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Original Research Article

Polymorphism and allelic variation of domain I of the *Plasmodium falciparum* apical membrane antigen-1 (ama1) gene and status of *Plasmodium vivax* infections in parasitized isolates from Buea, Cameroon

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Malaria still remains a scourge of humanity despite the significant impact of the currently used drugs and vector control measures. Only a very successful vaccine can ensure a long term effective control of this disease. This study was aimed at assessing the (i) antigenic variability of domain I of the AMA1 vaccine candidate gene in falciparum isolates from Buea, Cameroon (ii) status of *Plasmodium vivax* infections in this study area using nPCR and RFLP. Parasitized blood samples were collected from 139 adults/children with uncomplicated malaria for microscopy and genetic analyses by nPCR and RFLP. 113 (81.9%) of the participants were febrile and 25 (18.1%) afebrile. While Fever was directly associated with parasitaemia (p=0.001) haemoglobin concentrations were inversely correlated to the latter (p=0.05, r= -0.032). Digestion of the isolated AMA1 fragments with *Mse1*, *Bfcu1* and *Ssp1* revealed the presence of the K1, HB3 and 3D7 alleles respectively in 15%, 9% and 3.4% of the samples analyzed. These sites were absent in 71.6% of the samples. One *Plasmodium vivax* isolate was detected in 138 samples. Our findings show that the *P. falciparum* AMA1 gene from malaria parasites circulating in Buea is highly polymorphic with the three major allelic forms present. *Plasmodium vivax* infections are almost inexistent in Buea. The results herein obtained will be relevant for AMA1-based malaria vaccine development that will be globally effective as well as for the National malaria treatment guidelines.

Key words: Malaria, *Plasmodium falciparum*, *Plasmodium vivax*, apical membrane antigen, vaccine candidates, AMA1 alleles, polymorphism.

INTRODUCTION

Malaria, a serious tropical disease caused by the apicomplexan parasite of the *Plasmodium* genus and transmitted by the female Anopheles mosquitoes, remains a scourge of humanity despite the deployment of artemisinin and partner drugs and vector control measures over the last decade and which have substantially reduced the global burden of the disease (Mbengue et al., 2015; Dondorp et al., 2017). As of 2016 malaria was found to be endemic in 91 countries with an estimated death toll of 445,000 and 216 million cases (WHO, 2017). Ninety-one percent of the deaths occur in the WHO African Region. In high malaria transmission areas, pregnant women, HIV/AIDS patients and children under 5 are particularly susceptible to the infection and more than 70% of all malaria deaths occur in this age group (WHO, 2016; Conway et al., 2015). *Plasmodium falciparum*, the dominant malaria parasite

specie in the African continent, accounts for 99% of the cases in sub-Saharan Africa (WHO, 2017). Current efforts to curb the disease have been through the use of artemisinin combination therapies (ACTs), vector control measures and surveillance. These measures have substantially reduced the malaria death burden by 33% from 665000 in 2010 to 445000 in 2016. Though these measures need to be sustained, the emergence of artemisinin resistance in South East Asia and resistance to its partner drugs seem to be a problem as there is now fear of widespread resistance as was the case with chloroquine (Slater et al., 2016). With yet no alternative at sight the gains so far made may easily be reversed in case of widespread occurrence of resistance to artemisinin and partner drugs. Most notorious infectious diseases have been eradicated through the use of vaccines. Vaccine development against malaria unlike other past infectious diseases that were eradicated through a vaccine is challenging because of various reasons (Chattopadhyay and Kumar, 2009; Hyde, 2007). One of these is the genetic polymorphisms of the parasite antigens that help them to evade the host's protective immune response and so hampers the development of an effective vaccine against this disease (Drew et al., 2012). So far the most advanced malaria vaccine is RTS,S ("Mosquirix"). This circumsporozoite (CS) recombinant protein-based malaria vaccine only provides partial protection in young children with a decreasing efficacy after a year of administration (EMA, 2015; Conway et al., 2015; RTS,SCTP, 2014). While efforts are being made to improve the RTS,S there is also the need to seek other avenues. A number of malaria vaccine candidates based on *P. falciparum* antigens have been identified and successfully undergone clinical trials (Conway, 2015; Sagara et al., 2009; Tamminga et al., 2011). One of these leading malaria vaccine candidates is the Apical Membrane Antigen 1 (AMA1) which is a membrane protein expressed by the *falciparum* merozoite stage (Conway, 2015). AMA1 aids in blood-stage replication and antibodies raised against it help inhibit erythrocyte invasion (Zhu et al., 2016; Drew et al., 2012; Kennedy et al., 2002; Hodder et al., 2001). The protective efficacy of this vaccine candidate has successfully been tested in various models and it has been shown to be a target of naturally acquired human immunity (Fowkes et al., 2010; Stanisic et al., 2009; Polley et al., 2004). However, the polymorphic nature of AMA1 can mediate immune escape and strategies need to be put in place to overcome this antigenic variation in order to prevent any escape. A vaccine based on a single AMA1 allele has recently been shown in a phase 2 vaccine trial of children aged 1-6 years in Mali to elicit 64% protective efficacy against malaria; with no significant efficacy against malaria episodes due to other alleles (Thera et al., 2011). It is therefore imperative to assess the allelic variation of the PfAMA1 antigen in various geographical regions for an AMA1 based vaccine development that will give large scale coverage. This study aimed at assessing the allelic variation of the AMA1 antigen gene in field isolates from Buea in the South West Region of Cameroon and assess the status of *Plasmodium vivax* infections in this region.

