Journal of
Pharmacology & Clinical Research
ISSN: 2473-5574

Research Article
Volume 10 Issue 1 - March 2024
DOI: 10.19080/JPCR.2024.10.555779

J of Pharmacol & Clin Res

Diversity of Plasmodium falciparum Parasite Responsible for Malaria Infection detected at the Level of Peripheral and Cord Blood Samples from Pregnant Women with and without HIV Infection in the Kumba District Health Area, South West Region of Cameroon

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Submission: February 22, 2024; **Published:** March 07, 2024

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Abstract

Malaria elimination in pregnancy may be threaten by selective drug pressure especially among HIV positive pregnant women. The movement from Tenofovir-Lamivudine-efavirenz to Tenofovir-Lamivudine-dolutegravir regimen among HIV-positive pregnant women may have an effect on Plasmodium falciparum parasite diversity. Parasite genetic diversity has been incriminated as a major cause of parasite adaptation, virulence, morbidity and even resistance. Therefore, this study evaluated HIV effect on P. falciparum parasite diversity among pregnant women.

Methods

A total of 40 P. falciparum malaria positive samples were analyzed for the study: 26 peripheral positive samples from mothers who were HIV-negative, 10 peripheral positive samples from mothers who were HIV-positive and 4-cord blood samples from mothers who were HIV-negative were genotyped for msp1, msp2 and eba175 genes with the help of specific primers using the nested polymerase chain reaction technique.

Results

A total of 14 different genotypes of msp1, msp2 and eba175 responsible for P. falciparum malaria in this area. The average multiplicity of infection (MOI) for the mono-infected individuals was 0.22, co-infected was 0.791 and cord blood was 1.80. Majority of the study population were polyclonally infected with several strains of P. falciparum. Phylogenic analysis showing the distance tree of msp1 and eba175 shows similar strains of Plasmodium falciparum responsible for malaria in all the different groups. However, msp2 shows a homogeneous distribution of strains responsible for malaria infection among the mono-infected and co-infected individuals with a slight heterozygous strain with slight similarities among the cord blood infected individuals with an unbiased diversity of 0.520, 0.607 and 0.633 among the mono-infected, co-infected and cord blood infected individuals respectively.

Conclusion

HIV infection shows to have an influence on Plasmodium falciparum parasite genetic diversity. Majority of the individuals in both groups were polyclonally infected.

Keywords: Merozoite Surface Protein; Erythrocyte Binding Protein; Plasmodium Falciparum; Human Immunodeficiency Virus; Pregnancy

Abbreviations: EBA: Erythrocyte Binding Antigen; DNA: Deoxyribonucleic Acid; HIV: Human Immunodeficiency Virus; ITN: Insecticide Treated Bed Nets; MSP: Merozoite Surface Protein; nPCR: Nested Polymerase Chain Reaction; WHO: World Health Organization

Introduction

Malaria is a serious public health challenge internationally, especially in low and middle-income nations, with over half of the world's population at risk of infection [1]. The World Health Organization (WHO) recently reported a considerable reduction in worldwide malaria burden over the last 15 years, achieving the 2000 Millennium Development Goals. Fifty-seven nations have reduced malaria cases by 75%, in keeping with the World Health Assembly's goal for 2015 [2]. Malaria mortality rates in Africa have dropped by 66% across all age groups. The progress made in reducing malaria burden is credited to the huge deployment of effective prevention and treatment methods, notably insecticide-treated nets and the implementation of Artemisinin-based Combination Therapies (ACTs) as the first-line treatment[3]. Recently, the Cameroon government adopted the use of Tenofovir-Lamivudine-dolutegravir (TLD) as first line treatment of HIV in pregnant women opposed to Tenofovir-Lamivudine-efavirenz (TLE). This was based on findings which shows that TLD is more effective in preventing mother-to child transmission of HIV since it is capable of reducing viral load rapidly than TLE [4-6].

Plasmodium falciparum parasite: the most virulent of all the Plasmodium species is responsible for majority of malaria reported cases in Cameroon with a prevalence of about 30% [7]. In Cameroon where everyone is at risk of this infection and over 1,310,000 pregnancies per year were recorded between 2015 to 2019 and 201,000 resulted in abortions, malaria remains a major public health threat, with the entire country at risk of transmission [8]. In the tropics, climatic variables such as temperature and humidity influence the occurrence and spread of the malaria parasite. Changes in the altitudinal gradient and attitudes toward the use of insecticide-treated bed nets (ITNs) are two fundamental elements that contribute to the heterogeneity of malaria distribution in endemic populations [7]. Nevertheless, the introduction of combination therapies was to prevent the emergence of drug resistant strains as well as diverse strains of this parasite. However, this is not the case as several cases of diversity and drug resistant strains of *P. falciparum* have been reported in Cameroon [9-11].

Findings from previous studies shows that *P. falciparum* diversity plays a key role in its adaptation, morbidity and virulence. This specie of Plasmodium has been well studied and characterized. One of the primary antigens of *P. falciparum* merozoites requires a 175-kDa sialic acid-binding protein ligand to penetrate the host erythrocyte and this protein is termed as erythrocyte binding antigen 175 (EBA-175) [12]. EBA-175 is positioned in the micronemes at the terminal end of the merozoite and has been well defined as the ligand that binds glycophorin A (gyp A), which is found in the erythrocyte membrane [13]. The eba-175 gene is found on chromosome 7 and consists of four exons and seven sections labeled I through VII. Region III is positioned in the gene's center, and studies have revealed that it contains a highly dimorphic portion. This dimorphism is distinguished by the insertion of a segment of 423 base pairs (bp) in strain FCR3 (F-fragment) or a segment of 342 bp in strain CAMP. These two variations are conserved among *P. falciparum* strains, and given that the merozoites are haploid and eba-175 is a single copy gene, either one or the other segment is present in a unicolonial infection [14].

Merozoite surface protein 1 and 2 (msp1 and msp2) are also two main Plasmodium falciparum blood-stage malaria vaccine targets that play an important role in the identification of genetically different *P. falciparum* parasite subpopulations. They are engaged in erythrocyte invasion and are the target of immunological responses. Msp1 is a 190 KDa surface protein expressed by the msp1 gene located on chromosome 9. It has 17 blocks of sequences bordered by conserved areas [15]. Block 2, the most polymorphic region of msp1, is divided into three allelic families: K1, MDA20, and R033. Msp2 is a glycoprotein encoded by the msp2 gene on chromosome 2 that consists of five blocks, the central block being the most polymorphic. The msp2 alleles are divided into two allelic families: FC27 and 3D7/IC1 [15]. Msp1 and msp2 have been revealed to be highly polymorphic in diverse geographical locations in malaria-endemic countries [9,16]. In areas with intense malaria transmission, the probability of a subject becoming infected with the same parasite genotype is extremely low.

The genotyping of malaria parasite populations is still an important approach for determining the types and amount of parasite clones in an infection [15]. Cameroon has three main malaria transmission zones: soudano-sahelian, humid savannah, and forest zone [17]. However, among these zones are sub-zones with unique malaria transmission dynamics. Therefore, it is critical to define parasite populations in order to tailor malaria control and elimination measures. Malaria elimination will require knowledge of parasite genome variation in different geographical sites, as well as a greater understanding of the processes that govern gene flow between locations. However, understanding how and why numerous strains spread across diverse places is challenging due to the intricate interplay of selection pressure sources, including treatment characteristics, biological factors, transmission intensity, and treatment access. Therefore, this study investigated the diversity of *P. falciparum* parasite genetic diversity in pregnant women with and without HIV infection in the kumba heath district area.

