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RESEARCH ARTICLE

# Molecular profiling of human malaria parasites among individuals suspected for malaria in three geo-ecological facets of malaria transmission in Cameroon.

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Innocent Mbulli Ali[1,2], Keming Eva Mai[1], Marcel Nyuylam Moyeh[3], Indira Lontsie Saha[1], Darlin Bean Kaunda Nguene[1], Valery Pacome Tchuenkam[1], Giresse Lemogo[1], Mariama Mbouh[1], Arsene Dombou[1], Miriam Ngum Niba[1], Onesime Djerassem[1], Colette Noudou Nathan Belinga[1], Guillaume Ndjiofack Nguefack[1], Richard Folefock Ajua[4], Gustave Simo[1], Michel Noubom[5], Albert Zeh Meka[6], Leke Rose[2]

[1]Biochemistry, University of Dschang, Dschang, West region, 00000, Cameroon
[2]The Biotechnology Centre, The Biotechnology Centre, University of Yaounde 1, Yaounde, Centre region, 00000, Cameroon
[3]Biochemistry and Molecular Biology, University of Buea, Buea, Southwest, 00000, Cameroon
[4]Regional Technical Group for Malaria Control, Regional Delegation of Public Health West, Bafoussam, West Region, 00000, Cameroon
[5]Faculty of Medicine and Pharmaceutical Sciences, University of Dschang, Dschang, West Region, 00000, Cameroon
[6]National Malaria Control Program, Ministry of Public Health ,Cameroon, Yaounde, Centre Region, 0000, Cameroon

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## Abstract

Malaria still remains a major public health problem in Cameroon. Although *P. falciparum* is the predominant *plasmodium* species in the country, there is growing evidence of non-falciparum malaria especially vivax malaria which was previously thought to be absent. This suggests these infections are being underdiagnosed. However, there is limited data on the distribution of malaria parasite species in circulation in Cameroon posing a challenge in the true estimation of malaria burden. As a consequence, further investigation of the local epidemiology of malaria in Cameroon is important.

A cross-sectional study was conducted from June 2022 to August 2023 in three geo-ecological regions of Cameroon (Sahelian zone, Humid Forest and Western Highlands). Blood samples were collected from individuals suspected of malaria and dried blood spots (DBS) prepared. DNA was extracted from a total of 1390 DBS using Chelex-100 method and tested by nested PCR targeting the species-specific

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1. **Cynthia Awuor Odhiambo**, Kenya Medical Research Institute, Nairobi, Kenya

Any reports and responses or comments on the article can be found at the end of the article.

18srRNA gene.

A high prevalence of malaria (682/1390, 49.06%) was recorded. Single species infection accounted for 651/682 cases (95.45%) with *P. falciparum* mono infection being the most represented across all study sites (630/682, 92.38%). The proportion of all non-falciparum infections was 8.36% (57/682) and *P. ovalecurtisi* was the most frequent (20/57) followed by *P. ovale wallikeri* (18/57). P. malariae mono infection was the most common non-falciparum mono infection. *P. vivax* was detected exclusively as a coinfection with *P. falciparum* (2/682; 0.29%). Coinfections accounted for 31/682 cases (4.55%) with *P.falciparum*/*P. ovalecurtisi* identified as the most frequent. Significant determinants of malaria infection included age and geo-ecological zone. The age group 10-20 years was the most susceptible to malaria infection (AOR = 1.87, 95% CI = [1.06-3.30], p.value = 0.03) and the Sahelian zone the most at risk (AOR = 1.97, 95%CI = [1.41-2.70], p.value < 0.001).

**Keywords**
Malaria, nested PCR, non-falciparum species, geo-ecological zones, Cameroon.

This article is included in the European & Developing Countries Clinical Trials Partnership (EDCTP) gateway.

**Corresponding author:** Innocent Mbulli Ali (dr.alinn2022@gmail.com)

**Author roles: Mbulli Ali I**: Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Resources, Supervision, Validation, Visualization, Writing – Review & Editing; **Mai KE**: Data Curation, Formal Analysis, Investigation, Methodology, Visualization, Writing – Original Draft Preparation; **Moyeh MN**: Investigation, Methodology, Project Administration, Resources, Supervision, Writing – Review & Editing; **Saha IL**: Data Curation, Investigation, Methodology, Validation, Visualization, Writing – Review & Editing; **Kaunda Nguene DB**: Data Curation, Investigation, Methodology, Validation, Visualization, Writing – Review & Editing; **Tchuenkam VP**: Data Curation, Formal Analysis, Investigation, Methodology, Project Administration, Supervision, Validation, Writing – Review & Editing; **Lemogo G**: Data Curation, Investigation, Methodology, Validation, Writing – Review & Editing; **Mbouh M**: Data Curation, Investigation, Methodology, Project Administration, Validation, Writing – Review & Editing; **Dombou A**: Data Curation, Formal Analysis, Investigation, Methodology, Project Administration, Visualization, Writing – Review & Editing; **Niba MN**: Data Curation, Investigation, Methodology, Project Administration, Validation, Writing – Review & Editing; **Djerassem O**: Data Curation, Investigation, Methodology, Validation, Writing – Review & Editing; **Belinga CNN**: Data Curation, Investigation, Methodology, Validation, Writing – Review & Editing; **Nguefack GN**: Investigation, Methodology, Validation, Writing – Review & Editing; **Ajua RF**: Investigation, Methodology, Resources, Writing – Review & Editing; **Simo G**: Investigation, Methodology, Project Administration, Resources, Supervision, Writing – Review & Editing; **Noubom M**: Investigation, Methodology, Project Administration, Resources, Supervision, Writing – Review & Editing; **Meka AZ**: Methodology, Project Administration, Resources, Supervision, Validation, Writing – Review & Editing; **Rose L**: Conceptualization, Investigation, Methodology, Project Administration, Resources, Supervision, Writing – Review & Editing

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## Background

Despite substantial progress in the fight against malaria disease achieved through vector control, accurate diagnosis and effective treatment, malaria still persists as a major public health threat with devastating impact on human wellbeing especially in sub-Sahara Africa ((Mwenda *et al.*, 2021). Six malaria causing human Plasmodium species have been identified*; Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale curtisi, Plasmodium ovale wallikeri, Plasmodium malariae*, and *Plasmodium knowlesi* (Schindler *et al.*, 2019) – *Plasmodium falciparum* and *Plasmodium vivax* present the greatest threat (WHO, 2023). In 2023, there were an estimated 263 million cases of malaria recorded globally and 597,000 deaths, with the World Health Organization African Region accounting for about 94% global cases and 95% global malaria deaths. Populations such as children under 5 years of age, pregnant women, refugees, migrants and internally displaced persons are the most susceptible to malaria infection (WHO, 2024).

The severity of malaria infection may differ between Plasmodium species with *P. falciparum* accounting for the majority of malaria-related morbidity and mortality in sub-Saharan Africa (Garrido-Cardenas *et al.*, 2023; Phillips *et al.*, 2017). *Plasmodium vivax,* the most widely distributed malaria causing specie contributes to malaria burden in tropical and temperate zones yet it is considered a neglected specie (Xie *et al.*, 2020). Malaria control efforts primarily target *P.falciparum* and this has resulted in a decline in *P. falciparum* malaria burden, with a relative increase in the proportion of malaria cases attributed to *P. vivax* in malaria endemic regions (Abebe *et al.*, 2023). It has been shown that in areas where the two species co-exist, malaria elimination is more complicated because *Plasmodium vivax* exhibits higher resistance to control efforts (Howes *et al.*, 2015; Schwabl *et al.*, 2024). In Africa, *P. vivax* infection has been thought to be very rare or absent because of the absence of the Duffy antigen on the surface of red blood cells of black Africans (Howes *et al.*, 2015). However, increasing new reports on *P.vivax* infections in Duffy negative inhabitants of Cameroon and other African countries challenges existing knowledge on the epidemiology of vivax malaria in the region (Abate *et al.*, 2022; Djeunang Dongho *et al.*, 2021; Fru-Cho *et al.*, 2014; Russo *et al.*, 2017). This suggests that this parasite is well underdiagnosed and may be a significant cause of persistent malaria infection associated with a significant public health burden. *P. ovale* species now recognized as two sub-species; *P. ovale curtisi* and *P. ovale wallikeri* generally considered benign and less prevalent has been reported to be associated with severe disease complications and death (Kotepui *et al.*, 2020). Unlike *P. falciparum, P. vivax* and *P. ovale* can form hypnozoites in the host liver, making the prevention and treatment efforts challenging due to their ability to cause relapsing infections (Xie *et al.*, 2020)**.** *P. malariae* often co-existing with *P. falciparum* is also considered less prevalent and is often associated with very low parasitemia that can persist for years and sometimes lead to health complications such as anaemia and nephrotic syndrome (Nguiffo-Nguete *et al.*, 2023; Sendor *et al.*, 2023). To attain the WHO malaria control and elimination goal of 2030, non-falciparum malaria should be given attention especially in areas where they have been thought to be absent or less prevalent (Sendor *et al.*, 2023; WHO, 2021). Understanding the epidemiology and clinical characteristics of the often neglected *P. vivax* and other non-falciparum species in sub-Saharan African countries is vital for developing and executing appropriate control strategies specifically designed to address challenges posed by these parasites.

