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Haplotyping of *Plasmodium falciparum* Thrombospondin-Related Adhesive Protein Coding Gene in Isolates from Buea, Cameroon

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Abstract: Knowledge of the genetic diversity of leading malaria vaccine candidate genes in various geographical regions is very essential for the development of a very successful malaria vaccine. This study aimed at assessing the haplotype variation of *Plasmodium falciparum* thrombospondin-related adhesive protein (*PfTRAP*) gene in malaria isolates from Buea, Cameroon. Parasitized blood samples were collected on whatmann filter papers by finger pricks from 291 participants. *Plasmodium* infection was assessed by thick and thin blood films. DNA samples were isolated from blood spots using the chelex method and used for *falciparum* speciation and *PfTRAP* gene amplification by polymerase chain reaction. *PfTRAP* amplicons were digested using four different restriction enzymes. The *PfTRAP* sequences were amplified from 83 of 172 *falciparium* positive DNA extracts. Digestion of the gene with the *Bgl*II gave two of four possible variants {(+-; 2.4%), (--; 100%)}, three with *Ssp*I {(+-; 1.2%), (-+; 15%), (--; 80.7%)} and all four with *Afl*II {(+-; 71.1%), (++; 16.9%), (-+; 4.8%); (--; 7.2%)}. The single *Taq*$^{\alpha}$I site on the gene was present in 65% of the samples. Haplotype analysis showed that 14 of the 96 possible *PfTRAP* haplotypes were present in our isolates with haplotype 91 (----+-+) being the most prevalent (32%). We have demonstrated the various *PfTRAP* haplotypes circulating with low frequency in the malaria endemic region of Buea. These findings will add to the knowledge of the PfTRAP haplotypes circulating worldwide which is very vital for a TRAP-based/multi-antigen malaria vaccine development that will be globally effective.

Keywords: *Plasmodium falciparum*, Malaria, Vaccine Candidate, Haplotypes, Polymorphism

Introduction:

Efforts in recent years to curb malaria death toll has led to a significant reduction in the incidence of malaria especially in malaria stricken regions of Africa and south-East Asia. Presently malaria is endemic in 91 countries with about 216 million cases and an estimated death toll of 445,000; 90% of which are from Africa [1]. These deaths are caused mostly by the most deadly and most prevalent *falciparium* malaria parasite in the African continent. Various efforts to contain this disease have significantly reduced the recent annual malaria deaths to about 445,000 [1], down from the historic peak of 1.8 million in 2004 [2]. However, the advent of artemisinin resistant parasite strains in south East Asia [3], mosquito resistance to insecticides, non-compliance of the population to the use of the bed nets, reduction in funding for the fight against malaria, amongst others tend to pause a challenge to these efforts. Efforts to protect the most affected and vulnerable group which include children below the age of 5, pregnant women, HIV/AIDS patients and naïve travelers have also yielded much dividend but also remain compromised. Presently, the decline in malaria cases and deaths seem to have stalled as a result of these factors as the number of cases and deaths in 2016 slightly exceeds those in 2015 [1, 4]. Antimalaria drug resistance has been recurrent and with the development of parasite resistance to artemisinin in South East Asia in recent years leaves fears of widespread occurrence in sub-Saharan Africa as was the case with chloroquine and sulfadoxine-pyrimethamine (SP) in the 1950s and 60s [5]. No Asian artemisinin-resistance allele has yet been detected in the African continent though many other distinct Africa-specific alleles have been found with yet unkown functions [6]. There is therefore the need for novel therapeutic agents against malaria in order

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to preempt any eventual widespread artemisinin resistance in case the malaria-drug-resistance vicious cycle continues at a global scale.

A sustainable approach to malaria control is the development of a suitable vaccine that can confer full and long-lasting protection against all malaria populations. Till date none has been developed and certified in this line due to various challenges among which are the extreme genetic diversity among malaria parasite isolates observed in different parts of the world [7,8]. Of the malaria vaccine candidates based on *P. falciparum* antigens identified so far, slightly more than a dozen have undergone clinical trials [9, 10, 11, 12]. The circumsporozoite (CS) recombinant protein-based malaria vaccine RTS,S (RTS,S/AS01) ("Mosquirix") is the most advanced so far. However, it only provides partial protection against *falciparum* malaria in young children, short of full or complete protection to all the populations and shows a decreasing efficacy after a year of administration [13,14]. RTS,S comprises a portion of the *Pasmodium falciparum* circumsporozoite surface protein (CSP) sequence fused to the hepatitis B surface antigen and delivered with the adjuvant (ASO1). CSP and the thrombospondin-related adhesive protein (TRAP) are the two major *Plasmodium falciparum* sporozoite surface antigens on which experimental *falciparum* malaria vaccines are in advanced stage [9, 15].

Genetic diversity in these parasite antigens helps it evade the immune system and impose constraint on the development of an effective malaria vaccine. An antibody raised against an allele of a particular antigen may not be effective in blocking the invasion of red blood cells by a heterologous parasite strain that expresses a different allele of that same antigen. Consequently, a thorough genetic variability study on these *P. falciparum* vaccine antigen candidates in natural *P. falciparum* populations in various endemic areas is required prior to their inclusion in a cocktail for a very successful vaccine. The Thrombospondin-Related Adhesive Protein of *Plasmodium falciparum* (PfTRAP) is a component of the parasite micronemes, organelles that play an important role in apicomplexa adhesion and invasion, and is currently a promising target for malaria vaccine research. It is a sporozoite surface protein present in sporozoite and the erythrocyte stages of the parasite [16, 17]. TRAP functions in sporozoite gliding, motility and hepatocyte invasion. Previous findings suggested that it could be an important component of a multivalent malaria vaccine [18, 19, 20]. Currently, it is being tested in a number of vaccine formulations in various endemic areas with some promising results [21, 22, 23, 24]. Though the *PfTRAP* gene sequences of cultured parasites have been shown to be stable, sequences from field isolates have been shown to be polymorphic probably due to the host-immune response [25, 26]. These antigenic sites need to be represented if it has to be incorporated into a cocktail for an effective vaccine. Such a diversity-covering approach with the *P. falciparum* vaccine candidate antigens has been used for a number of vaccine formulations that have reached clinical trials [27, 28, 29]. Allele-distribution frequency genome-wide studies have shown that some of these genes are under balancing selection in endemic populations and may also be under current directional selection in local populations [30, 31]. Antigenic variation in relation to vaccination has been widely studied including clinical demonstration of allele-specific protection [32, 33]. Previous results predict allele-specific immunity to TRAP and CSP and these needs to be considered in designing vaccines based on these candidates [34]. Selection to maintain diversity operates more strongly on the *P. falciparum* TRAP as compared to the *P. falciparum* CSP [35]. The *Plasmodium vivax* TRAP has also been shown to be a suitable candidate for the development of an effective pre-erythrocytic stage vaccine based on this antigen [36]. Thus a TRAP-based vaccine could be very valuable for both *falciparum* and *vivax* malaria. In this study the genetic variability of the *PfTRAP* vaccine candidate antigen gene was assessed in *P. falciparum* isolates from the malaria endemic region of Buea in the South West Region of Cameroon. A good knowledge of the haplotypes circulating in various parts of the world is very vital for a TRAP-based or TRAP-based multi-antigen malaria vaccine development.

