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Development of Cooperative Primer-Based Real-Time PCR Assays for the Detection of *Plasmodium malariae* and *Plasmodium ovale*

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Plasmodium malariae and *Plasmodium ovale* are increasingly gaining public health attention as the global transmission of falciparum malaria is decreasing. However, the absence of reliable *Plasmodium* species-specific detection tools has hampered accurate diagnosis of these minor *Plasmodium* species. In this study, SYBR Green—based real-time PCR assays were developed for the detection of *P. malariae* and *P. ovale* using cooperative primers that significantly limit the formation and propagation of primers-dimers. Both the *P. malariae* and *P. ovale* cooperative primer-based assays had at least 10-fold lower detection limit compared with the corresponding conventional primer-based assays. More important, the cooperative primer-based assays were evaluated in a cross-sectional study using 560 samples obtained from two health facilities in Ghana. The prevalence rates of *P. malariae* and *P. ovale* among the combined study population were 18.6% (104/560) and 5.5% (31/560), respectively. Among the *Plasmodium*-positive cases, *P. malariae* and *P. ovale* mono-infections were 3.6% (18/499) and 1.0% (5/499), respectively, with the remaining being co-infections with *Plasmodium falciparum*. The study demonstrates the public health importance of including detection tools with lower detection limits in routine diagnosis and surveillance of nonfalciparum species. This will be necessary for comprehensively assessing the effectiveness of malaria interventions and control measures aimed toward global malaria elimination. *(J Mol Diagn 2021, 23: 1393—1403; https://doi.org/10.1016/j.jmoldx.2021.07.022)*

Human malaria is a life-threatening disease caused by five distinct *Plasmodium* species (namely, *Plasmodium falciparum, Plasmodium malariae*, *Plasmodium ovale, Plasmodium vivax*, and *Plasmodium knowlesi*).[1] Among these species, *P. falciparum* and *P. vivax* are the most prevalent and cause the most severe forms of the disease.[2,3] However, with the general global reduction in falciparum malaria, significant attention has been drawn toward the minor *Plasmodium* species: *P. malariae* and *P. ovale*.[4,5] Although these less prevalent species are generally associated with benign malaria,[6,7] recent reports have implicated them in major disease burden, such as severe anemia, acute respiratory distress syndrome, and acute renal failure.[8,9] Therefore, the availability of reliable tools for timely and accurate diagnosis of nonfalciparum malaria is necessary to inform appropriate treatment and effective management.

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Current methods for malaria diagnosis include microscopy, rapid diagnostic tests, and nucleic acid–based amplification tests (NAATs).[10] However, because of the morphologic similarities among *Plasmodium* species and the low parasite densities of *P. malariae* and *P. ovale* species in clinical isolates, both microscopy and rapid diagnostic tests remain unsatisfactory for routine detection of nonfalciparum *Plasmodium* species.[6,11,12] Efforts to address this diagnostic gap led to the development of highly sensitive and specific NAATs, including PCR and loop-mediated isothermal amplification.[12,13]

Several NAATs, involving the use of TaqMan probes and SYBR Green, have been developed for the detection of nonfalciparum species.[14–17] Although these NAATs have improved sensitivity, lower detection limits, and higher specificity compared with microscopy and rapid diagnostic tests,[13] the formation and propagation of primers-dimers remains a major sensitivity and specificity limiting factor, especially at low target concentration.[18,19] Attempts to address primers-dimers over the years led to the development of cooperative primers, which is the first technology that simultaneously curbs primer-dimer formation and propagation.[20] The cooperative primers were shown to significantly limit primer-dimer formation and propagation up to 2.5 million-fold compared with the conventional primers.[20]

A cooperative primer-based real-time assay [real-time quantitative PCR (qPCR)] has been developed for the detection of *P. falciparum*, and the assay was shown to have lower detection limit relative to its corresponding conventional primer-based assay.[20] In this study, SYBR Green–based qPCR assays were developed for the detection of *P. malariae* and *P. ovale* using cooperative primers that target the 18S ribosomal rRNA genes.

Materials and Methods

Ethical Approval

The study obtained ethical clearance from the ethics committees of the Ghana Health Service (GHSERC005/12/17), the Noguchi Memorial Institute for Medical Research (Institutional Review Board Certified Protocol Number 077/17-18), and the Kintampo Health Research Center (KHRCIEC/2018-10). A written informed consent was obtained from all participants and/or from parents or guardians of participants.

Development of *P. malariae* and *P. ovale* Cooperative Primer-Based qPCR Assays

The 18S rRNA gene sequences of *P. falciparum* (*XR_002273101.1*), *P. malariae* (*M54897.1*), *P. ovale curtisi* (*KF696371.1*), *P. ovale wallikeri* (*KF696364.1*), and *P. vivax* (*XR_003001225.1*) were retrieved from the National Center for Biotechnology Information database and aligned using Clustal Omega (*https://www.ebi.ac.uk/Tools/msa/clustalo*, last accessed May 12, 2021). Conserved genomic regions were selected for the design of the cooperative primers. Each cooperative primer consisted of a low melting temperature short primer and a capture sequence connected by two units of hexaethylene glycol (spacer 18). The process of annealing and extension of the cooperative primer has been previously described.[20] Attempts to develop assays consisting of both forward and reverse cooperative primers were unsuccessful for both *P. malariae* and *P. ovale*. Because neither the forward nor the reverse conventional primers, when used alone, would produce detectable primers-dimers,[21] a cooperative primer was paired with a conventional primer in both the *P. malariae* and *P. ovale* assays (Table 1). For *P. ovale* cooperative primer, one wobble base was introduced into capture sequence to ensure perfect complementarity to the two *P. ovale* subspecies: *P. ovale curtisi* and *P. ovale wallikeri*. The performance of the cooperative primers was compared with parallel assays containing the capture sequence of each cooperative primer adopted as the conventional primer and paired with the other conventional primer that was used in the cooperative assays (Table 1). The assays were compared using 10-fold serially diluted MRA-179 and MRA-180 plasmids (ATCC, Manassas, VA) for *P. malariae* and *P. ovale*, respectively, with concentrations ranging from 10^6 to 10^0 copies/μL. All primers used in the study were synthesized by Biosearch Technologies (Petaluma, CA).

