Predictive markers of transmission in areas with different malaria endemicity in north-eastern Tanzania based on seroprevalence of antibodies against *Plasmodium falciparum*

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Abstract

Background

Due to the scale-up of different interventions, Malaria burden declined significantly in many African countries between 2000 and 2015. As a result, some areas have become suitable for malaria elimination, and in such a situation, Due to the insensitivity of most commonly used methods, malaria transmission assessment is difficult. In north-eastern Tanzania, we tested for Plasmodium falciparum exposure by using serological markers.

Methods

A cross-sectional survey was conducted in Bondo, Tanga and Hai, Kilimanjaro between June and December 2014. A total of 788 participants were enrolled and screened for malaria and IgG antibodies against PfAMA-1 and PfMSP-1_{19} antigens using Enzyme-Linked Immuno-Sorbent Assay (ELISA). Malaria parasites were detected using polymerase chain reaction (PCR). The Mann–Whitney test was used to compare the Antibody levels between two independent groups (i.e. positive versus negative). The non-parametric Kruskal-Wallis test was used for comparisons between more than two age groups. Pearson's Chi-squared (χ2) test was used to compare proportions.

Results

Generally, malaria prevalence by PCR in two sites was 20.4% (161), with Bondo having a higher prevalence of 28.1% (n= 154) as compared to Hai 2.9%, (n= 7), χ^2=64.64, p<0.01. Anti-*PfAMA-1* and anti-*PfMSP-1_{19}* antibody concentrations were higher in malaria positive than malaria negative individuals, Mann-Whitney U test, p=0.07 and p=0.003 respectively. Antibody response against *PfAMA-1* was significantly different between the three age groups (Kruskal-Wallis test, p<0.001).

Conclusion

Plasmodium falciparum exposure immunological indicators have proven useful for explaining the dynamics of transmission, especially in low transmission environments like Hai.

Introduction

Malaria is still a public health problem facing sub-Saharan Africa and remains a major cause of morbidity and mortality in Africa. World Health Organization (WHO) data shows that Africa carries the highest burden of malaria with more than 70% of all malaria cases and deaths[1]. Each year, 10 to 12 million people contract malaria and more than 80,000 dies[2, 3]. *Plasmodium falciparum* is mainly responsible for 99.7% of estimated malaria cases. [4].

In many countries, local malaria transmission has decreased due to the extensive efforts being devoted to malaria control and elimination [5]. Despite Tanzania's large populations at risk for malaria, transmission varies between its regions significantly, with Plasmodium falciparum accounting for 96 percent of cases.. According to Tanzania HIV and Malaria Indicator Survey (THMIS), 2017 [6], malaria prevalence varies from < 1 percent in the highlands of Arusha to as high as 15 percent in the Southern Zone and 24 percent along the Lake and Western Zones. Immunity to *P. falciparum* malaria is poorly understood, however, evidence shows that antibody-dependent cellular mechanisms play a key role in immunity against *P. falciparum* malaria parasite [7, 8]. The rate of its development is believed to be associated with transmission intensity which is stage-specific and is rarely sterile[6]. In many epidemiological studies, the determination of malaria transmission has been based on the antibody levels against *P. falciparum* antigens [9]. Recent immunological studies revealed that antibodies against merozoite antigens act as biomarkers of malaria exposure and that, with increasing exposure and responses of higher levels, antibodies may act as biomarkers of protective immunity [10].

Apical membrane antigen 1 (AMA-1) is expressed on merozoites and sporozoites of *P. falciparum* as a type I integral membrane protein [11]while Merozoite surface protein1 (MSP-1), is a highly conserved protein among *Plasmodium* species as well as the most abundant protein expressed on the surface of merozoites[12]. The antigens MSP-1 and AMA-1 are promising vaccine candidates for *P. falciparum.* Antibodies against MSP-1 and AMA-1 antigens are potential markers of both exposure to *P. falciparum* and protection against the disease[7, 13] and have proven to be informative, in areas where transmission has dropped to low sustained levels, for monitoring the timing and magnitude of transmission reduction[13] as well as in obtaining epidemiological information in malaria control programmes[14].

In areas with low malaria transmission, it has become extremely difficult to detect changes in transmission intensity using conventional methods such as the entomologic inoculation rate (EIR) or malaria prevalence rates. Low transmission areas (low endemicity) sometimes have low mosquito density, below the detection limits of common mosquito trapping methods [15, 16] and the parasite prevalence also becomes less reliable [17–19]. Malaria serological markers may aid in estimating malaria transmission intensity [20–22]. Seroconversion rates may provide insight into recent changes in malaria transmission [23]. Due to the fact antibodies can persist for months or years after infection, seroconversion rates are less affected by the effects of unstable or seasonal transmission [20, 21]. We investigated the antibody response to recombinant AMA-1 and MSP-1 in individuals living in two regionally distinct malaria-endemic zones.

