of Zimbabwe Institutional Review Board and ethically approved by the Medical Research Council of Zimbabwe (MRCZ/A/1710). The study samples from students attending Bemberi primary school in Mt Darwin district in north eastern Zimbabwe were obtained from an ongoing study on schistosomiasis and malaria co-infection in an endemic area of Zimbabwe (Midzi et al., 2014).

The samples were collected during the malaria transmission season, between February and May. The study design comprised of a cross sectional survey of 203 children aged 6 to 14 and attending school in 2013 with a second follow-up survey and sample collection in 2015. Permission was obtained from the community leaders, provincial medical director, and district medical officer. Parents provided consent for participation of the children and the children signed an assent form after learning the aims, risks and benefits of the study. The primary inclusion criterion for the study included willingness to provide all required samples for parasitology and serology and those children who visibly appeared malnourished or presenting with any unrelated illness were excluded from participation in the study. All the children provided a stool and urine specimen over three consecutive days for detection of

schistosomiasis and soil transmitted helminth. Blood specimen were collected on the third day for malaria examination and plasma for immunological investigations. Children positive for any parasitic infection based on urine and stool microscopy received standard treatment of praziquantel at 40 mg/kg body weight and albendazole for the soil transmitted helminths infection. Prevalence of *S. haematobium* determined in 2013 and 2015 surveys was 33/121 and 31/80, respectively. Prevalence of malaria in the study subjects was 6/121 in 2013 and the target district reported *P. falciparum* malaria prevalence of 6.6/100 in 2015, as compared to much higher (>16.0/100) prevalence in the surrounding districts and the area ranks 9th out of top 20 malarious districts in Zimbabwe. The packed blood cells and plasma samples were kept frozen at −20°C in the field at a health facility then transported to the laboratory and kept at −80°C until used for the assays.

Rapid diagnostic tests and blood smear microscopy

Immediately upon collection, blood samples were tested for the presence of parasites using rapid malaria diagnostic test (Paracheck™). Thin and thick blood smears stained with 10 % Giemsa were prepared for detection of *P. falciparum* infection by microscopy. Children found to be positive by either rapid diagnostic test or microscopy were treated immediately at the local health facility with artemisinin-lumefantrine combination. Blood smears were re-examined by an expert microscopist from the National Institute of Health Research in Harare, Zimbabwe for quality assurance.

Recombinant antigens

Details of production, purification and immunogenicity evaluation of recombinant Pfs48/45 and Pvs48/45 are described elsewhere (Cao et al., 2016; Chowdhury et al., 2009). The protein purity was assessed by SDS-PAGE and Western blotting, and the protein concentration was measured by the bicinchoninic acid (BCA) method (Thermo Scientific).

ELISA

Plasma samples were analyzed by ELISA as described previously (Cao et al, 2016) to screen for antibody cross reactivity between Pfs48/45 and Pvs48/45. Briefly, ELISA plates (Immulon 4 HBX) were coated with 100 μl/well of 1 μg/ml antigen in 0.1 M carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C. After coating, the plates were washed three times with PBS 0.05% Tween 20 (wash buffer) and blocked with 5% non-fat milk in PBS for 1 hour at 37°C and washed three times with the wash buffer. Plasma samples were diluted 1:500 in the wash buffer containing 5% non-fat milk, and tested in duplicate. After incubation at 37°C for 1h in a shaking incubator, unbound antibodies were removed by three washes with wash buffer. Bound antibodies were detected with peroxidase-conjugated goat anti-human IgG and IgM (KPL; 1mg/ml stock diluted 1:5,000). The secondary antibody was allowed to react for 1 hour and then wells were washed three times with wash buffer. The amount of antibodies bound was quantified by adding 100μl ABTS peroxidase substrate (KPL) and the color development was measured after 20 minutes (VersaMax Elisa Reader, Molecular Device). A positive control was included on each plate to normalize readings of samples from plate to plate. Briefly, for any given ELISA, positive control readings were averaged for all the plates and used to find the differential for positive control for any given plate to apply as a correction factor for sample readings on the corresponding plate (Clark et

al., 2012). The negative cut-off was determined by averaging the negative control readings (pooled sera from 10 North American donors with no prior history of malaria or travel to malaria endemic region) and adding 2 standard deviations.