SYBR Green–Based qPCR Assays

The *P. malariae* and *P. ovale* SYBR Green–based qPCR assays were performed on the QuantStudio5 system (Applied Biosystems, Waltham, MA). All reactions were performed in a total volume of 15 μL containing 1× Luna Universal qPCR Master Mix (New England BioLabs, Hitchin, UK), 250 nmol/L of each of the cooperative and the conventional primers, and 3 μL of the template DNA. The cycling conditions for both assays consisted of 3 minutes at 95°C, followed by 45 cycles of 15 seconds at 95°C, 40 seconds at 50°C, and 40 seconds at 60°C. The specificity of the qPCR products was determined by analyzing the amplicons using the melting curves and on 1.5% agarose gel. The resulting gel was processed using the Amersham Imager 600 (GE Healthcare Life Sciences, Chicago, IL).

Analytical Specificity and Limit of Detection

The analytical specificity of the assays was determined using the National Center for Biotechnology Information Primer-BLAST tool (*https://www.ncbi.nlm.nih.gov/tools/primer-blast*, last accessed on May 12, 2021). Experimental specificity was also determined using genomic DNA of *P. falciparum*, *P. malariae*, *P. ovale*,

Table 1 Sequence of Oligonucleotides for Real-Time Quantitative PCR Assays

Assay	Target	Primer code	Sequence
Cooperative	*Plasmodium malariae*	PlasmoF	5′-TTATGAGAAATCAAAGTCTTTGGGTT-3′
		MalR3_Coop	5′-AAAACATTCTAATATTTTAATCA[Sp18][Sp18]GGGAAAAGAACGT-3′
Cooperative	*Plasmodium ovale*	OvaF_Coop	5′-CTGYTCTTTGCATTCCTTAT[Sp18][Sp18]GCTTAGACAATA-3′
		Plasmo2*	5′-AACCCAAAGACTTTGATTTCTCATAA-3′
Conventional	*P. malariae*	PlasmoF	5′-TTATGAGAAATCAAAGTCTTTGGGTT-3′
		MalR3	5′-AAAACATTCTAATATTTTAATCA-3′
Conventional	*P. ovale*	Ova_F	5′-CTGYTCTTTGCATTCCTTAT-3′
		Plasmo2*	5′-AACCCAAAGACTTTGATTTCTCATAA-3′
	Plasmodium falciparum	Pf_stRNA_F†	5′-AAGTAGCAGGTCATCGTGGTT-3′
		Pf_stRNA_R†	5′-TTCGGCACATTCTTCCATAA-3′

*Primer sequence has been previously published by Rougemont et al.[15]
†Primer sequence has been previously published by Heinberg et al.[22]
[SP18], spacer 18.

and *P. vivax*. The limit of detection and efficiency were determined using a 10-fold serial dilution of MRA-179 and MRA-180 plasmids for *P. malariae* and *P. ovale*, respectively. Each plasmid was diluted to obtain 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 copies/μL in tris-ethylenediamine tetraacetic acid buffer. All assays were performed in triplicate.

Clinical Samples

To validate the cooperative primer-based assays for the detection of *P. malariae* and *P. ovale* in clinical isolates, whole blood samples were obtained from individuals who presented with suspected malaria at the Ewim Polyclinic in Cape Coast ($n = 178$) and the Richard Novati Catholic Hospital in Sogakope ($n = 382$) between December 2017 and December 2018. Cape Coast is located in the Central Region of Ghana (Google Map Plus Code: 4Q74+Q9 Cape Coast), whereas Sogakope is located in the Volta Region of Ghana (Google Map Plus Code: 2H5Q+C9 Sogakope). Both study sites are meso-endemic for malaria, with all-year malaria transmission that peaks during the June-July rainy season.[23–25] Venous blood was collected from individuals who consented to participate in the study.

Detection of *P. falciparum*, *P. malariae*, and *P. ovale* in Clinical Isolates

Genomic DNA was purified from 200 μL of the venous blood using the QIAamp DNA Mini Kit (Qiagen, Manchester, UK) following instructions from the manufacturer. DNA was eluted in a total volume of 100 μL using elution buffer provided by the manufacturer (Qiagen). The purified genomic DNA was stored at −20°C until ready for molecular analysis. Identification of *Plasmodium* species was first performed by microscopy. Thick and thin blood smears were prepared at the time of blood sample collection and stained with 10% Giemsa for microscopy examination. The number of parasite-infected red blood cells was determined per 500 white blood cells. Parasite count per microliter of blood was determined using the standard leukocyte count of 8000 leukocytes per microliter of blood, as previously described.[26] The *P. malariae* and *P. ovale* were detected using the SYBR Green cooperative primer-based assays described earlier in this study, whereas *P. falciparum* detection was performed using a previously described SYBR Green qPCR protocol with primers targeting *P. falciparum* seryl-tRNA synthetase (*PF3D7_0717700*).[22] The specificity of the qPCR amplicons was determined using melt curve analysis. The resulting C_T values for positive samples were used to estimate parasite copy number per microliter using standard curves obtained from 10-fold serially diluted plasmids.