Methods

Study Area and period

The study area has been described elsewhere [8]. Briefly, the study was conducted within the Kumba health district area, Meme Division of the South West region of Cameroon. The study started on the 15th of March 2022 and ended 30th September 2022. Participants were enrolled from five different Health institutions: Regional Hospital Kumba, District Hospital Kumba-town, Presbyterian Hospital Kumba, Catholic Hospital Fiango and the Kossala Integrated Health Center (Figure 1).

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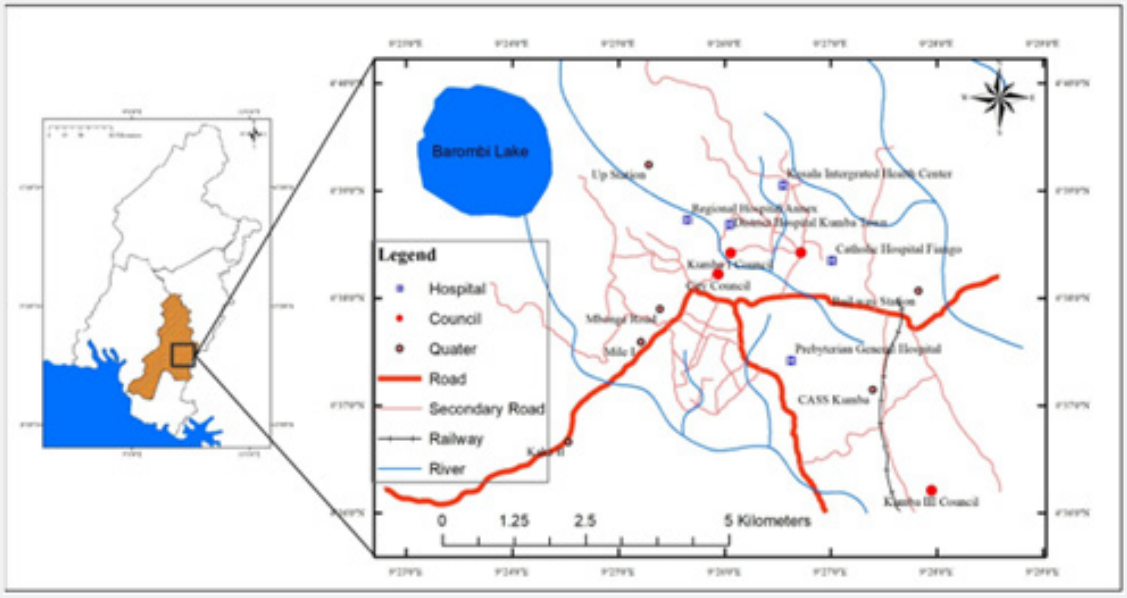

Figure 1: The map of Kumba showing all the different study sites where enrolment of participants was done.

Study Design and Target population

This was a hospital-based cross-sectional study. The study enrolled 10 HIV-positive pregnant women who were *P. falciparum* malaria positive by nested polymerase chain reaction (nPCR) at the level of peripheral blood, 26-HIV negative pregnant women who also were *P. falciparum* positive by nPCR and 4-HIV negative pregnant women who were *P. falciparum* positive at the level of cord blood.

Ethical consideration

Ethical clearance for this study was obtained from the Institute for Advanced Medical Research and Training (IAMRAT), College of Medicine, University of Ibadan, Nigeria (ID NHREC/05/01/2008a). However, ethical clearance from Cameroon was obtained from the Institutional Review Board of the Faculty of Health Sciences at the University of Buea (ID 2022/1655-02/UB/SG/IRB/FHS). Administrative clearance was obtained from the Ministry of Public Health, Regional Delegation of Public Health for the South West Region (ID R11 /MINSANTE/SWR/RDPH/PS/276/281). Further authorization was obtained from the Meme Health District service (ID 12/022/MINSANTE/RDPHSW/KHD/DMO/048). Directors of the health facilities involved also provided an authorization letter for the study to take place in their various facilities. Participants who wished to participate in the study had to first sign an informed consent form. For participants who were unable to read or write, the information was read out, and explained to them and their thumbprints were taken. The study considered pregnant women with and without HIV who were in the third trimester of pregnancy and were fully aware of their viral load counts.

Laboratory Procedures

Sample collection, processing and storage

During Labor, 3mL of mother venous blood was collected using a 5mL syringe and placed in an ethylene diaminetetraacetic acid (EDTA) tube. Immediately after delivery, the delivered placenta with the clamped umbilical cord was collected and the cord was cleaned and disinfected using povidone-iodine(betadine). Using a 5mL syringe, 3mL of cord blood was aspirated from the umbilical cord and placed in a sodium heparinized tube. This process was done immediately after the placenta was delivered when it was still warm to prevent clotting. The sample in the tube was then rotated gently to ensure it was properly mixed with the anticoagulant. The collected samples were transported to the lab for processing. In the laboratory, the maternal venous blood was used to prepare a thick film. A drop of the blood was placed on a clean slide, and rocked properly to lyse and expose the infected red blood cells. The slide was then allowed to air dry, and packaged to prevent contamination in preparation for further analysis.

The remaining whole blood sample for maternal venous blood and cord blood was then centrifuged to separate the plasma from the concentrated red cells (pellet). The plasma and pellet were then aliquoted into separate, and well-labelled Eppendorf tubes. The samples were then stored in a -20°C refrigerator in preparation for further analysis. After the field work, the samples were transported using a sample transportation box to the Immunology and molecular biology laboratory of the Biotechnology Center of the University of Yaoundé I for further analyses. Upon arrival, 50µL of the pellet for both the maternal venous sample and the cord blood sample was blotted on a 185mm Whatmann filter

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paper and allowed to air dry. The samples were then packaged and stored in separate, clean and well-labelled medicine zip-lock bags for downstream assays.

Extraction of Plasmodium DNA for molecular diagnosis

The DNA for the Plasmodium parasites was extracted using the hot Chelex method as described by Plowe et al1995[18]. A total of about 50µL of whole blood pallet sample was blotted on a 185mm Whatmann filter paper and allowed to air dry. This was later cut and placed in a 1.5mL Eppendorf tube. A total of 1mL 0.5% saponin was later added into the tube, inverted several times and allowed to incubate overnight (18-24hrs) at +4°C. The supernatant was discarded in 10% bleach and 1% phosphate buffer saline (PBS) was added to each Eppendorf tube. The tubes were capped and inverted several times and allowed to incubate at +4°C for 20minutes. The supernatant was removed and the filter paper was removed and placed in a clean labeled Eppendorf tube. A total of 150µL of nuclease-free water was added which was followed by the addition of 50µL of 20% heated Chelex. The tube was then vortexed vigorously for 30seconds and allowed to stand in a water bath for 10minutes at a temperature of 100°C. The supernatant was later transferred into a clean sterile tube and centrifuged at 14000 rotations per minute(rpm) for 2minutes. The supernatant was finally pipetted into a fourth final tube and stored at -80°C for further downstream analyses.

Nested polymerase chain reaction for the identification P. falciparum parasite

Nested PCR was done using the 18s rRNA primer which targets the multicopy 18s rRNA plasmodial gene. Using a modified protocol from Snounou et al., 1993 [19], the protocol for the PCR reaction was developed. For each sample, 14µL of PCR reaction was prepared consisting of 7µL of 2x OneTaq® quick-load master mix (New England Biolabs, Inc), 3µL of nuclease-free water, 0.5µL each of 10µM forward and reverse primers and 3µL of template DNA from the extract. For the nest-1 reaction of the PCR, templated DNA extract plus forward and reverse primers were used (rPLU5: 5′-CCTGTTGTTGCCTTAAACTTC-3′ and rPLU6: 5′-TTAAAATTGTTGCAGTTAAAACG-3′). For the nest-2 reaction of the PCR, the amplicons from nest-1 reaction were used as template DNA together with species-specific forward and reverse primers for *P. falciparum* identification (rFAL1: 5′-TTAAACTGGTTTGGGAAAACCAAATATATT-3′ and rFAL2: 5′-ACACAATGAACTCAATCATGACTACCCGTC-3′). Purified MRA 736G genomic DNA for Plasmodium falciparum while known positive samples for Plasmodium malariae and Plasmodium ovale were used as positive control. Nuclease-free water was used as negative control. Reaction conditions used for amplification are; For nest-1: initial denaturation: 95°C for 5minute in 1 cycle, denaturation: 94°C for 1minute, annealing: 58°C for 1minute, final extension: 72°C for 2minute in 25cycles and final extension: 72°C for 2minutes in 1cycle. For the nest-2, the reaction conditions remain the same as in nest-1. The nest-2 products were electrophoresed on ethidium bromide-stained 1.5% agarose gels and visualized on the Gel Doc™ XR+ System (Bio-Rad, USA). Samples were qualitatively identified as negative or positive. *P. falciparum* was identified with a band size of 205bp.