SDS-PAGE and Western blot analysis

SDS-PAGE and Western blotting was used to confirm antibody cross-reactivity between Pfs48/45 and Pvs48/45 *Plasmodium* protein antigens. Protein samples reduced with 5% 2-mercaptoethanol were fractionated by 10% SDS-PAGE and blotted on to nitrocellulose membrane. The blots were incubated with PBS containing 0.05% Tween 20 and 2% goat serum to block any non-specific sites and incubated with 1:200 dilution (PBS plus 0.01% tween 20, 2% goat serum) of test human sera for 2 hours. After washing with PBS containing 0.05% Tween 20, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-human IgG (1: 5,000) or anti-human IgM (1:20,000) and finally developed using ECL Prime Western blotting detection reagent (GE Healthcare).

DNA preparation and molecular diagnosis

Parasite DNA was extracted from 300 μl whole blood using the Qiagen FlexiGene DNA kit. DNA was suspended in 200 μl elution buffer and stored at −20°C. A nested PCR protocol (Mahajan et al., 2012) was used for molecular diagnosis of *P. falciparum* and *P. vivax*, First PCR employed pan-*Plasmodium* primers; PaF (forward) 5′-GAACGAGATCTTAACCTGCTAA-3′, and PaR (reverse) 5′-TCAGCACAATCTGATGAATCAT-3′. Forward and reverse primer pairs used for nested PCR were PfnF 5′-ACATAGGTAACTATACATTTATTCAGT-3′ and PfnR 5′-AGCATCAAAGATACAAATATAAGCA-3′ for *P. falciparum;* and PvnF 5′-GTGGGACTGAATTCGGTTGA-3′ and PvnR 5′-TACGAAACAGCAAGCTGAATCG-3′ for *P. vivax*. All the primers were reconstituted to a final concentration of 10 mM and stored at −20°C. The pan *Plasmodium* PCR mixture (50 μl) consisted of the following: 1 μl each of forward and reverse primers, 5 μl PCR 10X buffer (500 mM Tris.HCl, 20 mM MgCl2, 100 mM KCl, 50 mM (NH4)2SO4, pH 8.3), 1 μl 10 mM dNTPs, 0.5 μl *Taq* DNA polymerase, and 3 μl genomic DNA. For *Plasmodium* species–specific nested PCR, 1 μl of amplified pan PCR product was used as the DNA template and species specific pairs of primers. PCR was carried out using the PCR system (Gene Amp 9700, Perkin-Elmer, Waltham, MA) thermal cycler. The following PCR conditions were used: for pan *Plasmodium*-3 minutes of denaturation at 94°C, 40 cycles of 30 seconds at 94°C, 30 seconds at 57°C, and 30 seconds at 72°C, with a final extension time of 7 minutes at 72°C; and for nested PCR-3 minutes of denaturation at 94°C followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 30 seconds at 72°C, with a final extension time of 7 minutes at 72°C. Purified genomic DNA from *P. falciparum* (NF54 maintained in culture) and *P. vivax* (Sal 1 strain dried blood spots on filter papers obtained from MR4) were used as positive and negative controls to establish specificity of nested PCR. *P vivax* genomic DNA served as a negative control for *P. falciparum* nested PCR and *P. falciparum* genomic DNA as negative control for P vivax nested PCR. Amplified products were analyzed using 2% agarose gel and staining with ethidium bromide and photographed using BioRad GelDoc XR+.

Results

Analysis of sera for antibodies against Pfs48/45 and Pvs48/48 by ELISA

Sera/plasma samples collected in 2013 and 2015 from children attending Bemberi primary school in North-Eastern regions of Zimbabwe were analyzed for antibodies against Pfs48/45 protein. Afterwards, sera were tested by ELISA for recognition of Pvs48/45 antigen. ELISA with Bemberi 2013 and 2015 sera (n=181) screened for reactivity against *P. falciparum* using Pfs48/45 revealed 27 % (n=49) positivity (27.03% positive in 2013 and 27.24% positive in 2015). A pool of N. American normal human sera from ten donors with no past history of travel to malaria endemic areas was used as a negative control and used to assign a cut-off based on mean +2SD (0.26 for Pfs48/45 coated ELISA plates) to identify positive sera. ELISA results against Pfs48/45 for sera collected in 2013 and 2015 from participants are shown in Figs. 1A and 1B, respectively. The Pfs48/45 ELISA positive samples (n=49) were then screened for reactivity against *P. vivax* antigen Pvs48/45, together with an additional 31 other randomly selected samples to give a total of 80 samples. The negative control cut-off for Pvs48/45 was 0.19 and sixty samples were positive for Pvs48/45 positive by ELISA (Fig. 1A and B). Among Pfs48/45 positive samples a total of 36 (86% samples from 2013 collection and 61% from 2015 collection) showed positive reactivity with Pvs48/45 giving an overall average of 73%. Approximately a similar percentage and low ELISA positive reactivity was also observed for 24/31 randomly selected samples.

