THE ASSOCIATION OF *EBA-175* ALLELES WITH THE OUTCOME OF MALARIA IN NIGERIAN CHILDREN

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Abstract

Malaria remains a major cause of morbidity and mortality in Nigeria. *Plasmodium falciparum* erythrocyte binding antigen-175, *eba*-175, plays an important role in the invasion of host cells during falciparum malaria infection. It mediates erythrocyte invasion by sialic acid dependent binding to glycophorin A on erythrocytes. Dimorphic allelic segments, FCR-3 (F-segment) and CAMP (C-segment) have been found in the *eba-175* encoding gene and associations have been reported between the dimorphism and the clinical outcome of malaria in endemic populations. The possible associations of the dimorphism with the clinical outcome of malaria were investigated in Ibadan south-west Nigeria. Blood samples were obtained from 390 children categorized into clinical categories of asymptomatic controls and uncomplicated and severe malaria cases as defined by WHO. The allelic dimorphism of *eba-175* was analysed by nested polymerase chain reaction. Overall, the F-fragment was observed in a higher frequency than the C-fragment. Single infections were more frequent than mixed infections (F-/C-Fragments). The C-fragment and mixed infections were most common in the asymptomatic controls compared to the uncomplicated and severe malaria cases. The presence of C-fragments and mixed infections were significantly associated with the asymptomatic controls. The results from this study confirm the dimorphism of *eba-175* in the Ibadan south-west population. We conclude that the C-fragment and mixed infections are associated with asymptomatic malaria in children in Ibadan, south-west Nigeria.

Keywords: severe malaria, mixed infections, C-fragment, *eba-175*, Nigeria.

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Introduction

Malaria is a leading cause of childhood morbidity and mortality in most developing countries (World Malaria Report, 2012). The clinical outcome of malaria depends on complex interactions of host and parasite factors (Gupta *et al* 1994). The interaction between malaria parasite and the human host involves a number of interactions that result in the parasite evading the immune system (Plebanski *et al* 2002). The most common survival mechanism the parasites use in evasion process is its ability to undergo almost unlimited antigenic variation through changing the antigens on the infected erythrocyte surface. On the part of the parasite, the mechanism of antigenic variation is important for the survival of *Plasmodium falciparum* within the human host. It also promotes pathogenesis of malaria. The main limitation to the development of a vaccine against *P. falciparum* is the antigenic diversity related to *P. falciparum* polymorphisms. Various studies have typed different parasite genes of different *P. falciparum* isolates in sub-Sahara African countries to delineate molecular characteristics associated with different clinical presentations of malaria (Ntoumi *et al* 1995; Robert *et al* 1996; Smith *et al* 1999; Bendixen *et al* 2001; Amodu *et al* 2005; 2008). Thus, elucidating the genetic structure of parasite genes in *P. falciparum* isolates in a population has important implications for the understanding of malaria pathogenesis.

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Previous studies on the genetic diversity of some *P. faciparum* genes such as merozoite surface proteins (MSP-1 and MSP-2) have identified genotypes associated with clinical malaria and outcome (Engelbrecht *et al* 1995; Ntoumi *et al* 1995; Al-Yaman *et al* 1997; Konate *et al* 1999; Ofosu-Okyere *et al* 2001 Amodu *et al* 2005; 2008). One of the important *P. falciparum* antigens is the erythrocyte binding antigen-175 located in the microneme orgenelles at the apical end of merozoites.

The erythrocyte-binding antigen 175 kDa (*eba-175*) of *P. falciparum* is expressed on the merozoite surface and has been implicated in *P. falciparum* binding to erythrocytes, before the merozoite completely invades the erythrocyte. *Eba-175* is localized in the microneme, one of the organelles of the apical complex involved in merozoite invasion, and has been identified as the merozoite ligand that binds a sialic acid dependent site on glycophorin A (Cramer *et al* 2004; Perce-da-Silva *et al* 2011). The *eba-175* gene is located on chromosome 7 and is composed of 4 exons and 7 regions (region I-VII), it possesses 2 cysteine-rich segments (F1 and F2) located at the N-terminus in region II (Perce-da-Silva *et al* 2011). These cysteine-rich segments, F1 and F2 are responsible for glycophorin A binding to the erythrocyte membrane with few polymorphic regions. *Eba-175* region III is located in the central part of the *eba-175* gene (Perce-da-Silva *et al* 2011).

