High prevalence of *Plasmodium malariae* from hospital and communities in a high malaria transmission setting of Southern Nigeria.

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Abstract

Background

Plasmodium malariae is one of five malaria parasite species prevalent across sub-Saharan Africa, but with limited data on its prevalence and epidemiology across most endemic settings. Specific clinical diagnosis of *P. malariae* and other non-falciparum malaria parasites is uncommon and the most widely used malaria diagnostic tool, rapid diagnostic tests (RDTs), predominantly targets *P. falciparum* while nonspecifically identifying co-infecting species as 'pan-species'. Moreover, undetectable submicroscopic infections of these non-falciparum infections are likely and constitute a reservoir that could sustain transmission and challenge the achievement of elimination goals. This study aimed to explore prevalence of *P. malariae* infections in a high malaria transmission setting in Nigeria.

Methods

A preliminary survey was first carried out in two sites to compare prevalence of non-falciparum species in southern Nigeria, results of which informed subsequent sample collection. Study participants were recruited from health facilities in Lagos and Calabar between October to December 2017 and subsequently from health facilities in four communities in Akpabuyo local government area of Cross River State, Nigeria from October – November 2018, following community sensitization and consent. Pan species RDT (SD Bioline, Cat No 05FK60) was used for field diagnosis and Thick blood films were also collected for microscopic detection of malaria parasites. Dried blood spots (DBS) collected from all participants were used for molecular detection of *Plasmodium* species by real-time PCR techniques.

Results

Plasmodium falciparum was most prevalent in the study populations, 22 - 48% of all samples tested. From the primary survey, significantly higher prevalence of *P. malariae* and *P. ovale* were detected by qPCR in Calabar compared to Lagos. From a resampling of communities in Calabar, (N=798), *Plasmodium spp* infection prevalence by RDT was 23%. Non-falciparum infections detected was 12.5%, of which a median prevalence of 10.3% were infections with *P. malariae* (mixed and mono). The distribution of *P. malariae* infections between age groups (7.3 – 16.1%) and communities (6.2 – 12.1%) was heterogeneous. 32.9% of *P. malariae* infections occurred in the 11-15yrs age bracket, mostly as mixed infections. The odds of *P. malariae* as a mono infection

was highest in the 0-5yrs age group relative to the group with the least odds of mono infections (11-15yrs). *P. ovale* was detected at 2.3%, while no sample tested positive for *P. vivax*.

Conclusions

Overall, *P. malariae* was the most prevalent non-falciparum species and occurred mostly in the 11-15yrs age group, who are not targeted by some elimination strategies. Approaches for routine diagnosis of non-falciparum species in endemic settings will facilitate interventions towards pan-species malaria elimination.

Keywords: *Plasmodium malariae,* malaria control, elimination, non-falciparum, sub-Saharan Africa

Introduction

Global efforts at malaria elimination are focused on the most prevalent malaria parasite species – *Plasmodium falciparum* in sub-Saharan Africa (sSA) and *Plasmodium vivax* in Asia and south America (1). Three other human malaria parasite species are known, of which *Plasmodium malariae* followed by *Plasmodium ovale spp* are the most prevalent in malaria endemic regions including sSA (2). *Plasmodium malariae* in particular is clinically important and is characterized by quartan fevers due to its 72-hour blood stage life cycle (3,4). *P. malariae* infections are considered relatively benign due to low parasite densities and relatively less complicated pathologies (4). However, infection with *P. malariae* has been implicated with other severe pathologies such as anemia (5) and nephrotic syndrome in children and adults (6,7). With the dominance of *P. falciparum*, the biology and specific public health impact of *P. malariae,* has mostly been understudied as they most often occur as coinfections with other parasite species (3). Species-specific clinical diagnosis of *P. malariae and P. ovale* is uncommon in routine malaria diagnosis. The most widely used diagnostic tool, rapid diagnostic tests (RDTs), predominantly target *P. falciparum* while nonspecifically identifying co-infecting species as 'pan-species' (8). As a result, the burden and population at risk in most endemic areas remain largely unknown. A recent study reported persistent detection of *P. malariae* over a 22-year period in an area of declining *P. falciparum* infections in Tanzania (9), suggesting that decline of *P. falciparum* could provide a favorable ecological niche for other malaria parasite species as also recently reported with the zoonotic malaria parasite species *P. knowlesi* in Malaysia (10).

The continuous transmission of the non-falciparum species, their corresponding submicroscopic infections constitute a reservoir of infection that pose a significant challenge to achieving malaria elimination goals. Despite efforts at malaria elimination, there were an estimated 219 million cases of malaria worldwide in 2017 and approximately 25% were observed in Nigeria (1). Malaria transmission in Nigeria is perennial, mostly due to its climatic conditions, with about 85% of the population living in areas of mesoendemic transmission (11). *P. falciparum* accounts for over 95% of reported malaria cases in Nigeria and *P. malariae* is estimated to play a minor role, observed in approximately 9.8% of malaria cases as mixed infections (12). The objective of this study was to explore the prevalence of *P. malariae* infections in two geopolitical zones in southern Nigeria and to further characterize *P. malariae* infections in the area with high prevalence.

Methods

Study site and Plasmodium spp survey

The malaria survey was done in October to December 2017 in two study sites in southern Nigeria; Lagos in the south-west geopolitical zone and Calabar in the south-south geopolitical zone (**Figure 1A**). Lagos is the most populous city in Africa with a population of approximately 15 million, it experiences a tropical savanna climate as there is a significant precipitation difference between the wet season and the dry season. The wet season starts in April and ends in October, while the dry season starts in November and ends in March. The wettest month is June with a precipitation total of 315.5 millimetres (12.42 in). Located near the equator, Lagos has only a slight seasonal temperature variation, with high temperatures ranging between 28.3–32.9 °C (13). Calabar on the other hand features a tropical monsoon climate with a lengthy wet season spanning ten months and a short dry season covering the remaining two months. Temperatures are relatively constant throughout the year, with average high temperatures usually ranging from 25 to 28°C. Calabar averages just over 3,000 millimetres (120 in) of precipitation annually (14).