Materials And Methods

Study area

The study was conducted during April and December 2014 in two different areas of the Tanzanian mainland. The first site was Bondo in the Tanga region, inhabited by 7970 people [24]. Bondo lies at 309 meters above sea level, the coordinates of the area are 5°22'60" N and 38°34'60" E. The second study site

was Hai in Kilimanjaro region, located at the foot of Mount Kilimanjaro, with 67225 residents [14]and an elevation of 1000-1411 meters above sea level and the coordinates of 3° 12' 0" N and 37°13' 60" E. The selected study areas have diverse malaria transmissions and different ecologies and, malaria interventions have been differently deployed in these areas as well. Tanga used to be a high-endemic area in Tanzania and great effort has been done in controlling the disease. Kilimanjaro region is known to have very low transmission rates due to factors such as altitude and vector composition. Participant recruitment procedures and study design have been previously described [25], Figure 1.

Sampling procedure

Meetings were organized wherein the study staff explained the study and answered any questions in an open forum. Upon commencing participant recruitment, enrolment occurred in health facilities at each site. Participants were enrolled only after verification of potential subject eligibility, explaining the study in the Swahili language. Each member of the community had an equal chance of being selected as a participant. The list of all members in the village was listed and with a lottery method, each member of the population was assigned a number, after which participants were selected at random. The minimum age of the study participants was 2 years and above.

Sample size calculation

The sample size was calculated assuming the following parameters seroprevalence of anti-AMA/MSP 50% was used, P = 0.5 (proportion in the population), Power = 0.80, Alpha = 0.05 (two sided), Anticipated difference = 0.1, Alternative p = 0.4, Design effect = 2. Estimated required sample size in each site was, n = 194*2 = 388 per study, (Using STATA software). A minimum required sample was 776. In this study we enrolled 788 participants

Sample collection

A blood sample was obtained by finger prick. A portion of blood was used for malaria rapid test, which was performed by well-trained staff at each site. A blood spot was prepared for each participant, then dried and stored for further analysis. A 3.0 mm diameter circle of dried blood spot (equivalent to 2µl whole blood/1µl serum) was reconstituted in 200µl of sodium azide-phosphate buffered saline-tween (0.05%) (PBST/0.1% Azide). The solution is approximately 1:100 of whole blood which is equivalent to 1:200 dilution of antibodies concentration.

Enzyme-Linked Immuno-Sorbent Assay (ELISA)

Indirect immunosorbent Assay (ELISA)was performed using two *Plasmodium falciparum* surface antigens, *Plasmodium falciparum MSP* 1_{19} (Pf*MSP* 1_{19}) and *Plasmodium falciparum AMA-1*(*PfAMA-1*) [26]. Briefly, the recombinant antigens (MSP_1 and AMA_1) were coated to each well of flat bottom high binding microtiter plates (Greiner bio-one, Germany) at a concentration of 0.5µg/ml in sodium carbonate-sodium bicarbonate buffer (pH 9.5) and incubated at 4^0C overnight. The plates were then washed three

(3) times with phosphate-buffered saline and tween 20 (0.05%) and blocked with 1% (w/v) skimmed milk for 3 hours at room temperature. Samples and controls were then added in duplicate, positive controls were from pooled positive samples from highly endemic areas and negative controls were from European malaria naïve individuals. After three washes with Phosphate Buffered Saline (PBS), 50µl of horse-radish peroxidase-conjugated rabbit anti-human IgG diluted at 1:5000 in PBS was added to each well and incubated at room temperature for hours. O-phenylenediamine (Sigma-fast OPD) was used as the peroxidase substrate, the reaction was stopped by 2M sulphuric acid 15 minutes after adding the substrate. Optical density (OD) values were read using Microplate ELISA reader (Elx 808; USA), at the wavelength filter of 490nm.

Malaria parasite detection by polymerase chain reaction (PCR)

Parasite DNA was extracted using the simple chelex method, a dried blood spot of about 8mm in diameter was cut and placed in a 1.5 ml microcentrifuge tube containing 1ml of PBS with 10% saponin and incubated for 4 hours at a 4^0C fridge. The spot was then soaked in PBS for15 minutes at 4^0C, after the incubation, tubes were centrifuged, and the supernatant was discarded. 150µl of 6% chelex solution was then added and incubated at 95°C for 10 minutes with vertexing periodically through the incubation time. The final centrifugation for 5 minutes at high speed was done and the supernatant was transferred to a clean microcentrifuge tube and stored at -20^{0}C freezer until used.