MATERIALS AND METHODS

Ethics concern:

This study was approved by the Ethics Review and Consultancy Committee (ERCC) of the Cameroon Bioethics Initiative (CAMBIN) (Ref. No. 2016/01/1098). Written informed consent was obtained from all participants.

Study area:

The study was carried out at the Mount Mary Hospital in Buea, capital of the South West Region of Cameroon. Buea is a multi-ethnic town situated along the slope of Mount Cameroon with an estimated population of 81,478 inhabitants. This study area has previously been described [37]**.** The climate is of the equatorial type with temperatures varying between 18°C in August to 35°C in March. The mount Cameroon area has a long rainy season that starts in March and ends in late October (though the pattern seems to be changing slightly) with maximum rainfall of about 4,000 mm and a relative humidity of over 80% in August and September. Malaria in this region is meso-endemic and becomes hyper-endemic

during the rainy season with peak incidence between July and October. *Plasmodium falciparum* is the predominant parasite specie which accounts for 96% of malaria infection in this area [37, 38].

Subjects:
The study involved malaria positive volunteers aged between 6months and 65years. Participants were enrolled from amongst patients presenting to the outpatient unit of the Mount Mary hospital in Buea with malaria parasitemia. Written informed consent (or by proxy) was obtained from volunteers who were then made to fill a structured questionnaire prior to enrolment into the study. A total of 291 patients with malaria infection (confirmed by positive blood smear) participated in this study.

Malaria samples:
Blood samples were collected from patients for thin and thick blood films and for DNA analysis by fingerpricks. Screening for malaria was done by microscopic analysis of Giemsa-stained blood smears. For DNA analysis blot spots were collected on individually allocated sheets of Whatman (3MM) paper labeled with the subject name, code and date of collection. The spots were allowed to dry and were then transferred into individual resealable polythene bags containing desiccants and stored at room temperature until required for use. Samples were collected between June and November 2016.

Measurement of haemoglobin content:
In order to assess the anaemic status of participants which could be due to malaria, haemoglobin levels were measured using haemoglobinoter (STAT- Site MHgb metre, Stanbio Laboratory Texas, USA), as per the manufacturer's instructions.

Enzymes and reagents:
Restriction enzymes (*Bgl*II, *Ssp*I, *Afl*II/Acc651 and *Taq* alpha I) used in this study and PCR amplification reagents were from New England BioLabs$_{Inc.}$. (USA). Agarose and chelex-100 were both from Sigma (USA). Oligonucleotides were purchased from Inqaba Biotec Ind. Ltd (Pretoria, South Africa).

DNA extraction and speciation of *Plasmodium falciparum:*
Genomic DNA was extracted from dried parasitized blood spots on Whatman® (3MM) filter papers using the previously described Chelex extraction method [39]. Briefly, a piece of approximately 3 mm x 3 mm was cut from each blood spot and incubated at 4^{o}C in 1 ml of 0.5% saponin in a microfuge tube overnight. The saponin was then removed and 1 ml phosphate buffered saline (PBS) was added and further incubated at 4^{o}C for 30minutes. The filter papers were next transferred into a pre-heated (100^{o}C, heat block for 10minutes) solution containing 150 µl nuclease free water and 50 µl of 20% Chelex®-100 (Bio Rad Laboratories, Inc, California, USA). The tubes were spun at 11000 rpm for 2minutes and the supernatant (containing the genomic DNA) was transferred into new sterile microfuge tubes and stored at -20^{o}C until required for use. Genomic DNA samples of *Plasmodium falciparum* line 3D7 used as control in this study was provided by MR4, ATTC® Manassas Virginia. *Plasmodium falciparum* speciation was carried on the DNA extracts by Nested-PCR using the genus-specific primers (rPLU6F and rPLU5R) for the primary PCR and the specie-specific primers (rFAL1F and rFAL2F for *Plasmodium falciparum*) for the amplification of the 18s rRNA gene. These primers and PCR conditions have previously been described [40].

Amplification of *PfTRAP* sequences from *falciparum* positive samples:
The *PfTRAP* gene was isolated from the *Plasmodium falciparum* positive DNA extracts by primary and nested PCR using respectively the TRAP1 and TRAP2 and TRAP1A and TRAP2A sets of primers and conditions as described earlier [26]. Amplicons were confirmed on 1 and 2% ethidium bromide stained agarose gels respectively, for the primary and nested PCR products.

Restriction fragment length polymorphism analysis of *PfTRAP* amplicons:
Digestion of the nested products for the genetic variation of the *PfTRAP* gene made use of the following four enzymes: *Bgl*II, *Ssp*I, *Afl*II/*Acc*651 and *Taq alpha* I and as instructed by the manufacturers with some slight modifications. 0.4 µl of each enzyme, 1.5 µl of the corresponding buffer, 3.1 µl of nuclease free water and 10 µl (~200 µg) of nested PCR product in a total volume of 15 µl was incubated at 37°C (for 1hour for *Bgl*II and 3hours for *Afl*II/*Acc*651 and *Ssp*I). *Taq alpha* 1 was incubated at 65°C for 30mins. The digests were analyzed on a 2% agarose gel.

