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Harnessing Molecular Biotechnology to Optimize Malaria Diagnosis in Resource-Limited Settings

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ABSTRACT

Background: Malaria remains a major cause of morbidity and mortality, particularly among children under five in endemic areas. It is caused by different species of the *Plasmodium* genus. The World Health Organization recommends thick blood smear microscopy as the standard diagnostic tool, but its sensitivity is limited, especially in cases of low parasitaemia, non-falciparum infections, and when performed by unskilled personnel. Misdiagnosis is common in endemic areas, complicating disease management. Early and accurate diagnosis is essential to prevent progression, reduce deaths, and limit transmission. This study aimed to compare the diagnostic accuracy of microscopy, rapid diagnostic tests (RDTs), ELISA, and PCR, using PCR as the reference standard.

Methods: A total of 274 participants were recruited from three health centers. Samples were tested using microscopy, RDT, ELISA, and PCR. Diagnostic performance metrics—including sensitivity, specificity, predictive values, likelihood ratios, kappa statistics, and accuracy—were calculated. Subgroup analyses were stratified by gender, age, body temperature, symptom status, and collection site.

Results: ELISA reported the highest malaria prevalence, followed by RDT, microscopy, and PCR, with statistically significant differences across methods ($p = 0.0001$). Microscopy showed good sensitivity (82.8%) and specificity (80.6%), with moderate agreement with PCR ($\kappa = 0.60$). RDT demonstrated the best diagnostic agreement with PCR ($\kappa = 0.73$), high specificity (93.7%), and strong positive predictive value (85.1%). It also showed the highest positive likelihood ratio (PLR = 12.21), making it reliable for confirming malaria cases. ELISA exhibited the highest sensitivity (98.9%) and negative predictive value (96.9%), but its low specificity (56.0%) indicates a risk of overestimating cases due to antibody persistence. Its low negative likelihood ratio (NLR = 0.02) supports its utility for ruling out infection.

Conclusion: RDT emerged as the most diagnostically balanced method, offering high agreement with PCR and strong specificity. Microscopy remains valuable in skilled settings, while ELISA is better suited for surveillance than acute diagnosis due to low specificity. An integrated diagnostic approach leveraging the strengths of each method can enhance malaria control strategies in endemic areas like Buea, Cameroon.

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Introduction

Malaria is a life-threatening infectious disease caused by mosquito-transmitted parasites of the genus *Plasmodium*. It remains a significant public health concern in sub-Saharan Africa, where it is the second leading cause of death from infectious diseases after HIV/AIDS [1]. According to the 2024 World Malaria Report, an estimated 263 million malaria cases occurred in 2023 across 83 endemic countries, resulting in approximately 569,000 deaths. Children under the age of five are particularly vulnerable to malaria, accounting for 76% of the deaths due to malaria [1]. In addition to high mortality, the disease is a major cause of anemia and low birth weight in pregnant women, and it can impair cognitive and social development in affected children [2]. In Cameroon, approximately 4,000 malaria-related deaths are recorded annually, most of which occur in children under five [3]. The National Malaria Control Program (NMCP) reported over 3.1 million malaria cases and 3,863 deaths in 2021, accounting for 30% of health consultations and 14.3% of health facility deaths [4].

Accurate diagnosis is critical to effective malaria treatment, case management, and control strategies [5]. The conventional diagnostic method, microscopic examination of Giemsa-stained thick and thin blood smears, has been used for over a century. While microscopy is inexpensive and allows for species identification and parasite quantification, it requires significant training and expertise. Its sensitivity declines in cases of low parasitemia, mixed infections, and non-falciparum species [6]. Variability in skill among laboratory personnel can lead to inconsistent results and potential misdiagnosis.