Plasmodium nucleic acid amplification was conducted using genus-specific reverse and forward primers (rPLU6-5 ⊤ ∀ ∀ ⊤ G ⊤ $GCAG$ ⊤ ∀ ∀ $CG3$ and rPLU5-5ℂ TG ⊤ G ⊤ Gℂ ⊤ ∀ ACTℂ3) targeting small sub-unit ribosomal RNA (ssurRNA) of the parasite. A reaction mix of 20µl per sample was used, 5µl of template DNA extracted from participants whole blood plus 15µl of nuclease-free water, dNTPs, Taq enzyme, buffers, and salts. Amplification conditions were, 95ºC for 5 minutes followed by 30 cycles of 94^{0}C for 1minute, 58^{0}C for 2 minutes and 72^{0}C for 5 minutes then one final extension cycle at 72ºC for 10 minutes [27,28]. Amplification products were run in Ethidium bromide agarose gel (2%) electrophoresis at 120 volts, 50 watts and 120mA. The amplified bands were visualized under an ultra-violet light trans-illuminator.

Data analysis

All data were analyzed using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism8 software (San Diego, CA). Data cleaning was conducted before data analysis. Data were log-transformed before statistical analysis. After verifying that Optical density (OD) values were not normally distributed ($p<0.0001$; Anderson-Darling test), non-parametric tests were performed to compare the OD values. The Mann–Whitney test was used for the comparison of Antibody levels of two independent groups (i.e positive versus negative). The non-parametric Kruskal–Wallis test was used for the comparison of more than two groups (i.e age groups). Pearson's Chi-squared (χ^2) test was used to compare two proportions. All observed differences were considered significant at $p<0.05$. The cut-off for seropositivity among samples was determined as the mean OD of the negative control sera plus 3 standard deviations.

Results

Population characteristics and Malaria prevalence

The study enrolled a total of 788 participants, 239 (30.3%) from Hai and 549 (69.7%) from Bondo. Males were 283 (35.9%) and females were 505 (64.1%). About 405 (51.4%) participants had more than 15 years of age, 212 (26.9%) were between 5-15 years and 171(21.7%) were below 5 years. The malaria prevalence by mRDT was 8.6% (47) in Bondo and 0% in Hai, fisher exact test *p<0.001. By PCR, malaria prevalence was 20.4% (161), with Bondo having a higher prevalence 28.1% (n= 154) than Hai 2.9%, (n= 7), χ^2=64.64, p<0.01(Table 1).

Table 1: Prevalence of Malaria by serology, mRDT, Microscopy and PCR

		PfAMA-1 % (n)	*PfMSP-1$_{19}$* % (n)	mRDT % (n)	PCR % (n)
Bondo	Positive	34.2 (188)	36.6 (201)	8.6 (47)	28.1 (154)
	Negative	65.8 (361)	348 (63.4)	91.4 (502)	71.9 (395)
Hai	Positive	13.8(33)	17.2 (41)	0.0 (0)	2.9 (7)
	Negative	86.2(206)	198 (82.8)	100.0 (239)	97.1 (232)
Total	Positive	28.0(221)	30.7 (242)	6.0 (47)	20.4 (161)
	Negative	72.0(567)	69.3 (546)	94.0 (741)	79.6 (627)
χ^2 p-value		χ^2=34.66 p<0.001	χ^2=29.62 p<0.001	*p<0.001	χ^2=64.64 p<0.001

*Computed by Fisher exact test

Seroprevalence of Anti- *PfAMA-1* and *PfMSP-1$_{19}$* antibodies

Bondo had a higher seroprevalence 36.6% (188) for PfAMA-1 as compared to Hai 13.8% (33), Chi-square=34.66, p<0.01. Likewise, Bondo had a higher seroprevalence 201(36.6%) for PfMSP-I as compared to Hai 41 (17.2%) Chi-square test =29.62, p<0.01. In Bondo, participants with more than 15 years had a significantly higher seroprevalence of PfAMA-1 61.7% (116) (χ^2test =58.69, p<0.001) and PfMSP-1$_{19}$ 63.7 (128) (χ^2 =65.36, p<0.001) as compared to other age groups. Likewise, participants with 5-15 years and <5 years had a higher prevalence of malaria as measured by mRDT, χ^2 = 30.76, p<0.001 (Table 2).