MATERIALS AND METHODS

Study area/study population

This study was conducted in the multiethnic city of Buea, capital of the South West Region, located at the slope of Mount Cameroon. This study area with an equatorial type climate has previously been described (Takem et al., 2010). Malaria in this region is meso-endemic and becomes hyper-endemic during the rainy season with peak incidence between July and October (Achidi et al., 2012). *Plasmodium falciparum* is the predominant parasite specie which accounts for 96% of malaria infection in this area (Eyong et al., 2016). Male and female volunteers aged 6months to 65 years were enrolled from the outpatient unit of the Mount Mary hospital in Buea into the study after obtaining written informed consent (or by proxy) and made to fill a structured questionnaire containing information on participants. Individuals who tested positive for malaria by microscopy, with or without symptoms of malaria were recruited into the study.

Sample Collection

Blood samples were obtained from participants by fingerprick spotted on labeled slides and whatman filter papers for microscopy and genetic analyses respectively. Thin and thick film microscopic tests were carried out to assess the level of parasitaemia. The whatman filter papers containing the blood spots were air-dried and maintained at room temperature in individual resealable polythene bags containing desiccants until required for use. The anaemic status of participants which could be due to malaria was assessed by measuring the haemoglobin levels using haemoglobinoter (STAT- Site MHgb metre, Stanbio Laboratory Texas, USA) and according to the manufacturer's instructions.

Plasmodium DNA isolation and falciparum speciation

Plasmodium genomic DNA was extracted from whatman filter paper blood spots using chelex-100 and as previously described (Miguel et al., 2013).

Speciation

Assessment of the Plasmodium species in the DNA extracts and delineation of falciparum positive samples was done by Nested-PCR using the genus-specific primers (rPLU6F and rPLU5R) for the primary PCR to amplify the 18s rRNA gene and the specie-specific primers (rFAL1F and rFAL2F for *Plasmodium falciparum* and rVIV1 and rVIV2 for *Plasmodium vivax*) for the nested PCR to amplify the specie-specific sequences. These primers and PCR conditions were earlier described (Snounou et al., 1993). Amplicons were

Table 1. Variation of malaria parasitaemia load with socio-demographic and clinical parameters

Variables	Sub categories	N	%	MPD	P-value
Age group (years)	<5	26	18.8	648	
	5-9	9	6.5	932	
	10-14	12	8.7	1332	0.361
	≥15	91	66.0	1064	
Gender	male	56	40.6	944	
	female	82	59.4	1040	0.558
Anaemia status	anaemic	48	34.8	1060	
	non-anaemic	90	65.2	968	0.527
Febrile status	afebrile	25	18.1	736	0,001
	febrile	113	81.9	1060	

analysed on a 1.5% agarose gel.

Isolation of the AMA1 gene sequences.

The domain I of the *Plasmodium falciparum* AMA-1 gene sequences were amplified from the *falciparum* positive DNA extracts by primary and nested PCR using respectively the VM785/3 and VM990 and VM815 and VM990 sets of primers and conditions as earlier described (Ebrahimzadeh et al., 2014). Pure genomic DNA of P. *falciparum* 3D7, K1 and HB3 strains, obtained from the malaria Research and Reference Reagent Resource Center (MR4), American Type Culture Collection (Manassas, VA), was included to serve as positive controls during the amplification and restriction analyses. Amplicons were analysed on a 1% agarose gel.

Restriction analyses of the AMA1 gene sequences

Restriction fragment length polymorphism (RFLP) analysis of domain I of AMA1was carried out to screen for the three different alleles of AMA1 in Buea isolates. The isolated AMA1 gene fragments were digested with the restriction enzymes *Mse1*, *Ssp1* and *Bfcu1* (New England Biolabs (NEB)) for 60 minutes and according to the manufacturer's instructions with some slight modifications. The premix composition for digestion consisted of 0.4µl restriction enzyme, 2.0µl of 10x cutsmart buffer, 7.6µl autoclaved distil water and 10µl of nested PCR product making up a total volume of 20ul. Digestion with the above set of enzymes would generate respectively the K1 alleles (285bp), 3D7 alleles (400bp), and BH3 alleles (335bp). The digests were analysed on a 2.5% agarose gel.

Statistical analysis

Data were expressed as mean ± standard deviation (SD) for continuous variables and in percentages for categorical variables. All study data were reviewed before being entered onto a Microsoft Office Excel 2013 work sheet and later analysed with the statistical package SPSS version 20 (SPSS Inc, Chicago, USA). Chi square (χ^2) was used to test the independence of the sub categorical variable to malaria parasitaemia. Allelic frequency was counted directly and compared using Chi square test. A P-value ≤ 0.05 was considered statistically significant with confidence interval (CI) of 95.

RESULTS

Characteristics of study subjects

One hundred and thirty eight (138) subjects aged 6 months to 65 years and comprising 56 males (40.6%) and 82 females (59.4%) took part in the study. The general characteristics of these subjects are presented in Table 1. The geometric mean parasite density (GMPD) was 9784 parasites/µl of blood (range 800 to 16000 parasites/µl) and the mean haemoglobin concentration was 11.33 ± 1.83 g/dL (range 8 - 16.6 g/dL).

