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Original Research Paper

Medicine

GENETIC DIVERSITY OF TWO VACCINE CANDIDATE DIMORPHIC ANTIGENS (*ERYTHROCYTE BINDING ANTIGEN-175; MEROZOID SURFACE PROTEIN-3) OF PLASMODIUM FALCIPARUMFROM* UNCOMPLICATED AND SEVERE MALARIA PATIENTS IN SENEGAL

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ABSTRACT The 175 kDa erythrocyte binding antigen (*EBA-175*) and the merozoite surface protein 3 of *Plasmodium falciparum* are among the malaria vaccines candidate.. However, their polymorphism could play an important role in vaccine development. Therefore it becomes important to assess the polymorphism of these two malaria vaccines candidates in Senegal in the context of scaling up malaria control strategies. **Method:** To achieve our objective, dried blood spots were collected from patient with mild malaria during drug efficacy trial and from hospitalized patient with severe malaria in Pikine Teaching hospital. The allelic polymorphism of these two vaccines candidates was determined by nested PCR. **Results:** For *EBA-175* gene from uncomplicated malaria, our results showed that 47% (35/74) and 44% (33/74) of*malaria parasites* carried the C and F alleles respectively. However in patients with malaria treatment failure , we noted the presence of other minors alleles at 400 and 600bp (3.7%). For severe malaria, we noted 32% (8/25) and 60% (15/25) of the strains carried the C and F alleles respectively. In addition, our results showed that 8% of the strains carried both alleles C and F in our study population. For *msp-3 gene*, our results showed that 80.26% (61/76) and 56.25% (18/32) of parasites carried out the K1 allele for uncomplicated and severe malaria, respectively. Similarly, the*msp3_3D7* allele was noted at13.16% (10/76) and 40.62% (13/32) in patients with uncomplicated and severe malaria. The prevalence of strains carrying the two alleles(mixed) of*msp-3gene* was 6.58% (5/76) in uncomplicated malaria and 3.13% (1/32) in severe malaria. **Conclusion:** our results showed that the msp3_K1 allele was predominant in our study population; however for e*ba-175* gene, the F fragment was predominant in severe malaria cases. Our results suggested that *msp-3_K* 1 and *EBA-175_F* allelesshould be considered for any malaria vaccine development in Senegal.

KEYWORDS : Malaria, genetic diversity, MSP-3, EBA-175, PCR, vaccine.

INTRODUCTION

The persistence of malaria is partly due to antimalarial drugsresistance development to, mainly against drugs targeting the asexual erythrocytic stages of the parasite. This stage is also responsible for the pathological and clinical manifestations of the disease. Therefore there is an urgent need for malariavaccine that would be effective against these asexual stages. However, the effectiveness of this vaccine in a given region will depend on the genetic diversity of the circulating parasites. It has been shown that the genetic polymorphism of these parasites stages appears to play an important role in the escape mechanisms from the host immune system (1).

Several stage-specific *P. falciparum* antigens have been characterized as vaccine candidates through molecular epidemiology (2). However, the efficacy of these vaccine candidate antigens may be compromised by the genetic diversity of parasite antigens in a given locality.

The 175 kDa binding antigen (*EBA-175*) is a protein that plays a well-defined role in anchoring to glycophorin A (GpA) during the invasion of erythrocytes by parasites (3). It has been shown that this protein modifies the biophysical nature of the red blood cell, facilitating its entry by effectively reducing the energy barrier to invasion (4). The gene coding for *EBA-175proteine* is located on chromosome 7 and is composed of four exons and seven regions called I - VII which include three cysteine-rich regions (F1, F2 and C). A highly divergent dimorphic III region separates the two domains by inserting a 423 base pair segment in the FCR-3 strains (F-loop) or a 342 base pair segment in the CAMP strains (C-loop). The *EBA-175* antigen is dimorphic, consisting of the FCR-3 allele called the F-loop and the CAMP allele or C-loop (5). The role that dimorphism plays in host-parasite interaction is not fully understood but previous studies in Senegal have shown the predominance of the F fragment in two localities and the C fragment in another (6).