Genetic Diversity of msp-1, msp-2 and eba-175 (nested PCR)

The samples positive for only *P. falciparum* were selected to perform the genetic diversity of msp1, msp2 and eba175 markers using specific primers in a nested PCR rection. For each sample, 14µL of PCR reaction was prepared consisting of 7µL of 2x GoTaq® green master mix (Promega, USA), 3µL of nuclease free water, 0.5µL each of 10µM forward and reverse primers and 3µL of template DNA from the extract. For the nest-1 reaction of the PCR, template DNA extract plus forward and reverse primers were used (Table 1). For the nest-2 reaction of the PCR, the amplicons from nest-1 reaction were used as template DNA together with the different alleles specific forward and reverse primers for the different genes (Table 1). The entire mixture for MSP1, MSP2 and EBA175 was amplified under the following reactions conditions for both nest 1 and nest 2 (Table 2).

Table 1: Primer sequences for parasite genotyping of Plasmodium falciparum MSP1, MSP2 and EBA175.

			Primer Sequences	
MSP1 (block2)	MI-OF		5'CTA GAA GCT TTA GAA GAT GCA TTG3'	Nest 1
	MI-OMI-OR		5'CTT AAA TAG TAT TCT AAT TCA AGT GGA TCA3'	
	MI-K1(F)		5'AAA TGA AGA AGA AAT TAC TAC AAA AGG TGC3'	Nest 2
	MI-K1(R)		5'GCT TGC ATC AGC TGG AGG GCT TGC ACC AGA3'	
	MI-ROI33(F)		5'TAA AGG ATG GAG CAA ATA CTC AAG TTG TTG 3'	
	MI-ROI33(R)		5'CAT CTG AAG GAT TTG CAG CAC CTG GAG ATC3'	
MSP2 (block3)	M2-OF		5'ATGAAGGTAATTAAAACATTGTCTATTATA3'	Nest 1
	M2-OR		5'CTTTGTTACCATCGGTACATTCTT3'	
	FC27	M2-FCF	5′ ATATTAAGAGTGTAGGTGCARATGCTCCA-3′	Nest 2
		M2-FCR	5′ TTTTATTTGGTGCATTGCCAGAACTTGAAC-3′	
	IC/3D7	M2-ICF	5'AGAAGTATGGCAGAAAGTAAKCCTYCTACT-3′	
		M2-ICR	5′ GATTGTAATTCGGGGGATTCAGTTTGTTCG-3′	

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EBA175	EBA1		5'CAAGAAGCAGTTCCTGAGGAA-3'	Nest 1
	EBA2		5'TCTCAACATTCATATTAACAATTC-3'	
	EBA3		5'GAGGAAAACACTGAAATAGCACAC -3'	Nest 2
	EBA4		5'CAATTCCTCCAGACTGTTGAACAT-3'	

Table 2: The amplification conditions for nest 1 and nest 2 of MSP1, MSP2 and EBA175.

	Reaction conditions	
EBA175	Primary denaturation for 3mins at 94°C, followed by 30 cycles of second denaturation for 30sec at 94°C, annealing for 30sec at 54°C, extension of 2min at 68°C and final extension of 5min at 68°C.	Nest 1 and Nest 2
MSP1	Primary denaturation for 3mins at 94°C, followed by 29 cycles of second denaturation for 30sec at 94°C, annealing for 45sec at 50°C, extension of 2min at 68°C and final extension of 3min at 72°C.	Nest 1
	Primary denaturation for 3mins at 94°C, followed by 30 cycles of second denaturation for 30sec at 94°C, annealing for 1min at 42°C, extension of 2min at 72°C and final extension of 3min at 72°C.	Nest 2
MSP2	Primary denaturation for 3mins at 94°C, followed by 30 cycles of second denaturation for 30sec at 94°C, annealing for 1min at 42°C, extension of 2min at 65°C and final extension of 3min at 72°C.	Nest 1
	Primary denaturation for 3mins at 94°C, followed by 30 cycles of second denaturation for 30sec at 94°C, annealing for 1min at 50°C, extension of 2min at 72°C and final extension of 3min at 72°C.	Nest 2

The nest-2 PCR products were separated by electrophoresis at 100V on 1.5% molecular grade agarose gel, stained with ethidium bromide, submerged in 1× TBE (Tris–borate EDTA) buffer and visualized by UV transilluminator (BioDoc-It UVP, Cambridge, UK) [20], at 302nm on gel documentation system. The number and size of DNA fragments were estimated based on their mobility related to a 50bp DNA ladder. For the MSP1-k1, alleles of band sizes of 250, 210, 200 and 180bp were recorded, while MSP1-R033, just a single allele with a band size of 160bp was recorded. For the MSP2-FC27 allele, band sizes of 660, 640, 570, 480bp was recorded while for MSP2-3D7/IC allele, 510, 500, 470bp was recorded. For eba175, two alleles were recorded, FCR-3 and CAMP with band sizes of 795 and 120bp respectively.

Data processing and analysis

Data was coded, entered, and cleaned in Microsoft Excel spreadsheet version 16. Statistical analysis of the data was performed using Stata version.17 software [21]. Fisher exact test was used to ascertain the difference in the proportion between HIV positive and HIV negative group. The estimated marginal mean of genetic distance between the mono-infected and co-infected individuals was determined using the pearman's correlation test.

The presence or absence of reproducible bands on the gels was graded. Each band was thought of as a locus with two possible alleles. The msp1, msp2 and eba175 data were converted into a binary data matrix as discrete variables (1 = presence and 0 = absence). Analysis of diversity was performed using the combination of msp1, msp2 and eba175 data to uncover the extent of *P. falciparum* DNA polymorphisms and increase the reliability of the results. GenAIEx 6.51 software was used to determine parameters of genetic diversity: the percentage of polymorphic loci (PPL), Shannon's information index (SI), and the unbiased Nei's gene diversity index (H) [22]. Clustering analysis was implemented with MEGAEx-10, by computing the distance-tree based on the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm which uses Nei's Genetic Distance.

Multiplicity of infection

The mean number of *P. falciparum* genotypes per infected individual was used to calculate the multiplicity of infection (MOI). The MOI was determined by dividing the total number of *P. falciparum* genotypes for the same gene by the number of PCR positive isolates. Polyclonal infections were defined as isolates with more than one genotype after msp1,msp2 and eba175 amplification, whereas monoclonal infections were defined as the presence of a single allele [23].