Variation of parasitaemia with socio-demographic and clinical parameters

Malaria parasitaemia was independent of age, gender, ethnicity and anaemic status (Table 1). Febrile individuals had significantly higher parasitaemia loads compared to their afebrile counterparts (p=0.001). The mean parasite density was not significantly different among age groups (p=0.361). Haemoglobin concentration showed a significant inverse correlation with parasitaemia (p=0.01, r=-0.032).

Parasite species and strain diversity in Buea study area

Molecular determination of the infecting species of parasite was through the amplification of the 18s small subunit ribosomal RNA gene (ssrRNA) as described by Snounou et al. (1993). Plasmodium DNA was successfully isolated from all the 138 filter paper blood spots and gave the characteristic 1200 bp band specific to *Plasmodium* spp. F*alciparum* specie-specific gene sequences (205bp in length) were amplified from 97 of the 138 DNA extracts using the *P. falciparum* specie-specific set of primers. Vivax specie-specific gene sequence was obtained in only one of the 138 extracts using *P. vivax* specie-specific set of

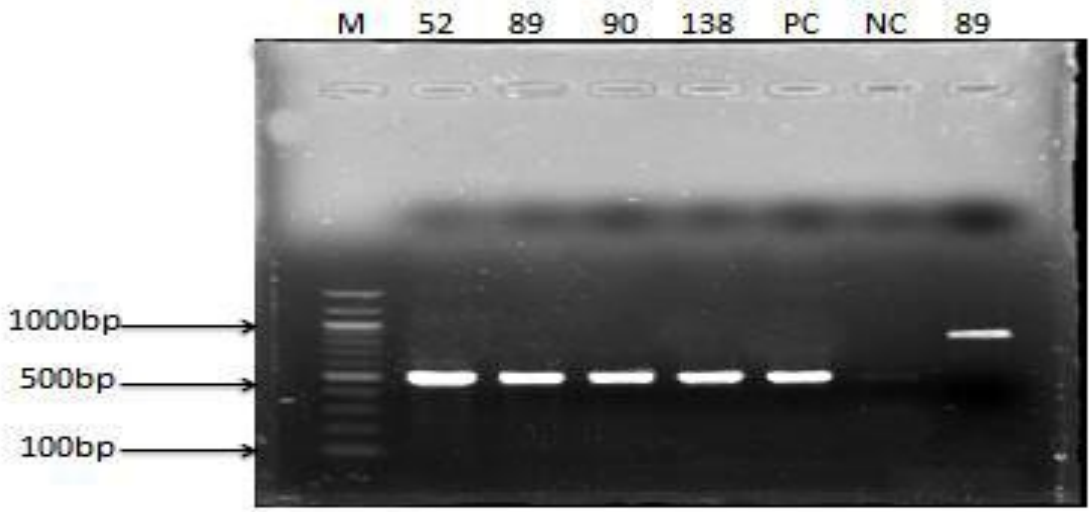

Figure 1: Agarose gel electrophoregram of AMA1 domain I amplicons with the expected banding pattern of 500bp. M=100bp Molecular weight marker (NEB); lanes (52, 89, 90, 138) = nested amplicons; Lane PC = +ve 3D7 control; lane NC = -ve control; lane 89 with band at 1200bp = primary amplicon (Plasmodium genus fragment); Numbers represent participants' codes.

primers. The other species (*Ovale* and *Malariae*) were not assessed in this study. The extracts were analysed on a 1.5% agarose gel alongside a 50bp molecular weight marker (NEB) and positive control samples obtained from MR4. This yielded the corresponding 205bp and 120bp bands respectively for the *falciparum* and the *vivax* species. No case of *falciparum-vivax* coinfection was observed with the 138 study cases.

Variation of the AMA1 gene sequences in isolates from Buea

A total of 175 *falciparum positive* extracts (the 97 extracts obtained above and 78 *falciparum* positive extracts isolated in a parallel study in our research group) were used for the isolation of the domain I of the AMA1 gene sequences by PCR. The sequences (500bp fragment sizes) were successfully amplified from 88 (50.29%) of the 175 falciparum positive extracts by nested PCR using AMA1 specific primer sequences. Eighty-seven (49.71%) of the positive extracts were refractory to amplification (Figure 1). In order to assess the diversity of the AMA-1 gene by RFLP, nested amplicons of the AMA-1 domain I gene from the 88 *P. falciparum* positive samples were digested using *Mse1*, *Bfcu1* and *Ssp1* enzymes and following the manufacturer's instructions. The 500bp long AMA1 domain I wild type gene sequence contains one *Mse1*, *Bfuc1* and *SspI* restriction enzyme sites. These sites are either present as in the wild type or absent as in the mutated form. The presence of these restriction sites gives three different restriction fragment length sizes (285bp, 335bp, 400bp; corresponding to the different allele types K1, HB3, 3D7 respectively) when digested respectively with these restriction enzymes. The digestion products of the AMA-1 gene were analysed on a 2.5% agarose gel and revealed the presence of the respective major band sizes of 285bp, 335bp and 400bp (Figures 2,3,4).

AMA-1 allelic frequency and polymorphism

Restriction analysis with the three enzymes showed that of the 88 field isolates digested, 3 field isolates had the *Ssp1* site present (3D7 allele), 8 had the *Bfcu1* site (HB3 allele) and 14 had the *Mse1* site present (k1 allele) (Figure 5). However, 63 field isolates had none of the restriction sites of these enzymes suggesting the loss of their restriction sites.