Genetic analysis of two different *P. falciparum* strains FCR-3 and CAMP identified highly dimorphic segments. The sequences of both strains are nearly identical, except for one dimorphic region in the FCR-3 strain (the F-fragment) and one in the CAMP-stain (the C-Fragment) (Perce-da-Silva *et al* 2007). The two fragments are inserted at slightly different positions in region III of the *eba-175* gene where the F-fragment is 91 amino acids upstream of the C-fragment. The fragments differ in length by 27 amino acids as a result of the different base pair lengths of the 2 fragments; 423 bp length for the F-fragment and a 342 bp length for the C-fragment. These two alleles are both conserved, and parasite strains being haploid possess either one or the other fragment (Perce-da-Silva *et al* 2011). The role of *eba-175* antigen dimorphism in host-parasite interactions is not fully understood.

The distribution of the two *eba-175* fragments has been studied in African populations showing a higher frequency of the F-fragment (Cramer *et al* 2004; Toure *et al* 2006; Soulama *et al* 2010). Cramer *et al* (2004) found that the C-segment was associated with fatal outcome in children with severe malaria in northern Ghana whereas mixed infections were more common in controls. Toure *et al* (2006) however found that the distribution of mixed infections were more associated with symptomatic malaria than asymptomatic malaria. The frequencies of the F- and C-fragments have been shown to vary in different ethnicities and populations, suggesting a functional role in the clinical outcome of malaria in different populations.

In this study, we compared the distribution of the F- and C-fragments of the *eba-175* gene of *P. falciparum* across three well-defined clinical categories – asymptomatic (ASM), uncomplicated (UM) and severe malaria (SM) in children in Ibadan, south-west Nigeria.

Materials and methods

Subject enrollment

The study was carried out in Ibadan, south-west Nigeria, a region that is holo-endemic for malaria. A total number of two hundred and ninety children presenting with fever, malaria parasitaemia and no clinical features suggestive of co-morbidity were recruited from the children's emergency ward and children out-patient clinic of the University College Hospital, Ibadan, and the Adeoyo Maternity Hospital, Ibadan. Asymptomatic subjects were recruited from two schools within the catchment area of the hospitals. Ethical approval was obtained from the joint University of Ibadan, University College Hospital, Ibadan, and the Oyo State Ministry of Health Ethical Review Committee. Informed consent was obtained from the parents or guardian of the patients prior to enrollment.

Blood collection

About 500µl of venous blood was collected into sterile EDTA tubes for parasitological and haematological assays. For parasite DNA analysis, blood was spotted on Whatman 3 mm chromatography paper and dried at room temperature. Malaria parasites were examined on a thick and thin Giemsa-stained blood films. Parasitaemia were quantified relative to 250 leucocytes (white blood cells, WBC) on thick films and estimated as parasites per ìl of blood assuming a mean of 8000 WBC per ìl of blood.

Parasite DNA extraction – gene amplifications

Parasite DNA was extracted from blood spots on filter paper using the simple methanol extraction method as described elsewhere (Lin *et al* 2004). Amplification of DNA was done by nested PCR. The primary PCR was designed to amplify the entire coding region of the gene using the primer pairs:

EB-F 5' CAAGAAGCAGTTCCTGAGGAA-3' (forward primer) and EB-R 5'-TCTCAACATTCATATTAACAATTC-3' (reverse primer) and the second set of primers EBnF 5'-GAGGAAAACACTGAAATAGCACAC-3' and EBnR 5'-CAATTCCTCCAGACTGTTGAACAT-3'.

Both primary and nested PCRs were performed in a final volume of 25µl containing 2.5µl PCR buffer, 100µM dNTPs (dATP, dGTP, dTTP, and dCTP), 2.5mM $MgCl_2$, 0.75 units of *Taq* polymerase, 12.5 pM of each primer and 5µl of extracted DNA (for the primary PCR). DNA was denatured at 94°C for 5 minutes (5 seconds for the nested PCR), followed by 35 cycles of amplification (denaturation at 94°C for 10 seconds, annealing at 57°C for 30 seconds and extension at 72°C for 40 seconds). This was followed by incubation or final extension at 72°C for 3 minutes. 2µl of the primary PCR product were re-amplified in the nested reaction using the following family-specific primers. PCR products were subjected to electrophoresis on 1.2% gel and visualised by trans- illumination with ultraviolet light after staining with Ethidium bromide. Fragment sizes were calculated for size polymorphisms relative to the standard size marker (100bp DNA ladder) using the BioDocAnalyzer computer software package.