Statistical Analysis

Data analysis was performed using IBM SPSS Statistics version 26 (International Business Machines Corporation, Armonk, NY), GraphPad Prism version 8.0.2, (GraphPad Software Inc., San Diego, CA), and Microsoft Excel 2016 Software (Microsoft Corporation, Redmond, WA). Probit analysis was used to estimate the limit of detection of the assays at 95% CI. Statistical significance for the proportion of positive samples between the two study sites was determined using χ^2 test or Fisher exact test, as appropriate. Parasite load across three or more groups was compared using the Kruskal-Wallis test, and where differences were observed, pair-wise comparisons were conducted using the *U*-test. Statistical significance for all analyses was considered at $P < 0.05$.

Results

Analytical Sensitivity, Specificity, and Limit of Detection

SYBR Green–based qPCR assays for the detection of *P. malariae* and *P. ovale* were developed using cooperative primers. Analysis of amplicons using melt curves (Figure 1,

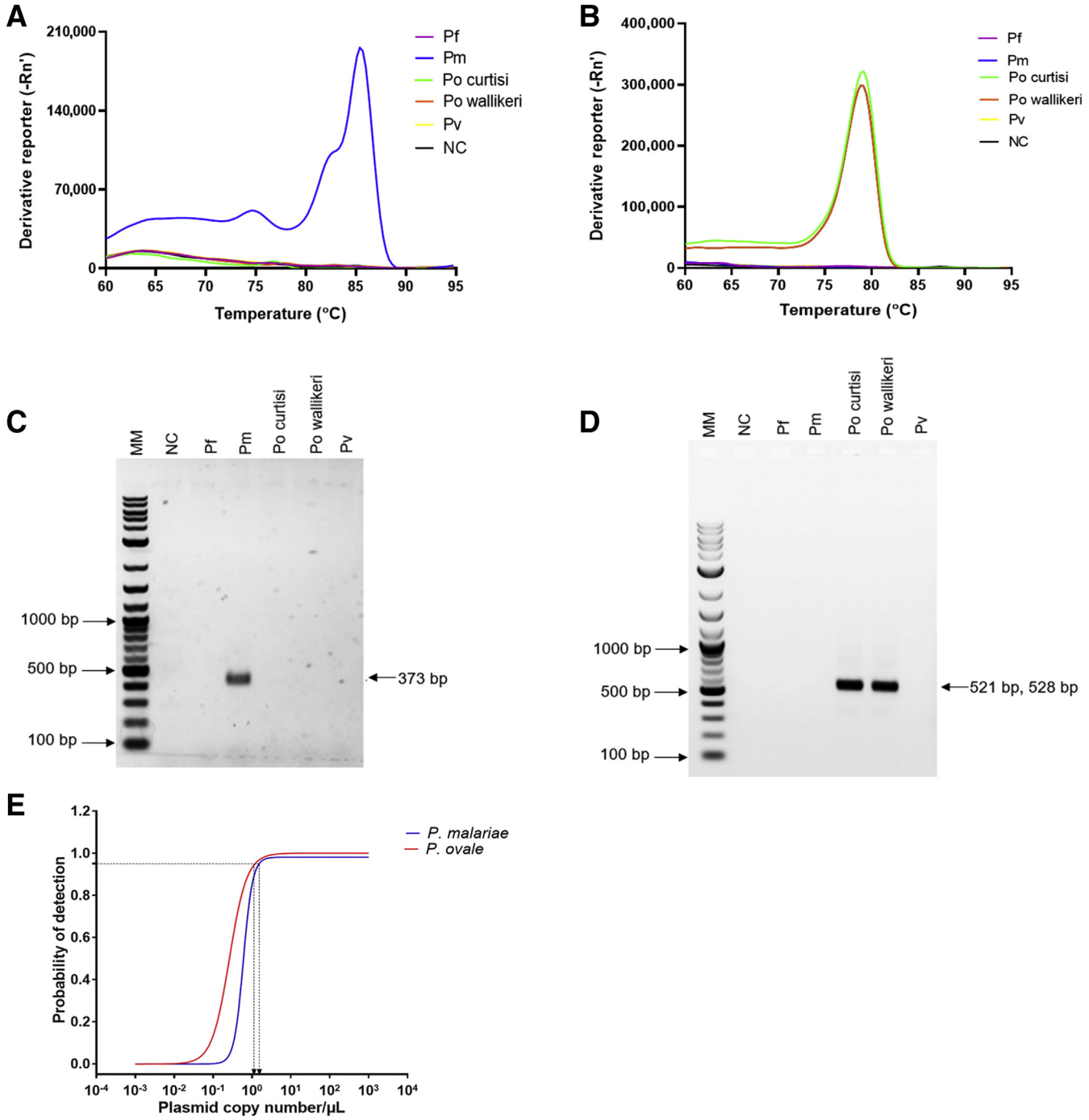

Figure 1 The specificity and detection limits of *Plasmodium malariae* and *Plasmodium ovale* assays. Assays were performed using *Plasmodium falciparum* (Pf), *P. malariae* (Pm), *P. ovale curtisi* (Po curtisi), *P. ovale wallikeri* (Po wallikeri), and *Plasmodium vivax* (Pv) genomic DNA. **A:** Melt curve for *P. malariae* (blue). **B:** Melt curves for *P. ovale curtisi* (green) and *P. ovale wallikeri* (orange) assays. Other colors represent the *P. falciparum*, *P. vivax*, and nontemplate control (NC). **C:** Separation of the resulting *P. malariae* real-time quantitative PCR (qPCR) amplicons on 1.5% agarose gel. **D:** Separation of the resulting qPCR amplicons for *P. ovale* subspecies on 1.5% agarose gel. Expected amplicon sizes for *P. ovale curtisi* and *P. ovale wallikeri* were 528 and 521 bp, respectively. **E:** The detection limits for *P. malariae* (blue curve) and *P. ovale* (red curve) assays were determined using a 10-fold serial dilution of plasmids. Plasmid concentrations were log-transformed and then analyzed using probit analysis. The probability of detection was plotted against the plasmid copy numbers/µL of the DNA. Molecular weight marker (MM) shown in bp.

A and B) and gel electrophoresis (Figure 1, C and D) showed that both assays were specific to the selected 18S rRNA genomic region with no cross-reactivity to other *Plasmodium* species. The melting temperature values of the assays were 85.60 ± 0.46°C and 79.44 ± 0.17°C for *P. malariae* and *P. ovale*, respectively (Table 2). Using 10-fold

Table 2 Real-Time Quantitative PCR Assay Details and Efficiencies

Assay	Slope	Intercept	R^2	Efficiency, %	Amplicon length, bp	Melting temperature, °C*
Plasmodium malariae	−4.4	39.10	0.99	68.1	373	85.60 ± 0.46
Plasmodium ovale	−4.0	38.73	0.99	76.9	528†, 521‡	79.44 ± 0.17

*Mean melting temperature for technical replicates.
†Expected qPCR amplicon length for *P. ovale curtisi*.
‡Expected qPCR amplicon length for *P. ovale wallikeri*.

serially diluted plasmids, the detection limits estimated at 95% confidence level for the *P. malariae* and *P. ovale* assays were 1.0 plasmid copy/μL (95% CI, 0.94−1.06) and 1.0 plasmid copy/μL (95% CI, 0.96−1.04), respectively (Figure 1E). The amplification efficiencies of the assays were 68.1% and 76.9% for *P. malariae* and *P. ovale*, respectively (Table 2).

