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Research Article

Performance and Comparative Evaluation of a Novel Diagnostic Assay, Novaplex™ Malaria Assay, against Routine Diagnostic Techniques in the Detection of Different Plasmodium spp. in Kenya

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Abstract

Background: Accurate and rapid diagnosis of malaria is crucial for effective treatment and control. More so, accurate species identification is central in guiding treatment strategies across infections with different species of Plasmodium. This study aimed to evaluate the performance of a novel malaria diagnostic kit, Novaplex™ Malaria Assay, compared to routine diagnostic techniques currently in use, including microscopy, rapid diagnostic tests (RDTs), and polymerase chain reaction (PCR) in malaria diagnosis.

Methods: A total of 142 suspected malaria cases from Matayos, a malaria endemic zone in Kenya, were sampled. Whole blood samples were collected, Plasmodium parasite positivity and species identification were performed using microscopy, rapid diagnostic kits, the Novaplex™ malaria diagnostic assay, and qPCR. Sensitivity, specificity, positive predictive value [PPV], negative predictive value [NPV], accuracy, and agreement [Cohen's kappa] were calculated to assess the diagnostic performance of the Novaplex™ kit against the rest of the techniques.

Results: Our analyses demonstrated that the Novaplex™ malaria assay yields a superior outcome compared to microscopy or mRDTs in terms of sensitivity, accuracy and NPV. The assay showed an overall diagnostic agreement with qPCR. Also, the kit showed an almost similar performance to qPCR in species identification. Using qPCR as the comparator "gold standard" test for the analysis, the sensitivity and specificity of the Novaplex™ assay was 94.8% and 100% respectively, while the sensitivity of microscopy and RDT was 63.7% and 61.5% respectively. The positive and negative predictive values were 100% and 53.9% respectively, for the Novaplex™ assay. This was in contrast to NPV values for microscopy and RDT which were 12.5% and 11.9% respectively. The accuracy of the Novaplex™ assay was recorded at 95.8% having a substantial agreement with qPCR at k=0.679 (95% CI: 0.442 to 0.917). The level of accuracy for Microscopy and RDT was determined to be 65.5% and 63.4% respectively, with a slight agreement with qPCR at k=0.148 (95% CI: 0.047 to 0.248), and k=0.136(95% CI: 0.042 to 0.230) respectively.

Conclusion: These findings demonstrate that the Novaplex assay outperformed microscopy or RDTs, showing comparable performance to qPCR in the identification and speciation of Plasmodium species in malaria infections. The high sensitivity, specificity, and overall agreement highlight the potential of the Novaplex assay as a reliable diagnostic tool for malaria. Implementation of this assay in routine clinical practice could improve the accuracy and efficiency of malaria diagnosis, leading to timely

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and appropriate treatment, enhanced surveillance, and effective control measures. Further validation studies and field evaluations are warranted to confirm the feasibility and cost effectiveness of this diagnostic assay in diverse malaria-endemic low resource settings.