SDS-PAGE and Western blot analysis

Specific human lgG and lgM responses to *P. falciparum* antigen Pfs48/45 and *P. vivax* antigen Pvs48/45 was confirmed in 23 randomly selected samples (ELISA positive for Pfs48/45 and Pvs48/45) using SDS-PAGE and western blot analysis using recombinant Pfs48/45 and Pvs48/45. Serum samples that produced double bands or a single band of apparent molecular weight of 50 kDa were considered to have anti-Pfs48/45 and anti-Pvs48/45 antibodies. Figs. 2A and 2B show Western blot reactivity of 14 representative samples to reduced forms Pfs48/45 and Pvs48/45 proteins, respectively. These studies also revealed some discrepancies: one ELISA positive sample (#33) was negative by Western blot analysis for both Pfs48/45 and Pvs48/45 and a second sample (#34) that was ELISA reactive with both antigens did not recognize Pvs48/45 by Western blotting.

PCR detection of human *Plasmodium* species

Pan *Plasmodium* PCR and nested PCR were used to identify *Plasmodium* species using DNA extracted from whole blood samples (n=27) available for individuals who had revealed ELISA positivity for both Pfs48/45 and Pvs48/45. Pan-*Plasmodium* PCR sense and antisense primers used were designed based on 18s ribosomal RNA gene that has 100% sequence homology for *P. falciparum* and *P. vivax*. In order to detect species of *Plasmodium*, primers specific for *P. falciparum* and *P. vivax* were used in a second nested PCR amplification. PCR amplification using *P. falciparum* and *P. vivax* species specific primers gave specific amplicons of expected size (Figs. 3A and 3B for *P. falciparum* and *P. vivax*, respectively). PCR analysis revealed 24 out of 27 participants positive for *P. falciparum*, and only one participant revealed a PCR band for *P. vivax*. This individual also showed *P. falciparum* specific band, suggesting co-infection with both species. One sample was found

to be negative by nested PCR for both *P. falciparum* and *P. vivax*. The specificity of nested PCR was established using standard *P. falciparum* (lab grown NF54 isolate DNA) and *P. vivax* (Sal 1 strain from MR4).

Discussion

The most widely distributed malaria parasite in the world is *P. vivax*, but it was believed to be "absent" from Africa, because of the fact that majority of the Africans are Duffy negative. The high prevalence of Duffy negativity in Africa provided a rationale for excluding the possibility of *P. vivax* transmission in Africa, since the Duffy antigen is the only known receptor for *P. vivax* infection. However, a recent paper (Howes et al., 2015) reviewed evidence sources such as local clinical case reports, entomological and serological studies that contradicts this general perspective. Other reports also described the presence of *P. vivax* parasites in Duffy negative individuals in Africa and Latin America (Cavasini et al., 2007; Ryan et al., 2006), and it has been proposed that the parasite is in the process of developing mechanisms that permit the infection of people that do not express the Duffy antigen on their red blood cells. Such findings highlight the requirement for a vibrant study of the prevalence and population dynamics *of P. vivax* in Africa, using accurate molecular species diagnostic tools (Culleton et al., 2008).

