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TITLE: Novel Malaria Multi-Epitope Vaccine Design

PRINCIPAL INVESTIGATOR: Dr. Kwadwo Asamoah Kusi

CONTRACTING ORGANIZATION: Noguchi memorial Institute for Medical Research (NMIMR)

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Malaria is a serious infectious disease threat to U.S. military personnel deployed in malaria endemic areas, and an efficacious vaccine is not yet available. To date, most vaccine candidates that have entered clinical trials, whether subunit or whole organism, have components derived from single parasite strains and have largely not been successful due to the strain-dependent features of the elicited immune response. We proposed to identify conserved immunodominant HLA A and B-restricted T cell epitopes from four leading *Plasmodium falciparum* candidate antigens and utilize a self-assembling protein nanoparticle (SAPN) technology to produce multi-antigen, multi-epitope subunit malaria vaccines. We have completed recruitment and blood sample collection from 300 study subjects. DNA from all 300 subjects have been purified and HLA typing of all subjects has been completed. Interferon-gamma/Granzyme B FLUOROSpot T cell assays with 15mer overlapping peptide stimulation of subject PBMCs has been completed. Of the 300 subject samples analyzed, 252 assays have passed based on our assay quality control criteria. Of the 252 passed assays127 HLA-typed subject samples yielded a positive IFN-ɣ/Granzyme B response to at least one of the 31 peptide pools. High resolution HLA typing was successfully done for 291 of the 300 subjects. This report will also present assay data on parasite diversity analysis, blood film microscopy and ELISA to assess anti-CSP antibodies as an exposure proxy.

15. SUBJECT TERMS

Malaria, Plasmodium falciparum, immunity, vaccine, multi-epitope, nanoparticles, Human leukocyte antigen (HLA), T cell, apical membrane antigen 1 (AMA1), Circumsporozoite protein (CSP), thrombospondin related anonymous protein (TRAP), Cell traversal for ookinetes and sporozoites (CelTOS), conserved epitopes,

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Table of Contents

1. Introduction

The most advanced malaria subunit vaccine, RTS,S, shows modest antibody-mediated efficacy of up to 50% over one year in children from endemic areas [2, 3] and is currently being further evaluated in pilots in three African countries. It is also known to be more effective against parasite that are closely related to the vaccine strain [4]. Despite the significant impact that RTS,S could potentially have in people entering malaria-endemic regions, it currently does not meet the WHO and US military requirements for a malaria vaccine, which are achievement of at least 80% efficacy against multiple *Plasmodium falciparum* (Pf) strains for at least one year [5]. Radiation-attenuated sporozoites (RAS) induce 100% protection in humans, with protection mediated mainly by CD8+ T cell-killing of malaria-infected hepatocytes expressing human leukocyte antigen (HLA) Class I-restricted malaria antigen epitopes [6, 7]. Identification of protection-associated HLA-class 1 restricted epitopes with wide population coverage within selected antigenic targets will greatly aid the development of novel subunit, multi-epitope pre-erythrocytic vaccines with similar efficacy.

While Pf3D7-based subunit vaccines potentially protect against infection with the homologous 3D7 parasites to some degree, they have not been shown to be very effective against genetically divergent parasite haplotypes. Protective immune responses are most likely induced against a mix of strain-specific and strain-transcending epitopes within single or multiple antigens and a focus on strain-transcending epitopes, such as those that are conserved across different parasite strains, will yield broad protection. Our strategy is to identify HLA class I-restricted epitopes within selected malaria antigens, particularly those that are conserved among strains, in individuals in malaria-endemic regions and assemble these into a multi-epitope vaccine. We will focus on the HLA supertypes A*01, A*02, A*03, B*07 and B*44 as these are known to be very predominant within the global population [8]. We propose to utilize a self-assembling protein nanoparticle (SAPN) technology to produce multi-antigen, multi-epitope malaria vaccines based on conserved immunodominant peptide sequences from four leading Pf antigens (PfCSP, PfAMA1, PfCelTOS and PfTRAP). We will test the hypothesis that SAPNs that display conserved HLA-class 1 restricted T cell epitopes from multiple malaria antigens can elicit strain-transcending cellular responses in the liver and protect against pre-erythrocytic stage of Pf development and prevent blood stage infection and/or clinical disease. Our goal is to develop a clinically relevant novel multi-antigen, multi-epitope malaria vaccine that can induce broad protection against multiple malaria parasite strains in genetically diverse human populations.