Comparison of the Cooperative and Conventional Primers

To evaluate the performance of the cooperative primer-based assays, the cooperative primers were compared with their corresponding conventional primers using plasmids. The C_T values observed for the conventional primer were relatively lower compared with its corresponding cooperative primers (Table 3). Despite this observation, the cooperative primer-based assays detected as low as 1.0 copy/μL, whereas the conventional primer-based assays had a detection limit of 10.0 copies/μL for both the *P. malariae* and *P. ovale* assays (Table 3). Separation of the resulting qPCR amplicons on a 1.5% agarose gel showed primers-dimers as the concentration of *P. malariae* and *P. ovale* decreased for the conventional assays (Figure 2). On the contrary, no observable primers-dimers were generated for both the *P. malariae* and *P. ovale* cooperative primer-based assays, even at the lowest concentration of 1.0 copy/μL (Figure 2). Taken together, the data suggest that the cooperative primer-based assays have 10-fold lower limit of detection compared with using conventional primers.

Prevalence of *P. falciparum*, *P. malariae*, and *P. ovale* among Study Participants

The prevalence of *P. falciparum*, *P. malariae*, and *P. ovale* among study participants ($n = 560$) (Table 4) was assessed by microscopy and SYBR Green−based qPCR assays. The overall prevalence rates of *Plasmodium* species infection by microscopy were 67.4% (120/178) and 66.8% (255/382) in Ewim and Sogakope, respectively (Figure 3A). Using qPCR, the prevalence rates of *Plasmodium* species infection in Ewim and Sogakope were 91.6% (163/178) and 88.0% (336/382), respectively (Figure 3A). For species identification by microscopy, the prevalence rates of *P. falciparum*, *P. malariae*, and *P. ovale* for the combined study population were 66.4% (372/560), 2.5% (14/560), and 1.1% (6/560), respectively (Figure 3B). As expected for qPCR, higher prevalence was observed for each of the three *Plasmodium* species compared with microscopy. The prevalence rates of *P. falciparum*, *P. malariae*, and *P. ovale* using qPCR were 85.6% (479/560), 18.5% (104/560), and 5.5% (31/560), respectively (Figure 3B).

A total of 3.2% (18/560) of the participants were found to be negative by both microscopy and qPCR for all the three *Plasmodium* species. For discrepancies between microscopy and qPCR, 7.5% (42/560) of the microscopy-positive participants were qPCR negative for the three *Plasmodium* species. Also, 29.8% (167/560) of the qPCR-positive participants were undetected by microscopy. Among the qPCR-positive participants who were undetected by microscopy, 76.6% (128/167), 2.4% (4/167), and 1.8% (3/167) were found to harbor mono-infections for *P. falciparum*,

Table 3 Comparison of C_T Values for Cooperative and Conventional Primer Assays

	Plasmodium malariae		*Plasmodium ovale*	
Plasmid copies/μL	Conventional	Cooperative	Conventional	Cooperative
10^6	9.83 ± 0.07	11.15 ± 0.15	12.94 ± 0.01	13.70 ± 0.04
10^5	13.57 ± 0.18	17.53 ± 0.28	16.60 ± 0.21	17.38 ± 0.07
10^4	16.96 ± 0.39	21.90 ± 0.36	21.46 ± 0.11	22.65 ± 0.05
10^3	21.22 ± 0.38	27.22 ± 0.25	26.11 ± 0.15	27.46 ± 0.13
10^2	25.31 ± 0.22	33.00 ± 0.45	28.15 ± 0.13	29.65 ± 0.14
10^1	27.35 ± 0.09	38.28 ± 0.36	32.75 ± 0.16	34.20 ± 0.29
10^0	Negative	41.94 ± 0.54	Negative	38.77 ± 2.15

Data are expressed as means ± SD.