Consistent with World Health Organization (WHO) guidelines, Cameroon through the National Malaria Control Program (NMCP) implements different measures to combat malaria infection in the country (Tangi *et al.*, 2023). Key strategies include early and accurate parasitological confirmation of all suspected cases using light microscopy and rapid diagnostic tests before administering treatment (Apinjoh *et al.*, 2024). Although the diagnosis of malaria using light microscopy (LM) and rapid diagnostic tests (RDTs) is cost-effective, these methods suffer less sensitivity and lack of highly experienced microscopists who can distinguish between the different plasmodium species. As a result, this makes them less efficient at detecting non-falciparum malaria species which often occur at low parasite density infections and co-exist with *P. falciparum* (Abebe *et al.*, 2023; Sambe *et al.*, 2024). With the increasing reports on different malaria species in Cameroon, it is very crucial to accurately determine the epidemiology of these species using more advanced, reliable and highly sensitive diagnostic tools. Molecular methods such as PCR are highly sensitive and specific, offering a valuable solution to overcoming the drawbacks of LM and RDTs (Darko *et al.*, 2023), enabling detection of submicroscopic infections and coinfections that represent a crucial challenge to malaria control efforts (Kojom Foko *et al.*, 2022). The true distribution of malaria species in sub-Sahara African countries including Cameroon is not clearly established. Therefore, this study aimed to investigate the different malaria parasite species and distribution among suspected cases, as well as risk factors associated with malaria infection in three geo-ecological zones of Cameroon.

## Materials and method

### Study site

A hospital-based multicenter cross-sectional study was carried out from June 2022 to August 2023 with purpose to characterize the different malaria species distribution across three malaria endemic settings in Cameroon; Sahelian zone (Maroua), Humid Forest (Bertoua, Yaounde and Douala) and the Western highlands (Dschang and Bamenda).

Maroua located in the Far North region of Cameroon belongs to the Sahelian zone characterized by tropical Savannah climate with annual rainfall of <700mm/year (Antonio-Nkondjio *et al.*, 2019). Malaria in this region is highly endemic with seasonal malaria transmission that usually leads to outbreaks during the rainy season, which typically lasts 2–3 months (Kojom Foko *et al.*, 2022).

Bertoua, Yaounde and Douala are part of the Forest zone marked by hot and humid climate conducive to malaria transmission. This region experiences two rainy seasons and two dry seasons with annual rainfall ranging from 1500 to 5000 mm each year. Malaria transmission in this zone is considered holoendemic and perennial. The zone is characterized by a

variety of ecosystems including humid savannah, dense vegetation and large forests (Antonio-Nkondjio *et al.*, 2019; Kojom Foko *et al.*, 2022).

Dschang in the West and Bamenda in the North-West regions are located in the Western highlands, characterized by a temperate climate with rainfall lasting up to eight months. The vegetation in these areas is dominantly grasslands. They are classified as hypoendemic, with seasonal malaria transmission. The average annual rainfall is approximately 1800 mm (Antonio-Nkondjio *et al.*, 2019). The map below highlights the different study zones and sites (Figure 1).

## Ethical consideration

This study received ethical approval from the National Ethics Committee for Human Health Research (N◦2021/11/1407/CE/CNERSH/SP). Research authorizations were equally obtained from the Regional Delegations for Public Health and from the different health centers where the study was conducted. Prior to any study related procedure, written informed consent was obtained from participants, and from parents or guardians if minor after clearly explaining the potential harm and benefits of the study as well data privacy and confidentiality. This study was design under the terms of the declaration of Helsinki concerning human research.

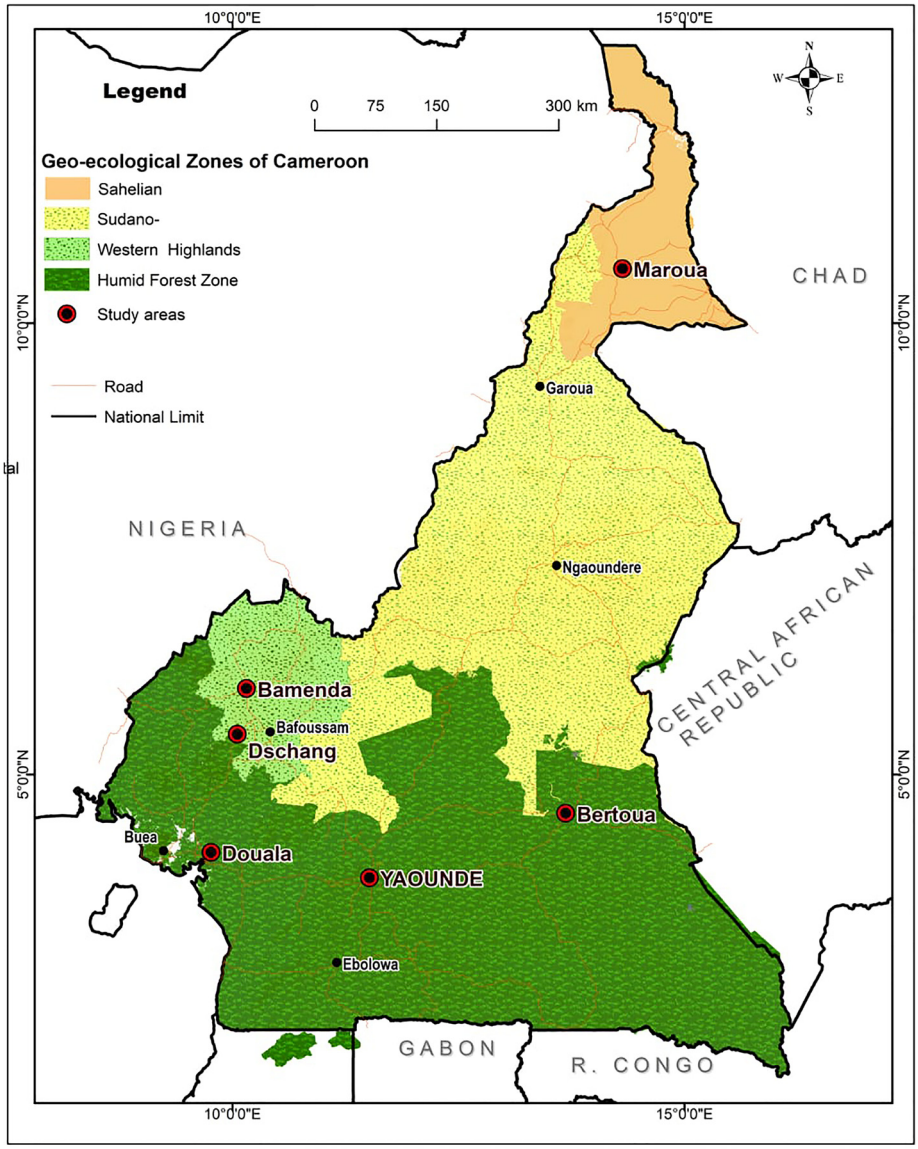



**Figure 1. Map highlighting the different study zones and study sites.** Three major geo-ecological blocks with different shades can be noted in the figure, with the Sahel ecology in the northern parts and the forest ecology in the southern and eastern parts. The western highlands contain a mix of color shades that illustrate the mosaic of ecologies in the western highlands of Cameroon.