Individuals reporting at health facilities were consented to participate in the study and screened for malaria with a pan species rapid diagnostic test (RDT) - SD Bioline, Cat No 05FK60. Dried blood spots (DBS) were also collected from each participant for molecular detection of *Plasmodium* species and thick blood film were made for microscopic diagnosis of malaria parasites. Following the preliminary hospital survey, a community survey was conducted in 2018 in Calabar, Cross River State.

The community study targeted Akpabuyo local government area (LGA) of Cross River State in the south-south geopolitical zone (**Figure 1B**), approximately 30km from Calabar Municipality. With an area of 1,241 km² and a population of 271,395 (2006 census), it is located at 4°88′N 8°30′E and situated close to the border with Cameroon (15). The inhabitants are predominantly Efik ethnic group (90%) with other ethnic minorities (10%). The climate is tropical with average temperatures ranging between 25°C - 29°C. It rains almost all year round with peak rainfall within the months of August to October, at approximately 400mm. Relative humidity is high with most months of the year recording a monthly mean value of 80% (16). Participants volunteered to report at health facilities for recruitment and RDT testing following prior community sensitization. Participant recruitment ran between October and November 2018. The age and sex were recorded for all those who consented for microscopy and molecular diagnosis.

Ethical considerations

Ethical approval was obtained from the respective authorities in Lagos and Cross River State, References – IRB/17/038, CRSMOH/RP/REC/2017/545 and CRSMOH/RP/REC/2017/809. Written informed consent was sought for adult participants while parental or guardian consent was sought for minors. Participants did not receive any retribution for participation in study. All individuals with positive malaria RDT were provided with recommended antimalarial drugs. Febrile individuals with negative RDT tests were referred to the health facility staff for further investigation.

Detection of Plasmodium spp parasites

Thick blood microscopy slides stained with 10% Giemsa solution were examined across 100 high power fields by two microscopists and parasite counts recorded per number of white blood cells observed. In case of 20% variance in counts between primary and secondary microscopist, the counts closest to that of a third reader was retained. A slide was declared negative only after observing no parasite in 100 microscopic high-power fields while parasite density was calculated using WHO protocols; number of parasitized erythrocytes against 8000 white blood cells (WBCs) (17), expressed as parasitaemia per microlitre of blood.

For molecular diagnosis of *Plasmodium spp*, DNA was extracted from DBS samples using the QIAamp DNA mini kit (QIAGEN, Germany) according to manufacturers' protocols. For the

preliminary survey, presence of *Plasmodium* species was detected using a commercial quantitative real-time PCR (qPCR) assay, Genesig® *Plasmodium* species kits (Primerdesign™ Ltd, UK) according to manufacturers' protocol. The Genesig protocol targets all 5 species of malaria with species-specific probe/primer mix. Subsequently, presence of *P. malariae* and *P. ovale* were detected using a custom qPCR assay targeting the *Plasmepsin* gene (18) as it showed comparable results with the Genesig® kit. A preamplification was done using 0.1µM of the forward and reverse primers for *P. malariae* and *P. ovale* (**Table 1**), 1× Qiagen Multiplex Master Mix (QIAGEN, Germany) and 5µL of template DNA in a 15µL reaction. The main amplification reaction contained 3µL of pre-amplified products in a 15 µL total reaction volume and was done using 1× qPCR Taqman Universal Master Mix (ThermoFischer Scientific, USA), 0.4µM of the forward and reverse primers and 0.2µM of the labelled probe. Preamplification was performed under the following conditions: initial denaturation for 5min at 95°C, then 14 cycles of denaturation for 30s at 95°C, annealing at 60°C for 1min and extension for 90s at 72°C; while the main amplification and detection were performed under the following conditions: initial denaturation for 10min at 95°C, then 40 cycles of denaturation for 10s at 95°C and 1 min at 60°C with data collection. *P. falciparum* was detected using a highly sensitive qPCR assay (varATS qPCR) targeting multi-copy subtelomeric sequences (19). Amplification reaction was done using 1× qPCR Taqman Universal Master Mix (ThermoFischer Scientific, USA), 0.8µM of the forward and reverse primers, 0.4µM of the labelled probe and 5µL of template DNA in a 15µL reaction. Amplification and detection were performed under the following conditions: pre-incubation at 50°C for 2mins followed by initial denaturation for 10min at 95°C, then 45 cycles of denaturation for 15s at 95°C and 1 min at 55°C with data collection.

Data analysis

Data analysis was conducted using Microsoft Excel spreadsheet, R statistical package (20) and Stata Version 13 (Texas, USA). Cross tabulations and t-test, Pearson Chi squared test (χ^2) or Fisher's exact test were used, where appropriate, to explore associations between *P. malariae* infection and demographic variables of interest.

