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Research Article

Characterizing the antibody specificity of sera from malaria-endemic African countries for sequence micro-heterogeneities within the dimorphic domain of Pf3D7-MSP2

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ABSTRACT

Background: Micro-heterogenicity of 25-mer segments within the dimorphic domain (Pf3D7-D) defined various alleles of Pf3D7-type MSP2. This study explored the specificity of African malaria-endemic antibodies (IgG) to this micro-heterogenicity.

Methods: We tested in ELISA immunoassays (standard and competitive ELISA) 12 synthetic 25-mer peptides representing each micro-heterogenous segment using sera from Mali, ML (N=75), Burkina Faso, BF (N=49) and Tanzania, TZ (N=37).

Findings: Overall, BF antibody samples had significantly higher affinities (p<0.0001) and the largest proportion (45%-65%) of positive responders for all the 25 mer-peptides. In contrast, ML samples had slightly lower antibody affinities and a smaller proportion of responders (15%-57%), whereas TZ sample demonstrated the lowest antibody affinities and fewest proportions of responders (3%-46%). Children 6-14 years-old exhibited higher antibody affinities and a larger proportion of responders (20%-80%) compared to ≤ 5 years-old (08-54%, p=0.001) and ≥ 15 years-old (05%-37%, p<0.0001). Furthermore, a large number of responders recognized at least one, if not all, of the 25-mer epitopes. The 25-mer peptides showed cross-inhibition with each other.

Conclusion: This work broadened our understanding of the specificity profiles of antibodies for the Pf3D7 allele epitopes and suggested the presence of multi-genotypic Pf3D7 infections in the population.

Keywords: Pf3D7-MSP2, Dimorphic domain, Micro-heterogeneity, African Malaria endemic countries, Immune sera

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INTRODUCTION

Plasmodium falciparum (Pf) merozoite surface protein 2 (PfMSP2) is a blood-stage protein that is characterized by unique domains and is a promising vaccine candidate [1]. The dimorphic (D) domain of MSP2 led to the definition of two allelic families of Pf, the 3D7-type and FC27-type alleles [2].

Immune responses against the native parasite protein and against synthetic peptide-mimics have demonstrated that the 3D7-D domain was the most strongly correlated with protection from clinical malaria in endemic areas, while suggesting a predominance of 3D7 parasite infections in endemic areas [3]. Mapping the B cell epitopes that characterize the D domains of the MSP2 allelic families in endemic blood samples has confirmed that 3D7 infections are predominant [4]. In addition, both proteins and synthetic peptides based on 3D7-D were antigenic and immunogenic for mice and humans [5].

On the other hand, while the 3D7-D domains demonstrate variety in their length and sequence, they also retain a high level of similarity [6]. Indeed, in a phase 2b blood stage vaccine study, it has been suggested that the conserved epitopes in the family-specific domains were the most important determinants of vaccine-effectiveness against new 3D7-type infections [7]. Furthermore, distinct serogroups of 3D7-type alleles have already been identified based on sequence similarities and antibody cross-reactivity [8]. All these earlier findings supported the notion that there is a multiplicity of infections and a diversity of 3D7-type msp2 allele genotypes in endemic regions [9].

How these regions of micro-heterogeneity in the 3D7 allelic domain "epitopes" shape immune responses in the naturally exposed population in endemic areas has not been previously described. Here, we analyzed the effects of these micro-heterogeneities by synthesizing 25-mer peptides based on the 3D7 allele sequences and assessing their seroprevalence with IgG (immunoglobulin G) antibody responses. Furthermore, we determined the cross-inhibition of these peptides against each other using sera from three malaria-endemic countries, including Mali (ML) and Burkina Faso (BF) in West Africa, and Tanzania (TZ) in East Africa.

We postulate that characterizing variations in the fine specificity of antibody responses for the 3D7-MSP2 allele types, as a result of sequence micro-heterogeneities, will strengthen future multiepitope, vaccine-candidate design strategies. For this purpose, twelve micro-heterogenous 25-mer motifs characterizing each Pf3D7-msp2 genotype [10], were selected and compared with each other in the GenBank database. The 25-mer peptides were then tested with ELISA to determine their recognition (antibody responses) by sera from ML, BF and TZ, where P. falciparum is endemic.