### Study design, recruitment of participants and data collection

This study was a multicenter hospital-based study and in each selected health facility, children and adult of both genders suspected of malaria were eligible for participation. Those who provided written consent were recruited in the study and seen only once. A brief pre-designed questionnaire was used to collect information including age, gender, neighborhood/village, ownership/usage of mosquito bed nets, febrility and education level via the open source Kobo collect software. Results of malaria smear and RDTs were reported to the participant and positive cases were referred to the prescribing clinician for treatment according to local guidelines. Excluded from the study were participants who decided to withdraw consent in the course of the study procedures. A total of 1,390 participants were enrolled in the study. Participation was voluntary.

### Sample collection procedure and analysis

Venous blood samples were collected from eligible participants who provided written informed consent. Briefly, the skin was cleaned and sterilized using an alcohol wipe and a sterile syringe was used for the procedure. The collected blood was then used to perform malaria RDTs, light microscopy and preparation of four dried blood spots using Whatman N°03 filter papers (DBS, Whitman 903 Protein Saver) for molecular assay. Spotted filter papers were air dried overnight away from direct sunlight, humidity and flies, individually packed in a plastic bag together with a desiccant and stored at room temperature to be transported to the research laboratory for subsequent analyses.

## Molecular identification of Plasmodium Parasite species

### DNA extraction

The Chelex® – Tween® 20 method was used to extract genomic deoxyribonucleic acid (DNA) from dried blood spots as described by Simon *et al.* (2020) with some modifications. Summarily, 2 x 6 mm DBS were punched out of each spotted filter paper sample into a deep well plate and 1 ml of 0.5% Tween®-20 added and allowed to shake overnight. Following this, the Tween-20 was aspirated and DBS incubated for 15minutes in 1ml of 1x PBS at 4°C. The PBS was then aspirated and 150 µl of 1:2 chelex®-100 solution was added in every well and incubated in the water bath at 95°C for 10 minutes. After centrifugation, the resulting DNA extract from each well was then transferred into a new labelled DNA loBind plate and stored at -20°C for subsequent use.

### Amplification of Plasmodium species DNA

Nested PCR amplification targeting the highly conserved regions of the small-subunit ribosomal RNA (18SrRNA) gene was used to detect and identify the different human malaria-causing Plasmodium species using an optimized protocol previously described by Snounou *et al.* (1993).

For the first nested PCR amplification reaction, a 25 µl reaction mixture was used; each contained 12.5 µl of One Taq® 2X master mix with standard buffer (New England Biolabs), 9.5♣l of molecular grade water, 0.5 µL of each *Plasmodium* genus-specific primers (Table 1) and 2 µl of DNA extract. All amplifications were carried out in a BIO-RAD T100™ Thermal Cycler with varying cycling conditions (Table 2). For the second reaction, nested amplification targeting the 18SrRNA gene for *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale* subspecies was performed in separate reactions. Each reaction included a specific primer pair targeting either *P. falciparum* (rFAL1/rFAL2), *P. vivax* (rVIV1/rVIV2), *P. malariae* (rMAL1/rMAL2), *P. ovale curtisi* (rOVA1/rOVA2), *P. ovale wallekeri* (rOVA1v/rOVA2v). A final volume of 25 ♣l containing 12.5 µL of One Taq® 2X Polymerase with standard buffer (New England Biolabs), 10.5 µL of molecular grade water**,** 0.5 µL of each *Plasmodium* species-specific primers, and 1 µL of Nest 1 amplicons was used. The reaction conditions of each PCR and the primer sequences used in this study are summarized in the tables below (Table 1, Table 2 and Table 3).

A common set of primer rPLU5 and rPLU6 were used for the primary amplification of the genus Plasmodium and specific nested primers for the different plasmodium species as shown on the table above.

### Agarose gel electrophoresis

The nested PCR amplicons were separated on a 2% agarose gel (Sigma, USA) stained with 3 µl of ethidium bromide in 40ml 0.5x TBE at 120 volts for 40 min. The bands were visualized under UV trans-illumination and photographed.

### Data management and statistics

Data from the study was recorded using Kobocollect software and subsequently downloaded in excel format for analysis. The Software R Studio version 4.3.2 was used to analyze the study data. R packages not included into the buildup of R was downloaded from the CRAN repository. The package readxl was used to import the data sheet into R studio. The epiR package was used to calculate prevalence, proportions across study groups and 95% confidence interval. The package ggplot2 was used to draw figures. Univariate and multivariable logistic regression models were used to determine the potential risk factors of malaria and was express as crude and adjusted odds ratio (COR and AOR). A P value $< 0.05$ was considered significant in all comparisons.

## Results

### Baseline characteristics of study participants

A total of 1390 samples were collected from consenting participants from three geo-ecological zones; Sahelian 289 (Maroua), Humid Forest 514 (Bertoua: 243, Yaounde: 100, Douala: 171) and Western highlands 587 (Dschang: 437, Bamenda: 150). Females accounted for 62.01% (862) of the total study population and males 37.99% (528). The majority of participants were aged above 20 (832; 59.86%) with mean age (25.6 ± 18.26). The overall median (interquartile range) age was 25 (12–35). Fever at the time of survey or 24 hours before survey was reported in 82.57% (1061) of participants. Education level varied from no formal education to Tertiary level of

**Table 1. Summary of primer pairs used in the identification of Plasmodium parasite species and expected band sizes of the target gene.**

| Species | Primer | Sequence (5'-3') | Target and size | |
|---|---|---|---|---|
| *Plasmodium genus* | rPLU5 | CCTGTTGTTGCCTTAAACTTC | 1200bp | |
| | rPLU6 | TTAAAATTGTTGCAGTTAAAACG | | |
| *Plasmodium falciparum* | rFAL1 | TTAAACTGGTTTGGGAAAACCAAATATATT | | 205bp |
| | rFAL2 | ACACAATGAACTCAATCATGACTACCCGTC | | |
| *Plasmodium vivax* | rVIV1 | CGCTTCTAGCTTAATCCACATAACTGATAC | | 120bp |
| | rVIV2 | ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA | | |
| *Plasmodium malariae* | rMAL1 | ATAACATAGTTGTACGTTAAGAATAACCGC | | 144bp |
| | rMAL2 | AAAATTCCCATGCATAAAAAATTATACAAA | | |
| *Plasmodium ovale curtisi* | rOVA1 | ATCTCTTTTGCTATTTTTTAGTATTGGAGA | | 787-789bp |
| | rOVA2 | GGAAAAGGACACATTAATTGTATCCTAGTG | | |
| *Plasmodium ovale wallikeri* | rOVA1v | ATCTCCTTTACTTTTTGTACTGGAGA | | 782bp |
| | rOVA2v | GGAAAAGGACACTATAAATGTATCCTAATA | | |

**Table 2. PCR conditions for Nest 1 amplification of *Plasmodium* genus.**

| Steps | Temperature/ °C | Time/mins |
|---|---|---|
| **Lid Heating** | 105 | / |
| **Pre-denaturation** | 94 | 3 |
| **Denaturation** | 94 | 1 |
| **Annealing** | 61 | 1 |
| **Elongation** | 68 | 1 |
| **30 cycles** | | |
| **Final extension** | 68 | 5 |
| **Hold** | 4 | ∞ |

**Legend:** The conditions for the amplification reaction were preprogrammed in the thermal cycler prior to amplifications. The ∞ sign indicates that samples should be held for a long time at 4°C.

education with the latter representing the highest proportion (398, 32.73%), followed by Secondary (352, 28.9%). 770 participants (64.01%) reported Mosquito bed net ownership and 593 (49.54%) reported to have used bed net the night before survey (Table 4).