Statistical analysis:
Study data were reviewed, entered into Microsoft Office Excel 2013 work sheet and exported to SPSS version 20 (SPSS Inc, Chicago, USA) for analysis. Association between qualitative variables was assessed using the chi square test while differences in group means were compared using the Student's t-tests or analyses of variance (ANOVA). A difference giving a P-value ≤ 0.05 was considered statistically significant.

RESULTS

Socio-demographic characteristics of study subjects:

Table 1 below summarizes the basic socio-demographic characteristics of the study population. Of the 291 subjects aged 6months to 65years that took part in the study 111(38.14%) were males and 180(61.86%) were females and the mean age was 23.67±17.39. Parasitemia ranged from 78,000 – 150,000 parasites/µL blood with geometric mean parasite density (GMPD) of 9995 parasites/µl of blood. The haemoglobin concentration varied from 8 - 16.6 g/dL with a mean concentration of 11.94 ± 1.78 g/dL. 47.4% of participants were anemic while 52.6% were non-anemic.

Table 1: Socio-demographic characteristics of study subjects

Variables		n	Percentage
Gender	Males	111	38.14
	Females	180	61.86
Anemia status	Not anemic	153	52.60
	Mild	25	8.60
	Moderate	113	38.80
Febrile Status	Afebrile	83	28.50
	Febrile	208	71.50
Parasitemia	Low	115	39.52
	Moderate	88	30.24
	High	88	30.24
Age groupings	<5	74	25.43
	5-9	52	17.87
	9-14	47	16.15
	≥15	118	40.55

Effects of socio-demographic parameters on parasitemia

The mean parasite density was significantly (p = 0.0002) different when compared amongst age groups with children having higher mean parasite density than adults. Parasitemia load was also significantly different (P=0.0001) between febrile and afebrile individuals with the febriles having relatively higher parasitemia loads. No significant association was observed between malaria parasitemia and gender (p=0.529) and anemia status (p=0.012). Though the geometric mean parasite density was higher for females than males it was not statistically significant. Fever showed a weak positive correlation with parasitemia (r = 0.435) while there was no significant association between fever status and gender (p=0.260) and between fever status and age groups (p=0.893). Equally there was no significant association between anemic status and gender (p = 0.191) or age categories (p = 0.090) (Table 2).

Table2: Anemic status of study participants against gender and age.

Parameter		N%	Anemia [n(%)]			P value
			Non anemic	Mild	Moderate	
Gender	Male	111 (38.1)	63 (56.8)	6 (5.4)	42 (37.8)	p = 0.191
	Female	180 (61.9)	86 (47.8)	19 (10.6)	75 (41.7)	
Age (yrs)	<5	74 (25.4)	46(62.2)	4(5.4)	24 (32.4)	p = 0.09
	5-9	52 (17.9)	32 (61.5)	3(5.8)	17 (32.7)	
	10-14	47 (16.2)	26(55.3)	4 (8.5)	17 (36.2)	
	≥15	118 (40.5)	49 (41.5)	14 (11.9)	55(46.6)	

Falciparum speciation and amplification of *PfTRAP*:

Plasmodium genomic DNA was isolated from all the 291 participants' Whatmann paper spotted blood samples. *Plasmodium falciparum* specie-specific gene sequences (205bp in length) were successfully amplified from 172 of the 291 samples using the *P. falciparum* species-specific set of primers. The extracts were analyzed on a 2% agarose gel and yielded sharp bands at molecular weights of 205bp on the electrophoregram corresponding to the *P. falciparum* species- specific gene sequences (Figure 1).

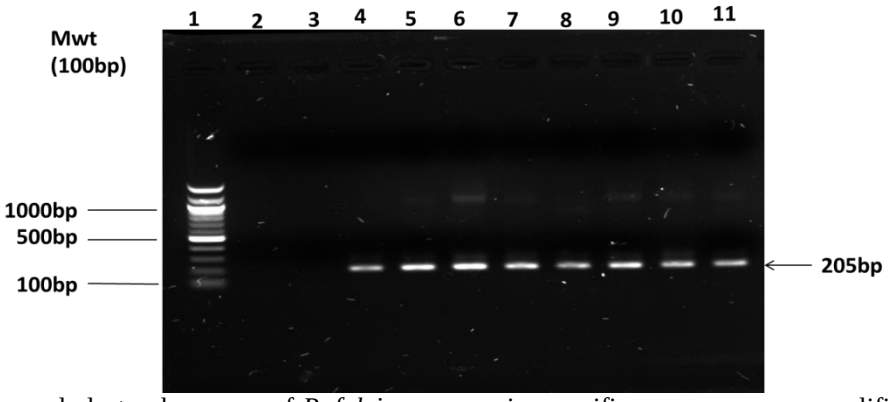

Figure 1: Agarose gel electrophoregram of *P. falciparum* species-specific gene sequences amplified by PCR from *Plasmodium* genomic DNA extracts using *falciparum* species-specific primers. Lane 1= 100bp ladder (Mwt), lane 2-3= negative control, lane 4= positive control (3D7), lane 5-11= DNA amplicons of field isolates.

The *PfTRAP* gene sequences were successfully amplified by nested PCR from 83 of the 172 *P. falciparum* genomic DNA samples using TRAP1 and TRAP2 forward and reverse primers respectively for the primary PCR and TRAP 1A and TRAP 2A forward and reverse primers respectively for the nested. Majority of the samples (89) were refractory to amplification despite repeated efforts to obtain amplicons. Nested PCR products were analyzed on a 1% agarose gel yielding the expected band size of 1638bp (size of the *PfTRAP* gene) (Figure 2).