Results

Demographic characteristics of the study population

A total of 40 *P. falciparum* malaria positive samples were analyzed for the study: 26 peripheral positive samples from mothers who were HIV-negative, 10 peripheral positive samples from mothers who were HIV-positive and 4-cord blood samples from mothers who were HIV-negative. The mean age of the HIV-positive population was 32±4.24(ranging between 26-41years) while that for the HIV-negative population was 30±24.43 (ranging between 15-38years). Greater proportion of the study population were married for both the HIV-negative (17/30:43.33%) and (9/10:90.00%) HIV-positive individuals. Majority (12/30:40.00%) of the HIV-negative population had tertiary education while most of the HIV-positive population (5/10:50.00%) had just secondary education with a disproportionate share of both study population being multigravidas. Also, most of the HIV-negative (17/30: 56.66%) and (10/10:100%) HIV-positive individuals testified of sleeping under the mosquito bed net with majority(7/10:70%) of the HIV-positive mothers on tenofovir-lamivudine-dolutegravir

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(TLD) regimen. Among the HIV-negative population, majority had received at-least 2-doses of sulfadoxine-pyrimethamine. There was a significant difference in the distribution of gravidity (p-0.019), and insecticide treated bed net use (p- 0.016) between pregnant women with and without HIV infection (Table 3).

Table 3: Demographic characteristic of the study population.

Variables	Frequency	HIV-negative (n=30) (%)	Frequency	HIV-positive (n=10) (%)	p-value	
Marital status	Single	13	43.33	1	10	0.07
	Married	17	56.66	9	90	
	Total	30	100	10	100	
Educational level	Primary	9	30	3	30	0.436
	Secondary	9	30	5	50	
	Tertiary	12	40	2	20	
	Total	30	100	10	100	
Gravidity	Primigravida	12	40	0	0	*0.019
	Multigravida	18	60	10	100	
	Total	30	100	10	100	
SP-dosage taken	1-dose	4	13.33	-	-	-
	2-doses	15	50	-	-	
	≥3 doses	11	36.66	-	-	
	Total	30	100	-	-	
Antiretroviral regimen	TLD	-	-	7	70	-
	TLE	-	-	3	30	
	Total			10	100	
Insecticide treated bed net use	yes	17	56.66	10	100	*0.016
	No	13	43.33	0	0	
	Total	30	100	10	100	

Distribution of the total haplotypes' frequencies of Plasmodium falciparum in all the different genes screened in the study population

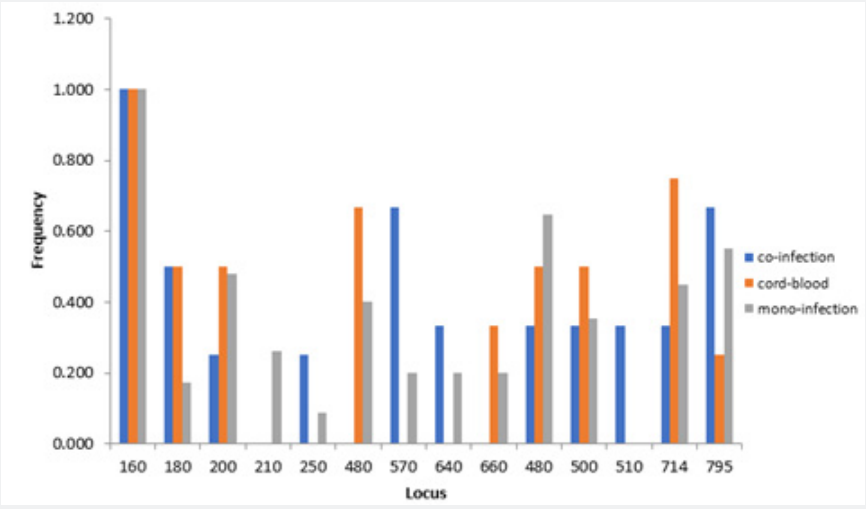

Figure 2: Distribution of the total haplotype frequencies of Plasmodium falciparum in all the different genes screened in the study population.

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A total of 14 genotypes (strains) of Plasmodium falciparum parasites were associated with malaria infection among our study population in Kumba. with a couple of these genotypes found just among the mono and co-infected population such as 640, 570, 250 and 210bp fragments, the 510bp was found just among the co-infected individuals. However, 480 and 660bp fragments were found only among the mono-infected and cord-blood infected individuals. In general, greater proportion of the strains were common across the cord, mono and co-infected individual with majority of the infections made up of Plasmodium falciparum 160bp genotype (Figure 2).

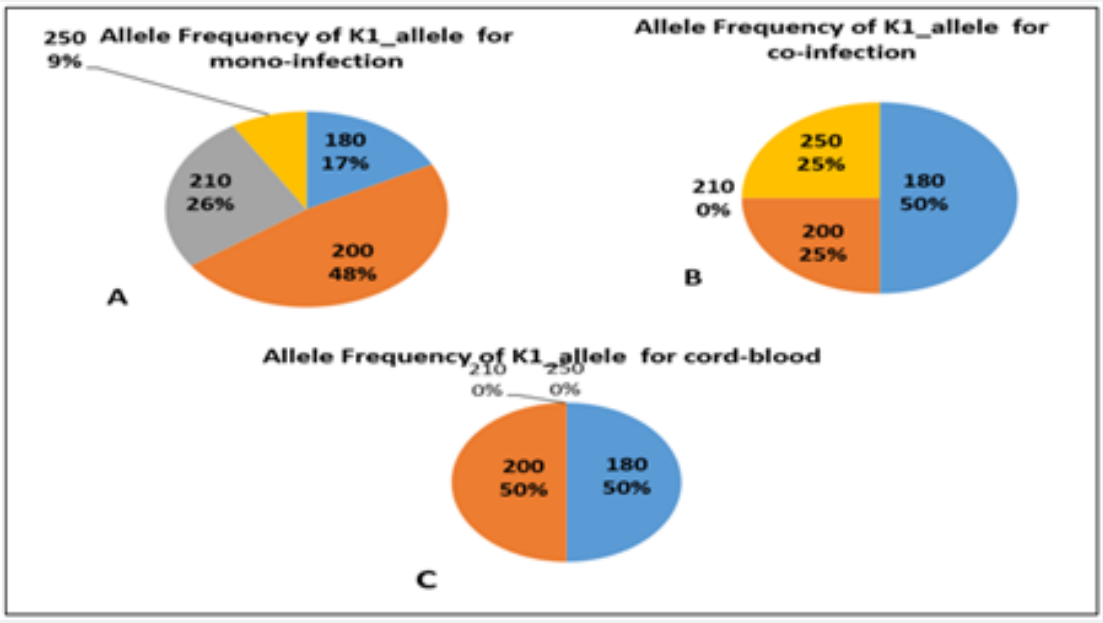

Figure 3: Frequency distribution of the K1 haploid alleles for malaria mono-infected, co-infected and cord blood infection among pregnant women in Kumba.

Frequency distribution of MSP1 alleles of P. falciparum parasite responsible for malaria infection among mono infected, co-infected and cord blood infection in the study population

The number of genotypes can be seen in Figure 3. In general, 5 genotypes were recorded for MSP1 with K1 family recording 4 sub-genotypes (250, 210, 200 and 180) and R033 family recording just one genotype (160). All the four genotypes of K1 were recorded among the mono-infected individuals with majority (48%) of the population infected with the 200bp genotypes of K1. Co-infected individuals recorded three out of the four genotypes with 50% of the population infected with the 180bp genotype. At the level of cord blood just two fragments out of the four fragments identified were recorded with equal proportion of the population infected with the 200 and 180bp genotypes. The RO33 family was found to be monomorphic with an amplified fragment of 160bp recorded among 92.3% (24/26) of the mono-infected samples, 90.0% (9/10) co-infected samples and 75.0% (3/4) cord blood samples used for the analysis.