To overcome these limitations, rapid diagnostic tests (RDTs) were developed as an easier alternative. RDTs detect parasite antigens such as histidine-rich protein II (HRP2) produced by P. falciparum, or enzymes like Plasmodium-specific lactate dehydrogenase (pLDH) and aldolase, which are common to all malaria species [7]. However, RDT performance is influenced by parasite density, geographic variation, and species prevalence. Their sensitivity decreases at parasite densities below 300–500 parasites/μL, and while they can detect approximately 100 parasites/μL, interpretation can be challenging [7]. RDTs may yield false positives due to HRP2 persistence post-treatment or false negatives due to HRP2 gene deletions and poor detection of non-falciparum species [8].

Molecular diagnostic tools, particularly polymerase chain reaction (PCR), offer high sensitivity and specificity, especially in detecting low-level and mixed infections [9]. However, PCR is expensive and requires advanced laboratory infrastructure, limiting its widespread use in low-resource settings. Enzyme-linked immunosorbent assays (ELISAs) have also been explored, particularly for detecting malaria antibodies [10]. While useful for epidemiological surveillance, ELISAs are less practical for routine clinical diagnosis due to delayed antibody production and cross-reactivity [10].

Despite decades of global control efforts, malaria continues to pose a major health threat, particularly in endemic regions like sub-Saharan Africa. Diagnosis remains a key challenge, with available tools varying widely in accuracy, cost, and feasibility [11]. Microscopy, while widely adopted, is labor-intensive and operator-dependent. RDTs are quick and user-friendly but may misclassify infections. PCR, though highly accurate, is resource-intensive. ELISA can detect previous exposure but lacks specificity for active infection. In many endemic areas, limited resources force health workers to rely on suboptimal diagnostic tools, increasing the risk of misdiagnosis, inappropriate treatment, and sustained transmission [12].

This study aims to compare the diagnostic performance of microscopy, RDTs, ELISA, and PCR for malaria diagnosis in Buea, Cameroon. By evaluating these methods against PCR as the reference standard, the study seeks to identify the most accurate and context-appropriate diagnostic approach to improve malaria detection and control in resource-limited settings.

Materials and Methods

Study Area and Design

This study was carried out in Buea, South West Region, Cameroon. Samples were collected from Solidarity Hospital, Regional Hospital and Muea Integrated Health Centre. This was a cross-sectional study where samples were collected from January to May 2025.

Study Population

A simple random sampling technique was used to select the study participants into the study. The study population comprised of individuals who were diagnosed of malaria whether they showed malaria sign and symptoms (Sick Subjects) or not (Semi-Immune Subjects). The study participants were between the ages of 0.1 to 90 years. The Inclusion criteria were participants who were diagnosed for malaria and gave their consent to take part in the study. Participants who were negative for malaria, sick of other diseases or did not give their consent to take part in the study were excluded.

Sample Size Calculation

The sample size was calculated using Cochran sample size formula for proportions [13].

$$n = \frac{(z_{1-\alpha/2} + z_{1+\beta})^2 \times [p_1(1-p_1) + p_2(1-p_2)]}{(p_1 - p_2)^2}$$

Where n is your sample size

Where p_1 and p_2= expected sensitivity of any two of the tests =0.90 and 0.75 respectively

Where $z_{1-\alpha/2}$ = confidence interval = 1.96 at 95% confidence

Where $z_{1+\beta}$ = power =0.84

$$n = \frac{(1.96 + 0.84)^2 x (0.90 x 0.10) + (0.75 x 0.25)}{(0.90 - 0.75)^2} = 96$$

Total Sample size = $\frac{n}{P}$

n = number of positive samples = 96

p = prevalence = 35% (0.35)

total sample size = $\frac{96}{0.35} = 274$

Sample Collection

After explaining the purpose of the research and obtaining a signed consent and a filled questionnaire, 2 ml of venous blood was collected from each participant and immediately used to make thick films and for RDT. The blood specimens were then dispensed into EDTA containers. Dry blood spot (DBS) were made on Whatman 903 filter paper so as to extract DNA for PCR test. The blood samples were then centrifuge and plasma collected for ELISA tests.

Microscopy

Microscopy was performed to detect malaria parasites, using thick smear stained with 10% Giemsa solution (Sigma-Aldrich, USA) for 15 min, allowed to air dry and subsequently examined by two trained microscopists. All the fields were examined for malaria parasite before declaring a slide negative.