The MSP-3 protein of *P. falciparum* is encoded by a single locus covering 1.1 kb of chromosome 10. MSP-3 is an approximately 43 kDa soluble protein associated with the merozoitemembrane

surface although it lacks a transmembrane recognition site or signal sequence for glycosyl-phosphatidyl-inositol anchoring. The amino acid sequence derived from MSP-3 predicts several domains (7): an N-terminal signal peptide sequence, a central domain of imperfect Ala heptad repeats, a second central domain rich in glutamine residues, and a C-terminal region containing a zipped leucine motif. While the C-terminal domain of MSP-3 is relatively conserved, the N-terminal region is polymorphic. As a result of this polymorphism, the *msp-3* gene can be found in two main types of alleles, K1 and 3D7 alleles (8). Previous studies in West Africa, including Senegal, have shown similar frequencies of these different allelic forms (9).These two antigens have been characterized as vaccine candidates and their efficacy may be compromised by genetic diversity in a given locality. Therefore it becomes important to determine the genetic diversity of *EBA-175* (Erytrhocyte Binding Antigen-175) and *MSP-3* (Merozoide Surface Protein-3) vaccines antigenesin hospitalized patients with severe malaria in Pikine teaching hospital (Pikine CHN) and patients with uncomplicated malaria in the central part of Senegal (Diourbel region)

MATERIALS AND METHODS

Study Areas

Samples from uncomplicated malaria patients were collected during antimalarial drug efficacy trial in Dioubel region located in the central part of Senegal. In Diourbel region malaria prevalence was 8.7% in 2019 (10). Samples from patients with severe malaria were collected in Pikine Teaching Hospital (PTH) located in the suburb of Dakar PTH is the reference centre for all severe malaria cases in this area. The department of Pikineis characterized by the presence of water basins witch is favourable for anopheles developmentand is a malaria hotspot area.

Study population

A total of 77 patientsall ages with uncomplicated malaria living in Diourbel region and 41 patients hospitalized in PTH for severe malaria were included in our study. In Diourbel region all patients included in our study had axillary temperature higher than 37°C,monospecificparasitemia higher than 1000 p/µl and no serious pathologies.Pregnant women and/or any patients who could not tolerate these drugs were excluded..For severe malaria cases, samples were collected from hospitalized patients for severe malaria in PTH. From all included patients dried blood spots (DBS) were collected and dried at room temperature away from dust and flies; and then stored in ziplock bags with desiccants. Collected DBS samples were shippedto the Parasitology-Mycology laboratory at UCAD for genotyping.

Extraction and amplification of *P. falciparum* DNA

P. falciparum DNA was extracted from DBS using the Chelex-100 method described previously (11).The extracted DNA was amplified by nested PCR with specific primers for each gene.

For the *msp-3* gene, the outerwas done with a 25 µl reaction mixture. This mixture consisted of 12.5 µl of One taq Quick-Load (M0486L), 0.5 µl of each forwardand reverseprimers (159F: 5' ATGTTGCTAGTAAAATTG 3' and 745R: 5' CATAACTAGAAGCTTCTTTTGC 3'), 9.5 µl of H20 and 2 µl of extracted DNA. For the nested PCRthe forward 216F: 5' GAATAATAATTCTCAAAATAGAAATG 3' and reverse 725R: 5' GCTTTTAAAACAGCTTGTTTGC 3' primers were used and the reaction mixture was similar to the outer except that the 2ul of the DNA are from the outer. The outer and nested PCR were done bya Bioradthermal cycler (C1000 Touch™ Thermal cycler) according to the following programs:

Outer PCR program formsp-3 gene

Steps	Temperature	Time
Initial denaturation	94 °C	1 min 30 s
Denaturation	94 °C	30s
Hybridization	54 °C	30 s
Elongation	68 °C	2 min
35 cycles from the 2nd stage onwards		
Final elongation	68 °C	5 min
Conservation	4 °C	∞