Results

Results of preliminary survey

A total of 243 RDT positive clinical samples were analyzed by qPCR for presence of *Plasmodium* species from Lagos and Calabar in the preliminary survey (**Table 2**). In Lagos, the proportion of qPCR detected *Plasmodium* species were 81.7%, 1.4%, 1.4% and 0% for *P. falciparum, P. malariae, P. ovale* and *P. vivax* respectively. The proportion detected by microscopy were 73.2% and 0% for *P. falciparum* and *P. malariae*, respectively. In Calabar, the proportion of *Plasmodium* species detected by qPCR were 80.8%, 9.9%, 5.8% and 0% for *P. falciparum, P. malariae, P. ovale* and *P. vivax* respectively; and proportion detected by microscopy were 76.7% and 5.8% for *P. falciparum* and *P. malariae* respectively. There was no significant difference between *P. falciparum* detected by qPCR and microscopy. Microscopy was less sensitive than qPCR in detecting *P. malariae* and *P. ovale*. qPCR detected more positive samples for these non-falciparum species and a higher prevalence in Calabar compared to Lagos (**Figure 2A-E**).

Summary of participants and prevalence of Plasmodium species in the south-south zone

Of the 799 participants recruited, results from all three diagnostic methods were obtained for 798. Male-female ratio was 44:56% respectively and the distribution of age groups was 12%, 16%, 21% and 51% for 0-5yrs, 6-10yrs, 11-15yrs and >15yrs respectively (**Table 3**). Prevalence of *P. falciparum* by qPCR ranged from 21.6% in Akansoko to 72.7% in Ifondo; prevalence of *P. malariae* ranged from 6.2% in Akansoko to 12.1% in Nkakat Eyamba; and prevalence of *P. ovale* ranged from 1.1% in Asabanka to 3.4% in Ifondo (**Figure 3B**). The two communities situated to the North of Akpabuyo LGA generally had more non-falciparum species than the two communities to the South (**Figure 4**). Prevalence of *P. falciparum* by qPCR ranged from 36.8% in the 0-5yrs age group to 66.1% in the 11-15yrs age group; prevalence of *P. malariae* ranged 7.4% in the 0-5yrs age group to 16.1% in the 11-15yrs age group; and prevalence of *P. ovale* ranged from 1.2% in the >15yrs age group to 4.2% in the 0-5yrs age group (**Figure 3C**). There was no significant difference in *Plasmodium* species detected in male or female participants (**Figure 3D**).

Overall, 23.3% (186/798) of the participant were positive for *Plasmodium* species infection by RDT. From the RDT positive individuals, *P. falciparum* was detected in 83.9% - 100%; pan-species mixed with *P. falciparum* was detected in 5.4% - 54.8%; and pan-species positive only was detected in 0% - 32.3% across the four sites. qPCR detected *Plasmodium* species in 42.4% (259/611) of the RDT negative samples, of which 236 (91.1%) were *P. falciparum*, 46 (17.8%) were *P. malariae* and 12 (4.6%) were *P. ovale*. Overall prevalence by microscopy was 18.5%

(148/798) (**Figure 3A**), with 73.6% (109/148) having parasite density <1000 parasites/µL; 21% (32/148) having parasite density between 1000 - 10000 parasites/µL; and 4.7% (7/148) having parasite density >10000 parasites/µL. Prevalence of *P. malariae* by microscopy was low, ranging from 0% - 4.3% across the four sites. Despite observing a higher proportion of *P. malariae* infections in the oldest age group >15yrs (**Figure 5**), the odds of having *P. malariae* as mono infection was highest in the 0-5yrs age group relative to the group with the least odds i.e 11-15yrs (**Table 4**). Similarly, the odds of *P. malariae* mono infections was highest in Asabanka relative to *P. malariae* mono infections in Ifondo community with the least odds. There was no significant difference in the proportions of *P. malariae* mono-infections detected amongst male and female participants.

Discussion and conclusion

Nigeria accounts for an estimated 25% of global malaria cases, mostly considered to be due to *Plasmodium falciparum* infection (1). The results here show a higher prevalence of non-falciparum species than previously reported. The prevalence of these other malaria parasite species was heterogeneous between communities and age groups. *P. malariae* infections were most prevalent in the 11-15yrs age group, which is currently not targeted by most elimination strategies. This concurs with the higher prevalence of *P. malariae* infections in older age groups reported in Papua New Guinea using similarly sensitive molecular method - post-PCR ligase detection reaction-fluorescent microsphere assay (LDR-FMA) compared with light microscopy (21). The results herein adds to data on prevalence of non-falciparum species in Nigeria which remains scanty (22–25) given the focus on *P. falciparum*. With the push for global eradication it buttresses the proponent that other human *Plasmodium* species should be brought to focus and included in malaria elimination strategies.

Non-falciparum malaria species are often found in mixed infections, but a significant number of mono-infections of *P. malaraie* was detected by qPCR. The odds of *P. malariae* mono infections were highest in the 0-5yrs age group, and next was the >15yrs group with the age groups in-between having lower odds. Children under 5 years are targeted by malaria elimination strategies and have poor immunity against falciparum malaria. *P. falciparum* elimination in this age group and the prevalence of mono-infections suggest support for the 'niche theory' in which non-