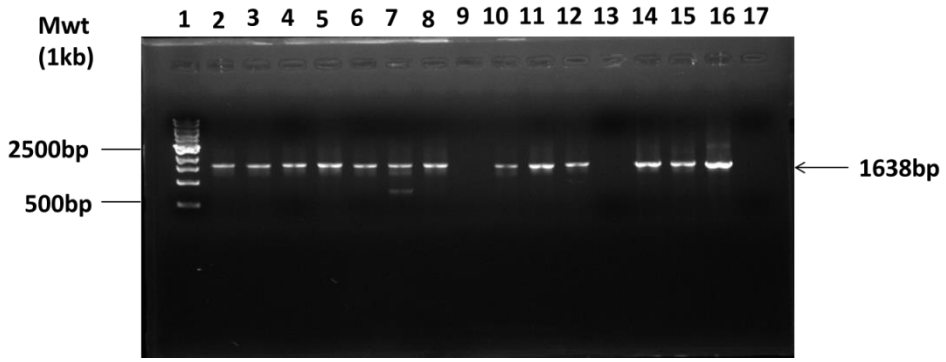

Figure 2: Agarose gel electrophoregram of PfTRAP sequences amplified from P. falciparum positive samples by nested PCR using TRAP 1A and TRAP 2A forward and reverse primers respectively. Lane 1= 1kb ladder (Mwt), lane 9 and 13= refractory to amplification, lane 2-15= PCR amplicons (PfTRAP sequences), lane 16= P. falciparum 3D7 (positive control), lane 17=negative control.

Digestion of *PfTRAP*:

The 1638bp long *PfTRAP* gene of *P. falciparum* contains two *Bgl*II, two *Afl*III/*Acc*651 and two *Ssp*I sites (the first at the 5′ end and the second at the 3′ end of the gene). There is only one *Taq*$^{\alpha}$ I site present in the gene. The 3' *Bgl*II and 3' *Ssp*I sites are mutually exclusive. Digestion of this gene with these four enzymes will give various fragment sizes (Supplementary data). When mutated these sites remain unrecognized by the enzyme and the presence (+) or absence (-) of the restriction enzyme sites will give different restriction fragment length sizes where in some cases the fragments may vary in sizes since they contain regions with repeats

Mutation of the *Afl*II sites on the *PfTRAP* gene:

*Acc*651 is an enzyme used in combination with *Afl*II to enhance *Afl*II activity. *Afl*III/*Acc*651 digestion of the 83 PCR amplified *PfTRAP* sequences gave various combinations of the presence/absence of the restriction sites (Figure 3). Polymorphism associated with *Afl*III/*Acc*651 revealed all four variants (+-), (++), (-+) and (--). 59 (71.1%) samples had intact 5′ site and no 3′site (+-), 14 (16.9%) samples had both sites intact (++), 4 (4.8%) samples lacked the 5′ but had the 3′site (-+) and 6 (7.2%) samples had both sites mutated (--). Overall, 73 (~88%) of the 83 field isolates from Buea had intact 5'*Afl*III/*Acc*651 sites and only 18 (~22%) samples had an intact 3'*Afl*III/*Acc*651 sites on the *PfTRAP* gene. The 3' site was thus highly mutated.

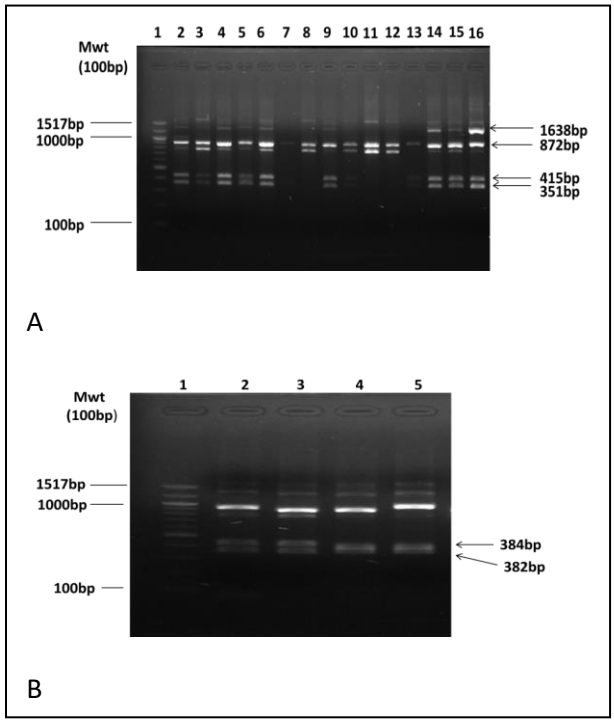

Figure 3: Agarose gel electrophoregram of *PfTRAP* amplicons digested with *Afl*II/*Acc*651 enzyme and depicting various combinations of presence/absence of *Afl*II/*Acc*651 restriction site**.** A: gel illustrating the +- and -- variants. Lane 1= 100bp ladder, Lane 2-5 and 8-16= *Afl*II/*Acc*651 digests, lanes 6 and 7 = positive and negative controls respectively. B: gel illustrating the -+ variant. Lane 1= 100bp ladder, Lane 2-5= *Afl*II/*Acc*651 digests.

Mutation associated with the *Ssp*I sites:
Digestion with the *Ssp*I restriction enzyme revealed the presence of three variants (+-), (-+) and (--) (Table 3). No case where both sites (++) were present was observed in any of the samples analyzed. 67 (80.7%) samples had both 5′ and 3′ sites mutated (--) while 15 (18%) lacked the 5′ site but had the 3′ site (-+) and only 1 (1.2%) sample had an intact 5′ site and a mutated 3′ site (+-) (Fig 4). Though the 5' *SspI* site was almost inexistent in these field isolates from Buea, the 3'site was found in 18% (15) of the samples. Both sites have thus been lost probably due to mutations.

Table 3: Distribution of restriction enzyme sites on *PfTRAP* genes isolated from samples from Buea.

Restriction enzyme	Restriction site	Number of samples containing site/percentage	Total samples with intact 5' site	Total samples with intact 3' site
*Afl*II/Acc651	+ + + - - + - -	14 (16.9) 59 (71.1) 4 (4.8) 6 (7.2)	73	18
*Bgl*II	+ + + - - + - -	0 2 (2.4) 0 81 (97.6)	2	0
*Ssp*I	+ + + - - + - -	0 1 (1.2) 15 (18) 67 (80.7)	1	15
Taq$^{\alpha}$I	+ -	50 (65) 33 (35)	50	33

++ = presence of both 5' and 3' restriction sites; +- = presence and absence of 5' and 3' restriction sites respectively; -+ = absence and presence of 5' and 3' restriction sites respectively; -- = absence of both the 5' and the 3' restriction sites.

