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Malaria and Typhoid Fever Co-infection Amongst Febrile Patients in Yaoundé, Cameroon: Implication in the Genetic Diversity of *Plasmodium falciparum*

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Abstract: Purpose: Malaria and typhoid are among the frequently reported infections in Cameroon and are becoming a major public health concern. The genetic profile of *Plasmodium falciparum* and its involvement in disease severity has been reported in recent studies. In order to better understand the mechanism behind the pathology of a parasitic disease, it's necessary to study and follow the genetic diversity within parasite population. Equally, to develop effective malaria control strategies and evaluate existing ones, it is important to determine the type of infection within a population. However, the genetic diversity of the parasite circulating in diagnosed cases of typho-malaria fever is yet unclear. We assessed the nature and extent of *Plasmodium falciparum* allelic diversity in the parasite population circulating in patients diagnosed with typho-malaria fever in two health facilities in Yaoundé, Cameroon. Methodology: In this cross-sectional study, thick/thin blood films from 178 febrile patients were examined using Microscopy for malaria diagnosis and acute sera were analysed using Widal agglutination test for typhoid fever diagnosis. The *msp2* gene of *Plasmodium falciparum* was amplified using nested PCR and descriptive statistics was used to determine and compare the parasite population genetic diversity, allelic frequencies, and multiplicity of infection. Findings: Of the 178 febrile patients, 28.65% (51/178), 16.29% (29/178) and 13.48% (24/178) were positive for malaria, typhoid, and malaria/typhoid respectively. *P. falciparum* and *S. typhi* were the major causes of fever, with both pathogens more likely to co-exist. The geometric mean parasitaemia in typho-malaria group of patients was 33700 versus 7305.11 in patients infected only with malaria parasite (*p value of* <0.05) A total of 145 and 127 DNA fragments were obtained in diagnosed cases of malaria mono- and co-infections respectively, giving rise to 11 different allele subtypes. In patients with typho-malaria infection, a total of 6 different *msp*2 alleles were recorded with allele 621 base pairs (25.98%) as a major subtype. A genetic diversity of 22.22% was observed in co-infected patients with multiplicity of infection (MOI) of 3.4. Conclusion: The reported high MOI and diversity of strains of *P. falciparum* in typho-malaria patients is a call for concern to malaria control stakeholders in Cameroon. The overall high genetic diversity of the parasite suggests that malaria transmission within the study population is still high. This study calls for an intensification of the malaria control strategies in Cameroon.

Keywords: Malaria, Typhoid Fever, Co-infection, Genetic Diversity, Multiplicity of Infection

1. Introduction

Malaria and typhoid fever have different transmission mechanisms and are caused by different pathogens, notably a parasite (*Plasmodium spp.*) and a bacterium (*Salmonella typhi and Salmonella paratyphi)* respectively. However, both diseases are among the most difficult tropical infectious diseases to deal with. Both diseases have been classified as disease of poverty and they affect mainly individuals living in sub-Saharan Africa [1]. In Cameroon, malaria and typhoid are among common infections frequently reported in health facilities and are becoming a major call for public health concern. These diseases present some common signs and symptoms with other viral and parasitic infections [2]. This homology in clinical feature of the two diseases pushes individuals who practice symptom-base treatment without prior diagnosis to treat one disease for the other. In malaria endemic areas with frequent reports of typhoid / enteric fevers, the lack of affordable accurate diagnostic tools for typhoid like real time PCR test [3, 4], nudges healthcare providers to regularly prescribe antimalarial treatment and antibiotics simultaneously. Such attitudes have further thwarted the significant effort to eradicate these infectious diseases endemic countries like Cameroon. Also, a complete understanding of the physiopathology of these diseases is lacking.

Malaria-related deaths have been associated largely with the parasite's ability to cause complications [5] including but not limited to anaemia [6], Malaria-associated acute respiratory distress syndrome (MA-ARDS) [7], Malaria-associated acute lung injury (MALI) [8], Splenomegaly [9-11], Malaria-associated acute kidney injury [12-14], just to name a few. This notwithstanding, many factors that can directly or indirectly contribute to malaria severity are still not clearly understood. Factors of disease severity that have been researched include: genetic profile of parasite population [1], epidemiology [15], health-seeking behavior and non-malaria co-morbidity [16]. It has been suggested that genetic diversity could increases the risk of being exposed to different genotypes of the parasite and this could eventually contribute in delaying development of immunity [17] which is crucial for survival from malaria-related deaths.