## Prevalence of malaria infection by PCR

A total of 1,390 dried blood spots were tested for malaria by nested PCR. Malaria infection was confirmed in 682 cases presenting an overall prevalence of 49, 06%. The highest proportion was recorded from the Sahelian zone with 163 cases (56.4%), followed by the Humid Forest 287 cases (55.84%) with contributions from Bertoua (92 cases, 37.9%), Yaoundé (49 cases, 49.0%) and Douala (146 cases, 85.4%). The Western highlands accounted for a total of 232 cases (39.52%), with 177 cases from Dschang (40.5%) and 55 cases from Bamenda (36.7%). Table 5 and Figure 2 present the prevalence of malaria across the different geo-ecological settings.

**Table 3. PCR conditions for nested amplification of species specific genes (*P. falciparum, P. vivax, P. malariae, P. ovale curtisi* and *P. ovale wallikeri*).**

| Steps | *P. falciparum* | *P. vivax* | | *P. malariae* | *P. ovale curtisi* | | *P. ovale wallekeri* | Time |
|---|---|---|---|---|---|---|---|---|
| **Pre-denaturation** | | | 94°C | | | 95 | | 3 mins |
| **Denaturation** | | | 94°C | | | 94 | | 1min |
| **Annealing** | 61°C | 60°C | | 54°C | | 56 | | 1min |
| **Extension** | 68°C | | | | | 72 | | 1min |
| | Number of cycles 30 | | | | | 25 Cycles | | |
| **Final extension** | 68°C | | | | | 72 | | 5mins |
| **Hold** | 4°C | | | | | | | ∞ |

**Legend:** The conditions for the amplification reaction were preprogrammed in the thermal cycler prior to amplifications. The ∞ sign indicates that samples should be held for a long time at 4°C.

**Table 4. Socio-demographic characteristics of study participants by Geo-ecological zone.**

| Variable | Sahelian Zone (n= 289) | Humid Forest (n= 514 ) | Western Highlands (n= 587 ) | Absolute frequency | Relative frequency (%) | 95% CI (%) |
|---|---|---|---|---|---|---|
| **Gender** | | | | | | |
| Female | 160 | 338 | 364 | 862 | 62.01 | [59.42-72.0 ] |
| Male | 129 | 176 | 223 | 528 | 37.99 | [ 35.39-40.69] |
| **Age category** | | | | | | |
| 0–4 years | 77 | 74 | 80 | 231 | 16.62 | [ 14.03-19.25] |
| 5–9 years | 21 | 31 | 34 | 86 | 6.19 | [ 3.59-8.82] |
| 10–20 years | 31 | 87 | 123 | 241 | 17.34 | [ 14.75-19.97] |
| 20 + | 160 | 322 | 350 | 832 | 59.86 | [ 57.27-62.49] |
| Mean age(± SD) | 21.4 (± 17.21) | 27.4 (± 17.99) | 26.1 (± 18.69) | 25.6 (± 18.69) | | |
| Range | 0-81 | 0-89 | 0-95 | 0-95 | | |
| Median Age(IQR) | 23(26) | 27.5(24) | 24(17) | | | |
| **Fever at time of survey or 24 hours before survey** | | | | | | |
| Yes | 247 | 268 | 546 | 1061 | 82.57 | [ 80.54-84.65] |
| No | 38 | 145 | 41 | 224 | 17.43 | [15.41-19.51 ] |
| **Level of Education** | | | | | | |
| Primary | 45 | 57 | 92 | 194 | 15.95 | [ 12.99-19.04] |
| Secondary | 64 | 95 | 193 | 352 | 28.95 | [25.99-32.03 ] |
| Tertiary | 81 | 108 | 209 | 398 | 32.73 | [29.77-35.81] |
| None | 97 | 82 | 93 | 272 | 22.37 | [ 19.41-25.45] |
| **Mosquito net ownership** | | | | | | |
| Yes | 263 | 241 | 266 | 770 | 64.01 | [ 61.26-66.87] |
| No | 21 | 91 | 321 | 433 | 35.99 | [33.25-38.85 ] |

| Variable | Sahelian Zone (n= 289) | Humid Forest (n= 514 ) | Western Highlands (n= 587 ) | Absolute frequency | Relative frequency (%) | 95% CI (%) |
|---|---|---|---|---|---|---|
| **Bednet use night before survey** | | | | | | |
| Yes | 212 | 219 | 162 | 593 | 49.54 | [ 46.62-52.53] |
| No | 73 | 113 | 418 | 604 | 50.46 | [ 47.54-53.45] |
| **Study sites** | | | | | | |
| Maroua | 289 | | | | 20.79 | [18.13-23.48] |
| Bertoua | | 243 | | | 17.48 | [14.82-20.17] |
| Yaounde | | 100 | | | 7.19 | [4.53-9.88] |
| Douala | | 171 | | | 12.3 | [9.64-14.99] |
| Dschang | | | 437 | | 31.44 | [28.78-34.13] |
| Bamenda | | | 150 | | 10.79 | [8.13-13.48] |

Legend: n = Number/counts, 95% CI = 95% confidence interval.

**Table 5. Malaria prevalence by nested PCR.**

| Zone | Study Site | Total samples | PCR Positives (n,%) |
|---|---|---|---|
| Sahelian zone | Maroua | 289 | 163 (56.4) |
| Humid Forest | Bertoua | 243 | 92(37.9) |
| | Douala | 171 | 146(85.4) |
| | Yaounde | 100 | 49(49.0) |
| Total | | 514 | 287(55,84) |
| Western Highlands | Dschang | 437 | 177(40.5) |
| | Bamenda | 150 | 55(36.7) |
| Total | | 587 | 232(39.53) |
| **Overall proportion** | | | **682 (49.06)** |

**Legend:** The prevalence of infection was determined as the proportion all PCR positive samples of all samples tested. n: number, PCR : polymerase chain reaction.

## Distribution of malaria parasite species by geo-ecological zones

Out of the 682 malaria cases detected by nested PCR, the majority were most commonly single species infections 651/682(95.45%). Five human malaria causing parasites were identified in the study population with *P. falciparum* being the most frequently detected across all study zones 657/682 (96.33%). There was a clear variation in parasite composition across the different sites. In the Sahelian zone, only *P. falciparum*, *P. ovale curtisi* and *P. malariae* were identified. The Western highlands recorded a broader parasite species diversity with *P. falciparum*, *P. malariae*, *P. ovale curtisi* and *P. ovale wallikeri* detected. The Humid forest zone showed the highest parasite diversity where all five species were detected; *P. falciparum*, *P. malariae*, *P. vivax*, *P. ovale curtisi* and *P. ovale wallikeri*. The Western highlands recorded the highest number of non-falciparum infections (Figure 3). As expected, *P. falciparum* mono infection was the most represented accounting for 630/682 cases (92.38%). This was followed by *P. malariae* mono infection observed in 8/682 cases (1.17%) and *P. ovale wallikeri* in 7/682 cases (1.03%). *P. ovale curtisi* mono infection detected in 6/682 cases (0.88%) was the least represented among single-species infection. *P. vivax* was only observed as a mixed infection with *P. falciparum* (2 cases 682; 0.29%). Coinfections accounted for 31/682 cases (4.55%). The study recorded 7 types of coinfections; 2 *P.falciparum/P.vivax*, 7 *P.falciparum/P.malariae*, 11 P.falciparum/P.ovale curtisi, 6 *P.falciparum/P.ovale walekeri*, 3 *P.ovale curtisi/P.ovale wallekeri*, 1