P48/45 proteins are highly conserved among *Plasmodium* species. Pvs48/45 shares 70.4% identity at the level of DNA sequence with other *Plasmodium* species. Greater homology is observed between *P. vivax* and *P. knowlesi* (84.0%), whereas *P. falciparum* shares 60.08 % homology with *P. vivax* at the level of DNA sequence, and 55% at the level of protein sequence (Arévalo-Herrera et al., 2015). Availability of recombinant Pfs48/45 and Pv48/45 proteins offered the opportunity to investigate the question of immune cross-reactivity between *P. falciparum* and *P. vivax* using human sera from a known, low to moderate *P. falciparum* transmission area. The cross-species immune reactivity was observed by the fact that 73 % anti-Pfs48/45 ELISA positive sera also reacted with recombinant Pvs48/45. A previous study using plasma from acute malaria infected patients living in central China where *P. vivax* is endemic has described cross reactivity of antibodies against two *P. vivax* antigens with *P. falciparum* homologous proteins, (Xia et al., 2015). They found an elevated level of antibodies to total *P. vivax* proteins and low levels of antibodies to total *P. falciparum* proteins in acute *P. vivax* infected samples, suggesting antibody cross reactivity between *P. vivax* and *P. falciparum* antigens.

In order to experimentally rule out local prevalence of *P. vivax* in our study area nested PCR detection method was used to analyze the parasite species in the blood samples evaluated by ELISA and Western blotting, As shown, 25 out of 27 samples analyzed by nested PCR showed the presence of *P. falciparum* infection and only 1 out of 27 samples showed presence of both *P. falciparum* and *P. vivax* specific PCR bands. Based on these results, we believe it will be incorrect to state that *P. vivax* is completely absent in Zimbabwe. However, propose that there is always a possibility that low level undocumented concurrent *P. vivax* infection may account for the apparent observed cross-reactivity demonstrated by our findings. However, it seems more reasonable to conclude that the higher sero-prevalence of Pvs48/45 positivity is more likely a reflection of cross-reactivity of antibodies elicited by

predominant *P. falciparum* infection in Zimbabwe. Tables 1 and 2 summarize data on reported cross-reactivity by ELISA and Western blot analysis and apparent lack of *P. vivax* infection by PCR analysis.

Officially, malaria in Zimbabwe is almost exclusively attributed to *P. falciparum*. Current diagnostic, treatment and surveillance systems in much of sub-Saharan Africa including Zimbabwe are not designed to identify or report non *P. falciparum* human malaria infections accurately. Recent reports on high occurrence of *P. vivax* in some parts of Africa, south of Sahara, it is possible that both Duffy negative and 1–5% of the human population who are Duffy positive might maintain the low level transmission of *P. vivax* (Culleton and Carter, 2012). In order to ascertain exposure of serum-donors to *P. vivax* malaria the reactivity of antibodies to Pvs48/45 protein was determined in the test serum by ELISA and Western blotting. Human IgG and IgM reacted with both Pfs48/45 and Pvs48/45 as shown by the ELISA data and Western blots suggesting that there might be undetected or underappreciated low level transmission of *P. vivax* malaria in some areas of Zimbabwe. With the reduction in malaria transmission globally and many countries heading towards malaria elimination, management of *P. vivax* malaria is gaining importance because of its unique inherent biological features (Barbosa et al., 2014). As the Zimbabwe national malaria control program is embarking towards elimination of malaria, the current data will be important in planning malaria control interventions as the strategy for *P. falciparum* malaria control may be different from *P. vivax* malaria control. For example, the current *P. falciparum* malaria control depends mainly on artemisinin based treatment while the elimination of *P. vivax* malaria requires robust surveillance for case recognition and radical management to prevent relapse malaria (Sharma et al., 2015).

The reactivity of the human sera with *P. vivax* antigen, Pvs48/45, measured by ELISA and Western blot using *P. vivax* antigen was likely due to the cross reactivity of antibodies elicited in response to *P. falciparum* exposure, rather than to exposure with *P. vivax*. The cross-reactivity of the antigens may have important consequences for species specific and species transcending immunity.