2. Keywords

Malaria, *Plasmodium falciparum*, immunity, vaccine, multi-epitope, nanoparticles, Human leukocyte antigen (HLA), T cell, apical membrane antigen 1 (AMA1), Circumsporozoite protein (CSP),

thrombospondin related anonymous protein (TRAP), Cell traversal for ookinetes and sporozoites (CelTOS), conserved epitopes,

3. Accomplishments

a. What were the major goals of the project?

Specific Aim 1: Prediction and identification of HLA A*01, A*02, A*03, B*07 and B*44 Pf epitopes from natural malaria infections	**Timeline Months**	**Site 1** (Partnering PI)	**Site 2** (Initiating PI)	**Status**	**Completion date**
Major Task 1: Regulatory approvals for recruitment of human subjects and use of animals.					
Subtask 1: Submit documents for local IRB review (NMIMR).	1-3	Dr. Kusi	Dr. Sedegah	completed	June 2019
Subtask 2: Submit documents for local IACUC and ACURO review (NMRC)	1-4		Dr. Sedegah	completed	June 2019
Subtask 3: Submit IRB approval and necessary documents for HRPO review.	4-5	Dr. Kusi	Dr. Sedegah	completed	August 2019
Major Task 2: Conduct of field studies (Ghana)					
Subtask 1: Undertake community entry procedures and recruit study subjects • Hold community durbar to explain purpose of study. • Recruit 300 study subjects and collect biological specimens.	6 6-7	Dr. Kusi		completed	February 2020
Subtask 2: Molecular determination of subject HLA supertypes and parasite genetic diversity	8-12	Dr. Amoah		HLA typing completed, parasite genetic diversity analysis on going; data on completed aspects is presented below	HLA typing completed in May 2020
Subtask 3: FLUOROSpot assays using CSP, AMA1, TRAP and CelTOS 15mer overlapping peptide pools (NMIMR)	8-12	Dr. Kusi	Dr. Sedegah	Overlapping peptides from the four study antigens procured and pools prepared at NMRC. Pooled samples were shipped to NMIMR for FLUOROSpot assays. Laboratory and data analysis for all 300 samples completed, results presented below)	

b. **What was accomplished under these goals?**

After scientific review and ethics approval by the NMIMR-IRB, the NMRC-IRB and the subsequent approval from the Human Research Protections Office (HRPO) of the US Army Medical Research and Development Command, we proceeded to have a community interaction to inform and educate the study community about the study objectives. We have since then successfully completed the recruitment of 300 study subjects and processed their blood samples at NMIMR. Biological specimen including PBMCs, packed cells and plasma samples have been archived. DNA from all 300 participants have been purified and high-resolution HLA typing for all 300 subjects has been completed. The data is currently being used for bioinformatic prediction of immunodominant peptides within the 4 test antigens and subsequent identification of conserved 9-10mer sequences for chemical synthesis. FLUOROSpot assays to determine the numbers of interferon-gamma (IFN-ɣ)- and granzyme B-secreting cells per million PBMCs have been completed. ELISA to assess anti-CSP antibody levels, detection of blood parasites by microscopy and PCR have also been completed. Parasite diversity analysis and gene sequencing of the four antigens (AMA1, CSP, TRAP, CelTOS) is on-going and is expected to be completed in the next two months.