MATERIALS AND METHODS

Blood samples and ethic statement

All samples used in this study were anonymized, stored at -80°C and have been used in our previous studies [11]. Venipuncture was performed to collect whole blood, sera fractions were then obtained by centrifugation and stored frozen at -80°C until use. For this study, samples from three endemic countries were selected: Mali, ML (adults and children, N=75), Burkina Faso, BF (adults, N=49) and Tanzania, TZ (adults, N=37). Samples from BF were collected in 1998 in the village of Goundry located 30 km from Ouagadougou (the capital city of Burkina Faso). Those from ML were collected from 2009 to 2011 in Kenieroba, a village located in the Bancoumana district, 73 km from Bamako (the capital city) and from Dangassa village in Kourouba town, 80 km from Bamako. Samples from TZ were collected from 1982 to 1984 during a large-scale community-based study undertaken in Ifakara village in the Kilombero District in Morogoro.

For BF, in 1998, no authorization was required for research study. For ML, ethical approval was obtained from the Faculty of Medicine, Pharmacology and Odonto-Stomatology (FMPOS) at the University of Bamako, Mali (N°0840/FMPOS). For Tanzania, ethical approval was obtained from the Commission for Science and Technology. Furthermore, written Informed Consent (IC) was obtained from each adult, and an Informed Assent (IA) or IC from a parent or legal guardian was obtained for each minor. Anonymized sera from healthy Swiss adults non-exposed to malaria and no malaria history and who gave their ICs to participate in malaria vaccine research (2012, study NCT01605786) were used as negative controls.

Synthetic peptides

The synthesis of long peptides was performed as previously described Balam et al. at the Department of Immunobiology, University of Lausanne, Switzerland, using an Applied Biosystem 431A instrument (Foster City. CA. USA). Fmoc chemistry and acetyl capping of unreacted peptide chains was performed. The 25-mers peptides were synthesized with the use of the MultiRespep Synthesizer (Bioanalytical Instrument, Intavis AG). The long synthetic peptides (LSP) spanned the entire sequence of the 3D7-D domain (3D7-D LSP); the twelve 25-mer peptides from MIP1 to MIP12 covered the micro-heterogeneity within the 3D7-D domain (Table 1). Peptide purity (>80%) was determined using analytical C18 HPLC and mass spectroscopy. After lyophilization, the peptides were dissolved in phosphate buffered saline, PBS (Gibco® Invitrogen™) at a concentration of 1 mg/ml and stored at -20°C.

Sequence alignment of the full length 3D7-dimorphic (3D7-D) and 25-mer micro-heterogeneous peptides covering the 12 3D7 types MSP2 allelic genotypes. Sequence names refer to Gen Bank accession numbers. Number and % of identical amino acids (aa)/25 aa shared among each of the 25-mer peptides (MIP2 to MIP 12) compared with the reference peptide MIP1 are determined for each micro heterogenous 25-mer peptide. The micro-heterogenous sequence is identified (in blue) in the reference 3D7-D sequence. The difference pertaining to aa is highlighted in red and underlined.

Table 1. Sequence of the full-length 3D7-D peptide and sequence alignment of the twelve of 25-mer micro-heterogenous peptides spanning the 3D7-type MSP2 alleles.