DISCUSSION

Despite the enormous challenges involved in developing a vaccine against malaria getting one remains very imperative given the continuous ability of the parasites to develop resistance against malaria drug regimens. Artemisinin and its partner drugs (ACTs) were a welcome relief after Chloroquine resistance went global and today with the emergence of artemisinin resistant strains of the malaria parasites in South East Asia leaves the global population at risk with no immediate alternative at sight. The current gains made by the use of these ACTs and other vector control measures (WHO, 2017) risk being reversed. The most advanced malaria vaccine candidate RTS,S ("Mosquirix") is short of conferring full and long-lasting protection against all populations. It only provides partial protection against *falciparum* malaria in young children with decreasing efficacy after a year of administration (EMA, 2015; RTS,S CTP, 2014). While further efforts remain

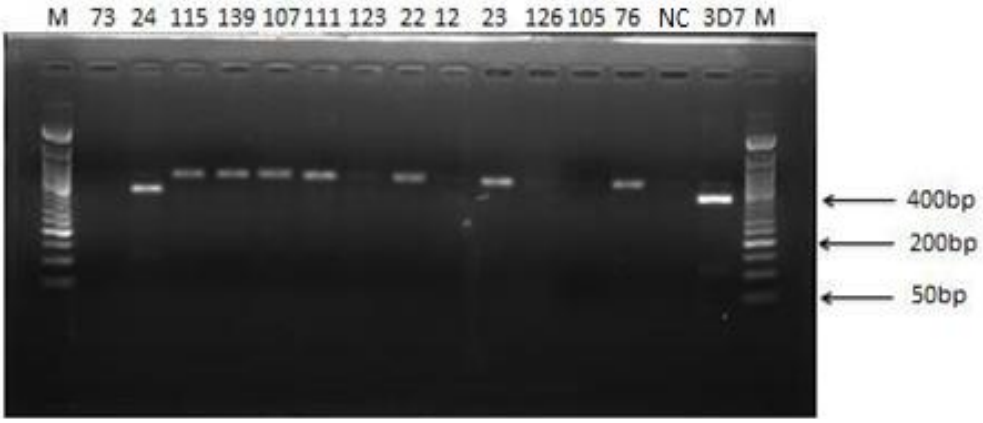

Figure 2: An electrophoregram of domain I of AMA1 amplicons digested with the restriction enzyme *Ssp1* and ran on a 2.5% agarose gel, showing the digested 400bp fragment corresponding to the 3D7 allele. M=50bp molecular weight ladder; numbers stand for participants' codes; 3D7 is the positive control; NC = negative control.

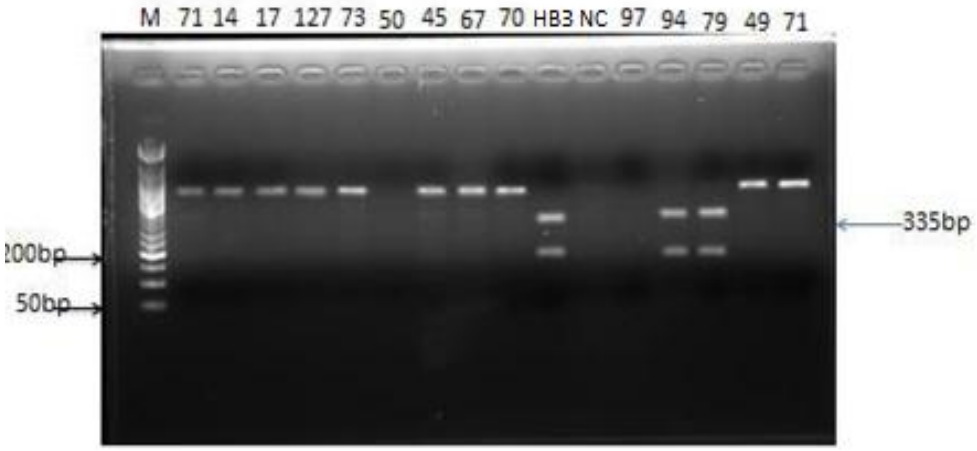

Figure 3: Agarose gel electrophoregram of AMA1 domain I amplicons digested with the *Bfcu1* restriction enzyme and showing the 335bp band size of the HB3 allele. M=50bp molecular weight ladder; numbers stand for participants' codes. HB3 = positive control sample; NC =

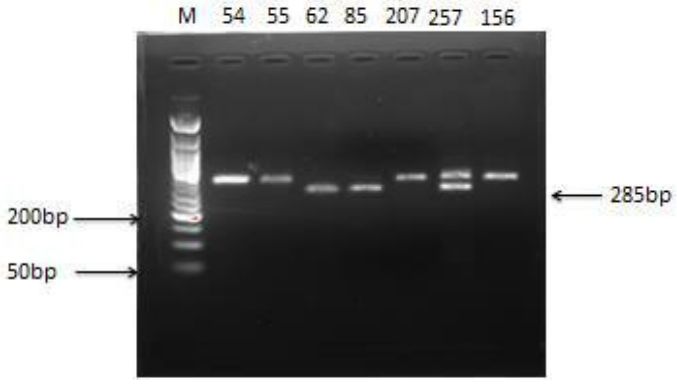

Figure 4: Agarose gel electrophoregram of AMA1 amplicons digested with the *Mse1* enzyme showing the 285bp band of the K1 allele of AMA1. M=50bp molecular weight marker; numbers stand for participants' codes.