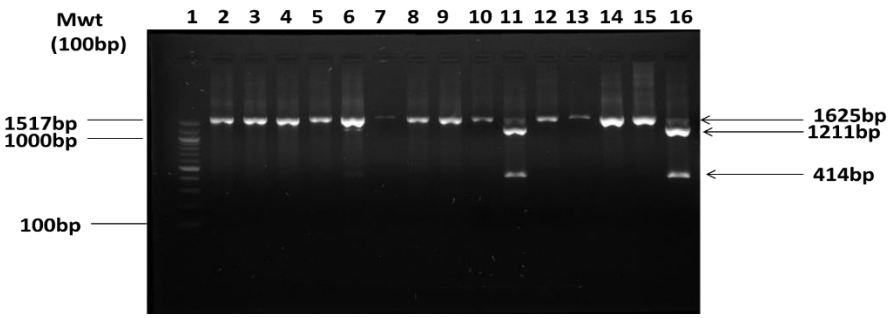

Figure 4: Agarose gel electrophoregram of *Ssp*I digested *PfTRAP* gene**.** Lane 1=100bp ladder, Lane 2-10, 12-15= *Ssp*I digests with band sizes at 1638bp (--), lanes11= *Ssp*I digests with band sizes at 1211bp and 414bp (-+), lane 16 = positive control (3D7). Note worthy is the fact that fragments at 1638bp and 1211bp can vary since they contain regions with repeats.

Polymorphism associated with *Taq*$^{\alpha}$I Enzyme:
Digestion with *Taq*$^{\alpha}$I enzyme showed that 50 (65%) samples possessed the *Taq*$^{\alpha}$I restriction site and 33 samples (35%) lacked the site (Figure 5; Table 3).

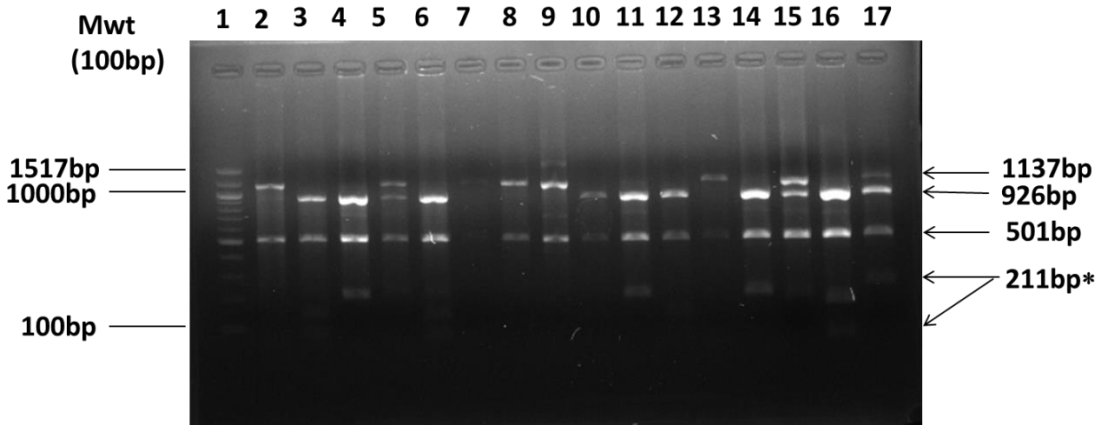

Figure 5: Agarose gel electrophoregram of *Taq*$^{\alpha}$I digested *PfTRAP* PCR products**.** Lane 1=100bp ladder, Lanes 2,5,8,9,13,15= *Taq*$^{\alpha}$I digests with band size of 1137bp and 501bp (-), Lanes 4,5,6,11,14,15,16,17= *Taq*$^{\alpha}$I digests with band sizes of 926bp, 501bp and 211bp (+). 211bp*= contain regions with repeats and size varies.

Mutation of the *Bgl*II site:
Digestion of the 83 PCR-amplified *PfTRAP* sequences with *Bgl*II enzyme revealed a predominance of a single band at 1638bp which corresponds to the absence of the restriction sites on both ends of the gene. Only two samples had an unmutated 5' site with mutated 3' site. Since the positive control (3D7) was also cut by this enzyme these two sites must probably have been completely mutated in these isolates (Table 3)

***PfTRAP* Haplotype analysis:**
The *PfTRAP* haplotype analysis was done by combination of various restriction enzyme sites. Particular haplotypes were defined by the sizes of the various restriction fragments obtained after digestion**.** The presence and absence of a particular restriction site was denoted by + and –, respectively. Combinatorial analysis of each + and – yielded a total of 128 possible haplotypes using the four sets of enzymes. The 3' *Bgl*II and the 3' *Ssp*I sites are mutually exclusive. Taking this into consideration implies that there are 96 possible haplotypes that can be delineated for the *PfTRAP* gene as 32 of 128 possible haplotypes contain both sites and are thus excluded (supplementary data). The 96 possible PfTRAP haplotypes range from haplotype 1 (+++-+++) to haplotype 96 (-------). Haplotype 1 is defined as *Bgl*II (++), *Ssp*I (+-), *Afl*II (++) and *Taq*$^{\alpha}$I (+) and haplotype 96 as *Bgl*II (--), *Ssp*I (--), *Afl*II (--) and *Taq*$^{\alpha}$I (-). No *Ssp*I (++) and *Bgl*II (++ and -+) variants were observed in the samples. Our analysis showed that 14 (14.6%) of the 96 possible *PfTRAP* haplotypes (Table 4) were present in samples from Buea with haplotype 91 (----+-+) being the most predominant**.**

Table 4: *PfTRAP* haplotypes present in *falciparum* isolates circulating in Buea and their predominance.

Haplotype number	Haplotype abbreviation	Frequency of occurrence of haplotype in samples.
91	----+-+	27 (32.5%)
92	----+--	12 (14.5%)
89	----+ + +	10 (12%)
90	----+ +-	8 (9.6%)
95	------+	6 (7.2)
84	---++--	3 (3.6%)
96	-------	3 (3.6%)
83	---+ + -+	3 (3.6%)
94	-----+ -	3 (3.6%)
44	+---+--	2 (2.4%)
93	-----++	2 (2.4%)
82	---+++-	2 (2.4%)
87	---+--+	1 (1.2%)
85	---+-++	1 (1.2%)

+ and – represent respectively the presence and absence of enzyme site. Order of enzyme sites: BglII, SspI, AflII/Acc651, Taq$^{\alpha}$I. It should be noted that there are two BglII sites, 2 SspI sites, 2 AflII sites and only one Taq$^{\alpha}$I site.