Full-length sequence of 3D7-MSP2 dimorphic (D) domain: AEASTSTSSENPNHKNAETNPKGKGEVQEPNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAEN SAPTAEQTESPELQS (95-183. 88 aa). Genbank accesion number: UDB86418)			
Sequence of 25-mer microheterogenous peptides	**Peptide name**	**Number (%) of identical aa**	**Genbank accession number**
PNHKNAETNPKGKGEVQEPNQANKE	MIP1(aa106-131)	Reference	XM_001349542
PNHNNAKTNPKGNGGVQEPNQANKE	MIP2	21 (83%)	DQ166534
PNHNNAKTNPKGNGGVQKPNQANKE	MIP3	20 (80%)	DQ174442
PNHKNAETNPKGKGEVQKPNQANKE	MIP4	24 (96%)	DQ162622
PNHKNAETNPKGKGEVQKTNQANKE	MIP5	23 (92%)	U07001
PNHNNAETNPKGKGEVQKSNQANKE	MIP6	22 (88%)	M73810
PNHNNAETNPKGNGEVQEPNQANKE	MIP7	23 (92%)	AJ318755
SNHNNAETNPKGKGEVQEPNQANKE	MIP8	23 (92%)	DQ166545
TNHNNAETNPKGNGKVQEPNQANKE	MIP9	21 (83%)	U07009
PNHNNAETNPKGKGQVQEPNQANKE	MIP10	23 (92%)	U16842
PNHNNAKTNPKGKGEVQKPNQANKE	MIP11	22 (88%)	AY534507
PNHNNAKTNPKGK-EVQEPNQANKE	MIP12	22 (88%)	DQ168571

ELISA assays

Indirect ELISA: ELISA was carried out (in triplicate) with the use of Maxisorp 96-well microtiter plates (Thermo scientific. Ref. 442404), which were coated with 50 µL/well using a 1 µg/mL solution of each 3D7-D LSP and a 5 µg/ml solution for the 25-mer peptides and stored overnight at 4°C. After blocking for 1 h at Room Temperature (RT) with PBSx1- 3% milk, plates were incubated for 2 h at RT with sera diluted to 1:200 in PBSx1-1.5% milk.

Goat anti-human IgG, conjugated with the Horseradish Peroxidase Enzyme (HRP), were used as secondary antibodies at a dilution of 1:2000 (Life technologies, Ref H10307) and incubated in each plate well for 1 h at RT. The reaction was developed using TMB substrate (BD OptEIA. cat 555214) for 30 min in the dark at RT. The reaction was then blocked using 1 M sulphuric acid (Merck. 1.00731.1000).

Optical Density (OD) was measured at 450 nm/630 nm using a TECAN NanoQuant Infinit M200 PRO spectrophotometer. ELISA results were considered positive if the sample mean OD were ≥ mean OD +3 SD of the negative control sample (Swiss naïve human sera, NHS).

Competition ELISA

ELISA competition assay was performed by incubating the competitor peptide with the sera at a dilution of 1:300, corresponding to 50% of the maximum signal of the competitor peptide, for 1 h at RT. The mixture was then added to wells coated with the peptides of interest. The plates were then incubated for 30 min at RT and the reactivity was determined as previously described in indirect ELISA. Each test was performed in duplicate. The percentage of inhibition in the presence of competitor peptide was calculated as 100 − [(mean antibody OD with competitor peptide (inhibited well)) × 100/mean antibody OD without competitor peptide (no inhibited well)].

Statistics

All ELISA data are presented as an average optical density (OD) value from triplicate wells. GraphPad Prism software, version 8.0 was used for the data analysis. The Mann-Whitney test was utilized for comparing two groups, and a Kruskal-Wallis test followed by Dunn's multiple comparison test was used when more than two groups were considered.

Fischer's exact and *Chi-square* tests were used to compare the proportion of responding sera between the groups, with a significance level at $p \leq 0$.

RESULTS

Regions of sequence micro-heterogeneity covering the 3D7-dimorphic domain

The 25-mer micro-heterogeneous peptide regions were identified and compared to the equivalent region (in blue) in the full-length sequence (88-residues, aa95-183) of the MSP2-3D7-D domain (D-LSP sequence) which is used here as reference [12]. This was performed to identify genotypic differences across the 11 PfMSP2-3D7 variants previously isolated from Papua New Guinea (PNG) samples (Table 1) [13]. In GenBank online data resources, we matched each 25-mer with the reference MIP1 and synthetized 12 peptides (MIP1 to MIP12) as representatives of each 3D7-MSP2 genotype variant. Furthermore, performing matching analysis of the 12 25-mer peptides with each other resulted in a 1-4 AA discrepancy with an identity ≥ 83% (Table 1).