Rapid Diagnostic Test

The RDT used was SD biolin malaria Ag p.f/p.v. The tests were performed by strictly adhering to the manufacturers' instructions.

Polymerase Chain Reaction (PCR)

Genomic DNA was extracted from dried blood spots (DBS) using the Chelex-100 resin method as described by Van Biesen et al., [14]. For PCR, 500 ng of genomic DNA was used with following

pair of primers for *Plasmodium falciparum*; rPLU6 (sense): TTAAAATTGTTGCAGTTAAAACG, rPLU5 (antisense): CCTGTTGTTGCCTTAAACTTC [15].

The primary PCR reaction was carried out in a 10 µL volume containing 5 µL of One Taq Quick-Load 2X Master Mix, 0.25 µL of each primer (10 µM), 2 µL of template DNA, and 2.5 µL of nuclease-free water. The following PCR conditions were used; pre-denaturation (94 °C for 3 min), denaturation (94 °C for 1 min), annealing (55 °C for 1 min), extension (68 °C for 1 min), number of cycles (25 cycles). final Extension (68 °C for 5 min). A PCR product of 1200bp was expected.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was used to detect antibodies against malaria parasite in the blood samples as earlier described with modifications [16]. Briefly, microtiter plates (Thermo Fisher Scientific, Roschester, NY, USA) were coated with 50ul of extracted malaria parasite protein antigen diluted in 0.1 M bicarbonate buffer, pH 8.5, and incubated overnight at 4°C. Plates were washed three times with 200 µL/well of wash buffer (PBS with 0.05% Tween-20), blocked with 200 µL/well of blocking buffer (PBS with 1% BSA), and incubated at 37°C for 1 h. The plasma samples (1:200 dilution) were diluted in antibody buffer (PBS with 0.5%BSA) and added to the plates (100 µL/well). The Plates were incubated for 1 h at 37°C . Afterwards, plates were again washed and 100ul of secondary anti-human IgG antibody conjugated to Alkaline Phosphatase (Calbiochem) diluted 1:10,000 (v/v) in antibody buffer according to manufacturer's instructions was added. After 1 hour, the plates were washed three times and incubated with 100 µL of substrate solution (20 mg of 4-nitrophenyl phosphate disodium salt hexahydrate diluted in 20 mL of Tris buffer) for 30 minutes at 37°C, protected from light. Finally, 50 µL/well of stop solution (3N sodium hydroxide) was added to stop the reaction and the absorbance in terms of Optical Density (OD) was read on a microplate reader at 405nm. These experiments were run in duplicates for each sample to improve reliability and accuracy. The samples were designated as positive if OD of the sample was greater than or equal to the mean OD of negative controls + 2 x Standard Deviation (2SD). The samples were designated as negative if the OD of the sample was less than the mean OD of negative controls + 2SD.

Measure of Diagnostic Accuracy

The sensitivity, specificity, positive predictive value, and negative predictive value for diagnostic test was assessed as follows;

$$\text{Sensitivity} = \frac{true\ positive}{true\ positive + false\ negative} \times 100$$

$$\text{Specificity} = \frac{true\ negative}{true\ negative + false\ positive} \times 100$$

$$\text{Positive predictive value} = \frac{true\ positive}{true\ positive + false\ positive} \times 100$$

$$\text{Negative predictive value} = \frac{true\ negative}{true\ negative + false\ negative} \times 100$$

Data Analysis

Data was analysed using descriptive statistics. Cohen's kappa coefficient was calculated using a 2x2 contigency table and used to compare the measure of agreements between microscopy and RDTs, PCR and in-house ELISA. The sensitivity, specificity, positive predictive value and negative predictive value were calculated using the formulae presented below by Medcal. Data analysis was performed with the aid of Microsoft excel (2010).