FLUOROSpot assay results: Each subject's PBMCs were tested against 15mer overlapping peptide pools covering the entire sequences of four malaria vaccine candidate antigens; CSP (a total of 9 peptide pools), AMA1 (12 peptide pools), TRAP (6 peptide pools) and CelTOS (4 peptide pools). About 4 x 10^5 PBMCs were suspended in 100 µl complete medium and incubated in wells of FLUOROSpot plates with antigen-specific peptide pools. For each stimulant, data was averaged for duplicate wells and expressed as the spot forming cells (sfc) per million PBMCs. Two criteria were used to identify positive responders; a stimulation index of 2 for any stimulant relative to the same subject's unstimulated PBMCs, and a sfc/million difference of 10 between stimulated and unstimulated PBMCs. Any subject that tested positive against one or more of all 31 peptide pools tested was designated as a positive responder to either IFN-ɣ or granzyme B.

Laboratory and data analysis for all 300 archived PBMC samples have been completed. Of this number, assays for 252 subjects (84%) passed our quality control check, which is subject PBMC positivity to the positive control (Con A or PHA). Of the 252 subjects with passed assays, 127 (representing 50% of subjects whose assays passed) made positive IFN-ɣ and/or granzyme B responses against at least one of the 31 peptide pools. A total of 125 subjects made positive IFN-ɣ responses and 22 subjects made positive granzyme B responses, with 20 subjects being dual responders. Subject SAN117 had the highest number of IFN-ɣ responses to 16 different peptide pools, followed by SAN118 with responses to 15 different peptide pools. Subject SAN117 again had the highest number of granzyme B responses to 20 different peptide pools, followed by subject SAN132 with responses to 18 different peptide pools.

Among the IFN-ɣ positive peptide pools, there were 183, 145, 39 and 38 positive responses against CSP, AMA1, TRAP, and CelTOS respectively. Also, there were 38, 35, 15 and 14 positive granzyme B positive responses against the CSP, AMA1, CelTOS and TRAP peptide pools, respectively, in the 22 responders Thus, CSP was the most recognized of the four antigens, followed by AMA1. The Cp9 pool had the greatest number of subject IFN-ɣ

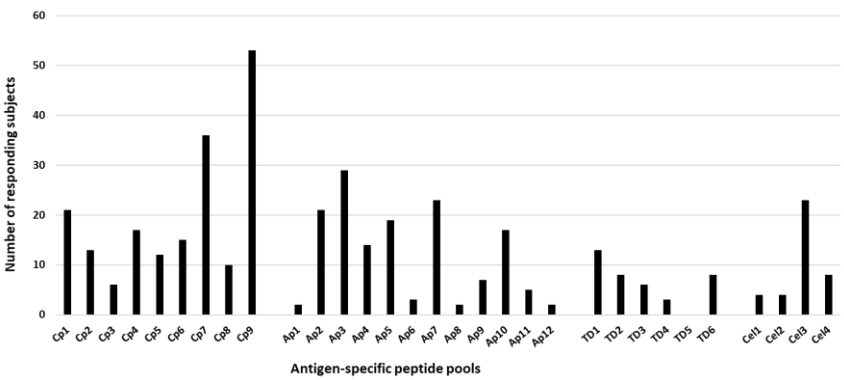

Figure 1. Total number of IFN-ɣ positive peptide pools from all passed assays. These are based on responses in 125 out of 252 subjects' assays that passed our QC and had at least one positive peptide pool response.

responses, followed by Cp7 pool (**Figure 1**). There was however no response in all subjects to the 5th peptide pool of TRAP (TD5).

For granzyme B, the Cel3 pool had the greatest number of subject responses, followed by the Cp6 and Cp7 pools, while there were no responses to the Ap8, Ap12 and TD1 peptide pools (**Figure 2**).