Fine specificity of antibody responses against the 3D7-D micro-heterogenous 25-mer peptides using sera from three malaria-endemic African countries

All 12 micro-heterogeneous peptides (MIP1 to MIP12) were tested in ELISA using African sera from Mali (ML), Tanzania (TZ), Burkina-Faso (BF). All 12 micro-heterogeneous 25-mer peptides were recognized to varying degrees across the three countries. Notably, BF sera exhibited significantly higher antibody levels (Figure 1A, $p<0.0001$) and a higher prevalence of responders ranging from 45% to 65% (Table 2) as compared to sera from ML donors, which showed marginally higher antibody levels and a slightly broader response range of 15% to 57%, relative to TZ samples, which had the lowest proportion of responders (03% to 46%) (Figure 1A).

The recognition profile for the 25-mer peptides was consistent between ML and TZ samples, with MIP1-MIP6 inducing higher antibody responses and MIP7-MIP2 showing lower antibody responses in both countries. However, certain peptides specifically MIP4 and MIP6 were similarly well-recognized in all three countries with the response proportion ranging between 35%-57% (Figure 1A, Table 2). Furthermore, aggregated data from positive responder samples in all three countries highlighted a broader recognition of peptide MIP1-MIP6 (Table 2). In some instances, some samples recognized several or even all twelve 25-mer micro-heterogenous peptides, with a higher prevalence in BF and ML donors (Figure 1B).

ELISA was performed using the 3D7-D LSP and the 12 micro-heterogenous 25-mer peptides (MIP1 to MIP12) with sera from Mali Tanzania and Burkina Faso. (A) Overall antibody Levels (mean OD value) against the micro-heterogeneity of the 3D7 are significantly disparate between countries, with higher levels in BF samples. Kruskal-Wallis test followed by Dunn's multiple comparison test was applied to compare OD values between the three country samples. (B) Responder proportions based on the number of recognized peptides regarding the country samples, Mali, Tz and BF. Ab, antibody; OD, optical density; ns, not significant; $^{***}p<0.001$; $^{****}p<0.0001$.

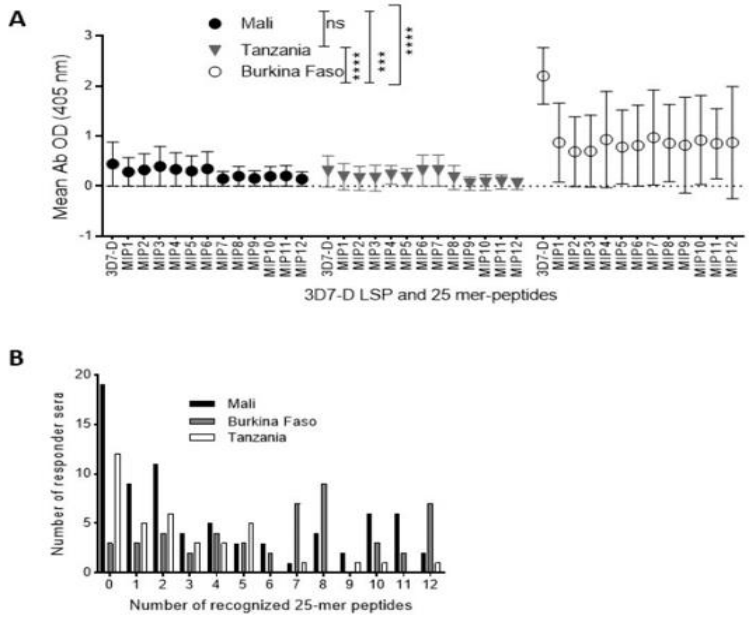

Figure 1. Antibody responses against the micro-heterogeneous regions of 3D7 across different endemic country.

ELISA was performed using sera from Mali, Tanzania and Burkina Faso and the 3D7-D LSP and the 12 micro-heterogenous 25-mer peptides (MIP1 to MIP12). The proportion of positive samples (responders) were determined for each country and Fisher's exact test were used to compare responder proportions between two countries. N, total donor samples; n, number of responders; %, percentage of responders; LSP, long synthetic peptide; a, b and c correspond respectively to Mali, Tz and BF donors; ns, not significant; $^{*}p \leq 0.05$; $^{**}p<0.01$; $^{***}p<0.001$; $^{****}p<0.0001$ (Table 2).