Nested PCR programformsp-3 gene

Steps	Temperature	Time
Initial denaturation	94 °C	1 min 30 s
Denaturation	94 °C	30s
Hybridization	46 °C	30 s
Elongation	68 °C	2 min
40 cycles from the 2nd stage onwards		
Final elongation	68 °C	5 min
Conservation	4 °C	∞

For the amplification of the eba-175 gene, the same reaction mixture was carried out as for the *MSP*-3 gene with the only difference the specific primers foreba-175 used: eba1 primers: 5' CAAGAAGCAGTTCCTGAGGAA 3'; eba2: 5' TCTCAACATTCATTAACAATTC 3' for the outer and eba3: 5' GAGGAAAACACTGAAATAGCACAC 3'; eba4: 5' CAATTCCTCCAGACTGTTGAACAT 3' for the nested PCRamplification. The following amplification program was used for the eba-175 gene eba-175 gene amplification program outer and nested

Steps	Temperature	Time
Initial denaturation	94 °C	1 min 30 s
Denaturation	94 °C	30s
Hybridization	54 °C	30 s
Elongation	68 °C	2 min
35 cycles for the first amplification the 2nd stage onwards		
Final elongation	68 °C	5 min
Conservation	4 °C	∞

For the nested 40 cycles were used from the 2nd stage onwards The nested PCR products were subjected to electrophoresis on 1,5% agarose gels (Gibco-BRL) in Tris acetate-EDTA buffer for various lengths of time depending of the predicted size of the PCR products and visualized withethidium bromide by an imaging system, the **Geldoc™ EZ Imager (Biorad).** A ladder of 100 base pair (Pharmacia Biotech,Piscataway, NJ) was used as standards for estimation of the sizes of DNA fragments.

Data Interpretation

The *eba-175*and *msp-3* allelic family were categorized by their molecularweights and considered the same if their molecular weights were approximately within 10 bp (12). Positives controls (MRA 159, MRA 531) were used during the PCR reaction. The detection of a single PCR fragment for each allele was classified as a single genotype infection.The *msp-3_K1* allele was identified as a single fragment atapproximately 500 base pairs, and the*msp-3_3D7* allele was identified as a single fragment atapproximately 400 base pairs. The CAMP allele was identified as a single fragment

atapproximately 714 base pairs, and the FCR3 allele was identified as a single fragment atapproximately 795 base pairs. Mixed infections were defined as the simultaneous presence of the K1 and 3D7 alleles for the *MSP-3* gene or the F and C fragments for the *EBA-175* gene in the same sample. Any other minors alleles identified will be reported.

Data Analysis

Data obtained was entered and analyzedby Excel spreadsheet (2016). The studyarea was located using QGIS version 2.18.25 software. Rsoftware version 3.5.3 was used to perform the exact Fischer and t-test; GenAlexsoftware (13) version 6.503 allowed us to calculate the Genetic Distance of Nei. Epi info 7 allowed us to calculate the allelic frequencies. The expected heterozygosity is calculated by the formula: HE ="[n/(n-1)]1-∑pi2" (14); n is the total number of samples and pi is the allelic frequency for a given locus.HE varied between 0 (no allele diversity) to 1 (all sampled alleles are different) (15). For statistical tests the significance threshold is set at 5%.

RESULTS

Out of the 118 samples analysed, 77 were from Diourbel and 41 from Pikine. Among the the 77 samples collected at Diourbel, 35% (27/77) were treatment failures.

Characteristic of the study population

For uncomplicated malaria cases, the mean age was 16.22 years with a minimum age of 5 years and a maximum age of 70 years. The average parasite density was 36436 p/µl.

For severe malaria cases, the average age was 28.15 years with a minimum age of 5 years and a maximum age of 65 years. The average parasite density was 18645 p/µl.