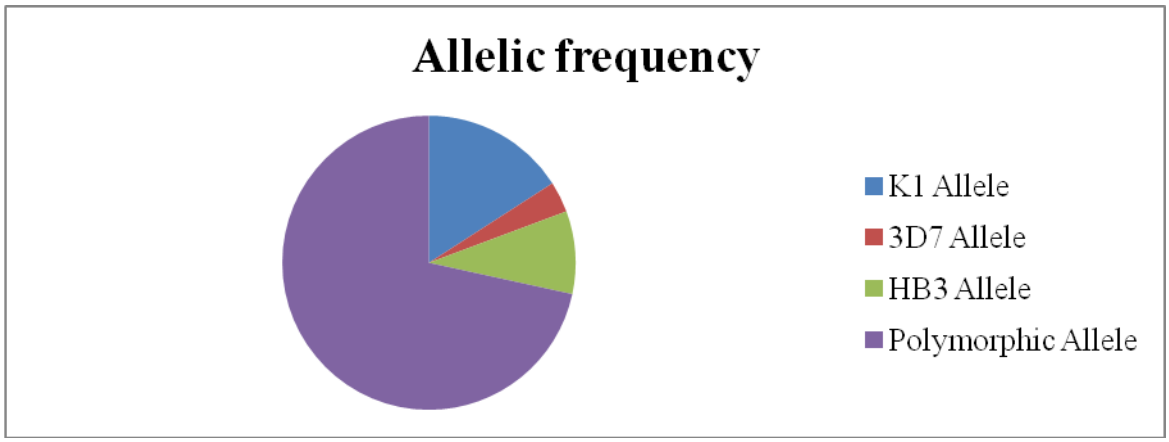

Figure 5: Frequency distribution of the major AMA-1 alleles in malaria field isolates from Buea

to be deployed to improve the efficacy of RTS,S, there is the need to scavenge other vaccine candidates especially those that have already gone through clinical trials for the development of a malaria vaccine. This study aims at assessing the genetic diversity of one of these vaccine candidates, the Apical Membrane Antigen 1 (AMA1), that have already successfully undergone clinical trials, in field isolates from Buea in Cameroon. Knowledge of the antigenic variants of any leading vaccine candidate antigen in various endemic areas is essential for the rational design of a malaria vaccine that can be effective across the globe.

The 138 subjects involved in the study had characteristics similar to those previously described (Sumbele et al., 2016; Kunihya et al., 2016). Anaemia prevalence (hemoglobin < 11.0 g/dL) was associated with decreasing age for children less than 5, though majority of the participants were non-anaemic. Parasite prevalence increased with age up to the 10-14 years ranged and then decreased, though not significant in both cases. Yeka et al. (2015) obtained similar results in Uganda although their increase in parasite prevalence up to the age of 11 and decrease were significant, probably due to a larger sample size.

The parasitized blood samples collected from the study participants were analysed for the presence of the two Plasmodium species, *falciparum* and *vivax*. Amplification of the various fragments of the two different species using previously described primers (Snounou et al., 1993) showed the presence of *falciparum* with the typical banding pattern of 205bp in 97 (70.3%) of the 138 malaria positive samples collected and only one sample (0.7%) of the total number gave the characteristic banding pattern of 120bp for *vivax*. This one *Plasmodium vivax* isolate, though not confirmed by sequencing, could not have been an artefact as the same band was obtained repeatedly alongside the genomic control from MR4. This further confirms recent findings from five epidemiological strata of malaria in Cameroon including the coastal strata under which this study site falls that *P. falciparum* was the only causative agent of clinical malaria and that *P. vivax, P. malariae, P. knowlesi* and the last of the malaria parasites of humans to be described, *P. ovale*, were all absent (Kwenti et al., 2017). These findings contradict those of Fru-Cho et al. (2014) who found substantial *Plasmodium vivax* (13 out of 87 malaria positive cases as determined by PCR) in this study area. The difference in these results could be that the *P. vivax* cases they got were imported cases or due to expansion of a founder effect which were not investigated. Moreover, their samples dated as far back as 2008-2009 (Fru-Cho et al., 2014). The fact that our results concord with those of Kwenti and collaborators show that *P. vivax* is not the cause of malaria in this coastal region and is not on the rise. However, the presence of this *vivax* isolate in this study which may be due to one of the reasons cited above is under further scrutiny in a larger scale study. Furthermore, the absence of Plasmodium *vivax* and *P. ovale in*fections within the coastal South-Western region of Cameroon which includes the present study area had previously been demonstrated about a decade ago (Bigoga et al., 2007). *Malariae* and *ovale* were not assessed in this present study. Though microscopy is the gold standard for malaria diagnosis the difference obtained with the PCR results further illustrates some of the short comings of malaria diagnoses in medical setups. The various malaria diagnostic methods have been elaborately discussed and compared and various reasons have been advanced for the disparity in the different diagnostic methods (Dinko et al., 2016; Rosanas-Urgell et al., 2010).