Table 2. Proportion of positive responders against the 25-mer long and 3D7-D peptides for the three countries tested.

Peptides	Mali (N=75) a		Tanzania (N=37) b		Burkina Faso (N=49) c		p value		
	n	(%)	n	(%)	n	(%)	a *vs.* b	a *vs.* c	b *vs.* c
3D7-D LSP	50	67	14	38	49	100	** ****	****	****
MIP1	29	39	11	30	32	65	ns	**	**
MIP2	36	48	6	16	23	47	***	ns	**
MIP3	43	57	8	22	22	45	***	ns	*
MIP4	36	48	13	35	28	57	ns	ns	ns
MIP5	29	39	9	24	32	65	ns	**	***
MIP6	31	41	17	46	27	55	ns	ns	ns
MIP7	11	15	17	46	29	59	***	****	ns
MIP8	18	24	8	22	26	53	ns	***	**
MIP9	13	17	2	5	32	65	ns	****	****
MIP10	21	28	3	8	29	59	*	***	****
MIP11	21	28	4	11	30	60	*	***	****
MIP12	16	21	1	3	25	51	**	***	****

Further analysis of five BF samples (donors D1-D5), which showed high recognition of the 3D7-D LSP, revealed variations in the Ab responses to the 25-mer peptides with in the same endemic area. Notably, donor N°2 (D2) showed higher antibody levels for (Figure 2A). In addition, purified antibodies (pAb) studied against the 3D7-D LSP from the D2 sample identified peptides MIP7 and MIP8 as being better recognized (Figure 2B). A competition ELISA with the pAb from D2 showed strong cross-reactivity between the micro-heterogeneous 25-mer peptides (Figure 3).

The highest recognizing serum donor N°2 (D2) as shown in Figure 2 was used to perform an inhibitory ELISA for all 12 of the 25 mer peptides. (A) Inhibition of the MIP1 peptide (the reference sequence) was achieved by using MIP7 and MIP8, the most recognized epitopes. MIP11 is the least recognized by pAb as shown in Figure 2. (B) MIP11, one of the least recognized peptides by pAb, is also inhibited by other poorly-recognized peptides, such as MIP1, MIP3 and MIP4. Naive Swiss donors (NHS) were used as negative controls.

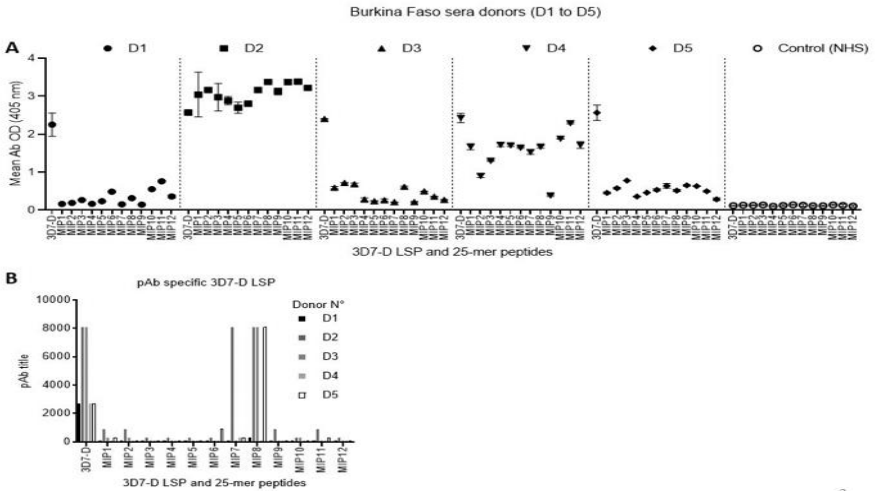

Figure 2. Purified antibody against the 3D7-D-LSP recognize the 25-mer micro-heterogenous peptides.