falciparum species become more relevant with the elimination of *P. falciparum*. Moreover, the age group with the highest prevalence of *P. falciparum*, 11-15years, have least odds for a mono infection. With higher falciparum immunity in the >15 years age group and low *P. falciparum* prevalence, mono-infections have a higher odd and raise a further question on the absence of cross-species immunity despite the high genomic similarity between *P. falciparum* and *P. malariae*. The extended 72-hour developmental cycle and the preference of the parasite to develop in older erythrocytes (likely to be removed by the spleen) could possibly affect the development of immunity by the human host to *P. malariae* (4), especially in the 0-5yrs age group. Here, the odds of *P. malariae* mono infection was lowest in Ifondo community, which had the highest prevalence of *P. falciparum* infections as detected by the highly sensitive qPCR assay. The dynamics of co-infections as elimination is implemented needs to be further evaluated across communities.
The preliminary survey showed a lower prevalence of non-falciparum species in Lagos compared to Calabar. This is not surprising despite having similar vector populations (26), as urbanization in Lagos metropolitan city with better housing and access to health services will limit malaria transmission. A consistent pattern of increased urbanization coincident with decreasing malaria transmission and elimination over the past century has been reported (27). Most of the non-falciparum infections in the urban area were submicroscopic and could only be detected using sensitive molecular methods. This emphasizes the challenges to malaria diagnosis with decreased prevalence and sub-microscopic infections. Sensitive and accessible diagnostic tools for detection of all human *Plasmodium* species therefore remain important. Microscopy, which is still considered the gold standard for malaria diagnosis, is laborious and time consuming for *Plasmodium* species detection and requires expert microscopists, which is rapidly becoming rare. Current antigen-based pan-species RDT also presents peculiar challenges, including low sensitivity and non-species specificity (28). The poor sensitivity for pan-species *Plasmodium* detection was recently demonstrated in an experimental human blood-stage model for *P. malariae* infection, where RDT targeting pan-genus lactate dehydrogenase enzyme – pLDH, remained negative despite the presence of symptoms consistent with malaria 72-hour prior to testing (29).

Most of the study participants reported no malaria specific symptoms. Therefore, most of the malaria infections detected by PCR were sub-microscopic. This may explain the high discrepancy between *Plasmodium* species detected by microscopy compared with PCR. Like for *P. falciparum*, ultra-sensitive molecular diagnostics that target multicopy genes will be relevant to assess the true

burden of non-falciparum malaria infections. The design of these will benefit from increased knowledge on the genomic diversity of these species across malaria endemic populations (30). This study did not detect *P. vivax* in the target populations, in spite of recent reports of *P. vivax* detection in Duffy-negative individuals in sub-Saharan Africa (31). Factors sustaining the low levels of *P. vivax* in some communities outside this study need to be determined. Therefore, studies to investigate and validate prevalence of non-falciparum species as well as associated risk factors in endemic populations need to be conducted.

In conclusion, the rate of non-falciparum species indicates that approaches for routine diagnosis in endemic settings should be encouraged to ascertain the true burden of these species as well as for administration of appropriate interventions towards pan-species malaria elimination.

Author Contributions

ECO conceptualized the study and is the principal investigator overseeing all aspects of the project, contributing to data curation, formal analysis and manuscript writing; AYO coordinated aspects of the investigation in the sites in Lagos; OO coordinated aspects of the investigation in the sites in Calabar; UDA and MM contributed to review and editing of the manuscript; AD contributed to funding of the research project and reviewing the manuscript; AAN contributed to conceptualization and supervision of the study, review and editing of the manuscript.

Competing Interests

The authors have no competing interests.

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Data Availability Statement

All data underlying the results are available as part of the article and no additional source data are required.