The domain 1 of the AMA1 gene sequences were next amplified from the 97 *falciparum* positive samples alongside 78 other *P. falciparum* positive DNA extracts obtained from the same study site within the same period in a parallel study. Half (88 extracts; 50.3%) of the total 175 *P. falciparum* positive DNA extracts gave the typical banding pattern of 500bp (size of domain I of the AMA1 gene) when amplified using AMA1 gene specific primers and the other half (87 extracts; 49.7%) was refractory to amplification for either of various reasons. This

amplification success rate is lower than that obtained in previous studies on this same domain I of the AMA1 gene from Iranian field samples (Ebrahimzadeh et al., 2014; Zhu et al., 2016). The difference could be explained by the differences in *falciparum* DNA extraction methods, working reagents and PCR conditions. While this study made use of chelex-100 to extract the *falciparum* DNA, Ebrahimzadeh and collaborators (2014) used fermentas genomic DNA purification kit and obtained a success rate of 79% while Zhu et al., 2016 used DNA Blood Mini kit and obtained 83% successful amplification. This difference in method could affect the yield and the degree of amplification. Mutations in the primer binding sites as well as the presence of PCR inhibitors might also have played a role. The PCR-amplified AMA1 fragments subjected to restriction fragment length polymorphism analysis using the *Mse1*, *Bfuc1* and *SspI* restriction enzymes revealed the presence of the different allele classes K1, 3D7 and HB3 of AMA1 in the isolates from Buea in Cameroon. Digestion with *Mse1*, *Bfuc1* and *SspI* enzymes gave restriction fragment length sizes of 285bp, 335bp and 400bp corresponding to the different allelic types K1, HB3, 3D7 respectively. Three (3.4%) field isolates were present with the 3D7 allele, 8 (9.1%) with the HB3 allele, 14 (15.9%) with the k1 allele and 63 (71.6%) field isolates had none of the restriction enzyme sites present. This result shows that the domain I of the AMA1 gene from this study area is highly polymorphic and presents the three major allele classes K1, 3D7 and HB3 in concordance with previous findings. These three major allelic classes have been documented with different frequencies in various endemic areas including S.E Asia, central and West Africa, Brazil and Peru, India and Iran amongst others (Zhu et al., 2016; Soulama et al., 2011; Chenet et al., 2008; Garg et al., 2007; Ebrahimzadeh et al., 2014). Significant geographic variation in genetic diversity of this antigen has also been demonstrated between and within continents (Anderson et al., 2000; Chenet et al., 2007). Domain I of the AMA1 has been shown to have the highest genetic diversity (compared to the other domains) and to be the most important domain as it possesses the inhibitory epitopes of the AMA1 antigen and the cross inhibitory activities of antibodies (Zhu et al., 2016; Drew et al., 2012). The haplotype diversity of *P. falciparum* AMA1 has been widely studied in South East Asia and were characterized by five to six dozen SNP loci across the gene distributed mostly in the ectodomain of the AMA1 gene with domain I being the most polymorphic (Lumkul et al., 2018; Zhu et al., 2016; Escalante et al., 2001; Polley and Conway, 2001). Our result thus confirms this polymorphic nature of domain I of the *P. falciparum* AMA1 antigen gene with 71.6% of the isolates having lost the restriction sites due to mutations. While some of the above studies had varied frequencies of the three major allelic classes of AMA1 at various geographic areas, this study revealed a prevalence of the K1 allele with the 3D7 allele being the least frequent. This variation in prevalence of the three different major allelic forms across various geographic zones could be accounted for by differences in the prevalence of malaria parasites and malaria transmission intensity. This study indicates that these major allelic forms of the AMA1 antigen need to be considered in the design of an AMA1-based vaccine that will be globally effective against *P. falciparum* infections.

CONCLUSION

The present study describes for the first time the presence and frequencies of the three major allelic forms of the polymorphic and most important domain I of the AMA1 antigen gene in parasite populations from Buea in Cameroon. The K1 allele has been shown to be the most prevalent in this area with the 3D7 allele being the least represented. Results from this study also demonstrate the high degree of polymorphism in domain I of the AMA1 gene which has previously been shown elsewhere to be due to positive natural selection. These results on AMA1 allelic frequencies and polymorphism will be useful for AMA1-based malaria vaccine development. The study also shows that *Plasmodium vivax* is almost inexistent in Buea.

Ethical and administrative considerations

Ethical clearance for this study was obtained from the Ethics Review and Consultancy Committee (ERCC) of the Cameroon Bioethics Initiative (CAMBIN) (Ref. No. 2016/01/1098). Administrative clearance came from the South West Regional delegation of Public Health in Buea. Participants consented to the study by providing a written informed consent.

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Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of the paper.

REFERENCES

Achidi EA, Apinjoh TO, Anchang-Kimbi JK, Mugri RN, Ngwai AN, Yafi CN (2012). Severe and uncomplicated falciparum malaria in children from three regions and three ethnic groups in Cameroon: prospective study. Malar J., 11: 215.Crossref

Anderson TJ, Haubold B, Williams JT, Estrada-Franco JG, Richardson L, Mollinedo R, Bockarie M, Mokili J, Mharakurwa S, French N, Whitworth J, Velez ID,

Brockman AH, Nosten F, Ferreira MU, Day KP (2000). Microsatellite markers reveal a spectrum of population structures in the malaria parasite *Plasmodium falciparum*. Mol Biol Evol., 17:1467–1482. Crossref

Bigoga JD, Manga L, Titanji VPK, Coetzee M, Leke RGF (2007). Malaria vectors and transmission dynamics in coastal south-western Cameroon. Malar J., 6:5. Crossref

Chattopadhyay R, Kumar S (2009). Malaria vaccine: Latest update and challenges ahead. Indian J. Experimental Biol., 47: 527-536.Crossref

Chenet S, Branch OH, Escalante AA, Lucas CM, Bacon JD (2008). Genetic diversity of vaccine candidate antigens in *Plasmodium falciparum* isolates from the Amazon basin of Peru. Malar J., 7:93.Crossref

Conway DJ (2015). Paths to a malaria vaccine illuminated by parasite genomics. Trends in Genetics, 31(2): 97-107.Crossref

Dinko B, Ayivor-Djanie R , Abugri J, Agboli E, Kye-Duodu G, Tagboto S, Tampuori J, Adzaku F, Binka FN, Awandare GA (2016). Comparison of malaria diagnostic methods in four hospitals in the Volta region of Ghana. MWJ., 7:5.

