First Observations of *Plasmodium Malariae* and Its Evolution During the Parasitological Evaluation of the Long-term Village Scale Malaria Vector Control Program in the Balombo Area of Angola

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Abstract

Background: A long-term village scale vector control program was implemented in eight villages around the Balombo town (Benguela Province, Angola) to compare the efficacy of four methods of vector control inside houses including long-lasting deltamethrin insecticide treated nets (PermaNet® 2.0 model or P.2.0); association of P.2.0 and deltamethrin Insecticide Treated Plastic Sheeting (ITPS)-Zero Fly® model; deltamethrin ITPS-Wall Lining model alone; and 2 rounds of lambda-delta-cyhalothrin Inside Residual Spraying followed by installation of ITPS.

Methods: Cross sectional parasitological surveys (CSS) were done every two months. Thick blood films (TBF) were microscopically examined at the Medical Department of the Angolese Sonamet company in Lobito and a sample of them were double-checked in Yaoundé. *Plasmodium* species determination, parasitaemia and gametocytes presence, and evolution in time were analyzed.

Results: A total of 190 CSS was done between 2007 and 2011, *Plasmodium* spp. were observed in 5,431 of the 21,804 TBF done. *Plasmodium malariae* alone was observed in 22 TBF (0.4%) and mixed infections *P. falciparum* and *P. malariae* in 44 TBF (0.8%).

Conclusion: The very low frequency of *P. malariae* could explain the fact it is usually not reported in classical point prevalence surveys. Our study confirms the presence of this species in Angola, which must be known due to its special clinical impact, quartan fever, kidney failure, chronicity, symptomless carriers, persistence for several years with long term recrudescence and reported cases of resistance to classical ACTs. The prevalence of *P. malariae* decreased after implementation of vector control methods.

Background

A great amount of work was devoted to *Plasmodium malariae* since its description by Grassi and Feletti in 1890 under the name *Haemamoeba malariae* in ancient [1–10], until recent studies [11–16]. Considered as benign, this *Plasmodium* species has been used in experimental infections for the treatment of neurosyphilis [10].

P. malariae is a particular species, especially at the clinical level, with a 72-hr erythrocyte cycle, a slow development (15 days in both liver and *Anopheles* mosquito), and a quartan fever [17, 18]. The parasite density is always low compared to *Plasmodium falciparum* and it produces clinical relapses due to the asexual forms that persist in the host's blood [17]. Symptoms include nephropathies [19–23], a long asymptomatic persistence of several years before access to recrudescence [24] that may induce transfusional malaria [25], genetic polymorphism [26–29], transmission by palearctic or tropical vectors [30, 31], and due to its possibility to infect human and simian subjects [32], it was considered as an anthroponosis.

This plasmodial species has a scattered world-wide distribution in the malarious regions of Africa [33, 34], South America [35], Asia and Pacific [36] with a variable frequency in time and space according to the

technique of investigations and biological analysis. With the recent acceleration of malaria control operations, the frequency of *P. falciparum* is decreasing [37] and a special interest has to be given to other plasmodial species, including *P. malariae*, which could persist for many years [38], or even the whole life of infected persons [39].

The genome of *P. malariae* has recently been decrypted [40, 41], which explains a case of resistance with a clinical recrudescence after treatment with the combination of artemether-lumefantrine (a full course of 6 doses over 3 days, equivalent to a total dosage of 6.2 mg/kg of artemether and 37.4 mg/kg of lumefantrine). The explanation given was that the primary infection would have been polyclonal but the recrudescence due to a resistant clone [41].

In 1937, the death of a 27-years old man in India was reported following what appeared to be the third malaria crisis access, with no particularly high fever, but parasitological examination revealed a high density of *P. malariae*. Despite treatment with quinine (injection and oral), resulting in the disappearance of microscopically observed *Plasmodium*, the subject died possibly from heart problems [42].

Cases of glomerulonephropathy associated with *P. malariae* infections were also reported with a disease which may persist and evolve despite the apparent elimination of parasites and with detectable chronic renal disease observed even 3–5 years after the so-called primary infection [43].

Overall, *P. malariae* is still not well understood and it therefore seemed interesting to pay a particular attention to *P. malariae* in the framework of the study aiming at comparing the efficacy of four vector control methods in the Balombo area, Angola and to monitor its possible evolution with the implementation of vector control operations.