Statistical analysis

Descriptive statistics (means, standard deviations, medians, ranges) were computed for continuous variables while frequencies were computed for categorical variables. Three-group comparisons of continuous variables were computed using analysis of variance (ANOVA) while comparisons of categorical variables were done using the chi square (χ^2) test. The logistic linear regression technique was used to investigate the association between clinical severity and parasite genotype characteristics. Using multinomial logistic regression with the outcome as severity 'severe malaria', 'uncomplicated malaria', 'asymptomatic malaria (reference category), univariate logistic models were used to investigate the association of number of *eba-175* alleles, presence of F and C alleles with the outcome. Multivariate logistic models with the same outcomes and variables were conducted, after inclusion of age, sex and parasite density in the models. A p value < 0.05 was considered statistically significant. These analyses were done using *STATA Version 6.0* (StataCorp, College Station, Texas, USA).

Results

The study-population of 290 children comprised of 158 (54.5%) males and 132 (45.5%) females with a median age of 34 months. Based on the criteria of the World Health Organization, 88 were classified as acute uncomplicated malaria (UM), 120 as severe malaria (SM) and 82 as asymptomatic malaria (ASM). Asymptomatic malaria was defined as presence of asexual *P. falciparum* in peripheral thick blood smears, an axillary temperature of >37.5ºC and an absence of malaria-related symptoms. Uncomplicated malaria was defined as presence of asexual parasitaemia and a temperature of >37.5ºC without severe malaria symptoms. Severe malaria was defined as presence of asexual parasitaemia, haematocrit of <15% and unrousable coma which persisted for more than 30 minutes after a seizure.

The three categories of subjects differed significantly in age, parasite density and hematocrit. The geometric mean parasite density of the UM group was 7,040/µl, in contrast to 2,000/µl for the ASM group and 31,724/µl for the SM group (Table 1).

Table 1. Characteristics of subjects in the three clinical categories of malaria.

Characteristic	Category of malaria			*p*-value
	Asymp-tomatic	Uncom-plicated	Severe	
Number	82	88	120	
Sex (% male)	57.5	44.3	61	0.064
Age in months (mean, IQR*)	36.4	33.5	37.9	0.433
Temperature (^{0}C)	36.6	37.8	38	< 0.0001
Parasite density (geometric mean)	2000	7040	31724	<0.001
Packed cell volume (%)	31.2	30.7	19.2	<0.001
Height (cm)	86.7	90	88.5	0.442
Weight (Kg)	12.7	10.8	11.9	0.127

*Interquartile range.
p-value is for *chi*-square test (for categorical variables) or Kruskal-Wallis non parametric ANOVA (continuous variables).

Association of parasite *eba-175* genotypes with malaria category

The F-fragment was found in 242 (83.4%) of the study samples while the C-fragment was found in 113 (39%). Mixed infections containing both the C and F fragments were found in 64 (22%) of the samples.

The association of *eba-175* genotypes with malaria category was explored in two ways: (1) the association of the F and C fragments with malaria category was explored and (2) the association of mixed infections

with malaria category was also explored. The distribution of *eba-175* genotypes was significantly different between the three groups (Table 2). The frequency of the C genotypes was significantly different in the 3 groups; 48.8% in ASM, 39.8% in UM and 31.7% in SM (p = 0.049). The frequency of mixed infections also differed in the three groups: 34.1% in ASM, 22.7% in UM and 13.3% in the SM group (p = 0.002).

A logistic regression model (Table 3) with age, sex, parasite density and packed cell volume as covariates and using asymptomatic group as the reference category (data not shown) showed that when compared with the asymptomatic, the presence of the C-allele was associated with a 3.7 fold (OR = 0.375, p = 0.007) reduced risk of severe malaria. The presence of mixed infections was also significantly associated with a 3.2 fold (OR = 0.321, p = 0.005) reduced risk of developing severe malaria.