Ethical Considerations

The study received ethical approval from the Faculty of Health Science Institutional Review Board (FHSIRB), University of Buea, reference number 2024/2575-08/UB/SG/IRB/FHS of 30 October 2024. The study's objectives were explained, and the participant or the caregiver of each child provided consent by signing an informed consent form.

Results

Socio-Demographic Characteristics of Study Population

Of the 247 participants recruited for the study, 82 (29.9%) were obtained from Solidarity Health Foundation, 85 (31.0%) from the Buea Regional Hospital and the remaining 107 (39.1%) from Muea Integrated Health Center. All data is presented for all sites combined. Participants' age ranged between 3 weeks and 90 years with a median age of 24 years. Majority of the study populace were below 25 years (61.7%), followed by the 25 – 40 (27.4%), and 40 + (10.9%) age groups in descending order. Females were more than (51.8%) the number of males involved in the study (48.2%). Axial temperature of <37.5(54.1%) was greater than axial temperature >37.5 (45.9%) (Table 1)

Table 1: Socio-Demographic Characteristics of Study Participants

Variable	Sub-variable	Frequency	Percentage
Gender	Male	132	48.2
	Female	142	51.8
Age (years)	< 25	169	61.7
	25 – 40	75	27.4
	41+	30	10.9
Axial body temperature (°C)	<37.5°C	148	54.1
	≥37.5°C	126	45.9
Asymptomatic based on PCR		58	58.65
Symptomatic based on PCR		41	41.4
Hospital of Collection	Solidarity Health Foundation	82	29.9
	Buea Regional Hospital	85	31.0
	Muea Intergrated Health Center	107	39.1

Antibody Detection by ELISA

ELISA results showed that 175 samples had a mean OD value above the baseline and where designated as malaria positives while 99 samples had a mean OD value below the baseline value and where designated as negatives (Figure 1).

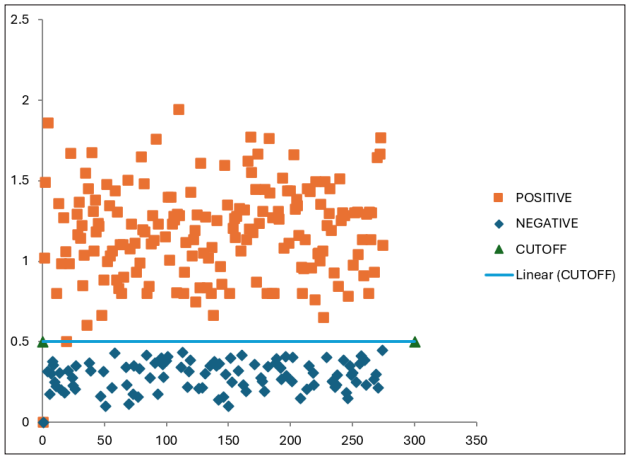

Figure 1: Absorbance (OD) at 405nm of the samples using ELISA. OD value of 0.5 (mean negative absorbance + 2SD) was considered the baseline value. Samples with mean OD above 0.5 were designated as positives while those with mean OD value below 0.5 were designated as negative for malaria parasite infection

Parasite Prevalence by Microscopy, RDT, PCR and ELISA
This study tested 274 blood samples using light microscopy of Giemsa-stained blood films, RDT (SD Biolin malaria ag p.f/pan), primary PCR and ELISA. The results are summarised in Table 2 and Figure 2; overall, 116 (42.5%), 87 (31.8%), 99 (36.1%) and 102(37.2%) samples were found positive by microscopy, RDT, PCR, and ELISA respectively.