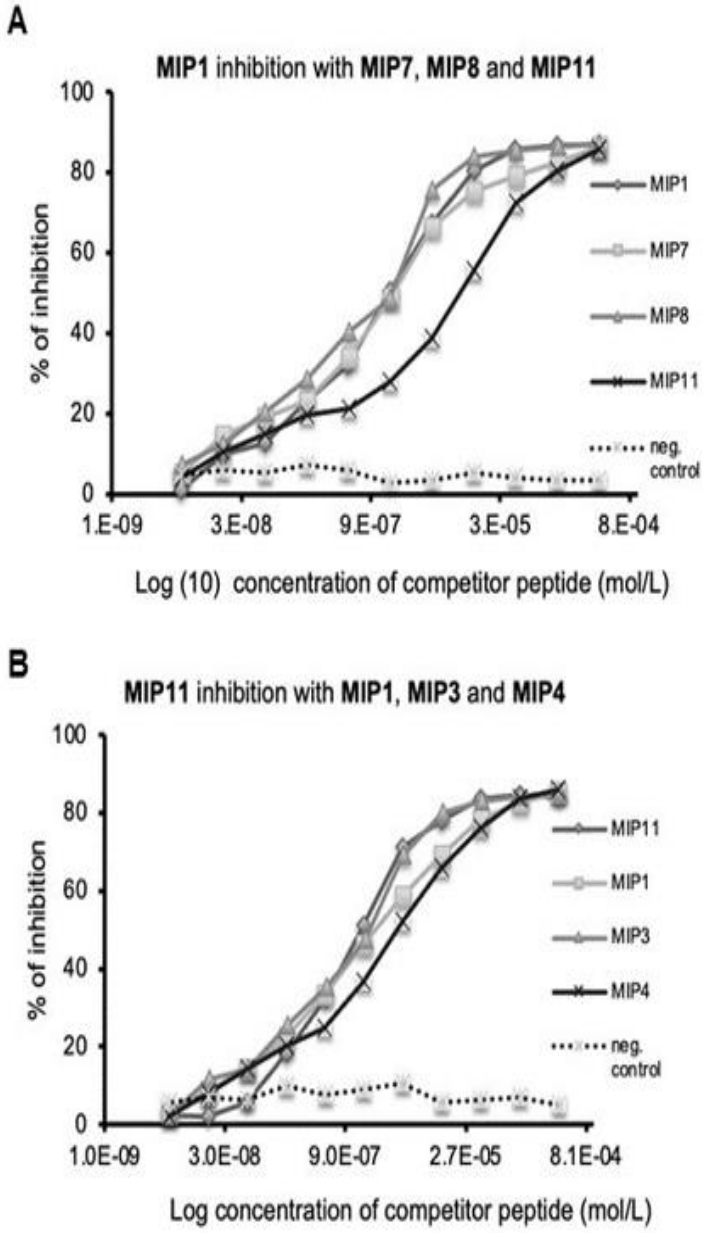

Figure 3. Cross reactive antibodies against the 25-mer micro-heterogenous peptides.

Fine specificity profile of Mali antibodies against the micro-heterogeneous 3D7-D domains heterogeneity with regards to age

We then examined antibody recognition of the 25-mer micro-heterogeneous peptides across three age groups (≤ 5, 6-14 and ≥ 15-years-old) from ML donors in order to determine relevance of age in antibody recognition. Notably, the 6–14-year-old groups showed significantly higher antibody levels (Figure 4A, $p<0.001$), with the proportion of positive responders ranging from 20%-80%, in contrast to the ≤ 5-year-old (8%-54%) and the ≥ 15 years old (5%-37%) (Table 3). However, the antibody levels were similar between the ≤ 5 and ≥ 15-year-old age groups. The recognition profiles across the MIP1-MIP6 peptides were consistent, with each age group showing a larger proportion of responders to MIP1-MIP6 than to MIP7-MIP12, which aligns with the overall trends observed in the samples from the three countries.

The same sera from Mali shown in Figure 1 were further analyzed according to age group: ≤ 5-years-old; 6-14-years-old and ≥ 15 years-old. (A) Antibody levels against the micro-heterogeneous region were significantly higher in the 6-14 age group as compared to the two other age groups. (B) Distribution of the number of responders regarding the number of the recognized 25-mer peptides for the three age groups. Ab, antibody; OD, optical density; LSP, long synthetic peptide; ns, not significant; $^{***}p<0.001$.