Frequency distribution of MSP2 alleles of P. falciparum responsible for malaria infection among pregnant women with and without HIV infection in Kumba

The study evaluated the 3D7/IC and FC27 family of MSP2. The 3D7/IC family recorded 3 genotypes as can be seen in Figure 4 with band sizes ranging from 510-480bp. A disproportionate share of the mono-infected samples (65%) was infected with the 480bp genotype. Co-infected samples recorded all the three genotypes with just a slight majority (34%) infected with the 480bp genotype. The cord blood infected samples recorded two out of the three fragments amplified (500 and 480bp) with both populations having equal distribution of the two genotypes. However, among the FC27 family, 4 genotypes were recorded as can be seen in Figure 5. All the 4 genotypes were recorded in the mono-infected samples with majority of the population (40%) infected with the 480bp genotype. Among the Co-infected individuals, just two out of the four genotypes with a disproportionate share (67%) infected with the 570bp genotype, while the cord blood infected samples recorded two of the four genotypes with a larger proportion of the samples (67%) infected with the 480bp genotype.

Frequency distribution of EBA175 alleles P. falciparum responsible for malaria infection among pregnant women with and without HIV infection in Kumba.

Figure 6 shows the genotypes recorded among EBA175 group. Two genotypes were recorded with band sizes ranging from 795-714bp. As shown by previous studies, two families make up the

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EBA175 group. The FCR-3 and the CAMP family. However, in our study just FCR-3 with a band size of 795bp was recorded while CAMP recorded a band size of 714bp. Among the mono-infected samples, a slight majority (55%) were infected with the F795bp genotype while among the co-infected samples, a disproportionate share (67%) was infected with the F795bp genotype. However, among the cord blood infected samples, greater proportion (75%) of the samples were infected with C714 genotype.

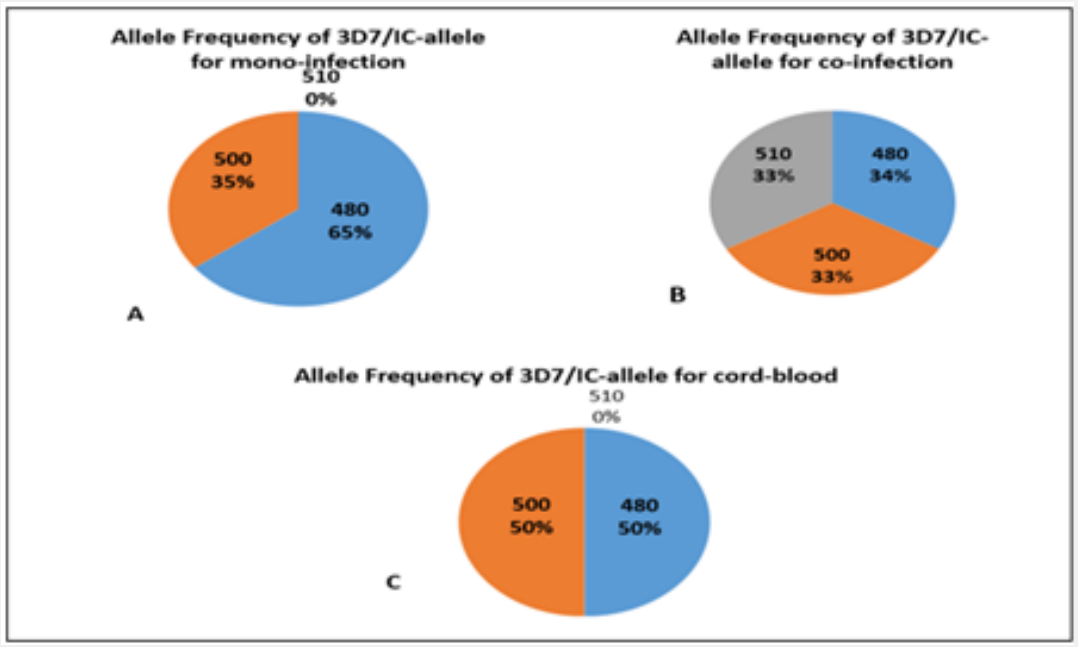

Figure 4: Frequency distribution of 3D7 haploid alleles among malaria Mono-infected, co-infected and cord blood infection among pregnant women in Kumba.

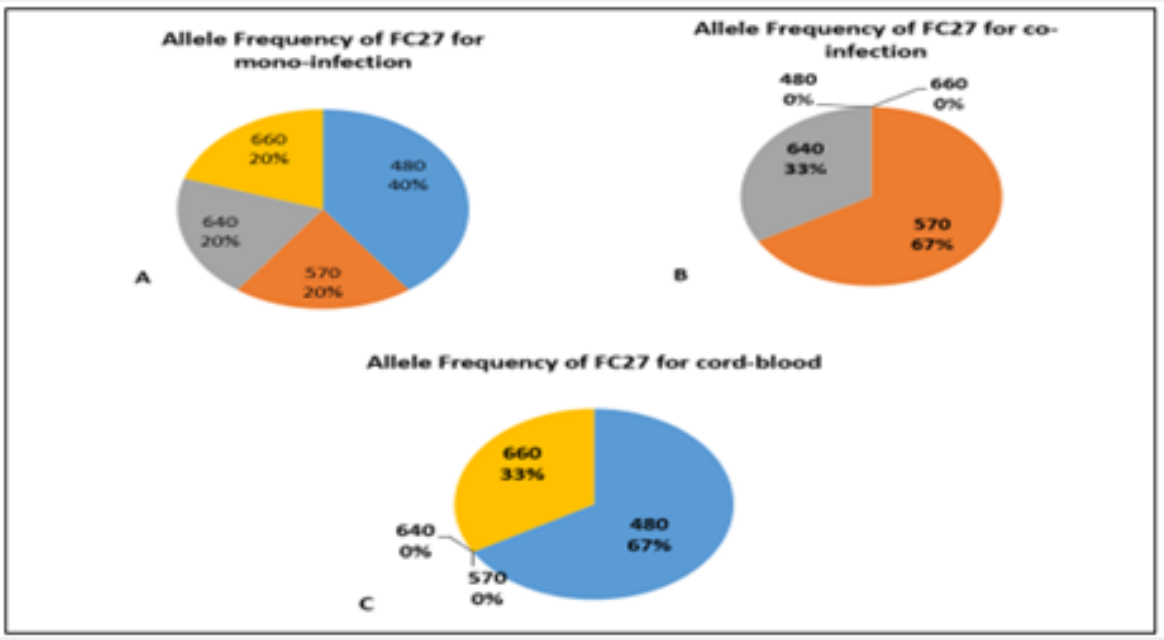

Figure 5: Frequency distribution of FC27 haploid allele among malaria Mono-infected, co-infected and cord blood infection among pregnant women in Kumba.

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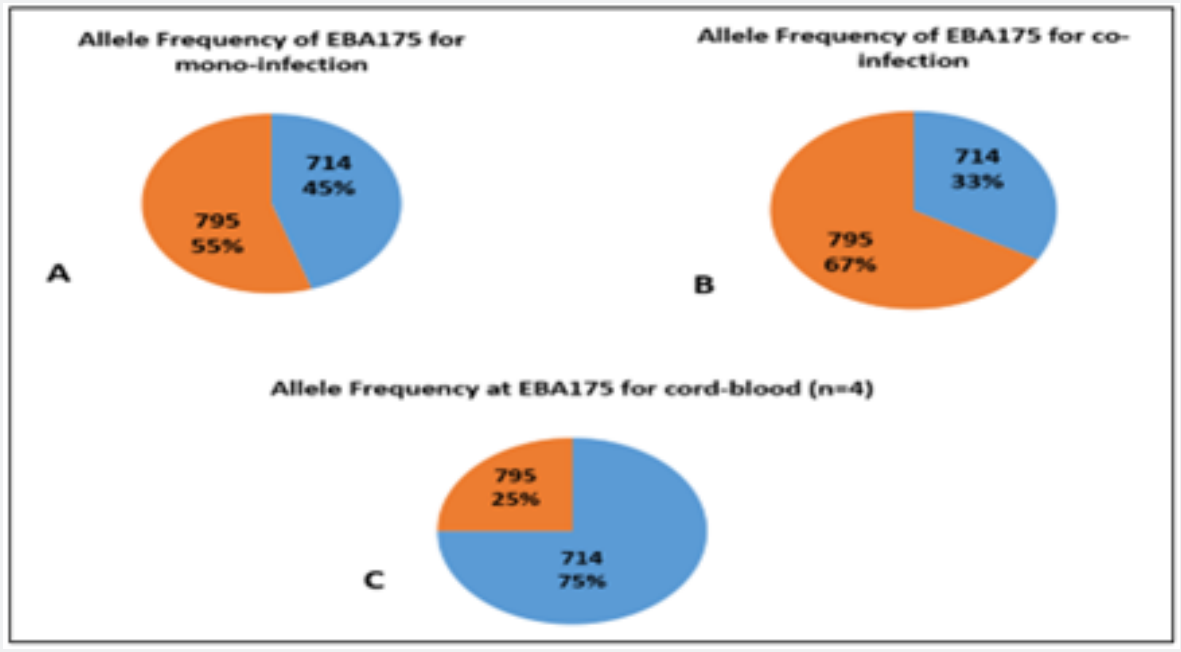