Table 2: Prevalence of Parasite Based on their Diagnostic Methods

TEST	No. of Samples	No. of Positives	No. of Negatives
Microscopy	274	116 (42.3%)	158 (57.7%)
RDT	274	87 (31.8%)	187 (68.2%)
PCR	274	99 (36.1%)	175 (63.9%)
ELISA	274	175 (63.9%)	99(36.1%)

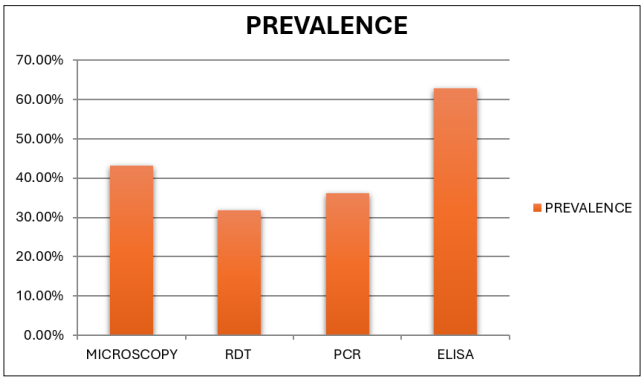

Figure 2: Prevalence of Malaria with Different Diagnostic Methods

Comparison Between the Diagnostic Methods Using PCR as Reference
The result of the performance of the microscopy and RDT is presented in Table 3 below. PCR was used as reference for the assessment of sensitivity of each method used in the analysis. This was explained as true positive if each methods tested positive as PCR for the same sample and as true negative, if each methods tested negative as PCR for same sample. The true positive, negative values, positive predictive value and negative predictive value are listed in Table 4. The ELISA has more false positives (77) than RDT (11) and microscopy (34) while RDT recorded higher true negative value (164) than microscopy (141) and ELISA (98).

Table 3: Comparison Of Microscopy, RDT and ELISA Using PCR as Reference

	Microscopy	RDT	ELISA
True positive	82	76	98
True negative	141	164	98
False positive	34	11	77
False negative	17	23	1

Sensitivity and Specificity of Microscopy, RDT and ELISA Using PCR as Reference
The comparative performances of the methods are presented in Table 4. Considering PCR as the reference method, the sensitivity and specificity of the RDT were found to be 76.8% and 93.7%, respectively, for microscopy, they were 82.8% and 80.6%, respectively while for ELISA they were 98.9% and 56.0% rspectively. The PLR of RDT (12.0) was higher than microscopy and ELISA (moderate evidence), indicating that RDT is slightly better at indicaing malaria if the test is positive. In addition, the NLR of the ELISA was 0.02, showing strong indication that a negative test suggests the absence of malaria. However, the NLR of microscopy was 0.2, suggesting that a negative test does not indicate the absence of malaria. Both the microscopy and ELISA showed a moderate agreement with the PCR results (kappa = 0.60 and 0.47, respectively) while RDT (kappa= 0.7) showed substantial agreement with PCR.

Table 4: Sensitivity and Specificity of Microscopy, RDT and ELISA Using PCR as Reference

Statistics	Microscopy	RDT	ELISA
Sensitivity (%)	82.83 (73.9 – 89.7)	76.8 (67.2 – 84.7)	98.9 (94.5 – 100)
Specificity (%)	80.6 (73.9 – 86.1)	93.7 (89.0 – 96.8)	56.0 (48.3 – 63.5)
PPV (%)	75.5 (69.3 – 80.9)	85.1 (76.1 – 91.1)	79.9 (77.1 – 82.4)
NPV (%)	86.6 (80.7 – 90.9)	89.6 (85.8 – 92.5)	96.9 (86.6 – 99.6)
PLR	4.3 (3.1 – 5.8)	12.21 (6.8 – 21.7)	2.3(1.9 – 2.7)
NLR	0.2 (0.1 – 0.3)	0.3 (0.2 – 0.4)	0.02 (0.0 – 0.1)
Accuracy (%)	81.5 (56.1 – 67.9)	88.3 (83.9 – 91.9)	83.5 (78.5 – 87.7)
Kappa value	0.60	0.73	0.47
p-value	3.6E-24	5.7E-32	8.8E-20

PPV: Positive Predictive Value, NPV: Negative Predictive Value, PLR: Positive Likelihood Ratio, NLR: Negative Likelihood Ratio. Data between parentheses are 95% CI.

Graphical Representation of the Statistical Results
Graphical representation showed that ELISA had the best sensitivity while RDT had the best specificity. RDT had the best PPV while ELISA had the best NPV. RDT had the best accuracy and PLR (Figure 3).