DISCUSSION:

Malaria vaccines may be the only ultimate solution to the malaria disease given the continuous vicious cycle of malaria-dry-resistance both with regards to the parasites and the vectors. Rationale design of a subunit malaria vaccine targeting any one of the leading malaria vaccine candidate antigens needs a very good knowledge of all the circulating antigenic variants in various endemic regions for a multivalent vaccine formulation that would be globally effective. The present study describes the diversity of the *P. falciparum* thrombospondin-related adhesive protein (*PfTRAP*) gene in field isolates from the malaria endemic region of Buea. The PfTRAP antigen is one of the 15 vaccine candidate antigens that successfully underwent clinical trials and is currently being tested with promising results in various vaccine formulations [24, 22, 23, 21]. Unlike other vaccine candidates this antigen (PfTRAP) has not been studied in details in naturally occurring infections. With increasing drug pressure especially in endemic areas these candidates need to be monitored for any change in the haplotype frequency for consideration in a vaccine formulation.

The genetic diversity of the *PfTRAP* gene was assessed in field isolates from Buea Cameroon by PCR and RFLP. Analysis of the results revealed that some of the restriction enzyme sites were lost on these isolates most probably due to mutations. While the two *Bgl*II and the two *Ssp*I sites on the gene were almost inexistent in these isolates, the 5' *Afl*II site was very little affected though its 3' site was also highly polymorphic**.** This shows that the *Ssp*I sites and especially the *Bgl*II sites on the *PfTRAP* gene from these field isolates have been seriously mutated and could no longer be recognized by these enzymes. Though these two enzyme sites were very polymorphic, polymosphism associated with the *Afl*II enzyme revealed the presence of all four variants ((+-; 71.1%), (++; 16.9%), (-+; 4.8%) and (--; 7.2%)) and the single *Taq*$^{\alpha}$I site on the gene was present in 65% of the 83 samples. This shows that some segments of the gene seem more susceptible to mutation than others. This confirms the suggestion from previous findings [25] that certain regions of the gene could tolerate point mutations while others could not.

Using a PCR-based haplotyping system for *PfTRAP* to detect changes in four restriction enzyme sites (*Bgl*II (2sites), *Ssp*I (2 sites), *Afl*II (2 sites) and *Taq*$^{\alpha}$I (1site)) and combining these sites taking into consideration the fact that the 3' *Bgl*II and the 3' *Ssp*I sites are mutually exclusive, the *PfTRAP* gene could be assigned to one of 96 possible haplotypes. Our analysis gave 14 haplotypes of *PfTRAP* out of the 96 in isolates from Buea. This shows that the *PfTRAP* haplotypes in this endemic area are present in low frequency. This concords previous finding which showed that most haplotypes on which the present *falciparum* vaccine candidates are based were found to exist at very low frequencies in the parasite populations [41]. Comparing this result with that of Robson and collaborators [26] who also used a PCR-

based haplotyping system for the *PfTRAP* gene and who obtained a haplotype variation of 20.83% (20 of the 96 possible haplotypes) using isolates from Mali, also shows that the sequence variation of the *PfTRAP* gene in this region is very low. The difference may be accounted for by the fact that the gene from isolates in Buea have been under intense drug combinations that might have removed some of the mutants from circulation just as it is known also to be responsible for the generation of mutant species. More so, the parasites in this region might not have been under intense drug pressure compared to those in Mali. The use of several drug combinations in most endemic areas since the advent of ACTs seems to exert a lot of pressure on the parasites. In a recent study, analysis of 32 single allele sequences of the entire coding region of the *PfTRAP* gene after direct PCR sequencing of DNA samples from 32 malaria infected patients from Thailand identified 23 different allelic haplotypes [42]. While this study was based on sequencing the whole length of the *PfTRAP* gene from only 32 patients, our study was based on restriction analysis of 83 PCR-amplified *PfTRAP* gene sequences from *falciparum* positive isolates. Though the present study involved a larger sample size as compared to the above mentioned study in Thailand, both results show a level of genetic differentiation of the *PfTRAP* (though at different frequencies) gene most probably due to strong diversifying selection acting on this gene. The most common haplotype in this study was haplotype 91 (----+-+; 32.5%) unlike the case of Mali where Robson and collaborators found haplotype 42 (+---++-) to be the most common. This difference clearly underscores the need for genetic analysis of these vaccine candidates in various endemic areas in order that all the allelic variants are considered for any vaccine development based on the candidate antigen. The genetic variability in T and B cell epitopes of the PfTRAP vaccine candidate antigen has also been assessed in a number of cases. The antigenic variability assessed at TRAP epitopes located at amino acid positions 78-88 and 504-513 (for T-cells) and 201-215, 421-435 and 461-475 (for B-cell epitopes) in Peruvian isolates showed highly conserved TRAP sequences [43]. Other epitopes in the above mentioned work of Ohashi and collaborators were also found to be conserved in Thailand. Though the haplotype frequency of *PfTRAP* gene in field isolates from this geographical region of Buea is low, further studies on the epitope and amino acid sequence variability of this antigen in this area will be necessary to throw more light on its diversity for implication in a TRAP-based vaccine development.

CONCLUSION:

The *PfTRAP* vaccine candidate genes from isolates in Buea have low haplotype frequency, a property relevant for a good vaccine candidate. However, more studies on its epitope and amino acid sequence variations in this endemic area are relevant for a conclusive definition of its diversity in this study area and its significance for a TRAP-based vaccine design.

Data Availability statement: The (table of all the possible haplotypes that can be obtained by combining the BglII, SspI, AflII and Taq$^{\alpha}$I restriction enzyme sites; table of different fragment sizes obtained upon digestion with each of the four enzymes) data used to support the findings of this study are included within the supplementary information file.

Conflict of interest: The authors declare that they have no competing interests.

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Authors' contributions: DLN designed and supervised the study, participated in data analysis and manuscript preparation. MMN, DNB and BTM participated in sample collection genetic analysis, data analysis, and manuscript preparation. ALZD participated in the genetic analysis and manuscript preparation. SMG participated in data analysis and manuscript preparation.