Figure 6: Frequency distribution of EBA175 haploid alleles among malaria Mono-infected and HIV and malaria co-infected pregnant women in Kumba.

Cluster analysis of MSP1 P. falciparum parasite genetic diversity among malaria Mono-infected and HIV and malaria co-infected pregnant women

Figure 7 shows an intermixed clone of *P. falciparum* K1-MSP1 originating from a common ancestry. From the diagram we can see that there was no difference in the strains responsible for malaria infection among the mono-infected, cord and co-infected samples. The same strains of Plasmodium falciparum are responsible for malaria infection in the mono-infected, co-infected and cord blood infected samples as we can see that same strains are distributed across all the two sub-clusters originating from a common ancestry. However, the co-infected sample M20 stands out separately from the rest of the different clusters. This may be a new strain but we can conclude because of the small samples size.

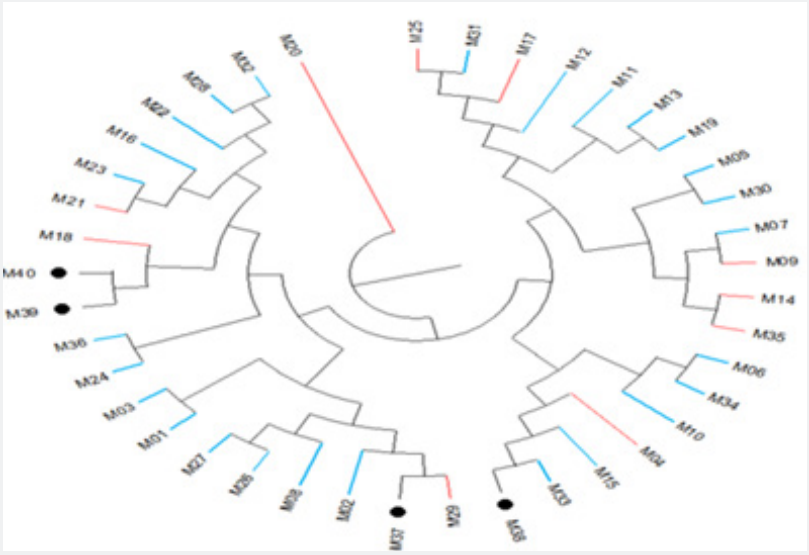

Figure 7: Distance tree of k1 allele of MSP1 gene showing similar clones of Plasmodium falciparum responsible for malaria infection among pregnant women in Kumba.

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Cluster analysis of MSP2 *P. falciparum* parasite genetic diversity among Malaria Mono-infected and HIV and malaria co-infected pregnant women

Figure 8 shows an intermixed of *P. falciparum* MSP2_3D7 clones responsible for malaria infection among the study population. However, there seems to be a slight difference in the clones responsible for vertical infection present in the cord blood. Even though all the cord blood samples are on a single cluster, C38 is distant away from the rest of the C39, C40 and C37 clones indicates that it might be different from the other clones. However, among the mono-infected and co-infected individuals, there is no difference in the strains of *Plasmodium falciparum* responsible for malaria infection in the two groups as we can see same strain spread across the two main clusters. Nevertheless, among the MSP2_FC27 clones, there may also be a slight difference in the clones responsible for vertical transmission of malaria. The close distance between the clones indicates that they are similar strains responsible for vertical transmission (Figure 9). These clones may be different from the clones responsible for malaria infection among the mono-infected and co-infected samples. However, among the mono-infected and co-infected samples, there is no difference in the clones responsible for malaria infection as both can be found distributed all across the different clusters.

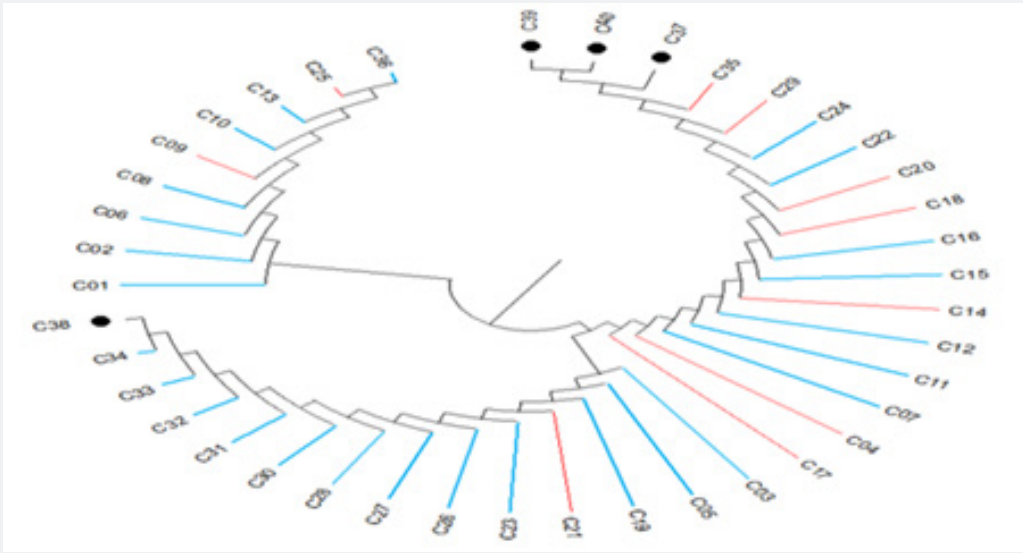

Figure 8: Distance tree of 3D7 allele of MSP2 gene depicting intermixed clones of P. falciparum responsible for malaria infection in the different study population.

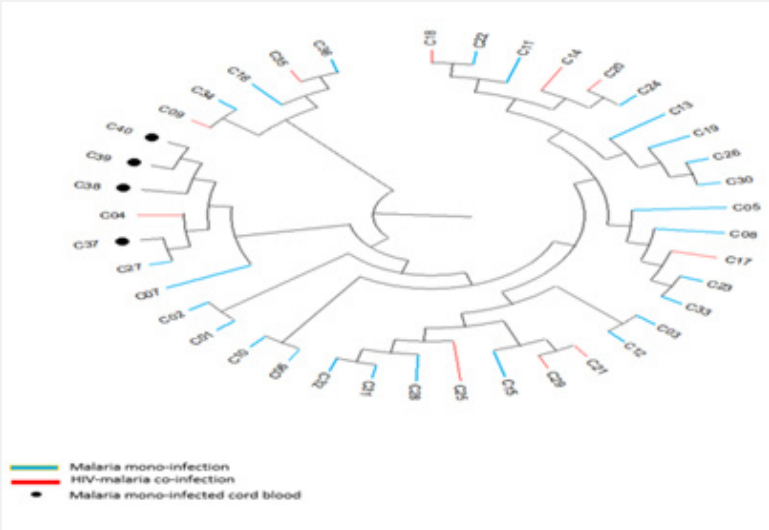

Figure 9: Distance tree of FC27 allele of MSP2 gene showing intermixed clones of P. falciparum parasite responsible for malaria in the three different study population.