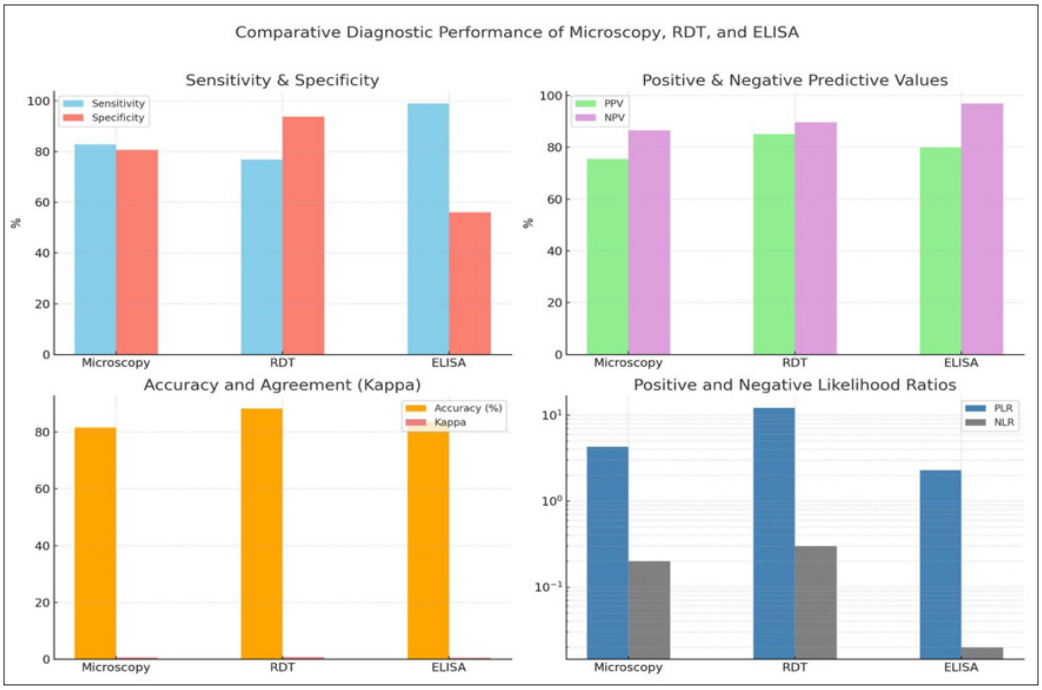

Figure 3: Graphical Representation of the Statistics

Performance According to Participants Characteristics of Microscopy, RDT and PCR

Microscopy showed slightly higher sensitivity in males (83.3%) compared to females (82.4%), with nearly identical specificity (81.0% vs. 80.2%). RDT outperformed microscopy in both genders, particularly in specificity (94.0% in males and 93.4% in females). ELISA showed the highest sensitivity across both genders (97.9% in males and 100% in females), with specificity (56% in both genders), The kappa statistic, was highest in RDT ($\kappa = 0.731$ in males and $\kappa = 0.717$ in females), followed by microscopy. ELISA had the lowest agreement.

Microscopy maintained consistent performance across age groups, with sensitivity ranging from 82.1% in adults aged 25–40 to 88.9% in those 41 years and above. RDT performance was stable, with high specificity (>93%) and moderate sensitivity across all age categories. Interestingly, ELISA maintained a sensitivity of 100% in adults aged 25–40 and 41+, but specificity (56%).
Across temperature for microscopy, sensitivity reduced from 84.0% in afebrile participants to 82.0% in febrile ones. RDT sensitivity similarly increased from 76.5% to 77.1%, while ELISA jumped from 98.0% to 100%.

Symptomatic individuals had higher detection rates across all tests. Microscopy improved from 76.0% in asymptomatic cases to 83.2% in symptomatic ones. ELISA maintained the highest sensitivity: 98.9% in asymptomatic and 100% in symptomatic patients. RDT performed similarly, with a noticeable drop in asymptomatic detection.