PCR efficacy

Amongthe 77 confirmed malaria by microscopy, 96% (74/77) were PCR positive for *eba-175* and 98.7% (76/77) for *MSP-3*. In contrast, fromthe 41samples collected from patients with severe malaria, 61% (25/41) were PCR positive for *eba-175* and 78% (32/41) for *msp-3*. The amplification failures could probably due to DNA extraction. All samples without PCR results were excluded from the analysis.

EBA-175 Allelic Distribution

Among the 74 *eba-175* positive samples from patients with mild malaria,,47% (35/74) carried out the C fragment, 44% (33/74) the F fragment, and 8% (6/74) are mixed alleles.Out of the 25 samples from severe malaria (SM) cases and *eba-175* positive samples after PCR, 60% (15/25) had the F fragment, 32% (8/25) the C fragment and 8% (2/25) mixed alleles.By comparing allelicdistribution between the two populations, our results shown that the F fragment was predominant in severe malaria cases while the C fragment was predominant in mild malaria cases with no significant different between the two populations. (Fisher's Exact Test: p-value = 0.703).

Allelic distribution of*msp-3 gene*

Out of the76 positive samples from mild malaria, 80.26% (61/76) carried outK1 allelic family, 13.16% (10/76), 3D7 allele and 6.58% (5/76) weremixed alleles.

Among the 32 positives samples collected from patient whith severe malaria, our results showed that 56.25% (18/32) carried outK1 allele, 40.62% (13/32) 3D7 allele and 3.13% (1/32) aremixed alleles.

By comparing the distribution of alleles between the two populations, our results shown the predominance of the *msp-3_K1* allele in both populations. However, K1 family allelic was more prevalent in simple malaria cases 80.26% compared to severe malaria cases 56.25% with significant difference (p=0.05%). The HE allele diversity index did not differ from one malaria attack to another according to the two genes (p-value >0,05, t-test).

A small genetic distance (Nei) of 0.053 was found between the parasites from patients with mild malariaand severe malaria.

Distribution of allelic family in treatment failure patients

By comparing the allelic frequency of samples with treatment failure between D0 and D-failed, our results showed that for the *eba-175* gene the FCR3 allele was predominant at D0 (63%) ; while we noted a frequency of 41% at D-failure.. In addition, our results showed for fragment C a frequency of 37% at D-failure and 33% at D0. Our results also showed a low frequency(0.18%) of *P.falciparum*strains carrying both alleles. However, our results showed the presence of other minor alleles at 400 and 600bp with a low frequency(3.7%).

For *msp-3* gene our results showed that the K1family allelic has been found at 77% atD0 and 81% at the day of failure;for 3D7 family allelic, a frequency 7% was found at the day of failure while we noted a frequency of 11% at D0. Similarly, our results showed that 11% of strains carried both alleles (mixed).

Allelic distribution fromsusceptible and tolerant/resistant plasmodial strains

By comparing allele frequency of*eba-175* gene between drug sensitiveplasmodial strains and tolerant/resistant strains to treatment; our results showed that FCR3 allele was predominant (53.1%) in the susceptible strains. In contrast, the CAMP family allelic was predominant (63%) in the tolerant/resistant (D-failure) strains. In addition, our results showed a low frequency (3.7%) of other minorsalleles at 400 and 600 bp in tolerant/resistant strains.

For the *msp-3* gene, K1 allelic family was predominant in bothsusceptible strains (80%) and drug tolerant/resistant strains (77%). MeanwhileLikewisefrequency of mixed infection (K1 and 3D7 alleles) are similar for the tolerant/resistant strains (11.5%), while for the susceptible strains the 3D7 allele (14%) is more represented than the mixed alleles (6%).

DISCUSSION

Despite all control and pre-elimination strategies implemented in Senegalese, malaria stillbe a public health problem. The complexity of malaria management could be due to the genetic diversity of Plasmodiumstrains. Authors have shown that genetic diversity is a major challenge for the effective development of malaria vaccines (16). Furthermore, it has been shown that the efficacy of a malaria vaccine in a given locality is highly dependent on the allelic forms of the strains circulating in the area (17). Therefore it becomes important to assess the polymorphism of two malaria vaccines candidates in Senegal in the context of scaling up malaria controland pre-elimination strategies.