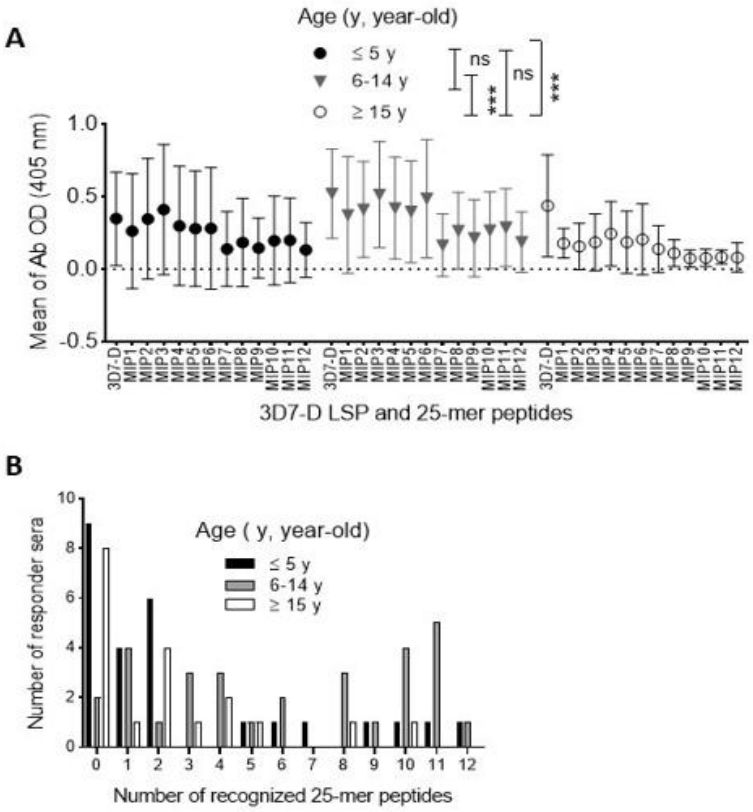

Figure 4. Mali antibody responses against the micro-heterogeneities of 3D7 according to age group.

Proportion of responder samples for each age group in Mali was determined. Fisher's exact test was applied to compare responder proportions between two age groups. N, total donor samples; n, number of responders; %, percentage of responders; LSP, long synthetic peptide; a, b and c correspond to the different age group (respectively ≤ 5, 6–14 and ≥ 15 years old); ns, not significant; $^{*}p\leq0.05$; $^{**}p<0.01$; $^{***}p<0.001$; $^{****}p<0.0001$ (Table 3).

Table 3. Proportion of Mali positive responders against the 25-mer long and 3D7-D peptides with regards to age-group.

Peptides	**≤ 5 y (N=26)a**		**6-14 y (N=30)b**		**≥ 15 y (N=19)c**		**p value**		
	n	**%**	**n**	**%**	**n**	**%**	**a *vs.* b**	**a *vs.* c**	**b *vs.* c**
3D7-D LSP	13	50	25	83	12	63	**	ns	ns
MIP1	6	23	16	53	7	37	*	ns	ns
MIP2	12	43	20	67	4	21	ns	ns	**
MIP3	14	54	24	80	5	25	*	ns	****
MIP4	8	31	21	70	7	37	**	ns	*
MIP5	7	27	19	63	3	16	**	ns	***
MIP6	6	23	20	67	5	26	***	ns	**
MIP7	2	8	6	20	3	16	ns	ns	ns
MIP8	3	12	12	40	3	16	*	ns	ns
MIP9	4	15	8	27	1	5	ns	ns	ns
MIP10	6	23	13	43	2	11	ns	ns	*
MIP11	4	15	15	50	2	11	**	ns	**
MIP12	4	15	10	33	2	11	ns	ns	ns