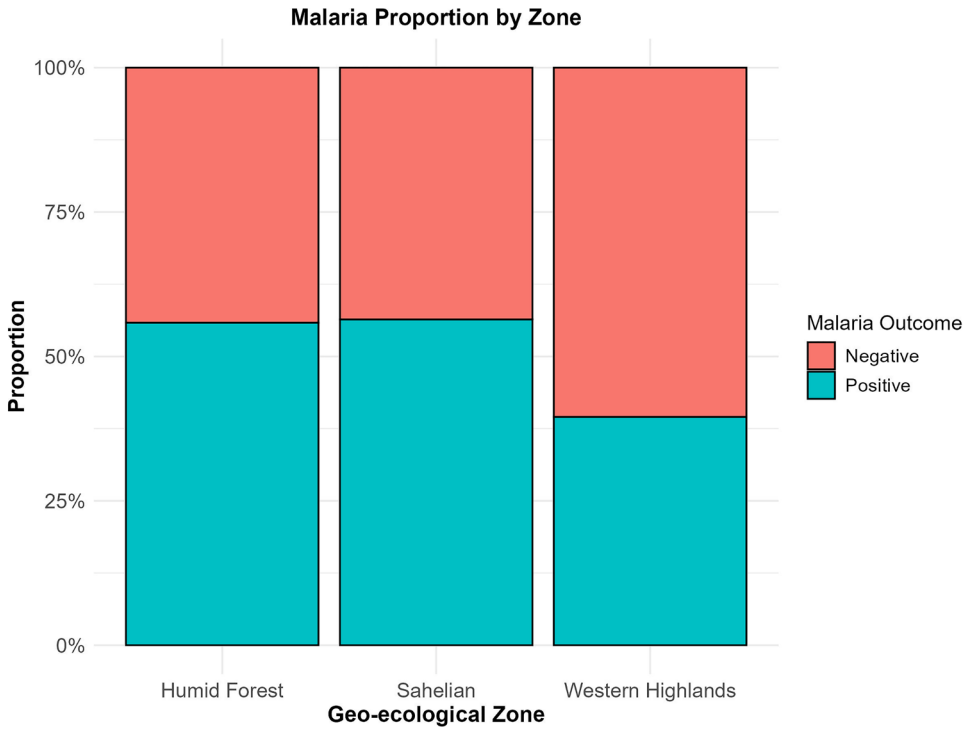



**Figure 2. Illustration of Malaria Infection Prevalence by Geo-ecological Zone.** The histograms show the proportions of negative (orange) to positive infections(green) infections plotted against geo-ecology.

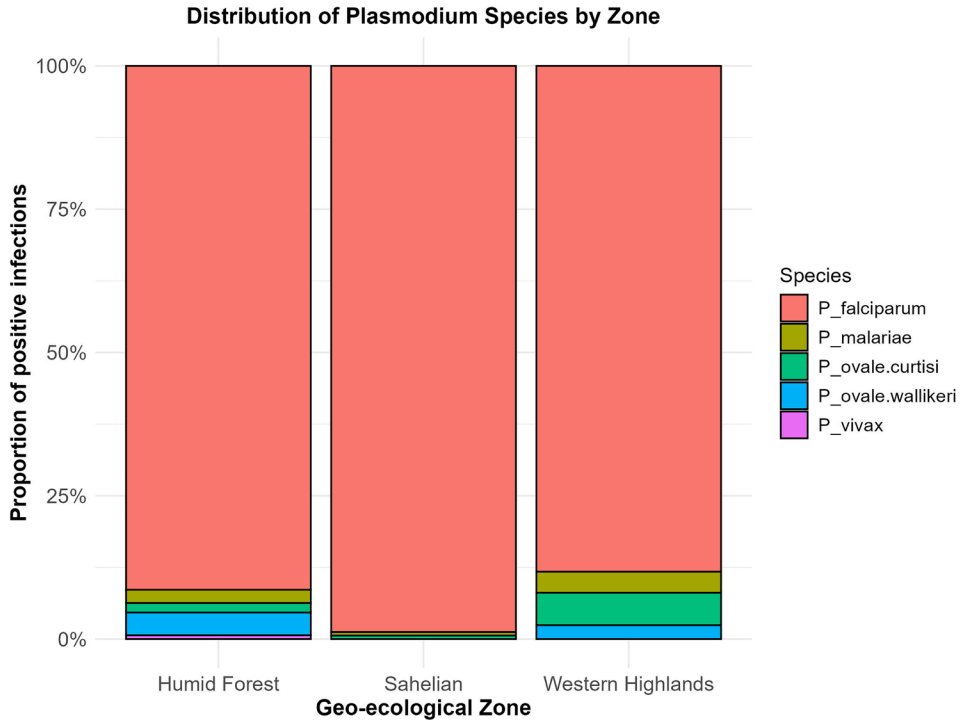



**Figure 3. Distribution of Plasmodium species by Geo-ecological zone.** This figure shows species composition diversity by geo-ecological zone. *Plasmodium ovale* parasites underwent further molecular processing to separate the two sympatric parasite species that do not recombine.

*P.malariae/P.ovale wallekeri* and 1 *P.falciparum/P.malariae/P.ovale wallekeri*. Double infections were detected in 30/682 cases (4.39%) with *P.falciparum* + *P.ovale curtisi* as the most common coinfection (11/682 cases; 1.61%). A single triple infection *involving P. falciparum* + *P. malariae* + *P. ovale wallikeri* was equally detected (1/682; 0.15%).

The proportion of non-falciparum infections was 8.36% (57/682). Among all non-falcipparum infections occurring as both mono and coinfections, *P. ovale curtisisi* was the most frequent (20/57; 35.09%), followed by *P. ovale wallikeri* (18/57; 31.58%), *P. malariae* (17/57; 29.82%) and lastly, *P. vivax* (2/57; 3.51%). Table 6 and Figure 3 present the different parasite species detected and their distribution across the different study zones.

## Risk factors associated with malaria infection

A total of six independent variables were considered for univariate logistic regression analysis to test for potential risk factors of malaria infection; gender, age, education level, bed net ownership, bed net use and geo-ecological zone.

### *Gender*

Majority of malaria confirmed cases in the study population came from males 265/528 (50.19%). Although the proportion of cases among males were higher than in females 417/862(48.38%), there was no statistically significant difference in malaria risk between them (COR = 1.08, 95% CI = [0.87-1.34], p.value = 0.512; AOR = 1.14, 95% CI; [0.89 – 1.46], p.value = 0.285). This showed that gender was not a major determinant of malaria infection in the study. Figure 4 shows the distribution of malaria infection in relation to gender.

### *Age*

Malaria proportion varied significantly across the different age groups. Age group 10–20 years had the highest proportion of infection 141/241(58.51%), followed by 20 years plus (20 +) with 396/832 cases (47.59%). Children less than 5 years of age (0–4 years) recorded 106/231cases (45.89%) and the age group 5-9 years, 39/86cases (45.35%). The risk of infection was higher in the age group 10–20 years compared to children aged less than 5 years (COR = 1.66, 95% CI = [1.16 - 2.40], p.value = 0.006, AOR = 1.88, 95% CI = [1.07-3.34], p.value = 0.03). Figure 5 illustrates malaria proportion with respect to age category.

### *Level of education*

Malaria infection varied among the different levels of education. Individuals in the Primary level accounted for the highest proportion of cases 96/194(49.48%), followed by the secondary with 168/352 cases (47.73%) and those who never had any formal education accounted for 122/272 cases (44.85%). Individuals who attended the tertiary level were the least affected with 149/398 cases (37.44%). Although individuals in the Primary level of education accounted for the highest proportion of cases, there was no statistically significant association with malaria infection (COR = 1.2, 95% CI = [0.83-1.74], p.value = 0.32, AOR = 1.28, 95% CI = [0.78-2.10], p.value = 0.32). Figure 6 shows the proportion of malaria infection in relation to education level.