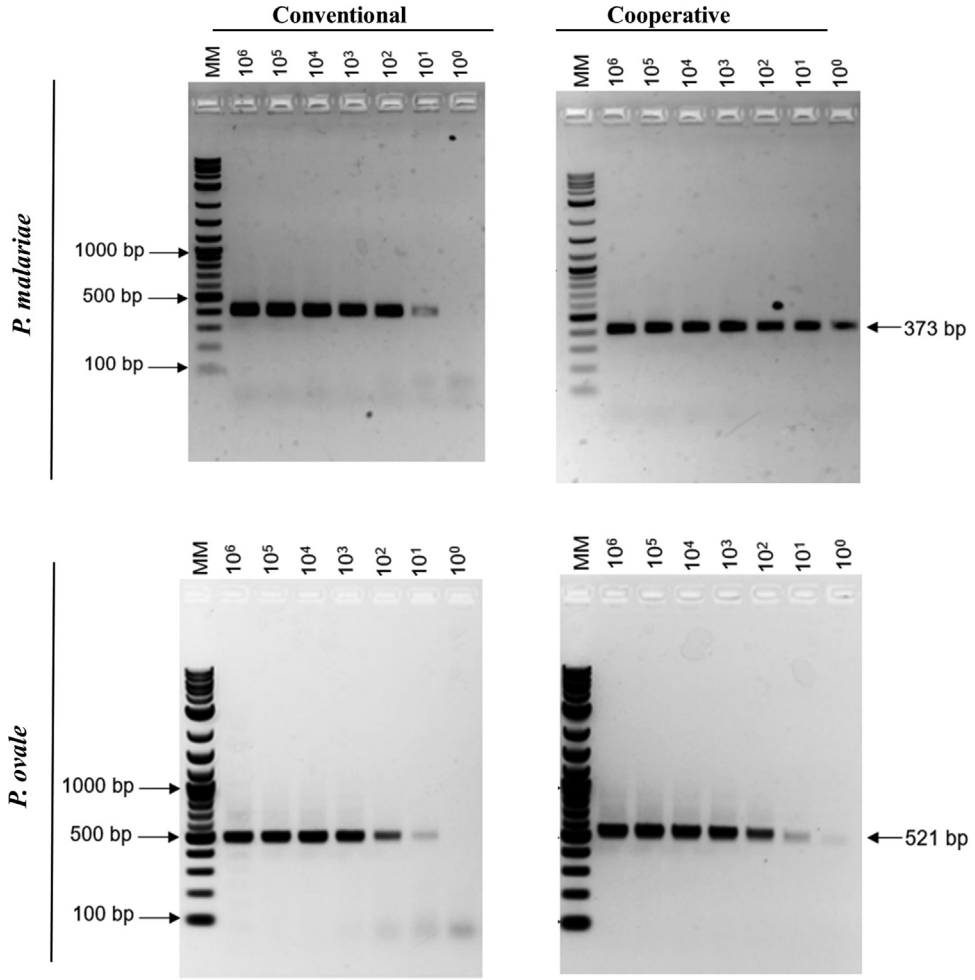

Figure 2 Comparison of cooperative and conventional real-time quantitative PCR (qPCR) assays. Assays were performed using serially diluted *Plasmodium malariae* (MRA-179) and *Plasmodium ovale* (MRA-180) plasmids. The qPCR amplicons were separated on 1.5% agarose gel. Molecular weight marker (MM) shown in bp.

P. malariae, and *P. ovale,* respectively, whereas the remaining 19.2% (32/167) harbored mixed infections of two or three *Plasmodium* species.

Among the qPCR-positive cases, *P. falciparum*, *P. malariae*, and *P. ovale* mono-infections in Ewim were 74.2% (121/163), 3.7% (6/163), and 3.1% (5/163), respectively (Figure 3C). In Sogakope, the prevalence rates of *P. falciparum*, *P. malariae*, and *P. ovale* mono-infections were 74.7% (251/336), 1.8% (6/336), and 0.0% (0/336), respectively (Figure 3C). There were no statistically significant differences in the distribution of *P. falciparum* ($P = 0.91$) and *P. malariae* ($P = 0.20$) mono-infections between the two sites. The proportions of *P. falciparum/P. malariae* mixed infection in Ewim and Sogakope were 14.7% (24/163) and 17.9% (60/336), respectively, whereas *P. falciparum/P. ovale*, *P. malariae/P. ovale*, and *P. falciparum/P. malariae/P. ovale* mixed infections were all <5.0% in both study sites (Figure 3C). Study participants were further stratified into five age groups, as previously described[14]; however, participants aged ≤5 years were classified as one group, and the prevalence of *Plasmodium* species was determined. The *P. falciparum* infection was highest among the age group of 6 to 10 years, whereas *P. malariae* infection was highest among age group of 11 to 20 years (Figure 3D). The *P. ovale* infection was found to be generally increasing with age (Figure 3D).