Acknowledgments

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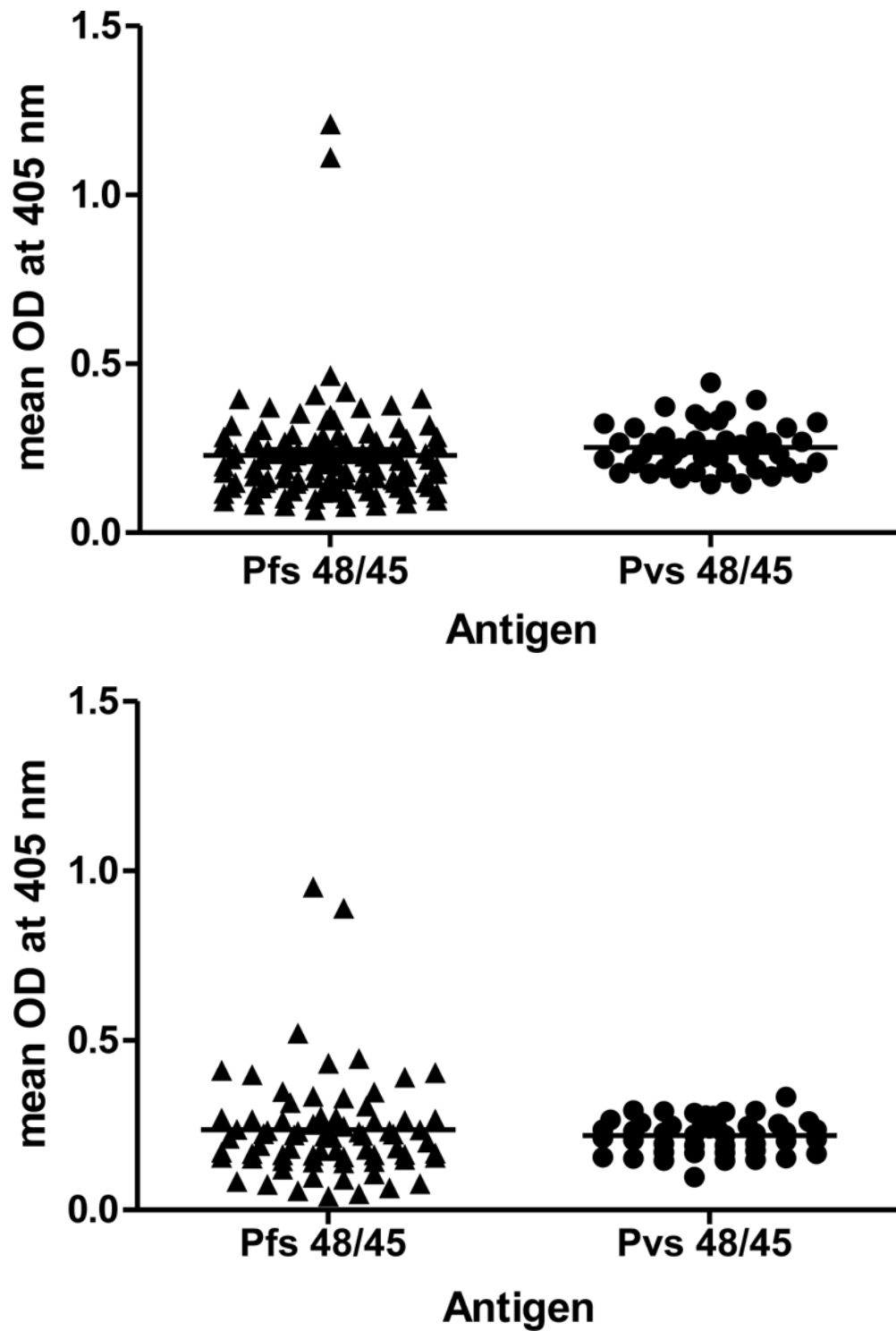
Figure 1. ELISA results for Pfs48/45 and Pvs48/45