Dondorp AM, Smithuis FM, Woodrow C, von Seidlein L (2017). How to Contain Artemisinin- and Multidrug-Resistant Falciparum Malaria. Trends in Parasitology, 33(5): 353-363.Crossref

Drew DR, Hodder AN, Wilson DW, Foley M, Mueller I, Siba PM, Dent AE, Cowman AF, Beeson JG (2012). Defining the Antigenic Diversity of Plasmodium falciparum Apical Membrane Antigen 1 and the Requirements for a Multi-Allele Vaccine against Malaria. *PLoS ONE,* 7(12): e51023. Crossref

Ebrahimzadeh A, Gharaei A, Saryazdi K (2014). Allelic Diversity of Polymorphic AMA-1 (Apical Membrane Antigen 1) Vaccine Candidate Antigen of *Plasmodium falciparum* in Two Populations of Imported and Indigenous Cases in South-East of Iran using Nested-PCR and RFLP. J Trop Dis., 2:149.

EMA (2015). First malaria vaccine receives positive scientific opinion from EMA, European Medicines Agency. Press release: EMA/CHMP/488348/2015.

Escalante AA, Grebert HM, Chaiyaroj SC, Magris M, Biswas S, Nahlen BL, Lal AA (2001). Polymorphism in the gene encoding the apical membrane antigen-1 (*AMA*-1) of *Plasmodium falciparum*. X. Asembo Bay Cohort Project. Mol Biochem Parasitol., 113: 279-287.Crossref

Eyong EEJ, Kengne-Ouafo AJ, Chounna PWN, Datchoua-Poutcheu FR, Wanji S (2016). Altitudinal variation in the parasitological and entomological indices of malaria around Mount Cameroon, South West Region of Cameroon. J. Parasitol. Vector Biol., 8(8): 74-85.

Fowkes FJ, Richards JS, Simpson JA, Beeson JG (2010). The relationship between anti-merozoite antibodies and incidence of *Plasmodium falciparum* malaria: A systematic review and meta-analysis. *PLoS Med.,* 7: e1000218. Crossref

Fru-Cho J, Bumah VV, Safeukui I, Nkuo-Akenji T, Titanji VP, Haldar K (2014). Molecular typing reveals substantial Plasmodium vivax infection in asymptomatic adults in a rural area of Cameroon. Malar J., 13:170. Crossref

Garg S, Alam MT, Das MK, Dev V, Kumar A, Dash AP, Sharma YD (2007). Sequence diversity and natural selection at domain I of the apical membrane antigen I among Indian *Plasmodium falciparum* populations. Malar J., 6:154; Crossref

Hodder AN, Crewther PE, Anders RF (2001). Specificity of the protective antibody response to apical membrane antigen 1. Infect Immun*.,* 69: 3286–3294. Crossref

Hyde JE (2007). Targeting purine and pyrimidine metabolism in Human Apicomplexans parasites. Current drug targets., 8:31-47.Crossref

Kennedy MC, Wang J, Zhang Y, Miles AP, Chitsaz F, et al. (2002). In vitro studies with recombinant *Plasmodium falciparum* apical membrane antigen 1 (AMA1): production and activity of an AMA1 vaccine and generation of a multiallelic response. Infect Immun*.,* 70: 6948–6960. Crossref

Kunihya IZ, Samaila AB, Pukuma MS, Qadeer MA (2016). Prevalence of Malaria infection and malaria anaemia among children attending Federal Medical Centre Yola, Adamawa State, Nigeria. The Int. J. Engineering Sci., 5(7):08-14.

Kwenti TE, Kwenti TDB, Njunda LA, Latz A, Tufon KA, Nkuo-Akenji T (2017). Identification of the *Plasmodium* species in clinical samples from children residing in five epidemiological strata of malaria in Cameroon. Tropical Medicine and Health, 45:14. Crossref

Lumkul L, Sawaswong V, Simpalipan P, Kaewthamasorn M, Harnyuttanakorn P, Pattaradilokrat S (2018). Unraveling Haplotype Diversity of the Apical Membrane Antigen-1 Gene in *Plasmodium falciparum* Populations in Thailand. Korean J Parasitol., 56(2): 153-165.Crossref

Mbengue A, Bhattacharjee S, Pandharkar T, Liu H, Estiu G, Stahelin RV, Rizk SS, Njimoh DL, Ryan Y, Chotivanich K, Nguon C, Ghorbal M, Lopez-Rubio JJ, Pfrender M, Emrich S, Mohandas N, Dondorp AM, Wiest O, Haldar K (2015). A molecular mechanism of artemisinin resistance in Plasmodium falciparum malaria. Nature, 520(7549):683-687.Crossref

Miguel RB, Coura JR, Samudio F, Suarez-Mutis MC (2013). 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., 55(3):205-208.Crossref

Polley SD, Conway DJ (2001). Strong diversifying selection on domains of the *Plasmodium falciparum* apical membrane antigen 1 gene. *Genetics*., 158: 1505-1512.