Table 4 Demographic Characteristics of Study Participants

Characteristic	Study site		Total
	Ewim	Sogakope	
Sample size, *n*	178	382	560
Sex, *n* (%)			
Female	94 (52.8)	177 (46.3)	271
Male	84 (47.2)	205 (53.7)	289
Age group, years, *n* (%)			
≤5	44 (24.7)	93 (24.3)	137
6–10	56 (31.5)	79 (20.7)	135
11–20	45 (25.3)	97 (25.4)	142
21–40	13 (7.3)	69 (18.1)	82
>41	20 (11.2)	44 (11.5)	64
Hemoglobin, g/dL*	10.6 (0.2)	10.3 (0.1)	

*Data presented as mean (SEM).

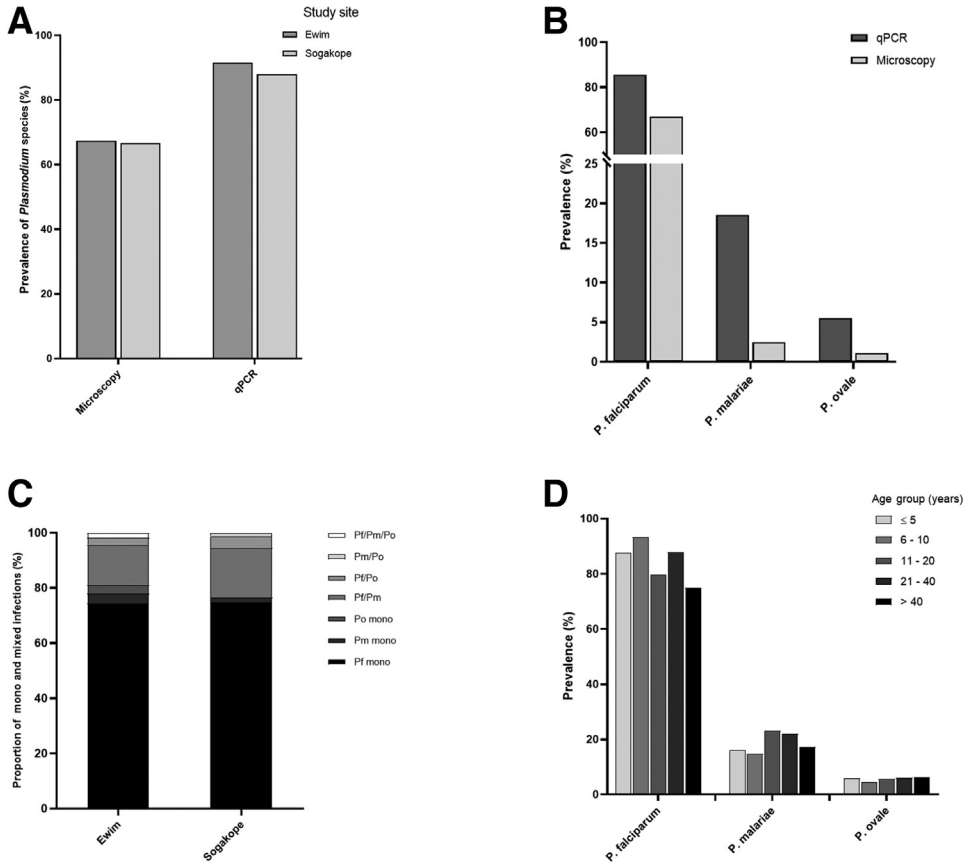

Figure 3 Prevalence of *Plasmodium* species among study participants. **A:** The prevalence of genus *Plasmodium* determined by microscopy and real-time quantitative PCR (qPCR) in Ewim and Sogakope. **B:** The prevalence of *Plasmodium falciparum* (Pf), *Plasmodium malariae* (Pm), and *Plasmodium ovale* (Po) determined by SYBR Green—based qPCR assays and microscopy among the combined study population. **C:** The proportion of *Plasmodium* species mono and mixed infections among study participants in the two study sites. **D:** The prevalence of *Plasmodium* species positive cases by age group (in years). The prevalence was determined by expressing the total number of positive cases as a percentage of the total sample size for each of the stratified age groups. $n = 560$ study participants (**A**); $n = 178$ in Ewim (**A**); $n = 382$ in Sogakope (**A**).

Quantification of Parasite Copy Number

The copy numbers of *P. falciparum*, *P. malariae*, and *P. ovale* were estimated for the positive clinical samples. Using qPCR, the median parasite loads of *P. falciparum* were significantly higher than those for *P. malariae* ($P < 0.0001$) and *P. ovale* ($P < 0.0001$). However, there was no significant difference between *P. malariae* and *P. ovale* parasite loads ($P = 0.16$) (Figure 4A). Across the stratified age groups, the median *P. falciparum* copy number among age group of 6 to 10 years was significantly higher compared with those for the age group of 0 to 5 years ($P = 0.04$) and the age group of 21 to 40 years ($P = 0.0002$) (Figure 4B). On the other hand, differences in the parasite loads for both *P. malariae* and *P. ovale* among the age groups did not reach statistical significance ($P > 0.05$ for both comparisons) (Figure 4B). Finally, the qPCR C_T values were correlated with parasitemia, as determined by microscopy (Figure 4C). As expected, there was a significant negative correlation between the qPCR C_T values and the log-transformed parasitemia ($r = -0.36$; $P < 0.001$), although the association was not strong.