Knowledge of the genetic diversity of *P. falciparum* populations is essential in distinguishing recrudescence from re-infection of the parasite and also determines the level of malaria transmission within a population [18]. This would provide a baseline data for any drug efficacy trials. Several studies have used merozoite surface proteins (*msp*) genotyping to determine the diversity and multiplicity of malaria infection and to eventually monitor intervention strategies [19]. Multiplicity of infection (MOI), defined as the number of genetically different strains of parasite that can cohabit a single host at moment of infection, is an important tool for monitoring malaria epidemiology. The polymorphic central domain of the gene encoding *msp*-2 can be grouped into two distinct families; 3D7 and Fc27 [19]. Allelic forms of this antigen gene have been reported in Cameroon [18, 20]. Genotyping this gene has been previously used to trace clones of parasites co-existing within a population in given period of time and to measure duration of infection [21, 22].

Genetic diversity and multiplicity of infection in Cameroon has been studied but there are very few data on this particular area of research, especially when genetic markers need to be considered in genotyping and epidemiological studies. Equally, the evidence of genetic diversity of the parasite circulating in diagnosed typho-malaria fever cases has not been reported. This study seeks to assess the nature and extent of genetic diversity of *Plasmodium falciparum* isolates in patients diagnosed with malaria and typhoid co-infection in two health facilities in Yaoundé, Cameroon.

2. Research Methods

2.1. Ethical Consideration

All patients referred to or attending the laboratory for medical diagnostic named "*Le Laboratoire Béthanie, Melen*" and the medical center named "*Centre de Soins d'Acacias*" in Yaoundé were assessed for eligibility and recruited in the study. This study was conducted as part of a community study aimed at evaluating the implementation strategy of ATCs in Cameroon. Ethics approval was obtained from the National Ethics Committee of the Cameroon's Ministry of Public Health (N°113/CNE/SE/2011). Approvals to collect blood samples and clinical data were obtained from the Directorates of both institutions. Signed informed consent or assent was provided by the participants or legal guardian.

2.2. Study Design

Participants for this cross-sectional study were assessed for eligibility and recruited from September, 2013 to February, 2014. The venipuncture technique was used for blood collection under aseptic conditions from all recruited participants. EDTA blood was used for preparation of blood films while plain tube blood was allowed to coagulate and then centrifuge at 45000 rpm for 5 minutes to generate serum for typhoid diagnosis.

The stained blood film was then examined microscopically for the detection of malaria parasites. Parasite density was considered as the average number of parasites per 200 white blood cells or per 2,000 red blood cells and converted to parasites/µl of blood using the patient's white blood cell count or hematocrit, respectively. Blood samples were spotted on filter paper (WHATMAN), air dried, and later used for molecular analysis at the Laboratory for Public Health Research Biotechnology, University of Yaoundé I.

Widal test was performed on acute serum using the Felix Widal agglutination kit (BIOLABO SA, France) containing coated somatic (O) and flagella (H) antigens for *S. typhi* and *S. paratyphi A, B* and *C*. The serum was then tested at a dilution of 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, in 0.9% normal saline and the results read immediately after. All positive results obtained through a slide test were confirmed

and quantified by the tube agglutination method. A positive Widal test was considered as one that gave a reaction titre greater or equal to 1/200 for *Salmonella* somatic and flagella antibodies [23]. However, the last test showing signs of agglutination was taken as the titre for that patient.

Parasite DNA was extracted from blood spots on filter paper using the Chelex® (Bio-Rad Laboratories, SIGMA) method as described elsewhere [24]. PCR amplification of the *Pfmsp-2* gene was done as described elsewhere [17] with slight modification. Amplification was performed using nested PCR in two turns. Each PCR cycle was performed in a total volume of 25 µl containing the following volumes:

Table 1. *Primer sequences and reaction conditions for the amplification of msp2 gene.*

PCR	Primers	Sequence		Conditions
msp2 outer	Forward (S3)	5'GAAGGTAATTAAAACATTGTC3'	Pre/Denaturing Annealing Elongation Final	95°C -3mins; 94°C -30sec, 42°C -60sec, 65°C -2minsx30cycles; 72°C -3mins, 4°C -hold
	Reverse (S2)	5'GAGGGATGTTGCTGCTCCACAG3'		
mp2 Inner	Forward (S1)	5'GAGTATAAGGAGAAGTATG3'	Pre/Denaturing Annealing Elongation Final	94°C -30sec; 50°C -60sec, 72°C -2minsx30cycles; 72°C -3mins, 4°C -hold
	Reverse (S4)	5' CTAGAACCATGCATATGTCC3'		

2.3. Data and Statistical Analysis

Data generated was entered into Microsoft Office Excel 2010 and the data was exported and analyzed using SPSS statistical software, version 16.0. In this study, a p-value < 0.05 was considered significant at 95 % CI. To determine the prevalence of Malaria and typhoid fever in the study population an Exploratory Data Analysis (EDA) was performed. The Chi-square test was used for intergroup differences between categorical variables.