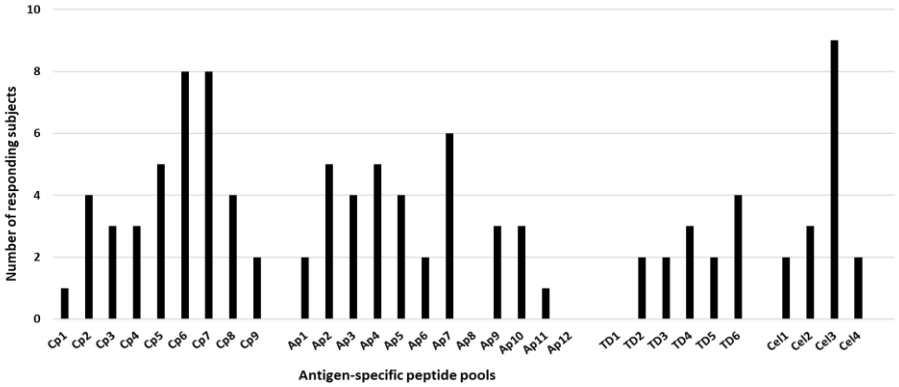

Figure 2. Total number of granzyme B positive peptide pools from all passed assays. These are based on responses in 22 out of 252 subjects' assays that passed our QC and had at least one positive peptide pool response.

The second round of sampling from the 127 subjects with positive responses has begun, and is expected to be completed in the next two months. PBMCs from collected samples will be isolated at NMIMR and subsequently shipped to NMRC in the USA for a second round of FLUOROSpot assays to deconvolute positive peptide pools.

HLA typing results: Human DNA was extracted from packed blood cells from the 300 subjects using the Flexigene DNA extraction kit. DNA aliquots were sent to ProImmune Inc. (UK) for high resolution HLA class I A and B typing using sequence-specific genotyping procedures. Two hundred and ninety-one (291) of the 300 subjects were successfully typed and within this subset of 291 subjects, the five HLA supertypes of interest to us, including HLA A01, A02, A03, B07 and B44, were the top five most frequently expressed supertypes (**Figure 3**), confirming our initial basis for selecting them for the design of broadly effective multi-epitope vaccines. Five (5) of the 108 subjects who made positive FLUOROSpot responses were amongst the 9 subjects with no HLA typing data available.

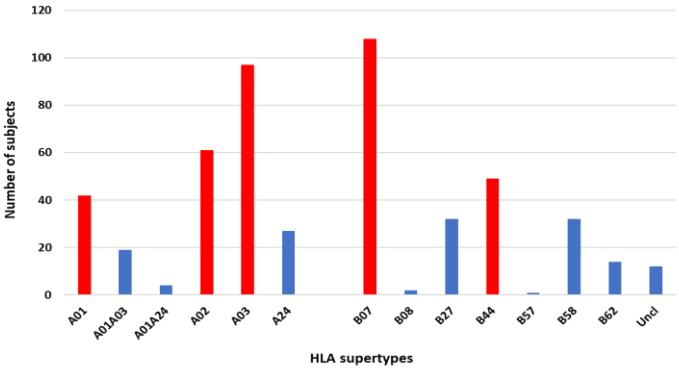

Figure 3. HLA supertype distribution amongst the 125 IFN-γ positive subjects. The five HLA supertypes of interest in the study are shown in red colour.

<u>Molecular detection and genotyping of *P falciparum*</u>: In order to identify subjects who have active *P. falciparum* infections, a nested PCR was used to amplify the *P. falciparum* 18s rRNA gene using DNA from the 300 subjects as templates. After the nest 2 reaction, an aliquot of amplified products was run in a 2% agarose gel with ethidium bromide and the gel visualized under a UV transilluminator. A representative gel is presented in **Figure 4**.