Discussion

Because of the limited geographic distribution and marginal contribution of *P. malariae* and *P. ovale* subspecies toward global malaria burden, these nonfalciparum species have not received much attention.[27] In 2018, the estimated non-falciparum malaria cases in sub-Saharan Africa were <1% of all malaria cases.[28] However, it is generally thought that the prevalence of these nonfalciparum species has been largely underestimated because of the lack of reliable diagnostic tools.[29,30] In addition, the few cases of *P. malariae* and *P. ovale* subspecies are usually detected as low density and mixed infections with the dominant *P. falciparum*, which further present an obstacle to routine diagnostic tools with poor sensitivity and limited specificity.[31–33] As such, there is the need for reliable diagnostic tools to accurately assess the burden of nonfalciparum species. In this study, cooperative primers were used to develop qPCR assays with improved detection limits for the detection of low-density *P. malariae* and *P. ovale* infections in clinical isolates.

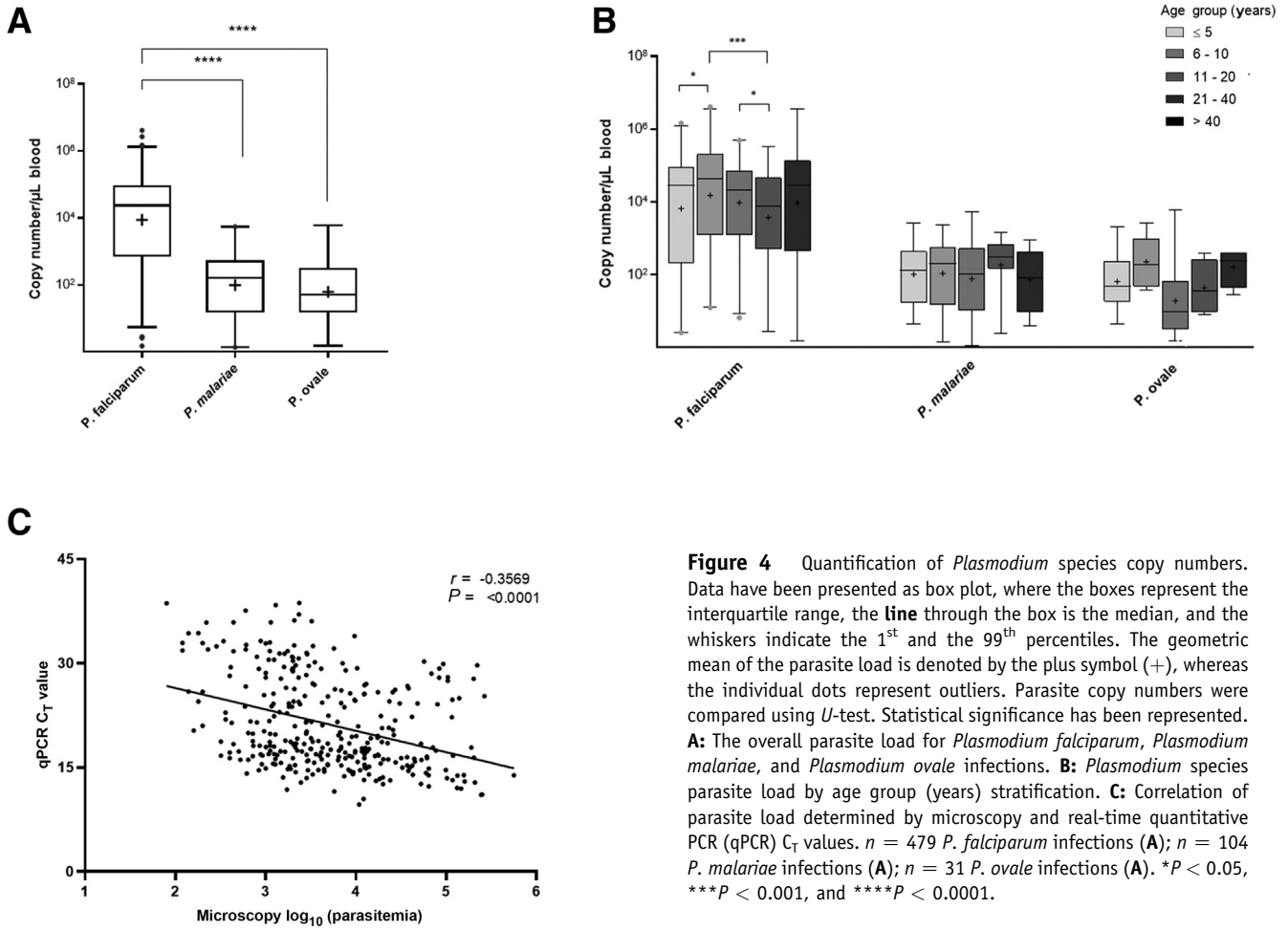

Figure 4 Quantification of *Plasmodium* species copy numbers. Data have been presented as box plot, where the boxes represent the interquartile range, the **line** through the box is the median, and the whiskers indicate the 1st and the 99th percentiles. The geometric mean of the parasite load is denoted by the plus symbol (+), whereas the individual dots represent outliers. Parasite copy numbers were compared using *U*-test. Statistical significance has been represented. **A:** The overall parasite load for *Plasmodium falciparum*, *Plasmodium malariae*, and *Plasmodium ovale* infections. **B:** *Plasmodium* species parasite load by age group (years) stratification. **C:** Correlation of parasite load determined by microscopy and real-time quantitative PCR (qPCR) C_T values. $n = 479$ *P. falciparum* infections (**A**); $n = 104$ *P. malariae* infections (**A**); $n = 31$ *P. ovale* infections (**A**). $^*P < 0.05$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