The allelic frequency which can be defined as the probability of having an allele (dominant or recessive) in a population was calculated by dividing the number of times the allele is observed by the total number of DNA fragments. The genetic diversity was obtained by dividing the total number of alleles by the total number of DNA fragments. Multiplicity of infection which is the average number of alleles per individual was calculated by dividing the total number of DNA fragments by the total number of samples [17]. The Chi-square test was also used to compare the allelic families of *msp2* gene in cases of malaria single infection and malaria-typhoid co-infection.

3. Results and Discussion

3.1. Results

3.1.1. Study Profile

A total of 178 febrile patients suspected for malaria and/or typhoid fever were included in this study. Among them, 41.57% of the study participants were males. The mean age was 23.4 ± 17.716 years and majority of the patients (41.5%) were within the age range of 12–30 years (Table 2). A mean temperature of 39.2°C was recorded among the study participants.

3.1.2. Prevalence of Malaria, Typhoid Fever and Typho-Malaria Within the Study Population

Malaria was the most prevalent disease in the study area. From the total 178 febrile patients 51 (28.65%) were positive for malaria only while 24 (13.48%) were positive for malaria and typhoid fever. Of the total study subjects, 29 (16.29%) patients were positive for typhoid fever while 74 (41.57%) were negative for both diseases (Table 2). Parasitic densities ranged from 560 to 77780 per µl of blood in patients with malaria infection only, with a mean of 7305.11, and standard deviation of 17851.77. Whereas, in co-infected patients, a geometric mean parasitaemia of 33700 per µl of blood was recorded in a range of 1950-187907. (Table 3)

3.1.3. Distribution of P. falciparum msp2 Alleles in the Study Population

The result for *Pfmsp2* gene amplification showed variation in the number and size of the bands from one sample to another. Most patients had two or more bands (Figure 1) with band size varying between 303 and 730 bp. A total of 145 and 127 DNA fragments were obtained in diagnosed cases of malaria mono- and co-infections respectively, giving rise to 11 different allele subtypes. The most predominant alleles were observed at 584bp (mono infection) and 621bp (co-infection) the least prevalent being allele 730 (mono-infection) and allele 303 (co-infection) subtype (Table 4).

Table 2. *Study profile and prevalence of malaria and typhoid fever in the study population.*

Sex	Age		Mean temperature	Malaria + (%)	Typhoid + (%)	Malaria + Typhoid (%)	Unknown etiology (%)
	Mean	SD					
Female *(N = 104)*	21.62	16.751	38.8	32 (17.97)	13 (7.30)	14 (7.87)	45 (25.28)
Male *(N = 74)*	37.22	19.221	39.7	19 (10.67)	16 (8.99)	10 (5.62)	29 (16.29)
(N = 178)	23.40	17.716	39.2	51 (28.65)	29 (16.29)	24 (13.48)	74 (41.57)

Table 3. *Parasite densities per µl of blood recorded in the study population.*

	Malaria positive (N = 75)	Range		Mean		Standard Deviation
		Minimum	Maximum	Statistical	Std. Error	
Malaria only	51	560	77780	7305.11	4207.703	17851.77
Typho-malaria	24	1950	187907	3.37 x10^4	1.85 x10^4	58434.01