Absorbance (405 nm) with Bemberi 2013 (A) and Bemberi 2015 (B) plasma samples reacting with recombinant Pfs48/45 and Pvs48/45 antigens.
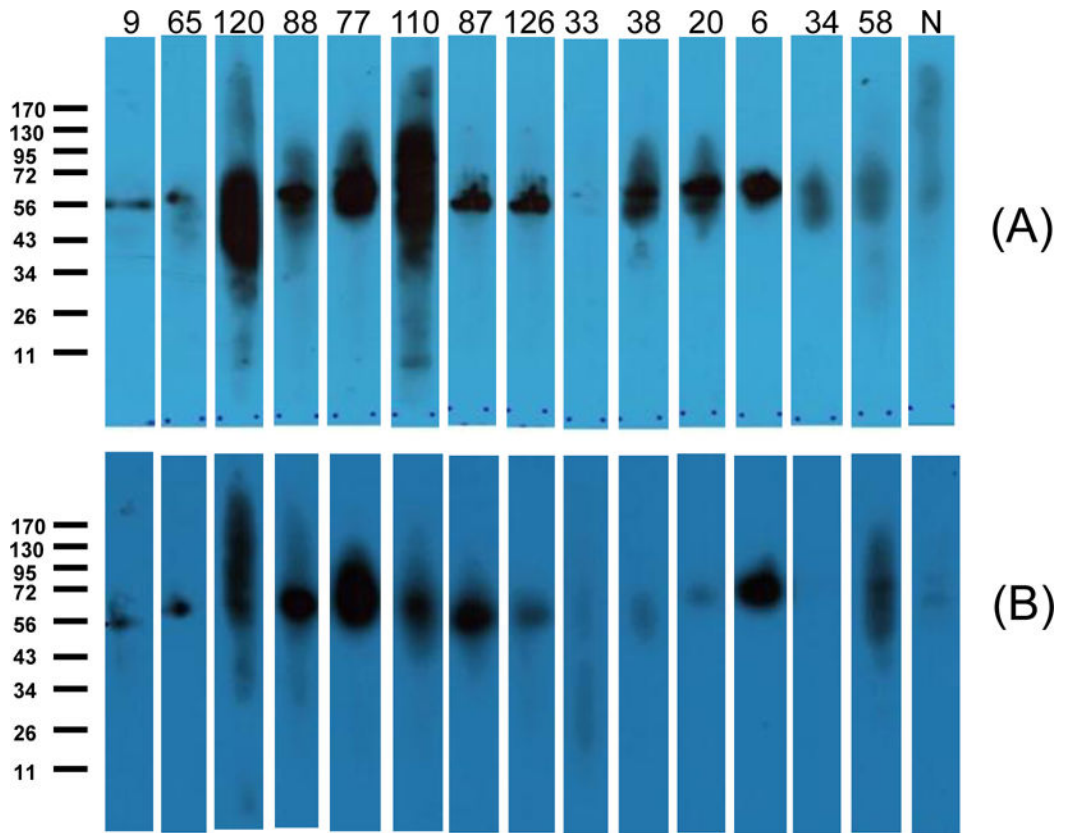
Figure 2. Western blot Analysis
Images of representative plasma samples (1:200 dilutions) reacting with Pfs48/45 (A) and Pvs48/45 (B).
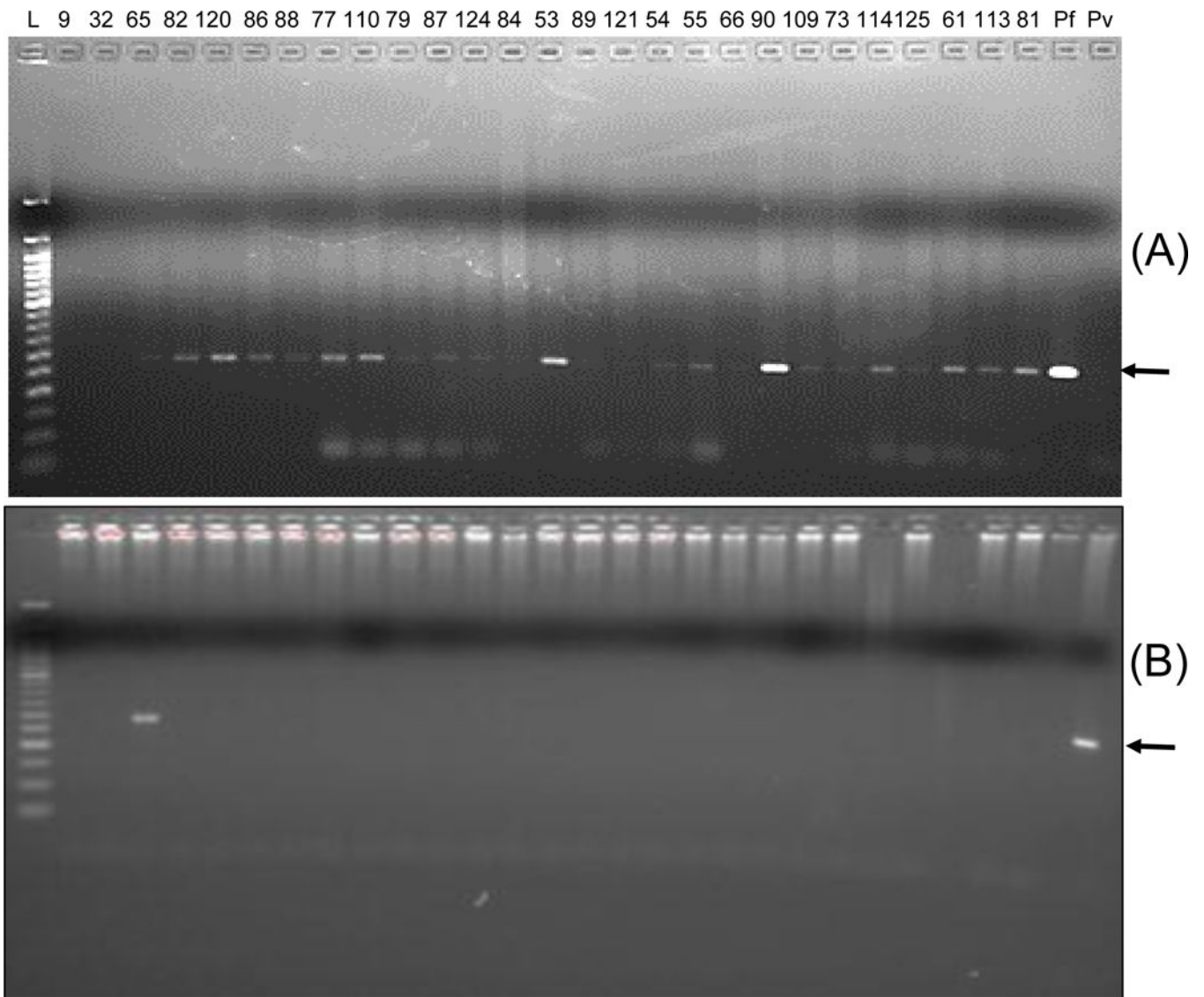
Figure 3. Results of nested PCR for *P. falciparum* (A) and *P. vivax* (B)
Lane marked L shows a 50 base pair molecular mass marker ladder and the number on top show the samples IDs of Bemberi 2013 participants. Lane marked Pf contained *P. falciparum* (NF54) genomic DNA and lane marked Pv contained *P. vivax* (Sal1 strain) genomic DNA.