Table 2: Age-specific Prevalence of Malaria by serology, mRDT, Microscopy and PCR

		PfAMA-1 % (n)	*PfMSP-1*$_{19}$ % (n)	mRDT % (n)	PCR % (n)
Bondo	Positive	34.2 (188)	36.6 (201)	8.6 (47)	28.1 (154)
	Negative	65.8 (361)	348 (63.4)	91.4 (502)	71.9 (395)
Hai	Positive	13.8(33)	17.2 (41)	0.0 (0)	2.9 (7)
	Negative	86.2(206)	198 (82.8)	100.0 (239)	97.1 (232)
Total	Positive	28.0(221)	30.7 (242)	6.0 (47)	20.4 (161)
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χ^2 p-value		χ^2=34.66 p<0.001	χ^2=29.62 p<0.001	*p<0.001	χ^2=64.64 p<0.001

*Computed by Fisher exact test

Anti-*PfAMA-1* and *PfMSP-1*$_{19}$ antibody concentrations

Anti-*PfAMA-1* titers were higher in malaria positive individuals (n=47) than in malaria negative individuals (n=741) (Mann-Whitney U test, p=0.07) (**Figure 2A**). Anti-*PfMSP*-1 antibody concentrations were significantly higher in malaria-positive individuals (n=47) than in malaria-negative individuals (n=741) (Mann-Whitney U test, p=0.003) (**Figure 2B**).

We went further to determine whether the two sites differed in antibody concentration.

and found that anti-*PfAMA-1* antibody concentrations, were higher among participants in Bondo (n=549) as compared in Hai (n=239), (Mann-Whitney U test, p<0.001) (**Figure 3A**). Anti-*PfMSP-1* antibody concentrations were higher among participants in Bondo (n=549) than those of Hai (n=239), (Mann-Whitney U test, p=0.01) (**Figure 3B**).

In assessing whether these differences were influenced by age, we calculated the differences among <5 years, 5 to 15 years and >15 years per site. Antibody response against *PfAMA-1* was significantly different between the three age groups (Kruskal–Wallis test, p< 0.001) **Figure 4A,** likewise, shows that *PfMSP-1*$_{19}$ was significantly different between the three-age group (Kruskal–Wallis test, p<0.001) while **Figure 4B** shows similar results when comparing each age category in Hai site. We also found significant differences in the anti-*PfAMA-1*antibody concentrations among the groups (Kruskal–Wallis test, p=0.004), as indicated in **Figure 4C.** Lastly, we also noted significant differences in the anti-PfMSP-

Wallis test, p=0.005) **Figure 4D**.

Discussion

The purpose of this study was to use immunological markers to investigate malaria transmission patterns in areas with diverse malaria endemicities. PCR, as well as the routine mRDT method, were used to detect malaria parasites. The results of this study revealed heterogeneity in malaria transmission across the study sites.

In this study, malaria prevalence by PCR in Bondo was 28.1%. Since Bondo is a malaria-endemic area, malaria transmission occurs nearly all year long with a peak period from April to June. In 2011 a study conducted in Tanga suggested a widening of the age group at risk for malaria infection to older children of 5–15 years [29]. No significant difference was observed in malaria prevalence among all age groups in the present study. A previous study conducted in two villages in the same region about 70 kilometers from the current study found a re-emergence of malaria despite previous reports of a decline in malaria [33] It is estimated that parasite prevalence at that time was 25% and it stayed there throughout 2016 [34]. PCR analysis of Hai found 2.9% parasite prevalence, thus remaining an area of low transmission and The mRDT test was negative, which suggests low-density parasite circulating in the population, similar to earlier findings [35]. There is, however, some evidence that individuals harbouring sub-microscopic parasites could be sources of new infections since mosquitoes can carry parasites with very low density. (< 5parasites/μl) [27,31,32], and hence, the use of a more sensitive diagnostic tool like PCR in clinical malaria diagnosis is necessary. Consequently, scientific evidence from these findings is consistent with the notion of mass drug therapy for individuals with microscopic parasites in light of efforts to eliminate malaria.

Antibodies to malaria antigens can explain long-term changes in malaria transmission dynamics [26]. To understand malaria transmission patterns immunological markers were used in parallel with the parasitological indicators. In Hai, the overall seroprevalence was 13.8% for *PfAMA-1* and 17.2% for *PfMSP-1*$_{19}$.

In 2009 a survey conducted in Moshi found low seroprevalence in children suggesting very low exposure to malaria parasite [36] In our study, Interestingly, when the age-dependent analysis was done, older children (5–15 years) had a relatively low seroprevalence to *PfAMA-1* antigens only as compared to younger children and Adults. This phenomenon is only seen when not is because older children experienced repeated malarial exposure as compared to younger ones most of whom were protected by treated bed nets. In populations with low immunity, such as young children, antibodies to MSP-1 act as a significant biomarker of malaria exposure and with increasing exposure the antibodies may contribute to protective immunity[10].