Supplementary Materials: Table A: Haplotype analyses. A table showing all the possible haplotypes that can be obtained by combining the restriction enzyme sites of the four enzymes (BglII, SspI, AflII and Taq$^{\alpha}$I). BglII, SspI and AflII enzymes have two restriction sites each on the TRAP gene; one at the 3'end and the other at the 5'end and Taq$^{\alpha}$I enzyme has only one site. Table B: Restriction fragment sizes. A table showing the different sizes of fragments that can be obtained upon digestion of the TRAP gene with each of the four restriction enzymes used in the study.

REFERENCES

1. WHO: World malaria Report 2017. Geneva: WHO; 2017. Available: www.who.int/malaria/publications/world-malaria-report-2017/en/
2. Murray, CJL., Rosenfeld, LC., Lim, SS., Andrews, KG., Foreman, KJ., Haring, D., Fullman, N., Naghavi, M., Lozano, R., Lopez, AD. Global malaria mortality between 1980 and 2010: a systematic analysis. LANCET. 2012; 379 (9814): 413-431.
3. Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, Lwin KM, et al. Artemisinin Resistance in *Plasmodium falciparum* Malaria. The New England Journal of Medicine. 2009; 361(5): 455-467.
4. WHO: World Malaria Report 2016: Geneva. WHO;2016. Available: www.who.int/malaria/publications/world-malaria-report-2016/report/en/
5. Trape, JF. The public health impact of chloroquine resistance in Africa. Am J Trop Med Hyg. 2001; 64:127
6. Menard D, Khim N, Beghain J, Adegnika AA, Shafiul-Alam M, Amodu O et al. A worldwide map of *Plasmodium falciparum* K13-Propeller Polymorphisms. N Engl J Med. 2016; 374:2453-2464.
7. 7.Chattopadhyay R. and Kumar S. Malaria vaccine: Latest update and challenges ahead. Indian Journal of Experimental Biology. 2009; 47: 527-536.
8. Hyde J. E. Targeting purine and pyrimidine metabolism in Human Apicomplexans parasites. Current drug targets. 2007; 8:31-47.
9. Conway, DJ. Paths to a malaria vaccine illuminated by parasite genomics. Trends in Genetics. 2015; 31(2): 97-107.
10. Tamminga C, Sedegah M, Regis D, Chuang I, Epstein JE, Spring M, et al. Adenovirus-5-Vectored *P. falciparum* Vaccine Expressing CSP and AMA1. Part B: Safety, Immunogenicity and Protective Efficacy of the CSP component. PLoS ONE. 2011; 6(10): e25868.
11. Oguru BR, Apollo OJ, Mckinney D, Okoth W, Siangla J, Dubovsky F, et al. For the MSP-1 Malaria Vaccine Working Group: Blood stage malaria vaccine eliciting high antigen-specific antibody concentrations confers no protection to young children in western Kenya. PLoS One. 2009; 4:e4708.
12. Sagara I, Ellis RD, Dicko A, Niambele MB, Kamate B, Guindo O, et al. A randomized and controlled Phase 1 study of the safety and immunogenicity of the AMA-1C1/Alhydrogel + CPG7909 vaccine for *Plasmodium falciparum* malaria in semi-immune Malian adults. Vaccjne, 2009; **27**:7292-7298.
13. EMA. First malaria vaccine receives positive scientific opinion from EMA, European Medicines Agency. 2015, Press release: EMA/CHMP/488348/2015.
14. RTS,S Clinical Trials Partnership. Efficacy and safety of the RTS,S/ASO1 malaria vaccine during 18 months after vaccination: a randomized, controlled trial in children and young infants at 11 African sites. PLoS Med. 2014; 11: e1001685.
15. Ewer k, Collins K, O'Hara G, Duncan C, Rowland R, Reyes-Sandoval A et al. Protection from malaria sporozoite challenge correlates with frequency of TRAP-specific CD8+ T cells secreting IFNγ. Malaria: New Approaches to Understanding Host-Parasite Interactions. 2010; s86.
16. Cowan G, Krishna S, Crisanti A, Robson K,. Expression of thrombospondin-related anonymous protein in *Plasmodium falciparum* sporozoites. Lancet. 1992; *339:* 1412–1413.
17. Rogers WO, Malik A, Mellouk S, Nakamura K, Rogers MD, Szarfman A, Gordon DM, Nussler AK, Aikawa M, Hoffman SL. Characterization of *Plasmodium falciparum* sporozoite surface protein 2. Proc Natl Acad Sci USA. 1992; 89: 9176–9180.
18. Khusmith S, Sedegah M, Hoffman SL. Complete protection against Plasmodium yoelli by adoptive transfer of a $CD8^+$ cytotoxic T cell clone recognizing sporozoite surface protein 2. Infect. Immune. 1994; 62: 2979-2983.
19. Alloueche A, Milligan P, Conway DJ, Pinder M, Bojang K, Doherty T, Tornieporth N, Cohen J, Greenwood BM. Protective efficacy of the RTS,S/AS02 *Plasmodium falciparum* malaria vaccine is not strain specific. Am J Trop Med Hyg. 2003; 68:97-101.
20. Wizel B, Houghten RA, Parker KC, Coligan JE, Church P, Gordon DM, et al. Irradiated sporozoite vaccine induces HLA-B8-restricted cytotoxic T lymphocyte responses against two overlapping epitopes of the *Plasmodium falciparum* sporozoite surface protein 2. J. Exp. Med. 1995; 182: 1435–1445.
21. Ogwang C, Afolabi M, Kimani D, Jagne YJ, Sheehy SH, et al. Safety and immunogenicity of heterologous prime-boost immunization with *Plasmodium falciparum* malaria candidate vaccines, ChAd63 ME-TRAP and MVA ME-TRAP, in healthy Gambian and Kenyan Adults. PLOS ONE. 2013; 8(3): e57726. Doi: 10.1371/journal.pone.0057726.
22. Afolabi MO,Tiono AB, Adetifa UJ, Yaro JB, Drammeh A, Nebie I, Bliss C, et al. Safety and immunogenicity of ChAd63 and MVA ME-TRAP in West African children and infants. Molecular Therapy. 2016; 24(8): 1470-1477.
23. Mensah VA, Gueye A, Ndiaye M, Edwards NJ, Wright D, Anagnostou NA, et al. Safety, Immunogenicity and Efficacy of Prime-Boost vaccination with ChAd63 and MVA encoding ME-TRAP against *Plasmodium falciparum* infection in adults in Senegal. PLOS ONE. 2016 ; 11(12): e0167951. Doi: 10.1371/journal.pone.0167951.
24. Mensah VA, Roetynck S, Kanteh EK, Bowyer G, Ndaw A, Oko F, Bliss CM, et al. Safety and immunogenicity of malaria vectored vaccines given with routine expanded program on immunization vaccines in Gambian infants and neonates: A randomized controlled trial. Front. Immunol. 2017; 8:1551. Doi: 10.3389/fimmu.2017.01551.
25. Robson KJH, Hall JRS, Davies LC, Crisanti A, Hill AVS, Wellems TE. Polymorphism of the TRAP gene of *Plasmodium falciparum.* Proc R Soc [Lond] B. 1990; 242: 205–216.
26. Robson KJH, Dolo A, Hackford IR, Doumbo O, Richards MB, Keita MM, Sidibe T, Bosman A, Modiano D, Crisanti A. Natural polymorphism in the Thrombospondin-Related Adhesive Protein of *Plasmodium falciparum*. Am. J. Trop. Med. Hyg. 1998; 58(1): 81-89.
27. Ellis RD, Wu Y, Martin LB, Shaffer D, Miura K, Aebig J, et al. Phase I study in malaria naïve adults of BSAM2/Alhydrogen1(R)+CPG 7909, a blood stage vaccine against *P. falciparum* malaria. PLoS ONE. 2012; 7(10): e46094
28. McCarthy JS, Marjason J, Elliott S, Fahey P, Bang G, Malkin E, et al. A phase I trial of MSP2-C1, a blood-stage malaria vaccine containing 2 isoforms of MSP2 formulated with Montanide(R) ISA 720. PLoS ONE. 2011; 6(9): e24413.
29. Elias SC, Collins KA, Halstead FD, Choudhary P, Bliss CM, Ewer KJ, Sheehy SH, Duncan CJ, Biswas S, Hill AV, Draper SJ. Assessment of immune interference, antagonism and diversion following human immunization with biallelic blood-stage malaria viral-vectored vaccines and controlled malaria infection. J. Immunol. 2013; 190:1135-1147.
30. Amambua-Ngwa A, Park DJ, Volkman SK, Barnes KG, Bei AK et al. SNP genotyping identifies new signatures of selection in a deep sample of West African *Plasmodium falciparum* malaria parasites. Mol. Biol. Evol. 2012; 29: 3249-3253.
31. Miotto O, Almagro-Garcia J, Manske M, Macinnis B, Campino S, Rockett KA et al. Multiple populations of artemisinin resistant *P. falciparum* in Cambodia. Nat. Genet. 2013; 45(6): 648-655.
32. Ouattara A, Takala-Harrison S, Thera MA, Coulibaly D, Niangaly A, Saye R, et al. Molecular basis of allele-specific