Of the 300 samples, 192 (64%) were positive for *P. falciparum*. The breakdown of parasite-positive subjects by month of sampling is presented in **Figure 5**. DNA from these parasite-positive samples were subsequently used to assess diversity in

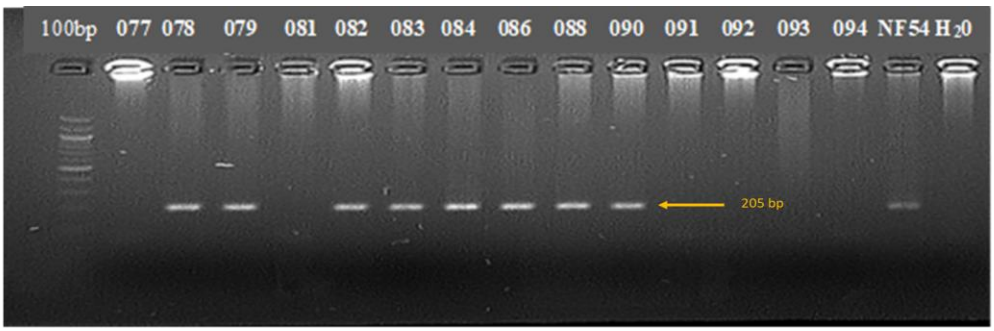

Figure 4. Representative gel for identifying *P. falciparum* 18s rRNA gene. Lane 1 is a 100 bp molecular weight marker, 077 to 094 are subject samples, NF54 is a positive control sample from the NF54 parasite variant and H_20 is a negative control

the *P. falciparum* merozoite surface protein 2 (msp2) gene by another nested PCR procedure with parasite family-specific primers.

This was to determine whether the identified parasites belonged to either the IC/3D7 or the FC27 allelic parasite families. Positive controls used were DNA samples from the 3D7 and DD2 (FC27 family) parasite variants. Representative gels for the IC/3D7 (**Figure 6**) and FC27 (**Figure 7**) family parasites are presented below. Of the 192 PCR-positive subjects, 62 had parasites that belong to the IC/3D7 family, 51 had parasites that belong to the FC27 family and 52 had mixed infections with both IC/3D7 and FC27 family parasites. 24 parasite positive samples did not yield any msp2 allele types

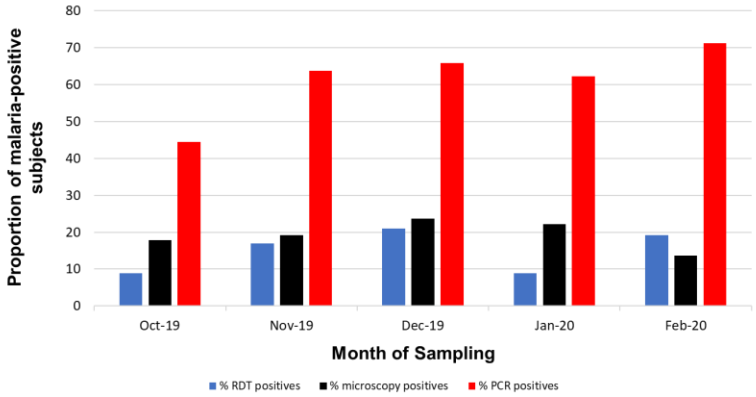

Figure 5. Prevalence of malaria parasites estimated by microscopy, RDTs and PCR.

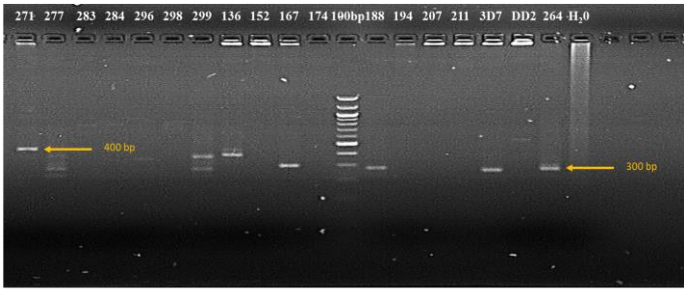

Figure 6. Representative gel for identifying IC/3D7 msp2 allelic family of *P. falciparum*. Lane 12 is a 100 bp molecular weight marker, lane 17 is a positive control DNA from the 3D7 parasite, lane 18 is DNA from the DD2 parasite as negative control and lane 20 is H_20 as a negative control.