Table 2. Association of clinical category of malaria with *eba-175* fragments.

msp-2 group	Asymptomatic malaria *n*=82	Uncomplicated malaria *n*=88	Severe malaria *n*=120	*p*-value
Mixed infection (%)	28 (34.1)	20 (22.7)	16 (13.3)	0.002*
F (% positive)	71 (86.6)	73 (83.0)	98 (81.7)	0.645
C (% positive)	40 (48.8)	35 (39.8)	38 (31.7)	0.049*

p value is for Fisher exact test * $p < 0.05$.
n = number of children in each clinical group.

Table 3. Association of clinical category of malaria with genotypes studied on multivariate analysis, controlling for age and parasite density.

Variable	Regression coefficient	SE[¥]	OR[#]	*p*-value
Uncomplicated malaria				
F-fragment	-0.136	0.451	0.873	0.764
C-Fragment	-0.422	0.326	0.656	0.196
Mixed infection	-0.524	0.364	0.592	0.150
Severe malaria				
F-fragment	0.101	0.453	0.904	0.824
C-fragment	-0.979	0.365	0.376	0.007*
Mixed infection	-1.136	0.408	0.321	0.005*

All regression models included adjustment for age and parasite density. The reference category (control group) for each model was the asymptomatic malaria group.
* $p < 0.05$.
[#]odds ratio, [¥] = standard error.

Discussion

This study evaluated the pattern of distribution of the F and C genotypes of the *eba-175* antigen and their associations with the clinical outcome of malaria in children in Ibadan.

Previous studies on the *eba-175* genotypes have shown an association with occurrence and disease outcome with varying results from different geographical regions (Cramer *et al* 2004; Toure *et al* 2006). The two *eba-175* fragments, F and C were found in all the three clinical categories of malaria; asymptomatic malaria, uncomplicated malaria and severe malaria, but the F-fragment predominated in the overall population (83.4%). Consistent with this result are two previous studies carried out in high endemic malaria areas in West Africa that showed a higher frequency of the F-fragment (Cramer *et al* 2004; Soulama *et al* 2010). This data however differs from results reported in low endemicity malaria populations of Sudan and Brazil, where C-fragment was reported to be the most frequent *eba-175* allele (Binks *et al* 2001). The high prevalence of the F-fragment in malaria endemic populations has been linked to acquired immunity (Smith *et al* 1999). However, the distribution of the F-fragment did not differ significantly between cases and controls.

Asymptomatic controls had the highest frequency of the two fragments and the mixed infections compared to the uncomplicated and severe malaria cases. The frequencies of mixed infections and C-fragment were significantly higher in the asymptomatic controls than in the uncomplicated and severe malaria cases. Previous studies of the genetic diversity of *P. falciparum*, using the merozoite surface protein (MSP)-1, have shown that mixed infections are predominantly found in asymptomatic subjects (Gupta *et al* 1994; Beck *et al* 1997; Zwetyenga *et al* 1998; Konate *et al* 1999). This is equally consistent with a previous study by Cramer *et al* (2004). Toure *et al* (2006) however found a higher frequency of mixed infections in symptomatic children than in asymptomatic children. The role of antigenic variation in predisposing an individual to either asymptomatic or symptomatic malaria is of great importance. There are two possible ways of getting mixed infection. Mixed infection could be caused by a single mosquito bite if the mosquito was carrying two parasite clones, each with the F- and C-fragment or it could be caused by two different bites from mosquitoes each carrying different clones of the parasite (Toure *et al* 2006). When an individual is exposed to the two parasite clones at once, there is the possibility for the host to develop immunity to both and remain asymptomatic.

In this study, the presence of C-fragment and mixed infections were significantly associated with protection against severe malaria, as children with the mixed infections and C-fragment were less likely to develop severe malaria thereby suggesting a role for *eba* dimorphism in the outcome of malaria in Ibadan. This differs from the finding of a previous study in Ghana by Cramer *et al* (2004), which reported an association of the C-fragment with fatality in severe malaria in Ghana. Generally, the differences observed in the distribution of *eba-175* fragments in different populations could be as a result of random shifts in parasite allele frequencies in the different populations (Cramer *et al* 2004). Another plausible explanation for these differences could be differences in host genetic background of different population studied. Hence genetic differences in the both the host and parasite factors may influence the outcome of clinical malaria.

Conclusion

Our study shows a significant association between *eba-175* alleles and the clinical outcome of malaria in Ibadan. There is therefore a need to study the distribution of *eba-175* alleles in different populations. These findings could prove useful in the development of malaria vaccine in the tropics.

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