**Table 6. Proportion of malaria infection by species and infection type.**

| Infection type | Species | Frequency (n) | Percentage (%) | 95% CI (%) |
|---|---|---|---|---|
| **Total proportion of malaria infection by PCR** | Positive | **682** | **49.06** | **[46.33-51.83 ]** |
| | Negative | 708 | 50.94 | [48.20-53.69] |
| **Single species** infection | *P.falciparum* | 630 | 92.38 | [90.62-94.14] |
| | *P.vivax* | 0 | 0 | [00-00] |
| | *P. malariae* | 8 | 1.17 | [0.00-2.93] |
| | *P. ovale curtisi* | 6 | 0.88 | [0.00-2.64] |
| | *P. ovale wallikeri* | 7 | 1.03 | [0.00-2.79] |
| **Total** | | **651** | **95.45** | **[94.13-97.02]** |
| **Double** infection | *P.falciparum + P. malariae* | 7 | 1.03 | [0.00-2.79] |
| | *P.falciparum + P. vivax* | 2 | 0.29 | [0.00-2.05] |
| | *P.falciparum + P. ovale curtisi* | 11 | 1.61 | [0.00-3.37] |
| | *P.falciparum + P. ovale wallikeri* | 6 | 0.88 | [0.00-2.64] |
| | *P.ovale curtisi + P. ovale wallikeri* | 3 | 0.44 | [0.00-2.20] |

| Infection type | Species | Frequency (n) | Percentage (%) | 95% CI (%) |
|---|---|---|---|---|
| | *P.malariae + P. ovale wallikeri* | 1 | 0.15 | [0.00-1.91] |
| **Total** | | **30** | **4.39** | **[3.08-5.96]** |
| **Triple** infection | *P.falciparum + P. malariae + P.ovale wallikeri* | 1 | 0.15 | [0.00-2.02] |
| **Total proportion of coinfections** | | **31** | **4.55** | **[3.11-6.39]** |
| **Falciparum infections** | *P.falciparum* | 657 | 96.33 | [94.64-97.50] |
| **Proportion of non-falciparum infections among all cases** | | | | |
| | *P. vivax* | 2 | 0.29 | [0.08-1.06] |
| **Non-falciparum species** | *P. malariae* | 17 | 2.49 | [1.56-3.96 ] |
| | *P. ovale curtisi* | 20 | 2.93 | [1.91-3.96] |
| | *P. ovale wallikeri* | 18 | 2.64 | [1.68-4.13] |
| **Total** | | 57 | 8.36 | [6.52-10.68] |
| **Proportion of non-falciparum species among all non-falciparum infections** | | | | |
| | *P. vivax* | 2 | 3.51 | [0.97-11.92] |
| | *P.malariae* | 17 | 29.82 | [19.53-42.66] |
| | *P. ovale curtisi* | 20 | 35.09 | [24.0-48.06] |
| | *P. ovale wallekeri* | 18 | 31.58 | [21.0-44.48] |

**Legend**: 95% CI : 95% confidence interval, PCR : polymerase chain reaction




**Figure 4. Proportion of malaria infection by gender.** The histograms show how malaria infections distribute by gender in the entire population. Slightly more males than females were infected.

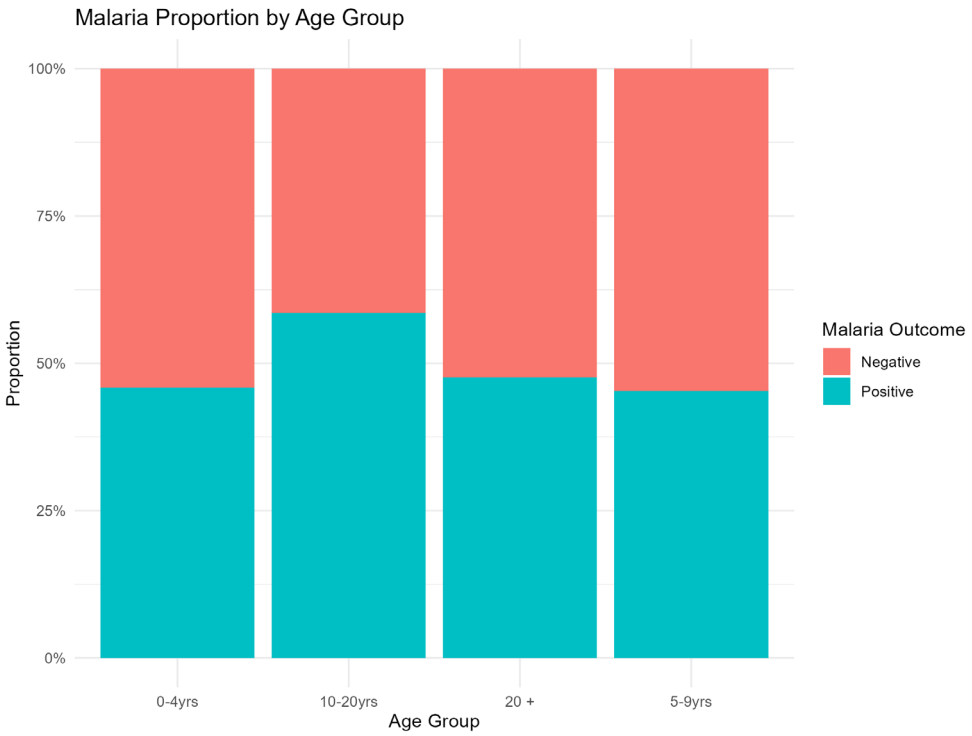



**Figure 5. Proportion of malaria infection by age group.** The histograms illustrate malaria infections distribute by group and highlight higher infection prevalence in the 10–20 years age group.

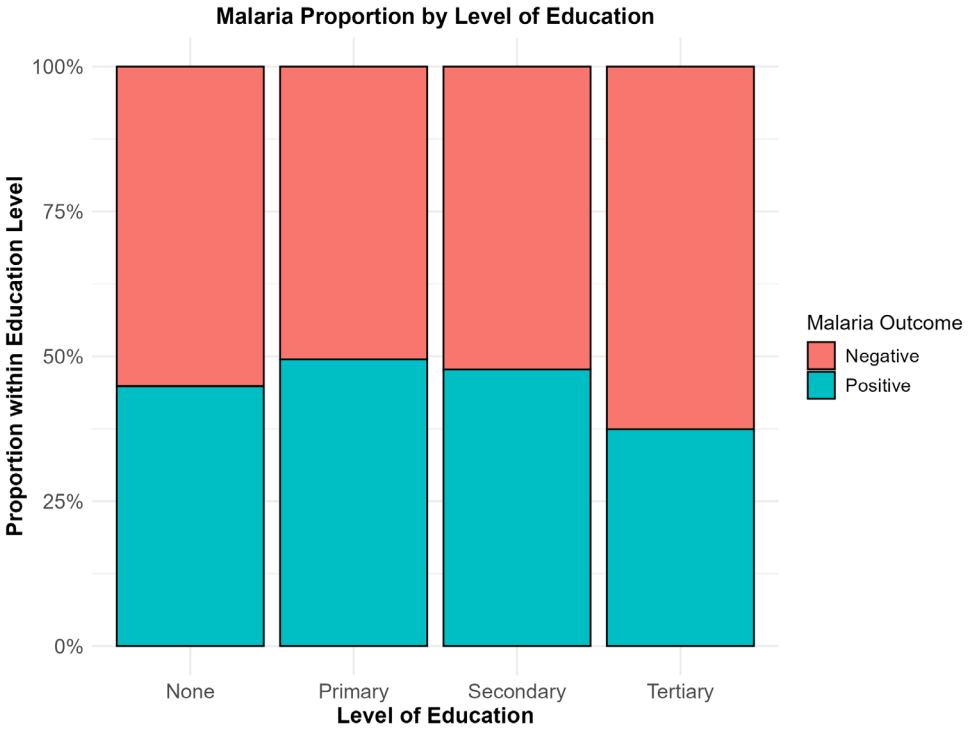



**Figure 6. Proportion of malaria infection by educational level.** The histograms show how malaria infection prevalence distribute by educational level and highlight lower prevalence in participants that attained tertiary education compared to the other education categories.

***Bed net ownership and use night before survey***

The proportion of malaria infection was surprisingly higher among individuals who reported bed net ownership 349/770 (45.32%) compared to those who never had bed nets 182/433 (42.03%) but there was no statistically significant difference in their association with malaria infection (COR = 1.14, 95% CI = [0.90 -1.45], p.value = 0.25, AOR = 0.86, 95% CI = [0.63-1.18], p.value = 0.35). Also, individuals who slept under bed net night before survey had a higher proportion of malaria 279/593(47.05%) compared to those who didn't 249/604(41.23%) but the difference was not statistically significant.