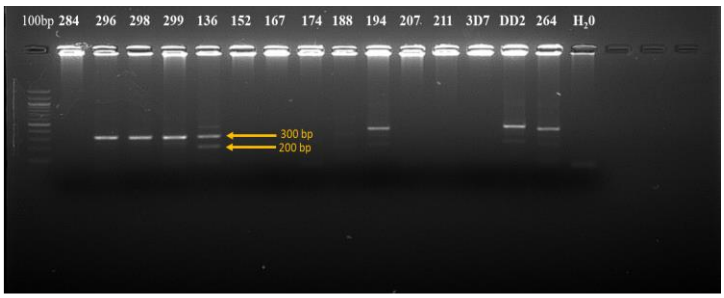

Figure 7. Representative gel for identifying FC27 msp2 allelic family of *P. falciparum*. Lane 1 is a 100 bp molecular weight marker, lane 14 is a negative control DNA from the 3D7 parasite, lane 15 is positive control DNA from the DD2 parasite and lane 17 is H_20 as a negative control.

Detection of blood parasites by microscopy and RDTs: Giemsa-stained slides from all 300 study subjects have been examined independently by two Microscopists for parasites. Fifty-three (53) of the 300 subjects (18%) had microscopic parasites, and a monthly breakdown of this is presented in Figure 5. Parasitemia ranged between 40 and 4,492 parasites per µl of blood, with highest levels of parasitemia occurring in December 2019. Presence of parasites in blood was also assessed using malaria-specific rapid diagnostic tests (RDTs). By this method, 16% of subjects were identified as carrying *P. falciparum* infections, and a monthly breakdown of these is presented in Figure 4. Thus, there was significant concordance of parasite positivity rates between microscopy and RDTs, but the PCR estimates were significantly higher, as expected.

Anti-CSP antibody ELISA results: We assessed the levels of anti-CSP antibodies in the 300 subject plasma samples as a proxy for exposure to malaria infectious mosquito bites. We first utilized a full-length recombinant CSP protein from the 3D7 parasite clone as the coating antigen for antibody measurement, but also used two different synthetic conserved peptides (24mer NANP repeat and 24mer NANPNVDP repeat) from the central conserved repeat region of the CSP antigen. The premise for the use of these conserved peptides for antibody measurement is that the full-length recombinant CSP antigen will have epitopes that are polymorphic. If a significant quantity of anti-CSP antibodies bind to polymorphic epitopes, the levels of antibodies measured with the recombinant antigen may not directly reflect transmission intensity but could be affected by the CSP variants of infecting parasites being very distant from the 3D7 clone CSP recombinant. The conserved peptides will however not differ between parasite variants, and antibodies against these conserved sequences will be consistent irrespective of the infecting parasite variants.

For the respective assays, ELISA plates were coated with each antigen and blocked with skimmed milk overnight. Plates were then incubated with diluted plasma samples from the 300 study subjects and bound antibodies detected using anti-human IgG conjugated to horseradish peroxidase. Plates were developed with TMB as substrate, color development stopped by the addition of sulphuric acid and the optical densities read at 450 nm. Optical density data was converted into titers using the standard pooled plasma from malaria-experienced persons which was run on each ELISA plate. Plasma from 16 malaria-naïve persons were included on multiple ELISA plates as negative controls, and the mean antibody titer plus three times the standard deviation of these negative control samples was estimated as the cutoff for seropositivity for antibodies to the respective antigens.

Antibody titers were generally higher against the recombinant antigen as expected, and lowest against the NANP-NVDP repeat region (**Figure 8**). Further comparison of antibody levels across the different sampling months show a distribution that mirrors parasite prevalence, especially as measured by RDT and microscopy (**Figure 9**). This was especially true for (recombinant vs peptides). The data suggest that the levels of antibodies against the three antigens are all useful as proxies for parasite prevalence, but CSP and the NANP repeat may be most suitable as appreciable levels of antibodies are made against these.