One of the major obstacles that limits the specificity, sensitivity, and detection limit of NAATs is the formation and propagation of non-specific products, such as primers-dimers, which result in false negatives or false positives.[18] Although several technologies have been described to mitigate this challenge, cooperative primers were the first technology that was shown to simultaneously inhibit the formation and the propagation of primers-dimers up to 2.5 million-fold compared with conventional primers.[20] This study describes the first report on the application of cooperative primers for the detection of nonfalciparum species. The data presented herein suggest that the cooperative primers had relatively higher C_T values than their corresponding conventional primers for a given concentration of target DNA. A possible explanation is that because the amplification process for the cooperative primer requires an initial binding of the cooperative sequence before the binding of the short low melting temperature primer to its complementary sequence, it is likely that this additional time may be a lagging phase that accounts for the differences in the C_T values between the cooperative and the conventional primers.[20] Notwithstanding this observation, the results show that the cooperative primers have at least 10-fold lower detection limit compared with their corresponding conventional primers. The lower detection limit of the cooperative primer-based assays may be explained by the ability of the cooperative primers to limit primer-dimer formation.[20,34]

In Ghana, *P. falciparum*, *P. malariae*, and *P. ovale* are the three *Plasmodium* species that have been implicated in clinical malaria.[35] The estimated national prevalence rates of *P. falciparum*, *P. malariae*, and *P. ovale* are 90% to 98%, <10%, and <2%, respectively.[35] However, different studies across various regions in Ghana have reported varying prevalence rates for the three *Plasmodium* species.[36–38] The data presented herein by microscopy show that the prevalence rates of *P. malariae* and *P. ovale* are comparable to the national prevalence.[35] Using qPCR analysis, the prevalence rates of *P. falciparum*, *P. malariae*, and *P. ovale* among the study population were 85.5%, 18.5%, and 5.5%, respectively. These prevalence rates of *P. malariae* and *P. ovale* are comparable to those in a

previous report,[36] but are about twofold higher than the reported national prevalence,[35] and higher than rates in studies conducted elsewhere in the country.[37,38] The higher prevalence reported in this study maybe due to the lower detection limits of the cooperative primer-based assays, even though these studies involve different study populations. An undetected population harboring nonfalciparum species is of great concern because these individuals potentially serve as parasite reservoir for sustained and long-term transmission of *P. malariae* and *P. ovale* subspecies.

In high transmission settings, malaria incidence generally peaks in the first few years and then declines and levels off in the later years.[39,40] Consistent with these reports, the results show that *P. falciparum* prevalence peaks at the age of 6 to 12 years and subsequently declines with increasing age. For nonfalciparum species, *P. malariae* was found to be most common among participants aged 11 to 20 years, whereas *P. ovale* prevalence was similar across the age groups. Other studies in Ghana,[38] Senegal,[41] and Kenya[33] also observed that children aged <15 years had a relatively higher risk of *P. malariae* infection than adults. Another study in Ghana found no *P. ovale* infection among participants aged ≥10 years, but found infection in younger participants.[36] In Indonesia, it was also observed that the older population, with a median age of 21 years, had higher *P. malariae* infections compared with the younger population.[35,42] These differences in the distribution of nonfalciparum species among different age groups could be due to underrepresentation of the different age groups and differences in malaria transmission intensity across the various study sites.[33,43]

The *P. malariae* and *P. ovale* infections are usually detected as coinfections with *P. falciparum*.[6,44–46] In areas of high transmission, *P. falciparum* has been reported to suppress the prevalence and the density of nonfalciparum species.[6,47,48] Consistent with previous reports,[33,36,38,44] the results show that the parasite density of both *P. malariae* and *P. ovale* parasites in all the mixed infection cases was lower than *P. falciparum*. Low density of nonfalciparum species in cases of mixed infection with dominant *P. falciparum* is likely to result in misdiagnosis and affect appropriate treatment recommendations.[49] Nevertheless, it could be argued that because artemisinin combination therapies are generally recommended for the treatment of both uncomplicated *P. falciparum* and nonfalciparum malaria,[50] such misdiagnosis would be of less importance for antimalarial treatment. However, with the recent reports of the increasing prevalence of nonfalciparum species despite artemisinin combination therapy treatment, accurate detection is necessary.[36,51–54]

In summary, the study shows at least twofold higher prevalence of *P. malariae* and *P. ovale* among study participants compared with the national prevalence in Ghana. This underlines the need for the employment of such detection tools with lower detection limits to accurately assess the burden of nonfalciparum species. The turnaround time of the current cooperative primer-based assays is comparable to conventional qPCR assays. However, it is also important to highlight that the cost of cooperative primers is relatively higher than their corresponding conventional primers. Notwithstanding the cost, the deployment of such detection tools will be helpful for reliable diagnosis and accurate surveillance of nonfalciparum species in a holistic approach toward malaria elimination.

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Author Contributions

F.A., P.K., Y.A., and G.A.A. conceived and designed the study; F.A., J.S., D.D., N.G.A., S.O.B., B.K.S.D., A.S., P.K., and J.D.C. collected data and performed the experiments; F.A. and Y.A. analyzed the data; F.A. drafted the manuscript; Y.A. and G.A.A. thoroughly edited the manuscript draft; Y.A., P.K., L.A.E., and G.A.A. supervised the study; all authors reviewed and approved the manuscript.

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