***Geo-ecological zone***

Geo-ecological zone was found to be significantly associated with malaria infection. Individuals in the Sahelian Zone had the highest proportion of malaria infection 163(56.40%), followed by the Humid Forest zone 287(55.84%) and lastly, the Western Highlands 232 (39.5%). The odds of malaria infection were higher in the Sahelian zone compared to the Forest zone (COR = 1.02, 95%CI = [0.77-1.37], p.value 0.877, AOR = 1.99, 95%CI = [1.43-2.78], p.value < 0.001). Table 7 summarizes the different socio-demographic variables examined as potential risk factors of malaria infection.

**Table 7. Risk factors associated with malaria infection.**

| Variable | Number of samples | PCR positive | PCR negative | COR; 95%CI | p.value | AOR: 95%CI | p.value |
|---|---|---|---|---|---|---|---|
| **Gender** | | | | | | | |
| **Female** | 862 | 417 (48.38) | 445(51.62) | reference | 0.512 | | |
| **Male** | 528 | 265 (50.19) | 263(49.81) | 1.08[0.87 – 1.34] | | 1.11 [0.87 – 1.42] | 0.39 |
| **Age category** | | | | | | | |
| **0-4yrs** | 231 | 106 (45.89) | 125 (54.11) | reference | | | |
| **10-20yrs** | 241 | 141 (58.51) | 100(41.49) | 1.66[1.16 -2.40] | 0.006 | 1.88 [1.07-3.34] | **0.03** |
| **20 +** | 832 | 396 (47.59) | 436(52.40) | 1.07[0.80 -1.44] | 0.645 | 1.07 [0.65-1.74] | 0.79 |
| **5-9yrs** | 86 | 39 (45.35) | 47(54.65) | 0.98[0.59-1.61] | 0.932 | 0.96 [0.51-1.80] | 0.89 |
| **level of education** | | | | | | | |
| **None** | 272 | 122(44.85) | 150(55.15) | reference | | | |
| **Primary** | 194 | 96(49.48) | 98(50.52) | 1.2[0.83-1.74] | 0.323 | 1.28 [0.78-2.10] | 0.32 |
| **Secondary** | 352 | 168(47.73) | 184(52.27) | 1.12 [0.82 -1.54] | 0.475 | 1.02 [0.62-1.67] | 0.94 |
| **Tertiary** | 398 | 149(37.44) | 249(62.56) | 0.74 [0.54-1.01] | 0.055 | 0.75 [0.46-1.21] | 0.23 |
| **Zone** | | | | | | | |
| **Humid forest** | 514 | 287(55.84) | 227(44.16) | reference | | | |
| **Sahel** | 289 | 163(56.40) | 126(43.59) | 1.02[0.77 – 1.37] | 0.877 | 1.99 [1.43-2.78] | **<0.001** |
| **Western highlands** | 587 | 232(39.52) | 355(60.48) | 0.52 [0.41 – 0.66] | **<0.001** | 0.91 [0.68-1.23] | 0. |
| **Bed net ownership** | | | | | | | |
| **No** | 433 | 182(42.03) | 251(57.97) | reference | | | |
| **Yes** | 770 | 349(45.32) | 421(54.68) | 1.14 [0.90 – 1.45] | 0.27 | 0.86 [0.63-1.18] | 0.35 |
| **Bed net use night before survey** | | | | | | | |
| **No** | 604 | 249(41.29) | 354(58.71) | reference | | | |
| **Yes** | 593 | 279(47.05) | 318(52.95) | 1.27[1.01-1.59] | **0.043** | 1.13 [0.83-1.54] | 0.44 |

**Legend**: Statistical significant at P < 0.05, PCR: Polymerase chain reactions, COR; 95% CI: Crude odd ratio with 95% confidence interval, AOR; 95% CI: adjusted odd ratio with 95 % confidence interval.

## Discussion

The primary objective of this study was to investigate the the species composition, distribution and risk factors of parasite species using nested PCR in three geo-ecological zones of Cameroon, with particular emphasis on non-falciparum and *Plasmodium vivax* in three geo-ecological zones of Cameroon.

Overall, the prevalence of malaria in this study was 49.06% (682/1390). This falls within the range of previously reported malaria prevalence in Cameroon which varies between 8.8% and 64.7% depending on factors such as geographic location, study population and methodological approach (Stopyra *et al.*, 2025).

Five human Plasmodium species were detected as both mono and coinfections; *P. falciparum* (657/682*), P. malariae* (17/682*), P. vivax* (2/682*), P. ovale curtisi* (20/682*) and P. ovale wallekeri* (18/682*).* This study revealed a broader range of parasite species compared to some previous studies, Kojom Foko *et al.* (2022) identified *P. falciparum, P malariae, P vivax and P. ovale curtisi* in a similar setting, Nguiffo-Nguete *et al.* (2023), reported the circulation of *P. falciparum, P. malariae* and *P. ovale, and* Stopyra *et al.* (2025) reported only *P. falciparum* and a single *P. vivax* infection in a Forest zone.

Single species infection was identified in 95.45% of all cases (651/682). *P. falciparum* mono infections accounted for 92.38% (630/682) of all cases and as expected, it was the main species responsible for majority of cases, which is consistent with historical and current data on the predominant parasite specie in a malaria endemic region like Cameroon and other sub-Saharan African countries (Akindeh *et al.*, 2021; Antonio-Nkondjio *et al.*, 2019; Kojom Foko *et al.*, 2022; Nguiffo-Nguete *et al.*, 2023).

The overall proportion of non-falciparum infections was 8.36% (57/682) detected either as mono or coinfections with cases attributed to *P. vivax, P malariae, P. ovale curtisi and P. ovale wallekeri. P. malariae* mono infection (1.17%, 8/682) was the most common non-falciparum mono infection followed by P. ovale wallekeri. This is similar to findings from some studies conducted in Cameroon and in the Democratic Republic of Congo (Mbama Ntabi *et al.*, 2022; Nguiffo-Nguete *et al.*, 2023) which reported *P. malariae* mono infection as the most frequent non-falciparum mono infection.

*P. vivax* infection was detected exclusively at a low frequency (3.51%, 2/57) as a coinfection with *P. falciparum*. This is consistent with reports from a study from the Centre region of Cameroon that detected a single case of vivax malaria as a coinfection with *P. falciparum* (Stopyra *et al.*, 2025) but different from some previous findings from Cameroon and other sub-Saharan African countries that reported a higher prevalence of *P. vivax* both as mono infections and as coinfections (Abebe *et al.*, 2023; Dieng *et al.*, 2025; Djeunang Dongho *et al.*, 2021; Fru-Cho *et al.*, 2014; Sambe *et al.*, 2024). In the past, *P. vivax* has been considered inexistent among sub-Saharan African populations due to the absence of Duffy antigens that serve as receptors for parasite invasion (Howes *et al.*, 2015; Tchuenkam *et al.*, 2025). Recent reports from Cameroon and other African countries have reported the circulation of *P.vivax* among Duffy negative individuals and interestingly, our study revealed the presence of 2 *P.vivax* infections from the Forest zone (Bertoua and Douala). This is consistent with previous reports from the same geo-ecological zone where the circulation of vivax in the zone was equally reported at a very low frequency (Dieng *et al.*, 2025; Kojom Foko *et al.*, 2022). This confirms the circulation of the parasite in this zone although at a low frequency. In the western highlands, particularly in Dschang this species has been reported at higher frequencies (Djeunang Dongho *et al.*, 2021; Russo *et al.*, 2017) but surprisingly, there was no evidence from this site with over 437 samples tested. This is similar to reports from Tchuenkam *et al.* (2025) who tested over 431 samples in the same area but no vivax was detected. The complete absence of *P.vivax* from zones where it has been previously reported could be explained by malaria transmission variations (Chouakeu *et al.*, 2023). The previous studies might have coincided with a higher vivax transmission season or a season where malaria control measures might have contributed to the low *P. vivax* transmission. These results emphasize the need for continuous surveillance of vivax malaria in areas where it has been thought to be absent, as it has been reported in other countries as a cause of severe malaria (Arya *et al.*, 2023; López *et al.*, 2023).