Table 7: Performance of Microscopy, RDT and ELISA According to Participants Characteristics

Subgroup	Method	TP	TN	FP	FN	Sensitivity	Specificity	Accuracy	Kappa	p-value
Gender_Male	Microscopy	40	68	16	8	0.833	0.81	0.818	0.621	0.00001
Gender_Male	RDT	37	79	5	11	0.771	0.94	0.879	0.731	0.0001
Gender_Male	ELISA	47	47	37	1	0.979	0.56	0.712	0.464	0.0001
Gender_ Female	Microscopy	42	73	18	9	0.824	0.802	0.81	0.602	0.0001
Gender_ Female	RDT	39	85	6	12	0.765	0.934	0.873	0.717	0.0001
Gender_ Female	ELISA	51	51	40	0	1.0	0.56	0.718	0.478	0.0001
Age_<25	Microscopy	51	87	21	11	0.823	0.806	0.812	0.607	0.0001
Age_<25	RDT	47	101	7	14	0.77	0.935	0.876	0.724	0.0001
Age_<25	ELISA	60	61	47	1	0.984	0.565	0.716	0.471	0.0001
Age_25-40	Microscopy	23	39	9	5	0.821	0.812	0.816	0.616	0.0001
Age_25-40	RDT	21	45	3	6	0.778	0.938	0.88	0.733	0.0001
Age_25-40	ELISA	27	27	21	0	0.983	0.562	0.72	0.481	0.0001

Age_41+	Microscopy	8	15	4	1	0.889	0.789	0.821	0.624	0.0029
Age_41+	RDT	8	18	1	3	0.727	0.947	0.867	0.701	0.0005
Age_41+	ELISA	11	10	9	0	1.0	0.526	0.7	0.449	0.0109
Temp_<37.5	Microscopy	42	73	18	8	0.84	0.802	0.816	0.615	0.0001
Temp_<37.5	RDT	39	84	6	12	0.765	0.933	0.872	0.716	0.0001
Temp_<37.5	ELISA	50	50	39	1	0.98	0.562	0.714	0.468	0.0001
Temp_≥37.5	Microscopy	40	68	16	9	0.816	0.81	0.812	0.608	0.0001
Temp_≥37.5	RDT	37	80	5	11	0.771	0.941	0.88	0.732	0.0001
Temp_≥37.5	ELISA	48	48	38	0	1.0	0.558	0.716	0.475	0.0001
Hospital_BRH	Microscopy	36	62	15	7	0.837	0.805	0.817	0.617	0.0001
Hospital_BRH	RDT	33	72	5	10	0.767	0.935	0.875	0.721	0.0001
Hospital_BRH	ELISA	43	43	33	1	0.977	0.566	0.717	0.471	0.0001
Hospital_ MHC	Microscopy	27	46	11	6	0.818	0.807	0.811	0.606	0.0001
Hospital_ MHC	RDT	25	54	3	8	0.758	0.947	0.878	0.728	0.0001
Hospital_ MHC	ELISA	32	32	26	0	1.0	0.552	0.711	0.467	0.0001
Hospital_SC	Microscopy	19	33	8	4	0.826	0.805	0.812	0.608	0.0001
Hospital_SC	RDT	18	38	3	5	0.783	0.927	0.875	0.723	0.0001
Hospital_SC	ELISA	23	23	18	0	1.0	0.561	0.719	0.479	0.0001

TP = True Positive, TN = True Negative, FP = False Positive, FN = False Negative

Discussions

Malaria remains a pressing public health concern in endemic countries like Cameroon, where accurate and timely diagnosis is critical for reducing morbidity, mortality, and transmission. In this study, we evaluated the diagnostic performance of microscopy, RDT, and ELISA against PCR as the reference standard across 274 samples, assessing prevalence, sensitivity, specificity, predictive values, likelihood ratios, accuracy, and agreement statistics.