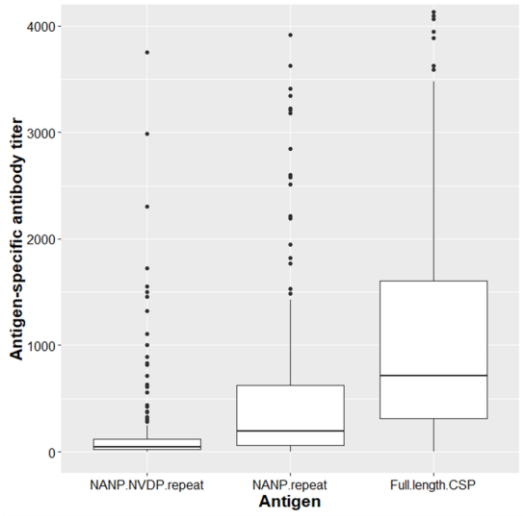

Figure 8. Antigen-specific antibody levels against the full length recombinant CSP and two conserved CSP peptides. Boxplots showing the median, lower and upper quartiles. Whiskers are 1.5 times the interquartile range. Outliers are shown as black dots

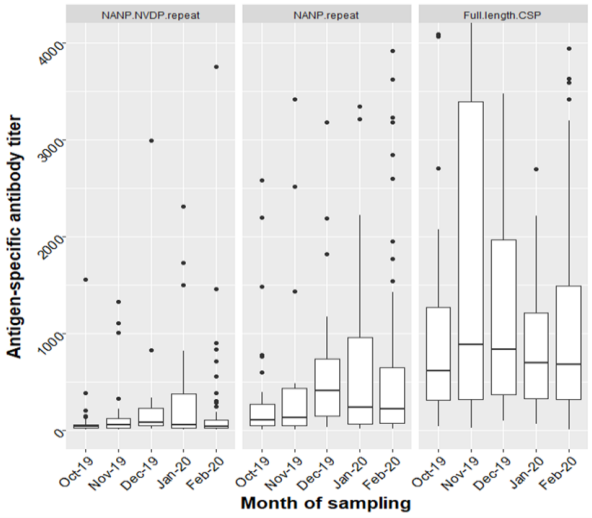

Figure 9. Variations in antigen-specific antibody levels over the 5 months. Boxplots showing the median, lower and upper quartiles. Whiskers are 1.5 times the interquartile range. Outliers are shown as black dots

c. What opportunities for training and professional development has the project provided?

The project currently has one Postdoctoral Fellow and three Research Assistants in full salary employment. In addition, the project has a number of laboratory staff who support various aspects of the conduct of project-related work and are paid allowances on the project. At the inception of the study, five field assistants were trained in the process of obtaining informed consent and two technical persons were given orientation regarding the taking of blood samples at the study site and the subsequent processing and packaging of samples for transport. Two persons involved in field activity coordination and making of community announcements for subject recruitment were engaged. We also had a trained counsellor as a member of the field team, to provide counselling support to study subjects who required counselling because of either their direct or indirect participation as study subjects. In the laboratory, the study procedures have been used to provide training for up to five research interns who are undertaking a mandatory one-year national service after completing their

BSc training in various fields in the biological sciences. There is also a plan to carve some minor projects related to this project for two additional undergraduate students in the coming year.

d. How were the results disseminated to communities of interest?

The study is on-going and there has not been an official presentation of study results to the community since the greater part of laboratory analysis still on-going. Summaries of study data, packaged in a non-technical format, will be shared with the community after the second sampling period and laboratory analysis are completed. The study team however has constant interaction with the community leaders and staff of the health facility in the study community with regards to some general observations the study has so far picked up, for the purposes of encouraging health service uptake and promoting openness with the community leaders.