Materials And Methods

Study sites

The Balombo study area in the Benguela district of Angola was already described [44]. This study was made in eight villages around the town of Balombo (12°21'S, 14°46'E) located at an altitude of 1,220 meters in a mountainous area of ancient volcanoes with a thermal spring at the west entrance to the city. The vegetation is a tropical savannah with shrub prairie, the original forest has been severely degraded for crop development and domestic use. There is a well-marked dry and cold season in June to August and abundant rains from November to April. Temperatures vary between a minimum of 10°-15°C in June-August and a maximum of 30–35°C.

Parasitological surveys

Parasitological surveys were carried out as part of a multidisciplinary evaluation of a vector control operation aiming at comparing four vector control methods, such as (1) PermaNet 2.0 Long lasting deltamethrin-impregnated mosquito nets (LLIN); (2) association of LLIN with Zero Fly®, deltamethrin insecticide treated plastic sheeting (ITPS) that are pinned on the hut walls; (3) deltamethrin treated ITPS

model Wall Lining (WL) model alone and also pinned on the walls; and (4) conventional lambdacyhalothrin indoor Residual Spraying (IRS) with two rounds/year (every six months), followed by the installation of ITPS to increase the long lasting efficacy of treatment. Each method was implemented in paired-randomized villages. Cross-sectional parasitological surveys were conducted on a regular basis every two months focusing on random samples of the population under 15 years of age. At the time of the field investigations, part of the population present in the village usually came to the team to have a blood test and, for obvious ethical reasons, a thick blood film (TBF) was prepared from each one of these volunteers and these TBF were colored in the field with Giemsa. Then, microscopic observations were done at the medical department of the Angolese Sonamet Company, which supported the study through their Malaria Control Program.

To compare the efficacy of each vector control method a subsample of children ≤ 15 years old was extracted from the original list to standardize the samples to be compared, but in the present study dealing with *P. malariae* the whole samples were considered. During the surveys a supplementary drop of blood was also obtained on filter paper for further immunological analysis in Montpellier, France for estimating the *Anopheles* biting pressure on the population before and after implementation of the four vector control methods [44]. Microscopy parasitological examinations were done by the same team throughout the study with a regular double-checking in Yaoundé, Cameroon of a sample of 10% of the slides. Results of these analyses were provided to the village health worker for further action to be taken in accordance with the National Malaria Control Program of Angola.

Statistical analysis

Data were analyzed and graphs constructed with GraphPad Prism6® software (San Diego, CA, USA). Distribution of parasitaemia were analyzed with the non-parametric Mann-Whitney statistical test.

Results

Prevalence of *Plasmodium malariae*

From February 2007 to December 2011, 190 field surveys were done and 21,804 thick blood smears were prepared from which 5,431 were positive for *Plasmodium* (24.9%), 5,365 with *P. falciparum* (98.8%), 22 (0.4%) with *P. malariae*, and 44 (0.8%) with a mix of *P. falciparum* and *P. malariae* (Table 1).

Table 1
Parasitological surveys done from 2007 to 2011 in 8 villages of the Balombo area.

Village	No surveys	No TBF done	No TBF +	No <i>P.f.</i>	No <i>P.m.</i>	No <i>P.f.</i> + <i>P.m.</i>
Caala	25	3,387	1,004	1,000	1	3
Cahata	25	2,873	833	819	7	7
Capango	24	1,856	314	312	1	1
Canjala	24	2,952	931	915	4	12
Chisséquéélé	23	2,551	463	460	2	1
Barragem	23	2,525	548	534	3	11
Candiero	23	2,761	524	523	0	1
Libata	23	2,899	814	802	4	8
Total	190	21,804	5,431	5,365	22	44
			(24.9%)	(98.8%)	(0.4%)	(0.8%)
TBF = thick blood film; TBF + = positive thick blood film; <i>P.f.</i> = <i>P. falciparum</i> ; <i>P.m.</i> = <i>P. malariae</i> .						

Parasitaemia of *P. malariae* alone or mixed infections *P. malariae* + *P. falciparum*

P. malariae parasitaemia, alone or associated with *P. falciparum*, were significantly not different with P value = 0.1127 (Fig. 1). For single or mixed infections, the geometric means were of 466 and 787, and the medians of 450 and 746 respectively (Table 2).