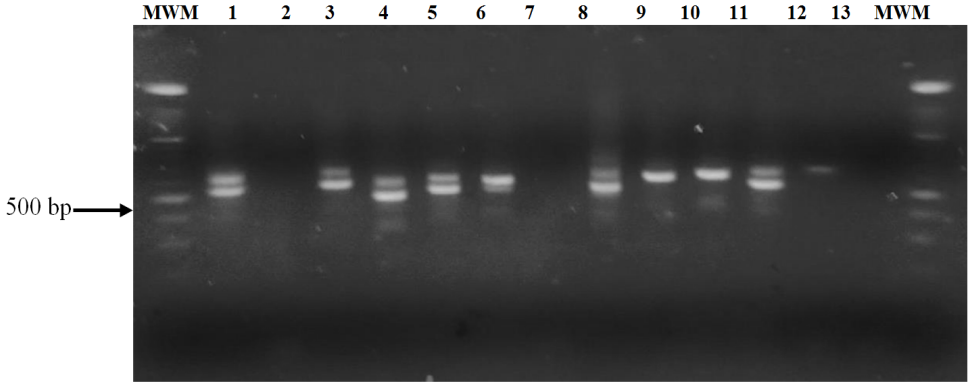

Figure 1. *Agarose gel electrophoresis analysis showing genetic diversity detected in the Plasmodium falciparum msp2 region. Most of the Pfmsp2 – positive samples had more than one band (indicating multiple parasite clones), with band sizes varying between 303 and 730 bp. MWM represent molecular weight marker, lane 1 = positive control; lane 13 = negative control; lanes 2 & 7 = Pfmsp2 – negative samples; lane 4, 8 and 11 = typho-malaria positive samples.*

Table 4. *Allelic frequency of P. falciparum.*

Allele type	*Pfmsp2*	
	Malaria + (%)	Typho-malaria
A303	-	05 (03.94%)
A362	10 (06.90%)	-
A412	37 (25.51%)	28 (22.04%)
A484	-	24 (19.90%)
A508	28 (19.31%)	-
A558	-	23 (19.11%)
A584	48 (33.10%)	-
A621	-	33 (25.98%)
A641	18 (12.41%)	-
A679	-	14 (11.02%)
A730	04 (02.76%)	-

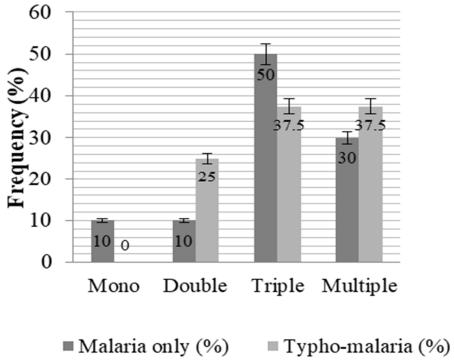

Figure 2. *Types of P. falciparum infection.*

The overall genetic diversity of *P. falciparum* in patients diagnosed with malaria infection only was found to be 19.35% while in patients with typhoid and malaria co-infection, a genetic diversity of 22.22% was recorded.

3.1.4. Multiplicity of Infection and Heterozygosity

Four types of *P. falciparum* infections were observed in the study population: simple, double, triple and multiple infections. Triple and multiple infections were found to be the predominant type of *P. falciparum* infection in co-infected patients (37.5 %). The prevalence of multiple alleles was higher in co-infected samples than in mono-infection. (Figure 2)

P. falciparum co-infected isolates in this study had higher rates of multiple genotypes infection with an overall average MOI of 3.4. The mean multiplicity of infection (MOI) in patients diagnosed with malaria infection only was found to be 3.1 and lower than the MOI of co-infected patients.

3.2. Discussion

Early diagnosis and prompt treatment of fevers, especially those of infectious origin, is one of the most important strategies for reducing disease severity, resistance and mortality. The prevalence of typhoid-malaria co-infections was determined and the genetic diversity of *P. falciparum* strains compared in confirmed cases of malaria versus typho-malaria co-infections in Yaoundé, Cameroon.

One of the most striking observation from our study is the 4.62-fold increase in parasitemia in typho-malaria group of patients compared to those patients who were infected only with malaria parasite (geometric mean parasitaemia of 33700 versus 7305.11 per µl of blood, *p value of <0.05*). Such significantly high parasitemia were also observed in other coinfections of *Plasmodium* with Soil Transmitted Helminth infections [25], HIV [26, 27], visceral leishmaniasis in mice [28] as well as from other countries like in Ghana [29]. This phenotype is hypothesized to be heavily driven by availability of iron [30].

The prevalence rate of co-infection with *Plasmodium* and *Salmonella* organisms detected within the study group was quite low (13.48 %) as compared to previous reports. Mbuh *et al* [31] confirmed this finding and postulated that most of the co-infections treated are based on methods of diagnosis

masked with clinical assumption which probably exacerbate the situation, since clinicians are often compelled to prescribe antimalarial or anti-typhoidal drugs even when malaria or serological test results are not suggestive of the diseases. However, Amah and co-workers [32] obtained a higher rate (47.9%). The decrease in the prevalence of typhoid may be due to the improvement in sanitation over the years and other factors directly associated to typhoid cases.

It has also been shown that acute malaria reduces antibody response to the somatic (O) antigen of *S. typhi* [33] and that hemolysis, which occurs during malaria infection, may predispose to *Salmonella* bacteremia [34]. However, we were unable to find any association between malaria and typhoid ($p > 0.05$; $\chi^2 = 10.33$), except that, the anemia in some of the co-infected participants could be attributed to the co-infection ($p < 0.05$; $\chi^2 = 0.901$). These results reflect what was reported elsewhere [35]; only that, no associations between both pathogens, were recorded at all.