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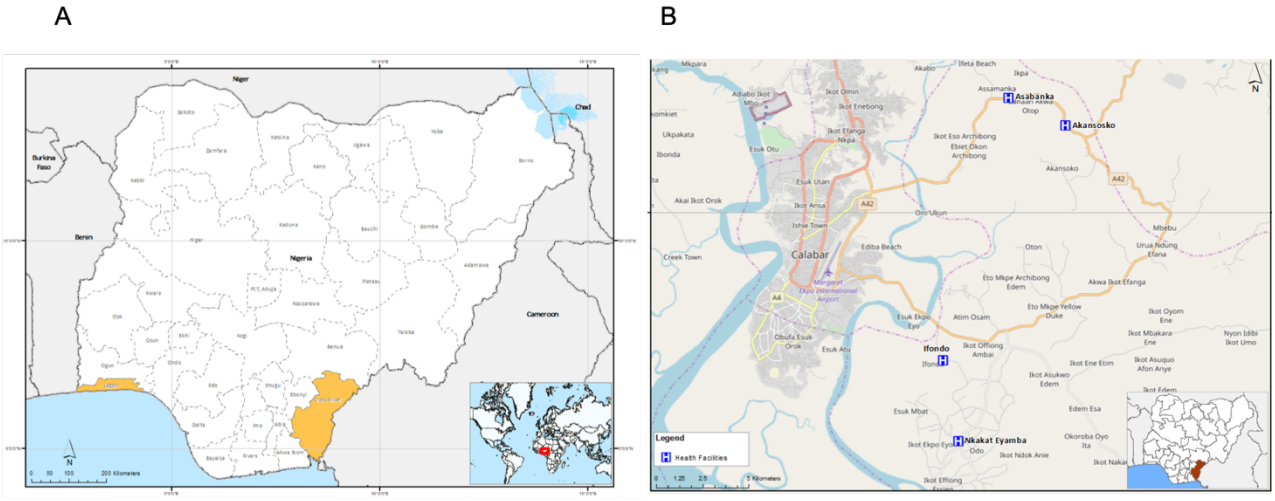
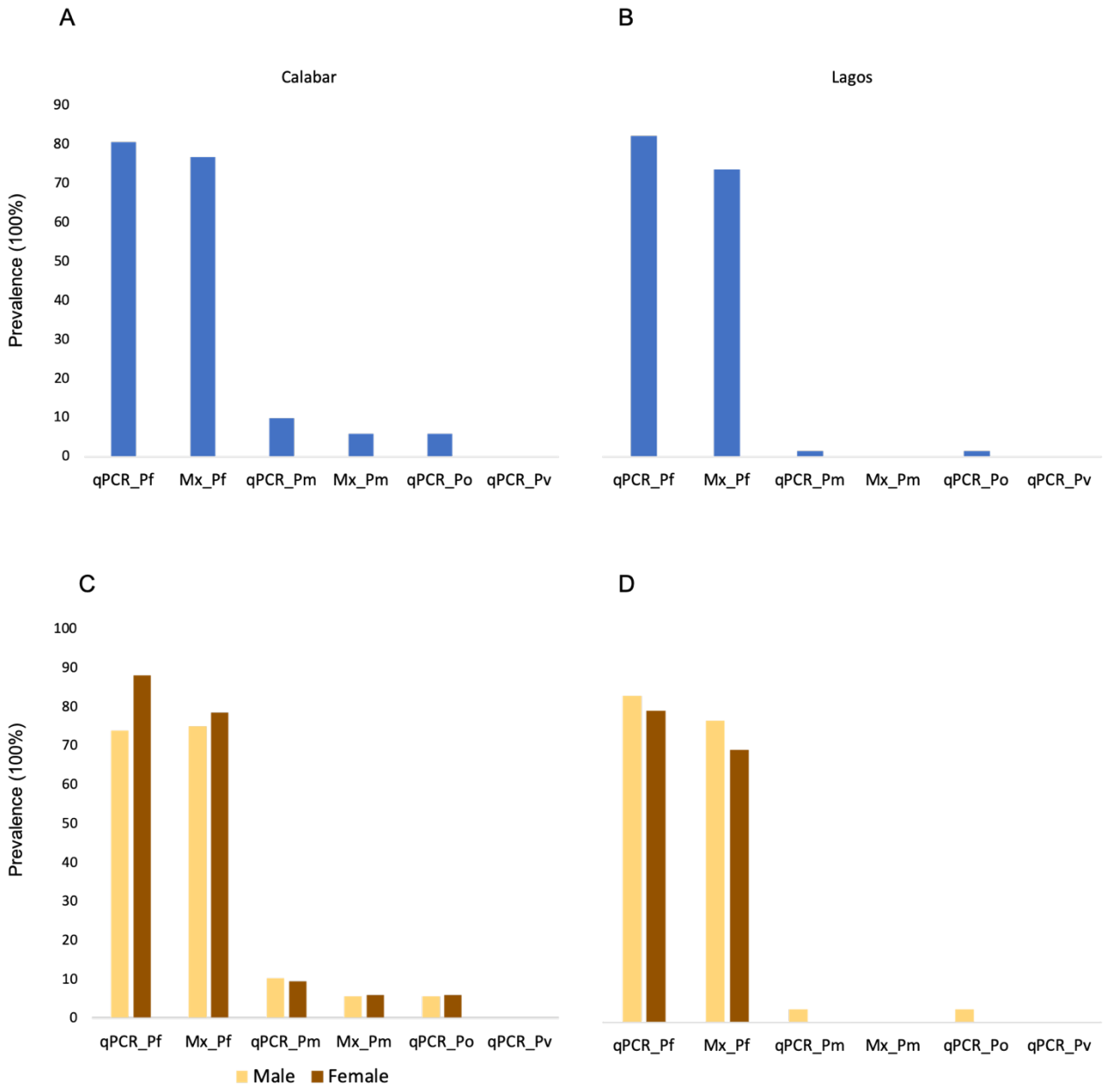


Figure 1: Map of Study sites in Nigeria showing A) Lagos (southwest) and Cross River (southeast) States; and B) Health facilities in Cross River State, where participants were resampled in 2018.


A
Calabar
Prevalence (100%)
90
80
70
60
50
40
30
20
10
0
qPCR_Pf
Mx_Pf
qPCR_Pm
Mx_Pm
qPCR_Po
qPCR_Pv
B
Lagos
qPCR_Pf
Mx_Pf
qPCR_Pm
Mx_Pm
qPCR_Po
qPCR_Pv
C
Prevalence (100%)
100
90
80
70
60
50
40
30
20
10
0
qPCR_Pf
Mx_Pf
qPCR_Pm
Mx_Pm
qPCR_Po
qPCR_Pv
Male
Female
D
qPCR_Pf
Mx_Pf
qPCR_Pm
Mx_Pm
qPCR_Po
qPCR_Pv