Table 2
Parasitaemia of *P. malariae* alone or associated to *P. falciparum*.

Indicator/ infections	<i>P. malariae</i>	<i>P. malariae</i> + <i>P. falciparum</i>
Arithmetic mean (parasites/μl)	1,065 ± 1,840	1,955 ± 4,008
Geometric mean	466	787
Median (sample size)	450 (n = 22)	746 (n = 44)
Minimum value (parasites/μl)	32	133
Maximum value (parasites/μl)	8,301	24,000

Influence of vector control methods

The presence of *P. malariae*, alone or associated with *P. falciparum*, according to the vector control method is reported in Table 3. Similar percentages of infections were found when considering *P. malariae* and mixed *P. malariae*+ *P. falciparum* with values ranging from 1% in villages with LLIN alone and IRS, 1.4% in villages with LLIN and Zero Fly, and 1.7% in villages with ITPS. While *P. falciparum* percentages vary between 98.3% and 99%. Besides, the number of infections with *P. malariae* alone or mixed with *P. falciparum* decreased drastically, by a factor of 4.5, after vector control operations with values of 54 out of 66 infections (82%) before to 12 (18%) after vector control implementation (Table 4). However, the parasitaemia of *P. malariae* infections alone or associated with *P. falciparum* did not change with vector control implementation as shown by P values of 0.0778 for *P. malariae* infections alone (median = 480 before and 233 after VC) and P value = 0.923 for mixed infections (median = 804 before and 853 after VC) (Fig. 2).

Table 3

Presence of *Plasmodium malariae* according to vector control method implemented (TBF = thick blood film; LLIN = Long lasting insecticide treated net; ITPS = insecticide treated plastic sheeting; ZF = Zero Fly model of ITPS; IRS = Inside residual spraying).

Method of vector control	No surveys	No TBF done	No TBF +	No <i>P.f.</i>	No <i>P.m.</i>	No of mixed <i>P.f.</i> + <i>P.m.</i>
LLIN alone	50	6,260	1,837 (29.3%)	1,819 (99.0%)	8 (0.4%)	10 (0.5%)
LLIN + ZF	48	4,808	1,245 (25.9%)	1,227 (98.6%)	5 (0.4%)	13 (1.0%)
ITPS alone	46	5,076	1,011 (19.9%)	994 (98.3%)	5 (0.5%)	12 (1.2%)
IRS then ITPS	46	5,660	1,338 (23.6%)	1,325 (99.0%)	4 (0.3%)	9 (0.7%)

Table 4

Prevalence of *P. malariae* alone (*P.m.*) or associated to *P. falciparum* (*P.m.* + *P.f.*) in surveys done before and after vector control (VC) implementation.

Vector control methods	Before VC		After VC	
	<i>P.m.</i>	<i>P.m.+P.f.</i>	<i>P.m.</i>	<i>P.m.+P.f.</i>
LLIN alone	8	6	0	4
LLIN + ZF	2	12	3	1
ITPS alone	4	11	1	1
IRS then ITPS	4	7	0	2
Sub-Total	18/22	36/44	4/22	8/44
(prevalence)	(82%)	(82%)	(18%)	(18%)
Total	54		12	
LLIN = Long lasting insecticide treated net; ITPS = insecticide treated plastic sheeting; ZF = Zero Fly model of ITPS; IRS = Inside residual spraying.				

Influence of the season

The analysis of *P. malariae* infections observed during the dry and rainy season surveys shows a similar overall number with respectively 30 and 36 infections, with twice less *P. malariae* alone infections in the rainy (32%) than the dry season (68%) (Table 5).

Table 5

Presence of *P. malariae* (*P.m.*) alone or associated to *P. falciparum* (*P.f.+P.m.*) according to season.

	Rainy season		Dry season	
<i>Plasmodium</i> species	<i>P.m.</i>	<i>P.f. + P.m.</i>	<i>P.m.</i>	<i>P.f. + P.m.</i>
No positive slides	7/22	23/44	15/22	21/44
(prevalence)	(32%)	(52%)	(68%)	(48%)
Total	30		36	

Influence of gender

The 66 infections of *P. malariae* alone or associated with *P. falciparum* included 28 men and 38 women. The parasite load of *P. malariae* were similar in men and women (P value = 0.14; respective median 480

and 299) and the parasitaemia of mixed infections *P. malariae*+ *P. falciparum* were also similar in men and women (P value = 0.93; respective median = 804 and 1,040) (Fig. 3).