Seroprevalence in moderate malaria transmission setting such as Bondo can play a small role in determining malaria transmission patterns although seroprevalence is almost two folds higher than Hai with the seroprevalence of 34.2% and 36.6% for *pfAMA-1* and *pfMSP-1*$_{19}$respectively. A slight decline in seroprevalence was observed in the study area when compared with previous studies [26,35], indicating a

long-term reduction in malaria parasite exposure, which may be attributed to intense malaria interventions in Tanzania [37,38].

To design and develop vaccines, it is important to know the epidemiology of the immune response to plasmodium falciparum. The malaria burden in Africa can only be determined through the use of current information about different malaria transmission areas. We analysed the results by stratifying age and relate with antibody concentrations against both *PfAMA-1* and *PfMSP-1*$_{19}$antigens. In both sites, the results show that overall antibody levels were low in children, compared to adults.

Study results showed that the overall concentration of pfMSP-1$_{19}$ (by PCR) was significantly higher in participants with positive malaria tests than in non-positive participants. As expected Bondo had significantly higher antibody concentrations against both antigens as compared to Hai. In Bondo, median OD was increasing with age as observed for both antigens. The results demonstrated that anti-*pfAMA-1* and anti-*pfMSP-1*$_{19}$ antibodies are reliable biomarkers for parasite exposure as well as vector activity and disease transmission in the study areas. Children with < 5 years present with low antibody titters suggesting a lack of recent malaria exposure and this makes the group vulnerable to the symptomatic manifestation of the disease. Earlier findings revealed that more than half of the participants reported being symptomatic and 14.1% were malaria positive by mRDT [21]. There is evidence of malaria transmission in low malaria-endemic areas, where traditional malaria indicators like prevalence and sporozoite levels may underestimate the burden of the disease.

Conclusion

The immunological indicators of Plasmodium falciparum exposure have been useful for explaining long-term changes in the dynamics of transmission particularly in areas of low transmission like Hai. Malaria prevalence in Bondo continues to be high, despite intervention and transmission at the submicroscopic level has been observed.

Abbreviations

CRERC: College Research and Ethics Review Committee

OD: Optical Density

AMA-1: Apical membrane antigen1

PCR: Polymerase Chain Reaction

ELISA: Enzyme-Linked Immuno-Sorbent Assay

ssurRNA: small sub-unit ribosomal RNA

PfMSP-1 Plasmodium falciparum Merozoite Surface Protein 1

PfAMA-1: Plasmodium falciparum AMA-1

Declarations

Patient consent for publication

Not required.

Patients and Public Involvement

Participants were not involved in the design of this study. Community leaders were involved during participant's recruitment. There is a plan to disseminate results to the participating sites.

Ethics approval and consent to participate.

Ethical approval was obtained from the Kilimanjaro Christian Medical University College Research and Ethics Review Committee (CRERC) with certificate number 658. Permission to conduct the study was sought from Handeni/Bondo and Hai district authorities. Written informed consent was obtained from all participants and from parents or guardians for children under 18 years of age who agreed to participate in the study.

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

RDK: Conceptualization of the study, data analysis, and writing the original draft of the manuscript; DCK: Funding acquisition, investigation, data analysis and review of the manuscript; JJM, AJN, FWM and JOC:

Interpretation of data and critical review of the manuscript; RAK: Overall study design and review of the manuscript

All the authors have read and approved the final version of the manuscript.