Of the 118 samples included in our study, 91.52% were positive for *msp-3* and 83.9% for *eba-175 genes by* PCR. The PCR negatives samples noted could probably due to the presence of chelex (PCR inhibitor) in extractedDNA (18).

By comparing allelicfrequency of *eba-175* gene in uncomplicated malariacases, results showed a predominance of the CAMP allele (47%). Similar results were found in Sudanese population with high prevalence of CAMP allele (73%) (19).

In severe malaria cases, we noted a predominance of the F fragment (60%) during our study. Similars results were noted on children with severe malaria in Ghana (20). Previous studies conducted in Senegal haveshowed a prevalence of F fragment in Pikine (72%) and Thies (63%) area (6).

By comparing the frequency of mixed alleles (presence of both F and C fragments in the same sample) frommild malaria and severe malaria, no significant difference was noted in our study area. . However in area of high malaria transmission, high frequency of mixed alleles was reported in severe malaria compared to asymptomatic malaria cases. The low frequency of mixed alleles (8%)notedin our study areas could probably be due to the lowmalaria transmission in study areas.

Despite the major alleles of the *eba-175* gene, our study showed the presence of other minor alleles at 400 and 600 bp. The *eba-175*_ 400 bpallelewas found at 10% in Senegal and Ghana (9). Furthermore, the presence of these minor alleles counterbalances the allelic dimorphism of *eba-175 gene. The* presence of these minor alleles could make malaria vaccine development complex, as it has been shown that the more polymorphic a vaccine candidate antigen is, the less effective it is at achieving vaccine efficacy (17).

For the *msp-3* gene, our results showed that K1 allele was predominant in uncomplicated malaria cases. Similar results were notedin 3 regions of Thailand (21). However in malaria hypo-endemic area, the 3D7 allele was predominant (22). This difference of family allelic distribution could probably due to differences in the level of malaria transmission in the different study areas.

In severe malariacases, our results showed a predominance of *K1* family allelic (56%) compare to 3D7 family (40%) with no significant difference. Similar results were noted in West and central Africa (9).

Furthermore, by comparing the frequency of*eba-175*family allelic for mildand severe malaria, no significant difference was noted. Our finding suggested that the eba-175 family alleliic does not play a role in the outcome of malaria disease. Similar results were noted for *msp-3* gene, K1 family allelic was predominant in uncomplicated malaria cases; while in severe malaria cases no significant difference was noted between K1 and 3D7 families allelic. Our resultssuggested that the occurrence of severe malaria is not associated with the presence of any of major msp-3 family allelic.

By comparing the genetic distance between our two parasite populations (simple and severe malaria), our results showed that the genetic distance was 0.053 and the indices of genetic diversity were not statistically different. Ours results suggested thatPlasmodium strains found in mild and severe malaria cases were genetically similar.

For uncomplicated malaria cases collected during drug efficacy trial, our results have shown predominance of CAMP family allelic in Plasmodium parasites collected at the day of treatment failure compare to parasites at day D0 (before any treatment).Similar results have been reported (23), authors have showed that parasites expressing a C fragment had a natural selective advantage over those with F fragment, which could explain the predominance of the C allele in parasites from patient with treatmentfailure.The presence of *eba-175*mixed alleles could indicate a more complex allelic structure for parasites found.at the day of failure.

CONCLUSION

Our results suggested that due to the low polymorphism noted, the *msp-3* gene could be consider for vaccine development in our study area compare to *EBA-175* where our results showed significant polymorphism with the presence of other minors alleles (400, 600 bp).

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Authors' Contribution

Magatte Ndiaye and Babacar Faye were responsible for the concept and the design of the study. MNdiaye, AichaDjigal, Isaac Manga collected samples for this study. M Ndiaye, A Djigal, Manga I were responsible for PCR analysis. M NdiayeandA Djigalprepare the manuscript.All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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