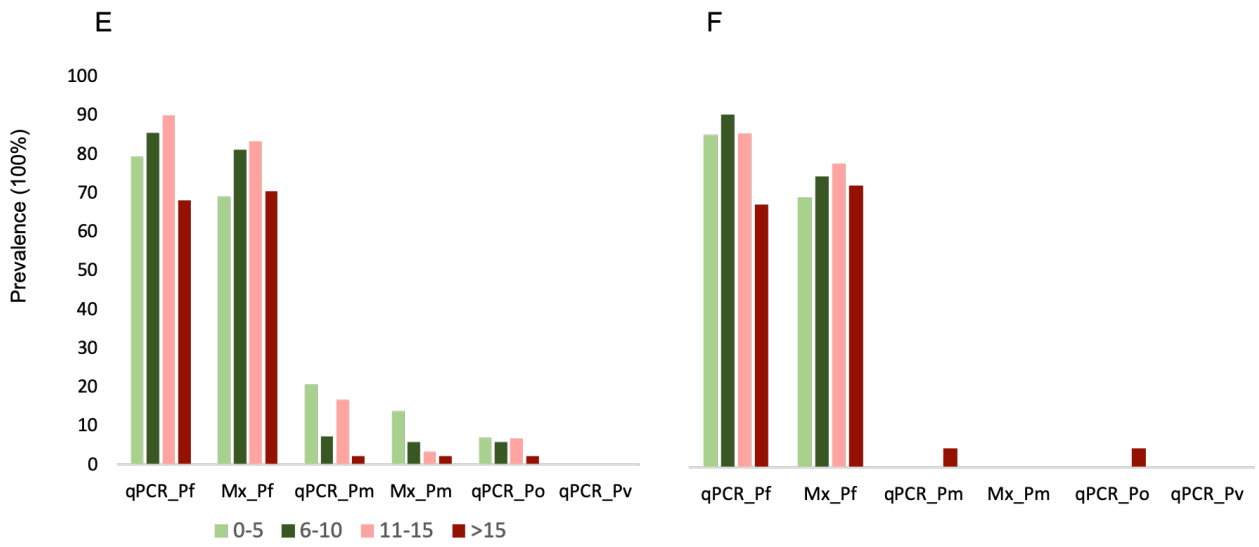
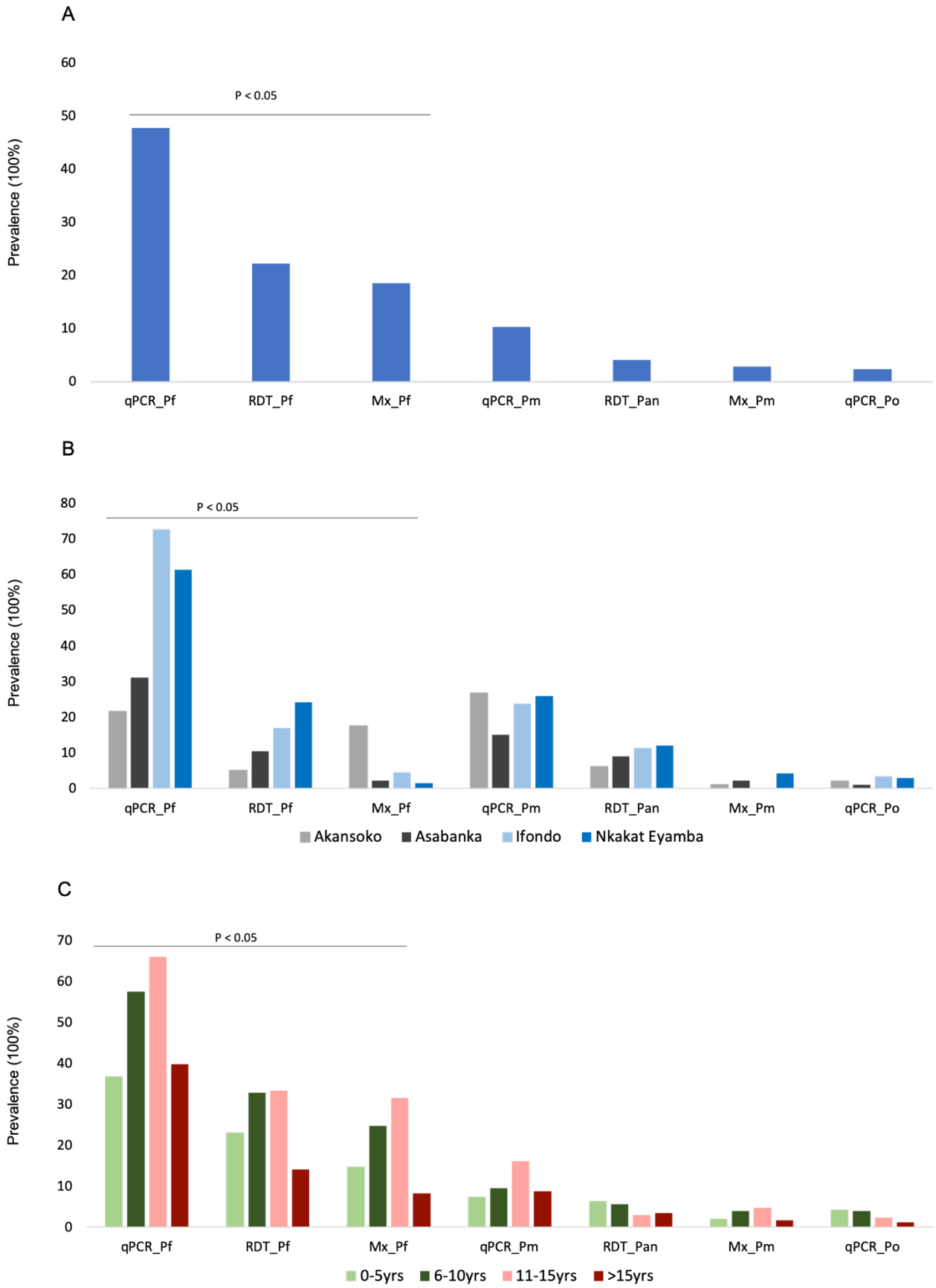


Figure 2: Prevalence of *Plasmodium* species in RDT positive individuals using different diagnostic methods shown overall in Lagos and Calabar (A and B), by gender (C and D) and by Age group (E and F). qPCR_Pf = *P. falciparum* detected by qPCR; Mx_Pf = *P. falciparum* detected by Microscopy; qPCR_Pm = *P. malariae* detected by qPCR; Mx_Pm = *P. malariae* detected by Microscopy; qPCR_Po = *P. ovale* detected by qPCR and qPCR_Pv = *P. vivax* detected by qPCR.