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Cluster analysis of EBA175 P. falciparum parasite genetic diversity among malaria Cord blood infected, malaria co-infected and mono-infected pregnant women

Figure 10 shows the genetic distance of EBA175 clones of *P. falciparum* responsible for malaria infection in the population across the three different samples. As we can see, there are two main sub-clusters which originates from a common ancestry. There is no difference in the clone responsible for malaria among the mono-infected and co-infected individuals as same clones can be seen spread out across the two main cluster. However, among the cord blood infected samples, C40, C39 and C38 seems to be similar clones as they all are located on a single cluster with C38 distant away from C 40 and C39 indicating a slight difference. C37 seems to be a different clone as it can be seen located on a different cluster from the rest. Double infection was the most dominant for msp1 infections amongst the mono-infected 76.92% (20/26) and co-infected 50% (5/10) individuals. At the level of the cord blood, 75% (3/4) of the individuals was infected with the R033 group (Table 4). However, for the msp2, greater proportion 53.8% (14/26) of infection among the mono-infected individuals was from the 3D7/IC group. More of FC27 30% (3/10) was responsible for malaria among the co-infected individuals and equal proportion of FC27 and double infections were found among the cord blood infections. The CAMP strain of eba175 was responsible for most of the infection 34.61% (9/26%) among the mono-infected and cord blood infected 75% (3/4) individuals. Double infection of CAMP + FCR3 was responsible 40% (4/10) of the infection among the co-infected individuals. The average MOI for the mono-infected samples was 0.22, co-infected was 0.791 and cord blood was 1.80.

Figure 10: Distance tree of EBA175 gene showing similar clones of P. falciparum parasite responsible for malaria infection in the study population.

Table 4: Genetic diversity of MSPI, MSP2 and EBA175 polymorphic regions at the different block fragments

Genes	Study population	Alleles	Allele sizes (bp)	No of samples positive (%)	MOI
MSP1	Mono infection	K1	180-250	2(7.69)	0.192
		R033	160	4(15.38)	
		K1+R033		20(76.92)	
		Total		26	
	Co- infection	K1	180-250	1(10.00)	0.5
		R033	160	4(40.00)	
		K1+R033		5(50.00)	
		Total		10	
	Cord infection	K1	180-250	0(0.00)	1.25
		R033	160	1(25.00)	
		K1+R033		3(75.00)	
		Total		4	

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MSP2	Mono infection	FC27	480-660	1(5.55)	0.388
		3D7/IC	480-510	14(77.77)	
		FC27+3D7		3(16.66)	
		Total		18	
	Co- infection	FC27	480-660	3(42.85)	1
		3D7/IC	480-510	2(28.57)	
		FC27+3D7		2(28.57)	
		Total		7	
	Cord infection	FC27	480-660	1(50.00)	3.5
		3D7/IC	480-510	0(0.00)	
		FC27+3D7		1(50.00)	
		Total		2	
EBA175	Mono infection	FCR3	795	6(30.00)	0.1
		CAMP	714	9(45.00)	
		FCR3+CAMP		5(25.00)	
		Total		20	
	Co- infection	FCR-3	795	2(20.00)	0.875
		CAMP	714	2(20.00)	
		FCR3+CAMP		4(50.00)	
		Total		8	
	Cord infection	FCR-3	795	0(0.00)	0.666
		CAMP	714	3(100.0)	
		FCR3+CAMP		0(0.00)	
		Total		3	

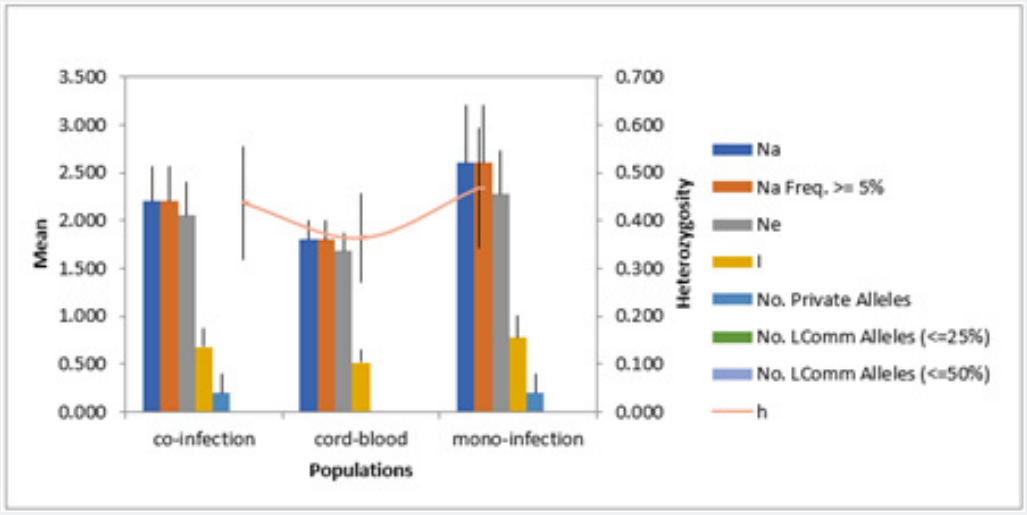

Figure 11: Allelic pattern of Plasmodium falciparum across the entire study population.

Distribution of Allelic pattern of Plasmodium falciparum across the entire study population

Figure 11 shows the allelic distribution pattern of *P. falciparum* across the three different groups of samples positive with *P. falciparum* malaria infection. The strains of *P. falciparum* parasite detected among the mono-infected and co-infected individuals are the same except for the strains responsible for cord blood infection, where there is a drop-in the strains responsible for infection.

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Genetic diversity of Plasmodium falciparum population by each antigenic marker in the different group of the study population.

Table 5 shows the level of diversity of *P. falciparum* among the mono-infected, co-infected and cord blood infected samples. As can be seen on the table, the diversity of *P. falciparum* parasite responsible for malaria infection in our study population, was more among the cord blood infected individuals, followed by the co-infected individuals and lastly the mono-infected individuals as can be seen especially from the Shannon information index which ranges between 0 to 1. The lesser the value indicate more diversity.

Table 5: Genetic diversity of Plasmodium falciparum population by each antigenic marker.

	Mono-infection	Co-infection	Cord blood infection
Shannon's information index (I)	0.778	0.682	0.517
Diversity (h)	0.467	0.436	0.364
Unbiased diversity (uh)	0.52	0.607	0.633

Relationship between the HIV status and genetic diversity of Plasmodium falciparum in the study population

Figure 12 evaluates the relationship between HIV status and the occurrence of *P. falciparum* genetic diversity among the study population. It shows that *P. falciparum* parasites among the HIV positive individuals is more diverse than among those who are mono-infected with just malaria alone even though it shows a weak correlation (r=0.248) which may be due to the sample size.

Figure 12: A correlation curve showing the relationship between HIV status and the genetic diversity of P. falciparum.