Prevalence rates differed significantly among methods ($p = 0.0001$), with ELISA recording the highest positivity rate, followed by microscopy, PCR, and RDT. ELISA's high positivity likely stems from its sensitivity to both active and past infections due to antibody or residual antigen detection. These findings align with prior studies [17,18]. RDT's low prevalence may be due to HRP2 gene deletions, low parasitemia, or inherent sensitivity limitations. Microscopy's higher positivity compared to PCR may reflect over-calling or observer bias, reinforcing the role of PCR as a confirmatory tool [19].

ELISA exhibited the highest sensitivity (98.9%), outperforming microscopy (82.8%) and RDT (76.8%). High sensitivity is crucial in screening programs to minimize false negatives. However, ELISA's specificity was lowest (56.0%) due to residual antigenemia or antibodies from past exposure, in contrast to RDT, which showed the highest specificity (93.7%), followed by microscopy (80.6%). The trade-off between ELISA's sensitivity and low specificity underscores its limited suitability for clinical diagnosis but strong value in surveillance.

Positive predictive value (PPV) was highest for RDT (81.5%), indicating better clinical accuracy in confirming infection. ELISA had the highest negative predictive value (96.9%), suggesting strong rule-out capability. This result is similar to what was obtained Kwenti and colleagues [20]. Correspondingly, ELISA had the best negative likelihood ratio (0.02), making it excellent for excluding infection, while RDT had the highest positive likelihood ratio (12.2), reflecting strong confirmation power.

RDT also had the highest diagnostic accuracy (88.3%) and agreement with PCR ($\kappa = 0.73$), followed by ELISA (83.5%, $\kappa = 0.47$) and microscopy (81.5%, $\kappa = 0.60$). As also shown by others, these data reinforce RDT's utility in clinical diagnosis [21]. All modalities showed statistically significant associations with PCR (RDT p = 5.7E-32), confirming the robustness of findings.

Subgroup analyses by gender, age, temperature, and hospital location confirmed the consistency of performance trends. ELISA maintained high sensitivity across all groups but had uniformly low specificity, particularly among older individuals, likely due to repeated exposure and lingering antibodies [22]. Microscopy showed improved sensitivity in adults ≥41 years, likely due to higher parasitemia aiding visualization. RDTs retained high specificity across age and sex groups, confirming their reliability and low operator dependence [23].

Febrile participants showed marginally improved test performance, particularly with microscopy and RDT, due to elevated parasitemia. Site-based comparisons showed that diagnostic performance varied across health facilities. BRH had the highest microscopy sensitivity

(85.7%), while RDT showed consistent performance across sites, with MHC achieving the best kappa value (0.701). These findings highlight the robustness of RDTs and the dependence of microscopy on laboratory quality and technician skill [24].

Prevalence data revealed ELISA as the most inclusive method, detecting the highest proportion of positives, likely due to its detection of past infections. In contrast, RDT underestimated prevalence, especially in asymptomatic cases. Subgroup analyses confirmed stable diagnostic trends across demographic and clinical strata, reinforcing the validity of findings.

RDT emerged as the most diagnostically balanced and field-appropriate tool. ELISA is best suited for surveillance and sero-epidemiological studies, while microscopy remains important for species identification and parasitemia monitoring in capable settings. A multi-tiered diagnostic strategy is recommended, incorporating RDT for rapid screening, ELISA for surveillance, and microscopy or PCR for confirmation and research.

Overall, the results emphasize that no single method is sufficient across all diagnostic needs. ELISA is ideal for surveillance and detecting asymptomatic carriers, particularly in high-transmission areas. RDTs provide a balance of high specificity, moderate sensitivity, and field applicability. Microscopy remains relevant for parasite quantification and species differentiation but is dependent on skilled personnel and quality equipment.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The research data are available upon request from the corresponding author.

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Author's Contribution
Conceptualization, JND; methodology, AYT, DLN, and SDG; validation, JND, AYT, DLN, SDG and VPKT; formal analysis, JND and VPKT; investigation, JND and SDG; writing—original draft preparation, JND, MT, and SDG; writing—review and editing, JND and VPKT. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest
The authors have no conflict of interest to declare.

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