A
Prevalence (100%)
P < 0.05
qPCR_Pf
RDT_Pf
Mx_Pf
qPCR_Pm
RDT_Pan
Mx_Pm
qPCR_Po
B
Prevalence (100%)
P < 0.05
Akansoko
Asabanka
Ifondo
Nkakat Eyamba
C
Prevalence (100%)
P < 0.05
0-5yrs
6-10yrs
11-15yrs
>15yrs

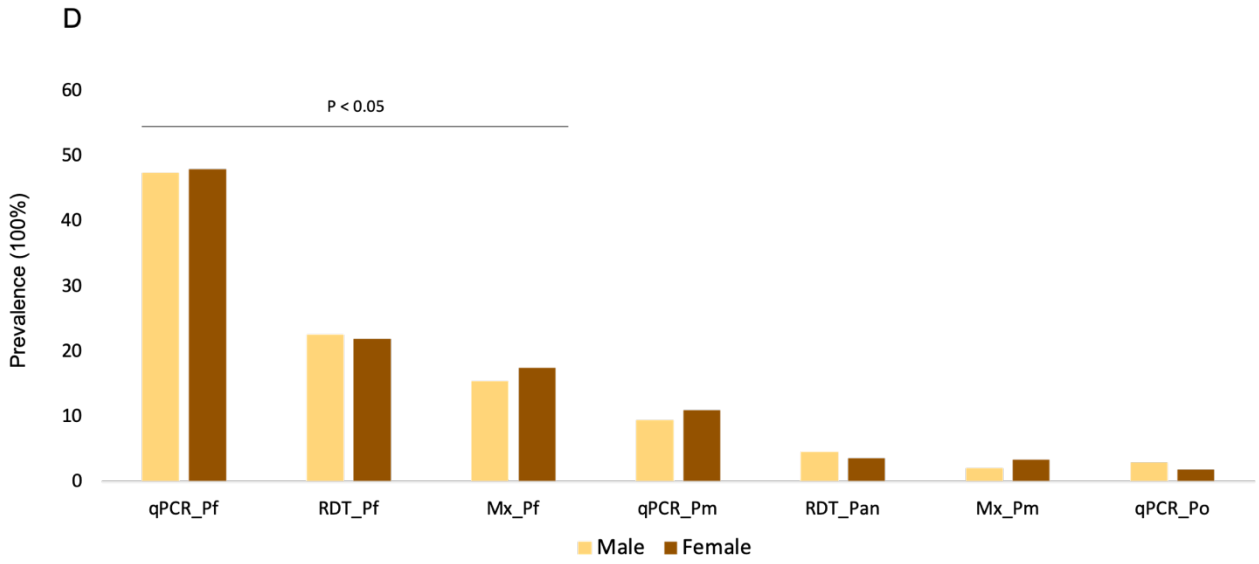


Figure 3: Prevalence of *Plasmodium* species by the different diagnostic methods (A) and further split by sites (B) Age groups (C) and Gender (D). qPCR_Pf = *P. falciparum* detected by qPCR; RDT_Pf = *P. falciparum* detected by RDT; Mx_Pf = *P. falciparum* detected by Microscopy; qPCR_Pm = *P. malariae* detected by qPCR; RDT_Pan = Pan species RDT; Mx_Pm = *P. malariae* detected by Microscopy; qPCR_Po = *P. ovale* detected by qPCR