*P. ovale curtisi* had the highest proportion among all non-falciparum infections, followed by *P.ovale wallekeri*. The two *Plasmodium ovale* sub-species, previously considered to be less prevalent in sub-Saharan Africa were distinctively identified in the present study. Kojom Foko *et al.* (2022) and Tchuenkam *et al.* (2025) equally reported the circulation of the two sub-types in similar study settings in Cameroon. Like *Plasmodium vivax, Plasmodium ovale* species though often neglected can also form hypnozoites in the host liver, making the diagnosis, prevention and treatment efforts challenging due to their ability to cause relapsing infections (Xie *et al.*, 2020; Zhou *et al.*, 2019).

The proportion of *P. malariae* in the study was 2.49% (17/682). *P. malariae* mono infections accounted for 1.17% of all cases which is lower than the 2.5% prevalence reported by Nguiffo-Nguete *et al.* (2023) and higher than the prevalence reported by Tchuenkam *et al.* (2025). *P. malariae* and *P. ovale* species have been considered as low-level parasites and with their increasing reports and prevalence, it is important to accurately map their distribution in Cameroon and contribution to the global malaria burden in order to achieve the malaria elimination goal.

Co-infections accounted for 31/682 cases (4.55%) with *P. falciparum/P. ovale curtisi* as the most common. These findings contrasts some reports from previous studies in Cameroon that detected *P. falciparum/P. malariae* as the most common coinfection (Feufack-Donfack *et al.*, 2021; Nguiffo-Nguete *et al.*, 2023). A triple infection involving *P. falciparum/P. malariae/ P. ovale wallekeri* was detected in Douala. Similarly, Nguiffo-Nguete *et al.* (2023) reported a similar type of triple infection.

Due to the low prevalence of non-falciparum infections and often existing as coinfections with *P. falciparum*, they are being underestimated in malaria control strategies in sub-Sahara Africa. In areas where *P. falciparum* co-exist with other species, control and elimination efforts are less effective and this can affect disease severity, diagnosis and treatment efficacy (Diagne *et al.*, 2024). Thus, there is need for a special attention to be given to non-falciparum infections and coinfections in the global fight against malaria.

The risk of malaria infection was significantly associated with age in the present study. Children aged 10–20 years were more likely to be infected with malaria compared to children less than 5 years. This can be attributed to malaria control campaigns mostly targeting children under 5 years of age and pregnant women and this is possibly resulting in a shift in malaria infection dynamics (Nguiffo-Nguete *et al.*, 2023). This is similar to a previous finding (Tangi *et al.*, 2023) that reported the age group 15–20 as the most at risk of malaria infection but disagrees with some recent reports, Kaghou *et al.* (2024) from the North west region of Cameroon found no significant association between age and malaria infection. Also, Mbohou *et al.* (2019) outlined that the risk of malaria infection decreases with increase in age in malaria endemic regions where repeated exposure results in better immunity in adolescents and young adults compared to younger children.

Another significant predictor of malaria identified in this study was geo-ecological zone. In Cameroon, malaria transmission varies across different ecological zones. As expected, the Sahelian zone reported the highest proportion of malaria infection and the odds of malaria infection was higher in this zone compared to the Humid Forest. This is due to its hyperendemic nature characterized by seasonal outbreaks of malaria favoring Anopleles mosquitoes breeding (Antonio-Nkondjio *et al.*, 2019; Chouakeu *et al.*, 2023).

Although males recorded a higher proportion of malaria in the study than females, there was no statistically significant difference in malaria infection distribution between males and females. This is consistent with findings from other studies (Mbohou *et al.*, 2019; Ngum *et al.*, 2023). In contrast, some studies from Cameroon and other African countries reported a significant association, with higher odds of malaria infection in either males or females (Ebhuoma *et al.*, 2022; Nyasa *et al.*, 2021; Quaresima *et al.*, 2021).

The prevalence of malaria infection in the study was unexpectantly higher among individuals who owned bed nets and equally among those who used bed nets night before survey than those not having or who never slept under bed net. This is similar to findings from a study in Cameroon (Nyasa *et al.*, 2021) but different from a report from Ethopia (Duguma *et al.*, 2022)

This study had some limitations. The low frequency of *P. vivax* observed in the study could be attributed to the fact that this was a hospital-based study that included only patients suspected of malaria. Vivax infection can have mild or unnoticeable symptoms and people with it may not have gone to the hospital. Studies investigating only clinical infections might therefore not reflect the full picture of vivax malaria incidence and transmission in the general population. Future studies should include community and asymptomatic samples.

## Conclusion

*Plasmodium spp.* infection still remains a serious health concern in Cameroon with *P. falciparum* contributing to the highest burden across all zones. This study revealed the circulation of five human parasite species in three malaria endemic zones of Cameroon; *P. falciparum, P malariae, P vivax, P. ovale curtisi and P. ovale wallekeri* and reaffirms the predominance of *P. falciparum* species in the country. The presence of *P. vivax* and other non-falciparum relapsing species at a lower frequency in Duffy-negative individuals emphasize the need for continuous surveillance of these neglected parasites in order to accurately define the burden of malaria in the country and to contribute to updated locally informed control and elimination strategies.

## Abbreviations

DBS: Dried blood spots

PCR: Polymerase chain reactions

18srRNA: 18 small sub-unit ribosomal ribonucleic acid

DNA: Deoxy-ribonucleic acid

NMCP: National Malaria Control Programme

LM: Light microscopy

RDTs: Rapid diagnostic tests

COR: Crude odd ratio

AOR: adjusted odd ratio

95% CI: ninety-five percent confidence interval

PBS: Phosphate-buffered saline

*P. falciparum: Plasmodium falciparum*

*P. vivax: Plasmodium vivax*

*P. malariae: Plasmodium malariae*

*P. ovale curtisi: Plasmodium ovale curtisi*

*P. ovale wallikeri: Plasmodium ovale wallekeri*

## Ethics approval and consent to participate

The study protocol was reviewed and approved by the National ethics committee for Human health research of Cameroon (N◦2021/11/1407/CE/CNERSH/SP). Written informed consent was provided by all study participants or from parents and legal guardians for minors before participation in the study. The study was designed under the terms of the declaration of Helsinki concerning human research.

## Data availability

Raw anonymised, de-identified data will be made available upon reasonable request to the corresponding author and with prior approval from the relevant institutions. The data contain identifiable information in the form of geographical coordinates and other personal information at this time. Please direct all requests to the corresponding author using the email provided (dr.alinn2022@gmail.com). Upon receipt, a determination will quickly be made and approvals sought from the academic institution and the relevant ethics review committee that reviewed the study.

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# Open Peer Review

## Current Peer Review Status: ✔

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✔ **Cynthia Awuor Odhiambo**
Kenya Medical Research Institute, Nairobi, Nairobi County, Kenya

The manuscript is well organized, with a clear and logical flow from the introduction through to the discussion. It examines the prevalence and distribution of malaria parasite species in Cameroon and draws attention to the circulation of non-falciparum species. This is a significant and timely topic, particularly in light of the increasing recognition of non-falciparum malaria across sub-Saharan Africa. The findings have clear public health relevance.
However, to strengthen the scientific rigor and reproducibility of the study, the authors should consider providing the raw data, including detailed PCR results and the questionnaire dataset. In addition, incorporating a month-by-month breakdown of total malaria cases would enhance understanding of infection patterns over time and add further analytical depth to the manuscript.

**Is the work clearly and accurately presented and does it cite the current literature?**
Yes

**Is the study design appropriate and does the work have academic merit?**
Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**
Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**
Yes

**Are all the source data underlying the results available to ensure full reproducibility?**
Partly

**Are the conclusions drawn adequately supported by the results?**
Yes

***Competing Interests:*** No competing interests were disclosed.

***Reviewer Expertise:*** Molecular biologist

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**