efficacy of a blood-stage malaria vaccine: vaccine development implications. J. Infect. Dis. 2013; 207:511-519.
33. Thera MA, Doumbo OK,Coulibaly D, Laurens MB, Ouattara A, Kone AK et al. A field trial to assess a blood-stage malaria vaccine. N. Engl. J. Med. 2011; 365:1004-1013.
34. McConkey SJ, Reece WH, Moorthy VS, Webster D, Dunachie S, Butcher G, et al. Enhanced T-cell immunogenicity of plasmid DNA vaccines boosted by recombinant modified vaccinia virus Ankara in humans. Nat. med. 2003; 9(6): 729-735.
35. Weedall GD, Preston BMJ, Thomas AW, Sutherland CJ, Conway DJ. Differential evidence of natural selection on two leading sporozoite stage malaria vaccine candidate antigens. International Journal of Parasitol. 2006; 37:77-85.
36. Nazeri S, Zakeri S, AbouieMehrizi A, Djadid ND. Naturally acquired immune responses to thrombospondin-related adhesion protein (TRAP) of *Plasmodium vivax* in patients from areas of unstable malaria transmission. Acta Tropica. 2017; 173:45-54.
37. 37.Takem NE, Achidi AE, Ndumbe PM. An update of malaria infection and anemia in adults in Buea, Cameroon. BioMed central. 2010; 3:121.
38. 38. Bigoga JD, Manga L, Titanji VPK, Coetzee M, Leke RGF. Malaria vectors and transmission dynamics in coastal south-western Cameroon. Malar J. 2007; 6:5. doi: 10.1186/1475-2875-6-5.
39. 39. Miguel RB, Coura JR, Samudio F, Suarez-Mutis MC. Evaluation of three different DNA extraction methods from blood samples collected in dried filter paper in *Plasmodium* subpatent infactions from the Amazon Region in Brazil. Rev. Inst. Med. Trop. Sao Paulo. 2013 ; 55(3):205-208.
40. 40. Snounou G, Viriyakosol S, Zhu XP, Jarra W, Pinheiro L, Rosario VE, et al. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Molecular and Biochemical Parasitology. 1993; 61: 315-320.
41. 41. Barry AE, Schultz L, Buckee CO, Reeder JC. Contrasting population structures of the genes encoding ten leading vaccine-candidate antigens of the human malaria parasite, *Plasmodium falciparum.* PLOS ONE. 2009; 4:e8497.
42. 42. Ohashi J, Suzuki Y, Naka I, Hananantachai H, Patarapotikul J. Selection on the Thrombospondin-Related Adhesive Protein (TRAP) gene polymorphism of *Plasmodium falciparum* in Thailand. PLOS ONE. 2014; 9(2): e90522. Doi: 10.1371/journal.pone.0090522.
43. 43. Chenet SM, Branch OH, Escalante AA, Lucas CM, Bacon DJ. Genetic diversity of vaccine candidate antigens in *Plasmodium falciparum* isolates from the Amazon basin of Peru. Malaria journal. 2008; 7:93. Doi: 10.1186/1475-2875-7-93.