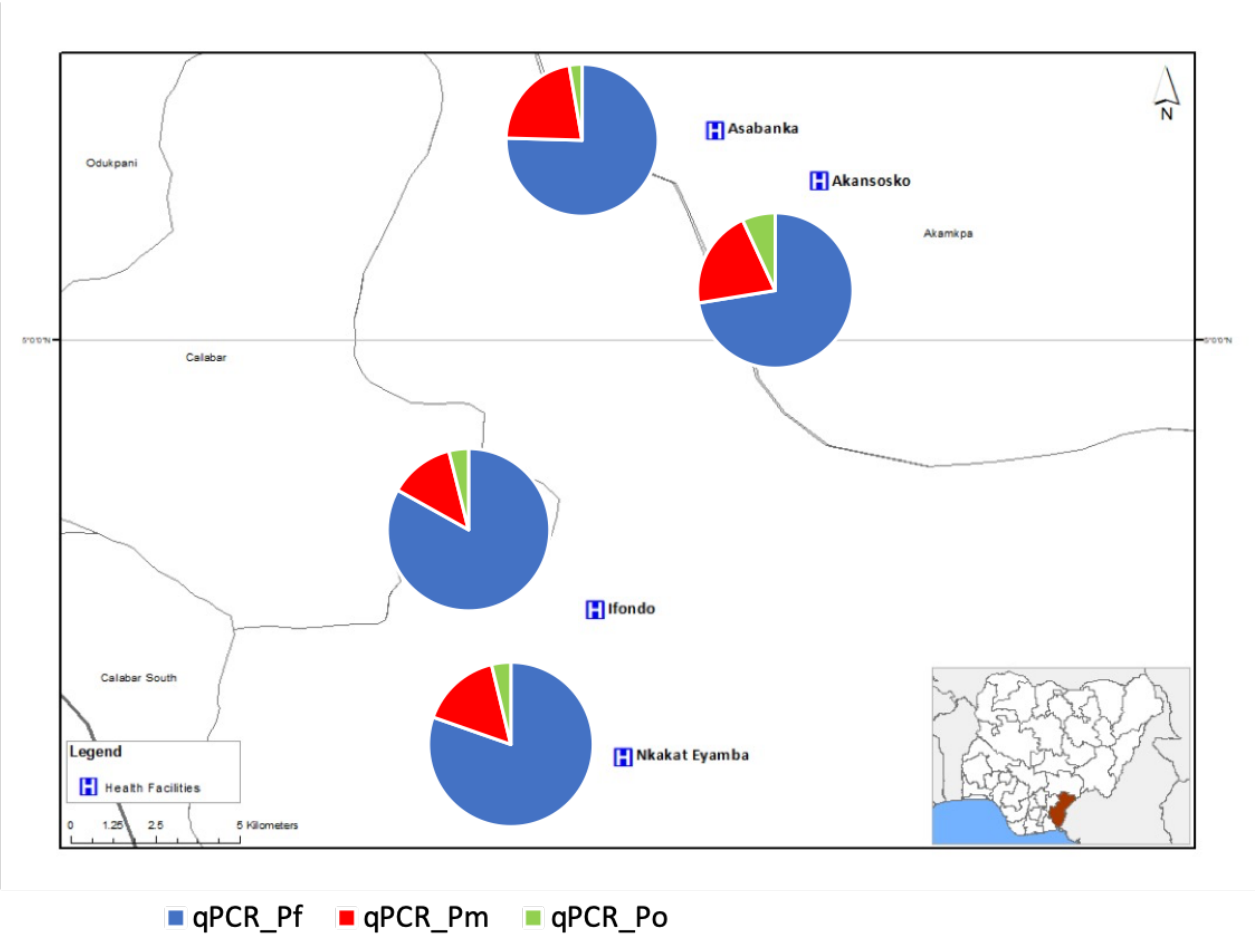


Figure 4: Map showing prevalence of *Plasmodium* species detected by qPCR in the four study sites. qPCR_Pf = *P. falciparum*; qPCR_Pm = *P. malariae*; qPCR_Po = *P. ovale*

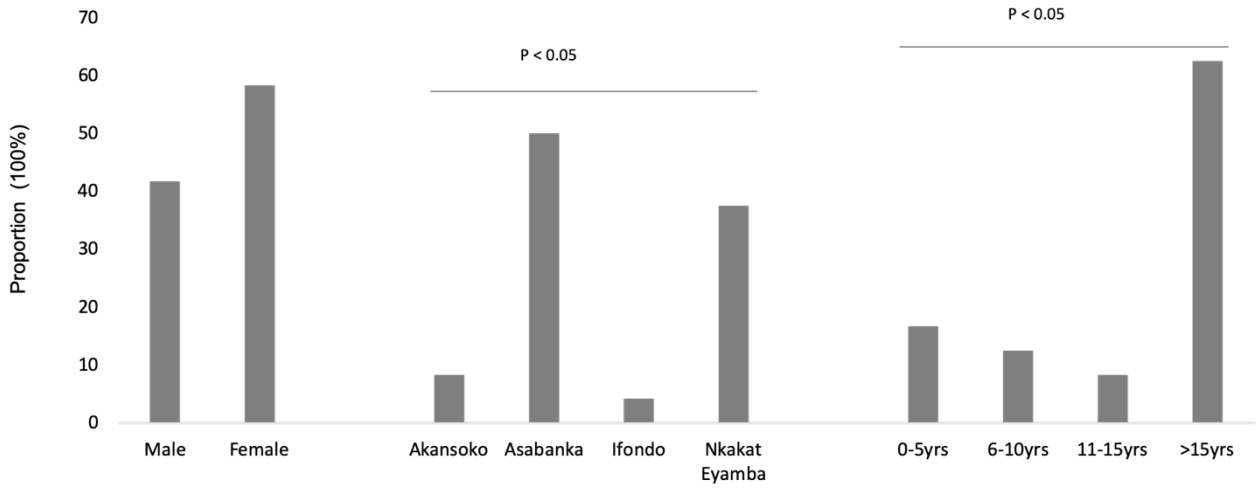


Figure 5: Proportion of *P. malariae* mono-infections detected by qPCR

Table 1: Primers and probes for molecular detection of *Plasmodium* species

Plasmodium spp	Primers and probe	Sequence 5′-3′
P. malariae	CCWR2K1_Fwd CCWR2K1_Rev CCWR2K1_Probe	TTCAGTCAGGATATGTAAAACAAAATTATTTAGGTA CCTACTTCCCCTTCACCATAAAACA TCGTCTAGTTCTATTACGTCATTTTC
P. ovale	CCRR9V5_Fwd CCRR9V5_Rev CCRR9V5_Probe	ACTCTTGGTTATTTGTCTGCACCTT CTATGTTACCATAAACAGGTTCTAAATCATCTGT TCAGTTGCTTCAACAAATTT
P. falciparum	*var*ATS_Fwd *var*ATS_Rev *var*ATS_Probe	CCCATACACAACCAAYTGGA TTCGCACATATCTCTATGTCTATCT TRTTCCATAAATGGT

Table 2: Summary of participants recruited in the preliminary survey in 2017

	Lagos	Calabar	Total
Total	71	172	243
Male	31	88	119
Female	40	84	124
0-5yrs	19	29	48
6-10yrs	19	69	88
11-15yrs	13	30	43
>15yrs	21	44	65

Table 3: Summary of participants recruited from the four sites in Calabar in 2018

	Akansoko	Asabanka	Ifondo	Nkakat Eyamba	Total
Total	97	266	88	347	798
Male	45	120	38	147	350
Female	52	146	50	200	448
0-5yrs	10	49	18	18	95
6-10yrs	6	71	9	39	125
11-15yrs	7	38	6	117	168
>15yrs	74	108	55	173	410

Table 4: Odds of *P. malariae* occurring as mono infections or mixed infections with other *Plasmodium spp.* in the different communities and age groups

	Mono infections	Mixed infections	Odds#	Odds ratio*
Akansoko	2	4	0.50	4.55
Asabanka	12	12	1.00	9.09
Ifondo	1	9	0.11	1.00
Nkakat Eyamba	9	33	0.27	2.45
0-5yrs	4	3	1.33	33.25
6-10yrs	3	9	0.33	8.25
11-15yrs	1	26	0.04	1.00
>15yrs	15	21	0.71	17.75

#Odds of *P. malariae* as a mono infection relative to as a mixed infection

*Odds ratio calculated relative to the least odds