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Figures

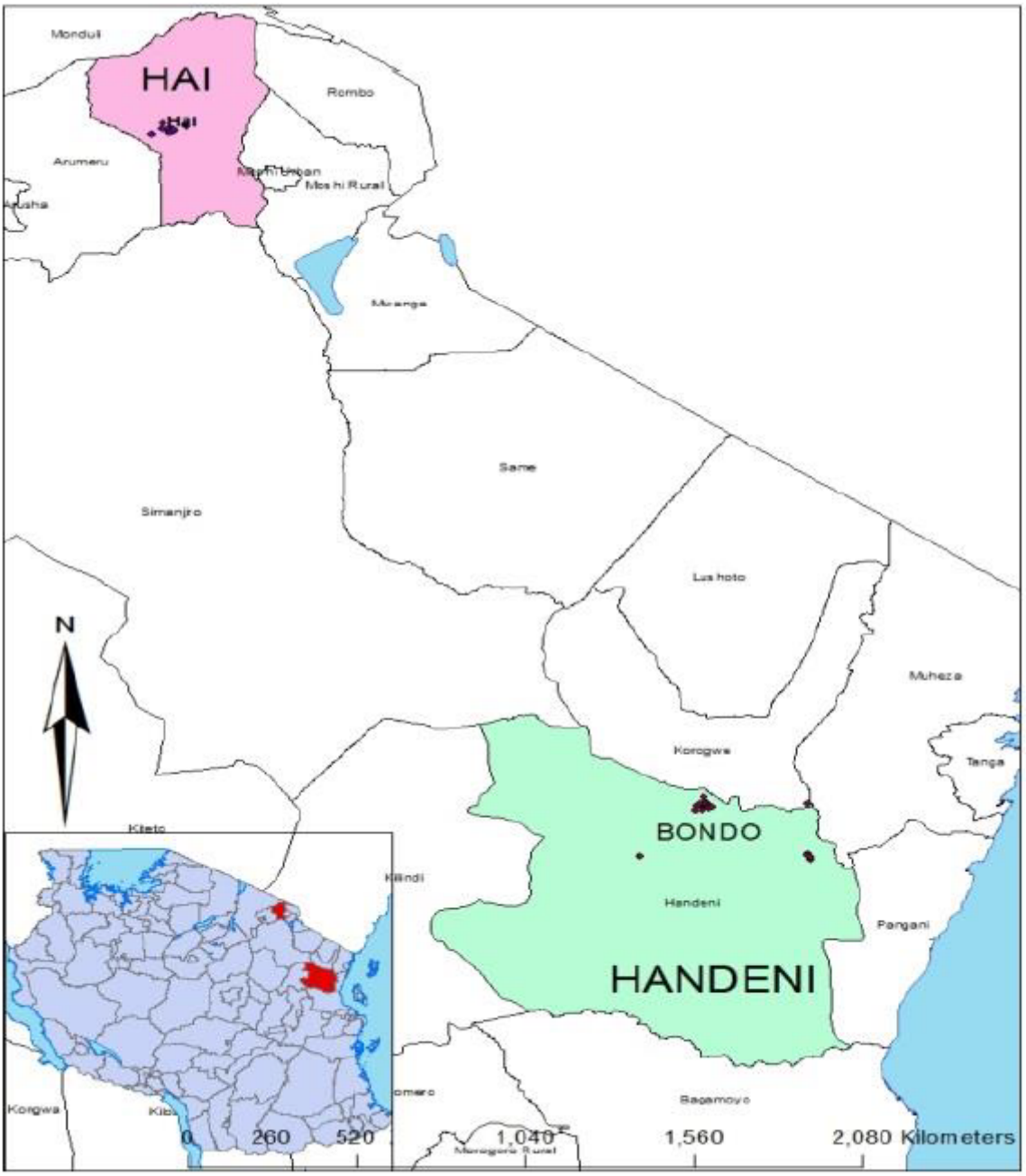


Figure 1

Map of Tanzania showing the study sites.