Table 1

Summary of the cross reactivity study using Bemberi 2013 serum samples

| Method	ELISA		SDS-PAGE and Western blot				*P. falciparum* Nested PCR	*P. vivax* Nested PCR
Antigen	Pfs 48/45	Pvs 48/45	Pfs 48/45		Pvs 48/45			
Secondary antibodies	Anti-human IgG/IgM	Anti-human IgG/IgM	Anti-human IgG	Anti-human IgM	Anti-human IgG	Anti-human IgM		
Participant								
9	+	+	+	+	+	+	–	–
58	+	–	+	+	–	+/–	+	–
32	+	–	–	–	+	+	–	–
65	+	+	+	+	+	+	+	+
82	+	+		+		+	+	–
120	+	+	–	+	+/–	+	+	–
86	+	+	+	+/–	+	+	+	–
88	+	+	+	+	+	+	+	–
77	+	+	+	+	+	+	+	–
110	+	+	+	+	+	+	+	–
79	+	+	+/–	+/–	–	+	+	–
87	+	+	+	+	+	+	+	–
126	+	+	+	+	+	+		
53	+	+	+	+	+	+	+	–
89	+	+	+	+/–	+	+	Faint bands	–
121	+	+	–	–	–	+	Faint bands	–
84	+	+	ND	ND	ND	ND	+	–
116	+	–	ND	ND	ND	ND	No sample	No sample
55	+	+	ND	ND	ND	ND	+	–
66	+	+	ND	ND	ND	ND	Faint bands	–
90	+	+	ND	ND	ND	ND	+	–
61	+	+	ND	ND	ND	ND	+	–
125	+	+	ND	ND	ND	ND	+	–
114	+	+	ND	ND	ND	ND	+	–