Influence of age

Our sample was not prepared to be representative of the village populations since it was intended to mainly evaluate the impact of vector control operations on *P. falciparum* infections in children (≤ 15 years). However, for *P. malariae* evaluation, 2 age groups were considered such as < 5 years old, considered as at-risk group, and > 5 years old. The results showed a similar number of infections in both groups with 11 carriers < 5 years old and 11 carriers > 5 years old for infections with *P. malariae* alone, and 20 carriers < 5 years old and 24 carriers > 5 years old for mixed infections *P. malariae*+ *P. falciparum*.

Discussion

According to the last WHO report [37], Angola had an estimation of 7 million malaria cases in 2018 and 7.4 million cases in 2019, and around 13,500 deaths in 2018 and 2019 with 100% *P. falciparum*. There is no report of the occurrence of *P. malariae* and *P. ovale* in the Balombo region of Angola, due to their rarity, although records have shown the presence of these *Plasmodium* species in Angola [45–49]. During a cross-sectional survey in northern Angola, *P. falciparum* was genotyped at resistance-associated loci in *pfprt* and *pfmdr1* by real-time PCR or by direct sequencing of amplicons. On a total of 3,316 blood samples collected, 541 (16.3%) were *Plasmodium* infected, out of them 477 (88.2%) were due to *P. falciparum* alone and 6.5% were mixed *P. falciparum* and *P. malariae* [47]. It is important to precise that *P. malariae* infections were reported by molecular methods and not observed with usual optical microscopy.

The five years longitudinal surveys in the eight villages around Balombo (Angola) confirmed the presence of *P. malariae* in the Balombo region of Angola and its very low prevalence, with 22 cases out of 21,804 thick blood smears observed (0.1%), and this could explain why this species is rarely reported in single sectional surveys, deserving continuous training of microscopic technicians [50]. For example, during continuous training operations for microscopists from health centers in Bengo, Benguela and Luanda Provinces in Angola, there was an increase in the sensitivity and specificity of examinations after training, while noting false positive *P. vivax* case in Bengo and *P. malariae* infection on a slide considered as negative [51]. Using combined techniques such as microscopy and PCR assays, the prevalence of *P. malariae* mono-infections at 1% was found in the Democratic Republic of Congo neighboring Angola [52].

In Angola, a cross-sectional survey conducted in 733 Chinese and Southeast Asian migrants showed that most infections were due to *P. falciparum*, although infection and/or exposure to *P. vivax* and *P. malariae* was also detected [48]. Based on these very few reports, more attention should be given to the detection of non-falciparum infections in order to know what and where plasmodial species are circulating in Angola, and more widely in the south of the Sahara region for a more precise diagnosis of patients in order to provide them an appropriate malaria prophylaxis [53]. In addition, it is important to know where this plasmodial species is circulating for the diagnosis of patients being examined in non-endemic areas, in order to avoid kidney failure or any other complications due to this plasmodial species.