Furthermore, results of all screening tests have been shared with the study subjects involved and where necessary, we have directed study subjects who required medical attention to the community health center for medical assistance. For study subjects who had malaria parasites in their blood at screening, the study paid for their malaria treatment by the health center.

e. What do you plan to do during the next reporting period to accomplish the goals?

For the next one-year reporting period, we expect to achieve the following;

i. Complete the bioinformatic analysis using HLA typing data and select 9-10mer predicted conserved epitopes that are present in positive peptide pools. Selected peptides will subsequently be chemically synthesized.

ii. Complete the second-round sampling of 127 of the original 300 study subjects to collect additional blood samples in Ghana for further analysis in the USA. Selection of the 150 subjects will be based on their having at least one of the five HLA supertypes of interest in this study, a positive IFN-ɣ and/or granzyme B response to at least one antigen-specific peptide pool from the earlier FLUOROSpot assays using 15mer-peptide pools and seropositivity for anti-CSP antibodies, which is our marker of exposure to infectious mosquito bites.

iii. Isolate PBMCs from the 127 subjects in Ghana and ship anonymized cryopreserved PBMC samples to the NMRC laboratories in USA for epitope identification.

iv. Perform FLUOROSpot assays at NMRC using the predicted/selected and chemically synthesized 9-10mer peptides, to identify conserved immunodominant epitopes from the four antigens.

v. Use identified conserved immunodominant peptides to design and produce the self-assembling nanoparticle-based multi-epitope vaccine candidates.

vi. Draft at least two manuscripts based on our accumulated data, and within the reporting period, get at least one of these manuscripts accepted and published in a peer-reviewed journal.

4. Impact

Nothing to report

5. Changes/problems

Nothing to report

6. Products

Nothing to report

7. Participants & other collaborating organizations

i. Individuals who have worked on the project

Name	Role	Research Identifier (ORCID ID)	Nearest person month	Contribution to project
Kwadwo Asamoah Kusi	Partnering PI	0000-0001-5483-9985	4.8	Overseeing project implementation and the day-to-day running of the project in Ghana, including the technical, logistical and financial aspects.
Linda Eva Amoah	Co-investigator	0000-0002-1652-8489	1.8	Overseeing the conduct of the molecular biology aspects of laboratory work (DNA purification, malaria parasite diversity analysis)
Augustina Frimpong	Postdoctoral Fellow	0000-0002-8637-6054	7.2	Assisting the partnering PI with day-to-day running of the project. She also manages project resources and ensures a steady progress of work.
Kwadwo Akyea-Mensah	Research Assistant	0000-0003-1772-2854	12	Responsible for performance of FLUOROSpot assays
Ebenezer Addo Ofori	Research Assistant	0000-0001-8163-1936	12	Responsible for performance of FLUOROSpot assays, also assists with blood sample collection duties at the field site.
Nana Aba Ennuson	Research Assistant	0000-0001-9430-6040	12	Responsible for performance of the molecular biology components (DNA purification, malaria parasite diversity analysis) of laboratory work
Eric Kyei-Baafour	Research assistant	0000-0001-6423-1885	1.8	Provides support for project related activities at the study site and also provides laboratory support
Alex Danso-Coffie	Research Assistant		1.8	Responsible for coordination of project related activities at the study site and for subject

				counselling services. Also provides laboratory support for PBMC cryopreservation

ii. Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

NO

iii. What other organizations were involved as partners?

Organization name: Walter Reed Army Institute of Research (WRAIR)

Location of Organization: 503 Robert Grant Avenue, Silver Spring, MD 20910

Partner's contribution to the project: Collaboration (subaward) with Dr. Evelina Angov as stated in SOW in

Major task 4 (year 2)

8. Special reporting requirements

This is a collaborative award, and as such, a duplicative report will be submitted by both the Initiating and Partnering PIs. The NMRC report was submitted and accepted in June 2020. This report therefore presents additional updates over the NMRC report.
Updated quad charts will also be uploaded along with the technical report.

9. Appendices

None