Very little information is presently available on the genetic variation of *P. falciparum* population circulation in confirmed cases of malaria and typhoid fever in Cameroon. The use of genetic polymorphic markers msp2 was conducted to gain insight into the genetic diversity of *P. falciparum* insolate in the population. The result for *Pfmsp2* gene amplification showed variation in the number and size of the bands from one sample to another. The total number of different alleles recorded in this study (Table 4) shows that the transmission level of malaria within the population is significantly high despite the measures put in place by the National Malaria Control Program (NMCP). This picture is similar to reports presented in other African countries such as Madagascar [36] and Kenya [22]. On the other hand, this study observed higher numbers of alleles than reported previously in the study area and in the central region of Cameroon [21, 37]. The genetic diversity of the parasite, expressed as expected heterozygote (*He*), signifies that *Plasmodium* population in Cameroon has a significantly high heterozygosity which may translate to the high transmission pattern observed in the country.

MOI assessments provide vital information concerning the intensity of malaria transmission and could be a useful tool for evaluating the impact of existing control measures. The mean multiplicity of infection (MOI) of *P. falciparum* strains in patients diagnosed with malaria infection only was found to be less (3.1) when compared to malaria co-infection (3.4). This could be an indication that the observed genetic diversity in the parasite population could be driven by the presence of other pathogens, which in turn may significantly influence the treatment outcome of malaria especially among children [17]. It has been suggested that a difference in the allelic families could arise as a result of considerable heterogeneity in parasite populations although this could also be due to immune selection [19].

In this study, triple and multiple infections were found to be the predominant in typhoid fever and malaria co-infected patients (37.5%). In patients diagnosed with malaria only, infection with three different alleles predominated. The prevalence of multiple alleles was higher in co-infected samples than in mono-infection (Figure 2). The high prevalence of multiple *P. falciparum* infections recorded in this study is broadly consistent with previous findings from other parts of Cameroon including Yaoundé [21, 37]. Such high allelic diversity recorded in this study may have a tremendous impact to increase the discriminatory ability of *msp*-2 to differentiate between recrudescence and re-infections. Therefore, this study emphasizes on the importance of genotyping of *P. falciparum* in effective malaria management, anti-malarial drug efficacy trials and control strategies in and out of Cameroon.

4. Limitations

The prevalence of typhoid fever in this study was based on Widal Agglutination test which has a low specificity. Beside cross-reactivity with other Salmonella species, Widal cannot clearly differentiate current infection from previous infection or vaccination.

5. Conclusion

Despite the increasing provision of treated bed nets, promotion of sanitation and hygiene in the city of Yaoundé, and transmission monitoring strategies, the prevalence of malaria and typhoid still remain significantly high. It is evident that *Plasmodium* infection is more likely to cause fever than *Salmonella* infection, and the rate of typho-malaria co-infections is quite high. Moreover, *Plasmodium falciparum* and *Salmonella typhi* are more likely to co-exist in febrile patients, leading to significantly high parasitemia which may be a predominant cause of fever in febrile patients. The overall genetic diversity of *P. falciparum* was quite high and this tends to be even higher in co-infected patients with a higher Mean Multiplicity of infection. The data generated by this study is valuable for guiding decision making by stakeholders to improve disease control strategies. The continuous evaluation of the parasite population could be useful in evaluation of implemented control strategies within the country and eventually prepare for disease outbreaks. Therefore, it will be important to conduct research that will capture more relevant data on the genetic diversity of *P. falciparum* isolates from other regions of Cameroon with different malaria epidemiology. Moreover, it is necessary to run longitudinal studies involving other biological molecules and markers of malaria transmission in order to better understand the strain variations associated with transmission intensity.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Dr Palmer Netongo Masumbe, designed the study,

supervised the research, laboratory analysis and revised the manuscript. *MacDonald Bin Eric* conducted the sample collection, laboratory & statistical analysis, and drafted the manuscript. *Dr Kamdem Donald Severin* facilitated patient recruitment, samples collection and revised the manuscript. *Dr Achonduh Olivia* and *Dr Chedjou Jean Paul* assisted in laboratory and data analysis. *Pr Wilfred Fon Mbacham* provided reagents and the space for laboratory analysis. All authors read and approved the final manuscript.

Data Availability

Most of the data generated from the research were used to support the findings of this study and they are subsequently included within the article.

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