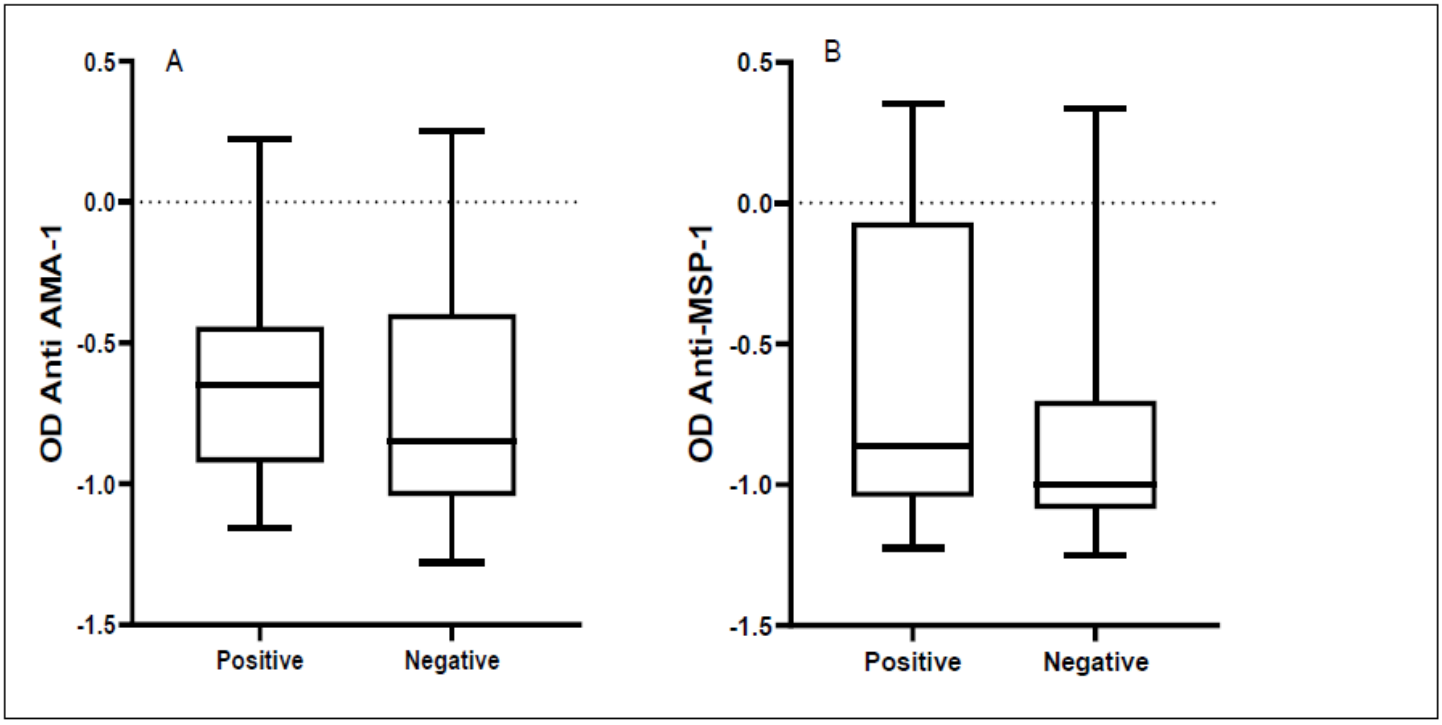
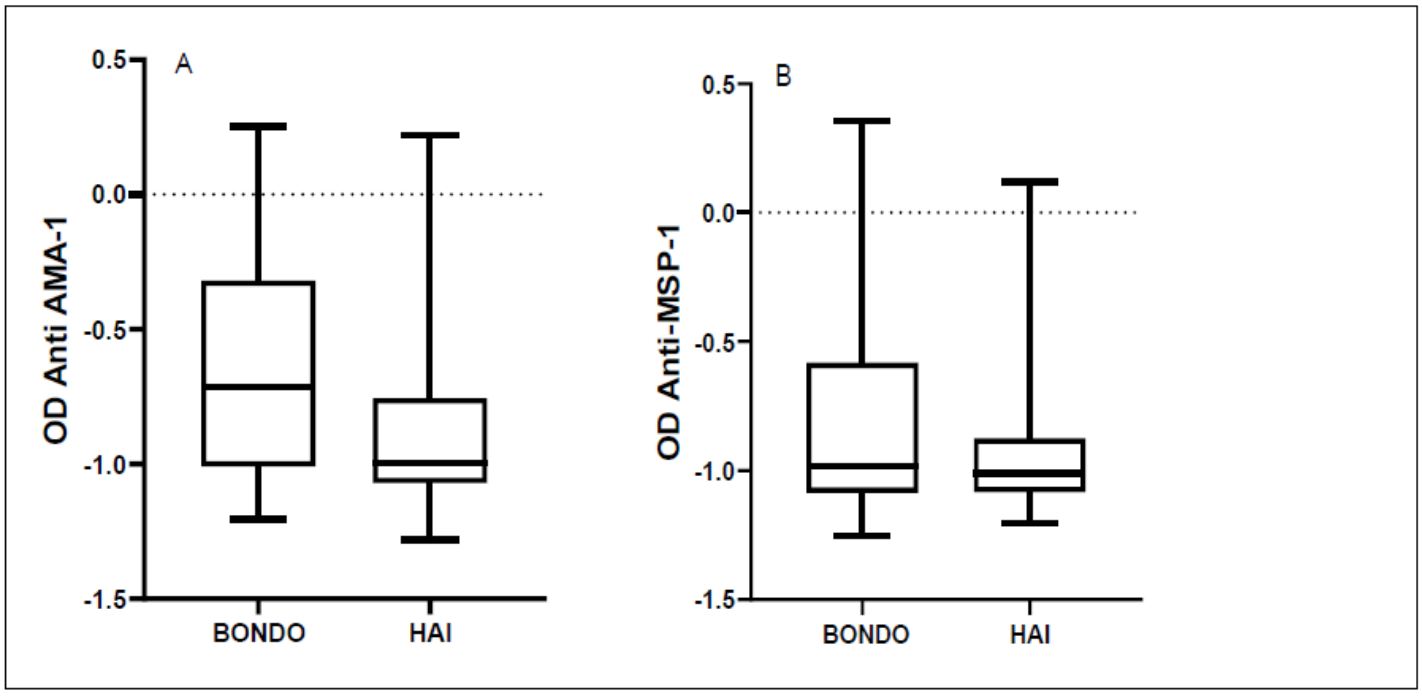


Figure 2

A graph showing mean OD values for PfAMA-1 (Figure 2 A) and PfMSP-119 (Figure 2B) among malaria positive and negative individuals. Presented in the Y-axis is the Log10 transformed OD values among malaria positives and negatives (X-axis).