Plasmodium infections with non-falciparum species, also referred to Non-Falciparum Malaria (NFM), are responsible for 25% of cases imported into Europe [49] and a retrospective analysis of the records from January 2006 to August 2016, recorded in a hospital in Portugal, revealed 19 cases of NFM among the 225 malaria cases including 12 cases from Angola. Out of these 19 NFM cases, 7 (37%) were due to *P. malariae*. The most frequent symptom was fever and the biological analysis showed thrombocytopenia. Treatments provided to these 19 NFM cases were quinine-doxycycline (11 patients), chloroquine (six patients) or artemether-lumefantrine (two patients) with cure in all cases. In Henan Province of China, three patients, including two returning from Angola and one from Equatorial Guinea, had symptoms such as irregular fever, headache, chills. Two cases had elevated total bilirubin and splenomegaly and were confirmed as *P. malariae* infection by microscopic examination. Both were cured with artemisinin-based combination therapy (ACT) [46], while the usual drug can have crucial side effect of G6PD deficiency and must be given only with strict medical care in main hospital. In Jiangsu Province of China, an observational study of imported malaria cases was carried out for the period of 2011–2014 [45]. Out of 1,268 imported malaria cases, *P. falciparum* cases accounted for 83.4% (n = 1,058), and it was noticed that the proportions of *P. ovale* and *P. malariae* increased during these four year-period. Therefore, this increase in rare *Plasmodium* species originating from south-Saharan Africa and Southeast Asia needs to be better monitored at all levels of health providers focusing on diagnosis and treatment of malaria. In addition, due to long latency periods and misdiagnosis of *P. malariae* and *P. ovale*, there is an increased risk of re-introduction of malaria in China, declared malaria-free by WHO in 2021 [45, 54]. A recent meta-analysis of the occurrence of *P. malariae* and *P. ovale* in the world revealed that 51% of these species were reported from the African region and each species was accounting for 3.16% and 1.69% respectively on this continent [55]. This prevalence of *P. malariae* is 10 times higher than the one found in this study (0.3% including mixed infections), which may be due to the fact that this species is often misidentified as *P. falciparum* or totally missed under microscopic examination due to the low parasite density compared to *P. falciparum* [56].

To improve the diagnosis of certain fevers, anaemia [57] and nephropathies, it is important to be aware of the presence of *P. malariae* and thus avoiding misdiagnosis [58] in endemic and non-endemic areas [59–61] with the critical issues of mistreatment [53, 62–67]. The persistence of *P. malariae* even after ACT treatment is of great concern [68, 69], as well as its well-known long chronicity [3] with an amazing persistence for several years, such as a *P. malariae* case reported 32 years after the initial infection of stay in malaria-endemic areas [70]. It was reported that infections have been transmitted by blood transfusion twelve years, twenty years and thirty years after infection of the donors [3].

In addition, it is important to highlight the fact *P. malariae* is a parasite of both man and chimpanzee [71] and with current and foreseen environmental changes and forest destruction, the risk of human infections could greatly increase as it was recently seen with *P. knowlesi* in Indonesia and Malaysia [72–80].

The fact that the prevalence of *P. malariae* could decrease with vector control implementation is an interesting information to be kept in mind for instance in the case of residual malaria, which is of great

concern [81], and malaria elimination [82, 83], which rightly concentrate on *P. falciparum* while *P. malariae* must not be forgotten when thinking to its long chronicity.

Conclusion

Malaria infections due to *P. malariae* are occurring in Angola, which is of main concern due to special clinical impact, quartan fever, kidney failure, chronicity, symptomless carriers, persistence for several years with long term recrudescence, reported cases of resistance to classical ACTs, and imported cases into other continents. Our study showed that the prevalence of *P. malariae* decreased with vector control implementation. This rare *Plasmodium* species must be better monitored in order to reduce residual malaria.

Declarations

Ethical Approval

This study was conducted in accordance with the Edinburgh revision of the Helsinki Declaration and was approved by the National Malaria Control Program of the Ministry of Health of Angola, the Ethical authority in charge of approving studies on malaria research in Angola. Written consent (signed by the head of each household) was obtained for all individuals enrolled in the study by the SONAMET Company - Malaria Control Program (MCP).

Consent for publication

All authors provided their consent for publication.

Availability of data

Data are available upon request to the senior author.

Competing Interests

The authors declare that they have no competing interests.

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Author's contributions

PC designed the study protocol; participated to field surveys, analyzed data and drafted the manuscript; MADS, VF, JCT carried out the field surveys. MADS read all blood slides. VF did initial and ongoing training, participated in laboratory analysis and double-checked part of the blood slides. SM did data

analyzes, drafted and finalized the manuscript. FM participated in data analysis and finalized the manuscript. All authors read and approved the final manuscript.

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Figures

Fig. 1

Figure 1

Distribution of parasitaemia (No of parasites/μl) and median (red line) of *P. malariae* alone or associated with *P. falciparum* (Pf + Pm).

Fig. 2

Figure 2

Evolution of *P. malariae* (Pm) load alone or associated with *P. falciparum* (Pfm) with median (red line) before and after vector control implementation.

Fig. 3

Figure 3

Parasitaemia due to *P. malariae* alone (Pm) or associated with *P. falciparum* (Pfm) with median (red line) according to the gender. M, men; W, women.