Discussion

The genotyping of malaria parasite populations is still an important approach for determining the types and amount of parasite clones in an infection [15]. Cameroon has three main malaria transmission zones: soudano-sahelian, humid savannah, and forest zone [17]. However, among these zones are sub-zones with unique malaria transmission dynamics. Therefore, it is so critical to define parasite populations in order to tailor malaria control and extermination measures. Therefore, the study investigated the influence of HIV on *P. falciparum* parasite genetic diversity in pregnant women and the first conducted in this locality among this target group. Out of the three different families amplified for msp1 (K1. R033 and MAD20), the MAD20 was eliminated because it produced very poor amplification bands. However, all the different sub-alleles of msp2 and eba175 produced clear and easy to interpret bands. Allelic genotyping reveals that *P. falciparum* responsible for malaria infection in Kumba is polymorphic in terms of msp1, msp2 and eba175. This provided a better understanding on the transmission dynamics of *P. falciparum* in this locality and an insight into the pathogenesis of malaria, the process of building antimalarial immunity and the

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mechanisms of resistance to therapy. Akindeh and collaborators reported that the number of coinfections inside a host could be a significant predictor of transmission intensity [10].

In this study, we analyzed samples from mono-infected, co-infected and cord blood infected individuals that were positive for *P. falciparum* malaria molecularly. Majority of the samples were included msp1, msp2 and eba175 *P. falciparum* infected (mixed/polyclonal). This finding is line with the report of Tewara et al., who reported that South west in general of which Kumba is part of, is among the malaria hotspot areas in Cameroon [24]. Our study population contains a total of 14 genotypes of *P. falciparum* responsible for malaria infection, with a slightly higher level of parasite genotype at the level of the co-infected individuals compared to the mono-infected individuals, with an average MOI of 0.79 and 0.22 respectively. msp1 recorded 4 genotypes, msp2 recorded 5 while eba175 recorded 2. The MOI of the co-infected individuals even though lower than that of the cord blood infected individuals, was higher than the mono-infected individuals confirming that HIV infection has an influence on *P. falciparum* diversity. The cord blood infected individuals recorded an average MOI of 1.80 which was much higher compared to the rest. This study showed a lower level of MOI in Kumba compared to the findings of other studies done in Yaoundé and Limbe which happens to be in the same malaria transmission zone in Cameroon [10,24,25]. The small number of individuals used in this aspect of the study may have contributed to the results observed in this study.

The genotyping success rate of msp1 alleles which were successfully amplified (K1 and R033), msp2 and eba175 for this study among the mono-infected individuals was 100%(26/26), 69.2%(18/26) and 76.9%(20/26) respectively. Among the co-infected individuals, it was 100% (10/10), 70% (7/10) and 80% (8/10) while among the cord blood infected individuals it was 100% (4/4), 50% (2/4) and 75% (3/4). Metoh et al., reported that *P. falciparum* field isolates from Cameroon showed a significant level of genetic variation in *P. falciparum* msp-1 and msp-2 markers [11]. These genes, which encode for specific functional proteins produced on the surface of the merozoite, appear to be critical for red blood cell invasion and are being studied as possible vaccination candidates as well as pharmacological targets for suppressing blood-stage replication [11]. Our study observed that more amplified products of msp2 were found compared to the msp1. This is in line with previous studies [26]. This could be explained by non-synonymous substitutions introduced in template DNA that could jeopardize the proper annealing of the primer at its binding site in the msp1 gene or by the fact that natural selection is more efficient when acting on msp-1 than msp-2 [27,28].

More alleles of msp1 were detected among the mono infected individuals compared to the co-infected individuals. While for the msp2, more alleles of the 3D7 family were detected among the co-infected individuals. the mono-infected individuals recorded more alleles of the FC27 family compared to their counterparts. However, a disproportionate share of the population was infected with the eba175_FCR-3 fragment among the co-infected individuals. This variation is consistent with the findings of other studies [29-31]. The genetic diversity of *P. falciparum* populations and the complexity of infection have been found to vary depending on the strength of transmission in different geographical areas and immune competence of individuals, with size polymorphism revealed by the size of the PCR generated fragment [32].

The distant-trees obtained with msp1_k1 and eba175 suggest clonal parasite population for mono-infected, co-infected and cord blood infected individuals with similar genetic background as they intermix across trees. Msp2_3D7 and msp2_FC27 trees shows an intermix of *P. falciparum* population between the mono-infected and the co-infected individuals but a homogeneous clonal population individualized and separated from the rest of intermixed parasites in other sub-trees, suggesting a different genetic background. These may have consequences in the detectability and diagnosis of new clones which may come with higher virulence. However, we cannot come to a proper conclusion since the sample population used was very small but these observations warrant in-depth investigation into how the different placenta barriers influence malaria diversity and the interest of targeting populations with different genetic background for diagnostic purpose.

These discrepancies were expected since dominant markers cannot distinguish between homozygotes and heterozygotes [33]. Despite these disadvantages, dominant markers are distinguished by their capacity to assess unbiased genetic variation and lack of a sequence requirement, making them appropriate for studies of species with little or no genetic information [33]. In natural populations of *P. falciparum* that reproduce sexually and asexually, have varied ecological niches, and/or cover a vast geographical range, high levels of genetic variability are often found [34]. Our findings recorded genetic polymorphism in both mono-infected, co-infected and cord blood infected samples with an increased in unbiased diversity among the co-infected and cord blood infected individuals. correlation analysis shows association between parasite genetic diversity and the HIV status of the study population even though it was weak. It was observed that diversity of *P. falciparum* parasite increases among HIV infected individuals. The results of the cluster analysis confirmed the assumption that the genetic ties between groups differed to some extent. Several studies have shown that drug pressure is one of the contributing factors to strain variations [35]. In Cameroon were recently there is a shift from TLE to TLD among HIV pregnant women together with the administration of cotrimoxazole as prophylaxis against malaria [5,36], the possibility of the existence of multiple strains of *P. falciparum* is common among HIV positive pregnant women. Our study shows the possibility but with a weak correlation which

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may be due to the smaller number of populations used. However, this information will be very helpful for future studies aiming to look at the diversity of this parasite in this area.

Strength and Limitations of the study

This is the first study looking at the diversity of Plasmodium falciparum among pregnant women by looking detailly at the different alleles of *P. falciparum* responsible for malaria in this locality with over 14 sub-strains of *P. falciparum* recognized to be responsible for malaria infection. One limitation of the study was that just one technique was used to analyze *P. falciparum* genetic diversity, the vast majority of surface markers were not included, and the sample size was modest. Because of these limits, the study's findings are not ideal for understanding the association between HIV status and genetic diversity. As a result, the study's conclusions should be regarded with caution.

Conclusion

HIV infection may have an effect on Plasmodium falciparum malaria parasite genetic diversity responsible for malaria infection among pregnant women in the Kumba Health District area with over 14 different genotypes of msp1, msp2 and eba175 responsible for *P. falciparum* malaria in this area. However, the diversity of *P. falciparum* malaria parasite observed was low in this area with msp2_3D7 and eba175_FCR-3 families being dominant among the co-infected individuals. However, MSP2 shows to be a better marker for diversity in this area compared to the MSP1 and EBA175.

Acknowledgement

Pan African University of Life and Earth Sciences Institute (Including Health and Agriculture),

University of Ibadan, Nigeria. Union for African Population Studies, University of Buea. The entire staff of the Regional Hospital Annex Kumba, District hospital Kumba town, Catholic Hospital Fiango Kumba, Presbyterian Hospital Buea Read Kumba, and Kossala Integrated Health Center Kumba. The entire field work team that assisted with the sample collection and slide examination. My profound gratitude to all the co-authors of this manuscript.

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DOI: 10.19080/JPCR.2023.09.555779

How to cite this article: Bekindaka N O, Abdel Aziz S-N, Awanakam Honore A, Esemu Livo F, Tanue Elvis A, et al. Diversity of Plasmodium falciparum Parasite Responsible for Malaria Infection detected at the Level of Peripheral and Cord Blood Samples from Pregnant Women with and without HIV Infection in the Kumba District Health Area, South West Region of Cameroon. J of Pharmacol & Clin Res. 2024; 10(1): 555779. DOI: 10.19080/JPCR.2023.09.555779