Method	ELISA		SDS-PAGE and Western blot				P. falciparum Nested PCR	P. vivax Nested PCR
Antigen	Pfs 48/45	Pvs 48/45	Pfs 48/45		Pvs 48/45			
Secondary antibodies	Anti-human IgG/IgM	Anti-human IgG/IgM	Anti-human IgG	Anti-human IgM	Anti-human IgG	Anti-human IgM		
78	+	–	ND	ND	ND	ND	+	–
91	+	+	ND	ND	ND	ND	ND	ND
109	+	+	ND	ND	ND	ND	+	–
81	+	+	ND	ND	ND	ND	+	–
113	+	+	ND	ND	ND	ND	+	–
124	+	No	ND	ND	ND	ND	+	–
70	–	+	ND	ND	ND	ND	ND	ND
64	–	–	ND	ND	ND	ND	ND	ND
31	–	+	ND	ND	ND	ND	ND	ND
42	–	+	ND	ND	ND	ND	ND	ND
38	–	–	ND	ND	ND	ND	ND	ND
7	–	+	ND	ND	ND	ND	ND	ND
48	–	–	ND	ND	ND	ND	ND	ND
62	–	+	ND	ND	ND	ND	ND	ND
54	–	–	ND	ND	ND	ND	ND	ND
72	–	+	ND	ND	ND	ND	ND	ND
69	–	+	ND	ND	ND	ND	ND	ND
60	–	+	ND	ND	ND	ND	ND	ND
71	–	–	ND	ND	ND	ND	ND	ND
75	–	–	ND	ND	ND	ND	ND	ND

ND: Not determined

Table 2

Summary of the cross reactivity study using Bemberi 2015 samples

Method	ELISA		SDS-PAGE and Western blot			
Antigen	Pfs 48/45	Pvs 48/45	Pfs 48/45		Pvs 48/45	
Secondary antibody	Anti-human IgG/IgM	Anti-human IgG/IgM	Anti-human IgG	Anti-human IgM	Anti-human IgG	Anti-human IgM
Participants						
110	+	+	+	High background	–	+/–
76	+	+	+/–	+	+/–	+
36	+	+	+	+	+	+
58	+	–	–	+	–	+
20	+	+	+	+	+	+
86	+	No sample	No sample	No sample	No sample	No sample
6	+	+	+	+/–	+	+
34	+	+	+/–	–	+	+
61	+	+	+	+	+	+
1	+	–	+	+	+	+
99	+	+	+	High background	+	+
32	+	–	High background	+/–	High background	+
98	+	–	+	+/–	–	–
7	+	–	+	+	+/–	+/–
93	+	+	ND	ND	ND	ND
77	+	–	ND	ND	ND	ND
96	+	+	ND	ND	ND	ND
94	+	+	ND	ND	ND	ND
27	+	ND	ND	ND	ND	ND
16	–	ND	–	–	–	ND
17	–	–	–	–	–	ND
21	–	+	ND	ND	ND	ND
120	–	–	ND	ND	ND	ND
29	–	+	ND	ND	ND	ND

Method	ELISA		SDS-PAGE and Western blot			
Antigen	Pfs 48/45	Pvs 48/45	Pfs 48/45		Pvs 48/45	
Secondary antibody	Anti-human IgG/IgM	Anti-human IgG/IgM	Anti-human IgG	Anti-human IgM	Anti-human IgG	Anti-human IgM
44	–	+	ND	ND	ND	ND
75	–	+	ND	ND	ND	ND
57	–	+	ND	ND	ND	ND
123	–	+	ND	ND	ND	ND
17	–	–	ND	ND	ND	ND
78	–	+	ND	ND	ND	ND
108	–	–	ND	ND	ND	ND
106	–	–	ND	ND	ND	ND
5	–	+	ND	ND	ND	ND
92	–	+	ND	ND	ND	ND
65	–	+	ND	ND	ND	ND
10	–	+	ND	ND	ND	ND
14	–	+	ND	ND	ND	ND
80	–	+	ND	ND	ND	ND
71	–	+	ND	ND	ND	ND
9	–	+	ND	ND	ND	ND
90	–	+	ND	ND	ND	ND
40	–	–	ND	ND	ND	ND
63	–	+	ND	ND	ND	ND
74	–	–	ND	ND	ND	ND

ND: Not determined