A graph showing mean OD values for PfAMA-1 (Figure 3A) and PfMSP-119 (Figure 3B) at Bondo and Hai sites. Presented in the Y-axis is the Log10 transformed OD values in two sites (X-axis).

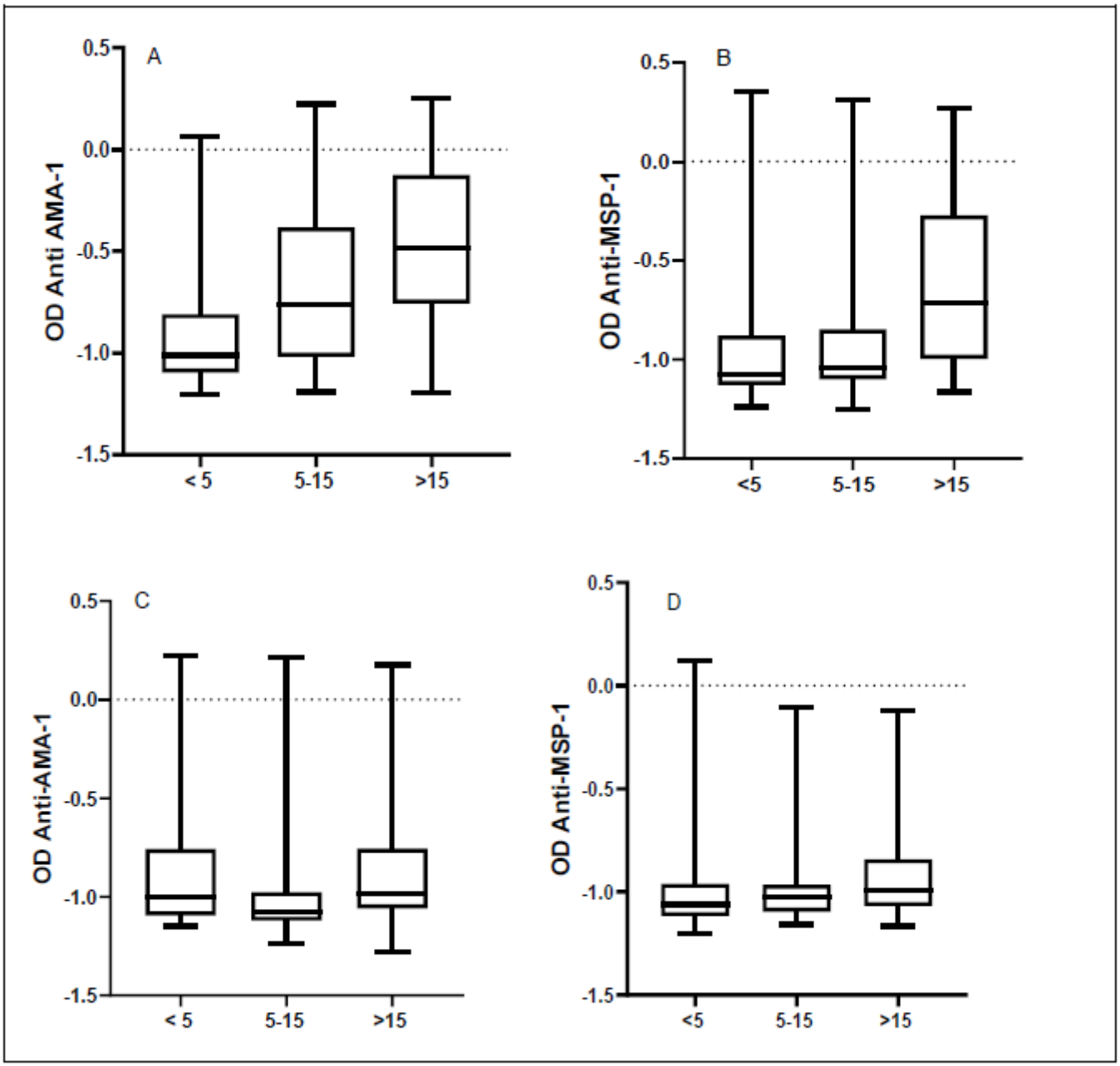


Figure 4

A graph showing mean OD values for anti-PfAMA-1 antibodies and anti-PfMSP-119 antibodies. Figure 4A and figure 4B show anti-PfAMA-1 and anti-PfMSP-119 in Bondo respectively. Figure 4C and figure 4D show anti-PfAMA-1 ad anti-PfMSP-119 antibodies in Hai respectively. Presented in the Y-axis is the Log10 transformed mean OD values in age groups (X-axis).