DISCUSSION

This study aimed to characterize the fine specificity profile of natural antibodies against micro heterogeneous epitopes, corresponding to the Pf3D7-D genotypes of MSP2, across several malaria-endemic areas and among various age groups. The results obtained may contribute to the characterization and selection of immunodominant epitopes in the Pf3D7-D domain of MSP2. In previous studies, we and others have characterized the full-length sequence of the MSP2-3D7-D domain and described the major epitopes for a single 3D7 genotype of MSP2, using sera samples from malaria-endemic areas and in mice [14]. Here, we refine the study of the MSP2-3D7-D domain by using micro- heterogenous 25-mer peptide fragment, representing this MSP2-3D7 genotype and 11 others that were previously identified [15]. We tested all these 12 micro-heterogeneous 25-mer peptides across three countries, finding that they were recognized to varying extents. Despite significant differences among the three countries, antibody responses in samples from Burkina Faso and Mali were significantly greater for most 25-mer peptides compared to those from Tanzania. These findings raise questions about whether the selected 3D7 allelic family variants of MSP2 that were chosen mare more prevalent in in ML and BF-countries in West Africa with similar climate (and malaria burden, than in TZ, located in East Africa [16].

Furthermore, the patterns observed in antibody responses and the prevalence of antibodies to specific25-mer epitopes across the three countries may support the hypothesis that Pf infection might frequently be caused by common 3D7 genotypes.

Analysis of individuals who recognized multiple peptides revealed significant differences in how the 3D7-MSP2 genotype variant epitopes were identified across the malaria endemic areas. In addition, the use of pAb further enhanced the detection accuracy of the 25-mer peptides [17].

Age is known to influence the outcome of malaria infection and naturally acquired immunity Yazdani et al. since immunity levels typically increase with age throughout childhood before reaching a plateau in adulthood [18]. Here, we found significantly higher antibody levels against 25-mer peptide in the 6–14-year-olds, followed by children under 5 years, with the lowest levels found in individuals 15 years and older. This pattern warrants further exploration.

The lower antibody responses in children (≤ 5 years) are consistent with the assertion of loss of maternal immunity and the absence of infection-specific immunity that has not yet developed at that age [19]. On the other hand, this does not fully account for the lower immunity observed in those over 15 year, suggesting that factors such as immune-senescence and/or aging may impact host immunity to parasitic infections [20]. However, data from this age group must be interpreted with caution, due to the small number of participants (only 19 samples).

Further study is needed to explain this finding or it is just a coincidence. Altogether, our findings further strengthen the notion that immunity levels against malaria is not only dependent on age and exposure, but also on the cumulative number of episodes of clinical malaria experienced by an individual. The micro-heterogeneity within the 3D7-D domain enhances natural immune responses against the 3D7-MSP2 genotype. We observed multi-epitope recognition in sera from African populations, suggesting the existence of distinct serogroups within the PfMSP2 3D7 allelic family. These findings imply that recognition of micro- heterogeneities may be dependent on the genetic background of the population and/or their exposure to the same allelic variants, highlighting the need to further investigate these micro-heterogeneities in the context of infections with multiple genotypic and localized epidemics.

This is particularly important as it has been suggested that antibodies induced against repeats and regions where this micro-heterogeneity occurs do not protect vaccinated children against new infections. Competing inhibitions of the 25-mer micro-heterogenous peptides with each other further support not only the similarity between sequences (which was ≥ 83%) but also the hypothesis that competitive interactions in multi-genotypic exposure to P. falciparum leads to cross- reactive immune reactions among the population. Indeed, some studies have postulated that pre-existing acquired immunity against either the 3D7 genotype or an ongoing infection can prevent novel infection from the same allelic family. Therefore, longitudinal investigations to understand the dynamics of antibodies, the prevalence of their recognition of these micro-heterogenous epitopes and their correlation with protection from clinical malaria in endemic population settings are warranted.

CONCLUSION

By profiling specific antibody responses against micro-heterogeneous fragments of the 3-D7 MSP2 allelic family, the most common parasite, we have created an opportunity to determine the optimal immunodominant epitopes for eliciting pan-reactive and specific antibodies against the parasite allelic family. Furthermore, this study advances the approach of selecting multi- epitopes, paving the way for the development of multivalent vaccines.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

SB and GP designed the experiment. SB, NCI, DK and GP performed most experiments, tests, and analyses. SB and GP wrote the manuscript.

NCI, DK, GA, MD, RM and IN contributed to antigen and sample processing and manuscript revisions. All authors read and approved the submitted version.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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