Polley SD, Mwangi T, Kocken CH, Thomas AW, Dutta S, Lanar DE, Remarque E, Ross A, Williams TN, Mwambingu G, Lowe B, Conway DJ, Marsh K (2004). Human antibodies to recombinant protein constructs of Plasmodium falciparum Apical Membrane Antigen 1 (AMA1) and their associations with protection from malaria. Vaccine, 23: 718–728. Crossref

Rosanas-Urgell A, Mueller D, Betuela I, Barnadas C, Iga J, Zimmerman PA, A del Portillo H, Siba P, Mueller I, Felger I

(2010). Comparison of diagnostic methods for the detection and quantification of the four sympatric *Plasmodium* species in field samples from Papua New Guinea. Malaria J., 9:361. Crossref

RTS,S Clinical Trials Partnership (2014). 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.*, 11: e1001685.Crossref

Sagara I, Ellis RD, Dicko A, Niambele MB, Kamate B, Guindo O, Sissoko MS, Fay MP, Guindo MA, Kante O, Saye R, Miura K, Long C, Mullen GE, Pierce M, Martin LB, Rausch K, Dolo A, Diallo DA, Miller LH, Doumbo OK (2009). 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. Vaccine, 27:7292-7298.Crossref

Slater HC, Griffin JT, Ghani AC, Okell LC (2016). Assessing the potential impact of artemisinin and partner drug resistance in sub-Saharan Africa. Malaria J, 15:10. Crossref

Snounou G, Viriyakosol S, Zhu XP, Jarra W, Pinheiro L, do Rosario VE, Thaithong S, Brown KN (1993). High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Molecular and Biochemical Parasitol., 61: 315-320.Crossref

Soulama I, Bigoga JD, Ndiaye M, Bougouma EC, Quagraine J, Casimiro PN, Stedman TT, Sirima SB (2011). Genetic Diversity of Polymorphic Vaccine Candidate Antigens (Apical Membrane Antigen-1, Merozoite Surface Protein-3, and Erythrocyte Binding Antigen-175) in *Plasmodium falciparum* Isolates from Western and Central Africa. Am J Trop Med Hyg., 4 84(2): 276–284.

Stanisic DI, Richards JS, McCallum FJ, Michon P, King CL, Schoepflin S, Gilson PR, Murphy VJ, Anders RF, Mueller I, Beeson JG (2009). Immunoglobulin G subclass-specific responses against *Plasmodium falciparum* merozoite antigens are associated with control of parasitemia and protection from symptomatic illness. Infect Immun., 77: 1165–1174. Crossref

Sumbele IUN, Sama SO, Kimbi HK, Taiwe GS (2016). Malaria, Moderate to Severe Anaemia, and Malarial Anaemia in Children at Presentation to Hospital in the Mount Cameroon Area: A Cross-Sectional Study. *Anemia*,

Takem NE, Achidi AE, Ndumbe PM (2010). An update of malaria infection and anemia in adults in Buea, Cameroon. *BioMed central*, 3:121

Tamminga C, Sedegah M, Regis D, Chuang I, Epstein JE, Spring M, Mendoza-Silveiras J, McGrath S, Maiolatesi S, Reyes S, Steinbeiss V, Fedders C, Smith K, House B, Ganeshan H, Lejano J, Abot E, Banania GJ, Sayo R, Farooq F, Belmonte M, Murphy J, Komisar J, Williams J, Shi M, Brambilla D, Manohar N, Richie NO, Wood C, Limbach K, Patterson NB, Bruder JT, Doolan DL, King CR, Diggs C, Soisson L, Carucci D, Levine G, Dutta S, Hollingdale MR, Ockenhouse CF, Richie TL (2011). Adenovirus-5-Vectored *P. falciparum* Vaccine Expressing CSP and AMA1. Part B: Safety, Immunogenicity and Protective Efficacy of the CSP component. *PLoS ONE,* 6(10): e25868.Crossref

Thera MA, Doumbo OK, Coulibaly D, Laurens MB, Ouattara A, Kone AK, Guindo AB, Traore K, Traore I, Kouriba B, Diallo DA, Diarra I, Daou M, Dolo A, Tolo Y, Sissoko MS, Niangaly A, Sissoko M, Takala-Harrison S, Lyke KE, Wu Y, Blackwelder WC, Godeaux O, Vekemans J, Dubois MC, Ballou WR, Cohen J, Thompson D, Dube T, Soisson L, Diggs CL, House B, Lanar DE, Dutta S, Heppner Jr DG, Plowe CV (2011). A field trial to assess a blood-stage malaria vaccine. N Engl J Med., 365: 1004–1013.Crossref

WHO: World malaria Report 2017. Geneva: WHO; 2017. Available: www.who.int/malaria/publications/world-malaria-report-2017/en/.

WHO: World malaria Report 2017. Geneva: WHO; 2017. Available: www.who.int/malaria/publications/world-malaria-report-2016/en

Yeka A, Nankabirwa J, Mpimbaza A, Kigozi R, Arinaitwe E, Drakeley C, Greenhouse B, Kamya MR, Dorsey G, Staedke SG (2015). Factors Associated with Malaria Parasitemia, Anemia and Serological Responses in a Spectrum of Epidemiological Settings in Uganda. *PloS One,* 10(3): e0118901. Crossref

Zhu X, Zhao Z, Feng Y, Li P, Liu F, Liu J, Yang Z, Yan G, Fan Q, Cao Y, Cui L (2016). Genetic diversity of the *Plasmodium falciparum* apical membrane antigen I gene in parasite population from the China –Myanmar border area. Infect Genet Evol., 39:155-162. Crossref