Keywords: Malaria; Novaplex; Species-specific; qPCR

Introduction

Malaria remains a health burden in many developing countries in spite of enormous investments in control efforts. The latest reports show that there were an estimated 249 million malaria cases in 2022 with an estimated 608 000 malaria deaths globally [1]. Majority of the burden lies in sub-Saharan Africa with the most vulnerable ones being pregnant women and children below the age of 5 years [2]. In Kenya there was an estimated 3.4 million new malaria cases annually with at least 291 reported deaths [1]. This burden coupled with the numerous reports of resistance to the current medical interventions makes malaria a primary concern for medical research. A critical aspect in the fight against malaria is accurate diagnosis followed by timely treatment with effective antimalarial drugs [3]. This was also set out in the WHO guidelines for malaria treatment which stipulate that parasitological confirmation of infection is required prior to treatment [4]. The current diagnostic techniques for malaria detection include clinical microscopy use of the rapid diagnostic test (RDT) kits and Polymerase Chain Reaction (PCR) techniques using plasmodium specific gene markers. These techniques are however plagued with drawbacks; need for trained personnel for microscopy delayed results when using PCR due to the duration of the assay thus not suitable for routine diagnosis and the inability to distinguish between the species of parasites in a mixed infection for RDTs. Microscopy is widely recommended as the standard tool for parasitological confirmation of malaria as it is highly adaptable to the poor and marginalized settings where majority of the cases occur. However, this technique is limited by the need for highly trained and experienced technicians who may not always be available. There is a shortage of skilled microscopists in Kenya which negatively affects the quality of test results [5]. It has also been noted that ensuring the quality of staining procedures for blood slides is a difficult task especially in rural settings which is a major drawback of microscopy as a stand-alone diagnostic tool for malaria infections [6]. This technique is also limited to relatively high parasitemia detection thresholds which means that individuals with sub-microscopic parasitemia often go untreated contributing to the ongoing transmission by malaria vectors [7]. Poor quality laboratory reagents and lack of good quality microscopes also hinder the use of microscopy for diagnosis. This clearly shows that microscopy needs to be supplemented by more sensitive diagnostic tests to reduce transmission rates. Rapid diagnostic test (RDT) kits used in malaria diagnosis have overcome some of these limitations. The RDTs can either detect Plasmodium falciparum infections or non-falciparum infections. Those that are HRP-2 based only detect P. falciparum whereas those that detect the rest of the antigens (pLDH aldolase) don't distinguish falciparum infections [8]. There are several shortcomings of using Pan RDTs in malarial diagnosis. The tests are incapable of detecting specific non-falciparum infections. The test cannot conclusively indicate which of the four common plasmodium species caused the non-falciparum infection limiting the quality of the results. Advantages of using RDT kits in malaria diagnosis include their specificity quick turn-around time and simplicity voiding the need for highly trained lab personnel. However, several reports of mutation/deletions in *Plasmodium falciparum Histidine Rich Protein 2* (Pfhrp2) and *Plasmodium falciparum Histidine Rich Protein 3* (Pfhrp3) genes from other malaria endemic countries including within Sub Saharan Africa resulting in false negative malaria diagnosis results when using RDT kits. There have been improvements to RDT kits especially RDT kits that use Pfhrp2 with development of ultra-sensitive kits which have lower limits of detection and are able to detect parasites even in instances of low parasitemia. However, Pfhrp2 is also known to be produced by gametocytes in a stage specific manner and this has been attributed to several cases of false positivity especially among patients treated with chloroquine and Sulfadoxine -pyrimethamine [9]. There is also variance in the analytical sensitivity of different hrp2-based and Pan LDH- based RDT with limit-of-detection for pLDH based RDTs being significantly higher than those of hrp-2 based RDTs [10]. This indicates the need to establish universal reference assays for diagnosis using RDTs. The Government of Kenya has utilized RDTs as its major diagnosis tool in its parasite-based diagnosis policy. RDTs are used as the first line diagnosis tool to allow the swift detection of malaria infections in the country [11]. However, each RDT performs differently in different regions in the country. The differences in performance can be attributed to population differences genetic variation of the parasite in different regions and the diagnostic skills of the medical health worker [12, 13]. As a consequence, the reliability of RDTs as a diagnostic tool is questioned and a need for more specific detection method warranted.

PCR depends on the use of primers that target specific genes on the parasite. Most PCR protocols target the 18S-rRNA gene on the parasite since it is a highly conserved region [14]. The target is however present in very low copy number and thus may not be sensitive in cases of low parasitemia. This drawback led to the development of protocols that target other sites that have a higher copy number and the use of non-

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ribosomal targets. A previous study by Amarall, et. al, 2019 was conducted to compare the sensitivity of PCR in detecting mixed infections using ribosomal and non-ribosomal targets. The ribosomal target was the 18S-rRNA gene and the non-ribosomal target was the Pfr364 and Pvr47 as defined by Demas. They concluded that the non-ribosomal targets showed a higher sensitivity for detecting mixed infections at 3P/ul [15, 16]. Since then, highly sensitive qPCR methods have been developed that can detect 0.002P/ul [17]. Targeting the genes on the parasite makes the method highly sensitive to mixed and single infections. The method is still however wrought with drawbacks, the most prominent one being the need for expertise while using qPCR. The procedure is also very expensive and needs special infrastructure, which may not be present in rural settings. While all these tests have been instrumental in diagnosis, a gap still remains in ensuring a quick and accurate, species-specific parasite diagnosis of patients with malaria. Determination of the specific species of plasmodium causing the illness is essential in guiding appropriate treatment strategies, assessing disease severity, conducting epidemiological surveillance, and monitoring drug resistance. It plays a pivotal role in improving patient outcomes, optimizing resource allocation, and advancing our understanding of malaria. Different species of Plasmodium exhibit variations in their pathogenicity and susceptibility to antimalarial drugs. Species such as *Plasmodium vivax* and *Plasmodium ovale* may lead to cases of complicated malaria making treatment difficult as they have dormant hypnozoites which cause relapses even after treatment. Their management requires additional treatment to eliminate the dormant liver-stage parasites (hypnozoites). Failure to identify the species correctly could result in inadequate treatment, leading to prolonged illness, increased parasite resistance, and potentially life-threatening complications. *Plasmodium falciparum*, the most common and deadly malaria parasite, is often resistant to certain antimalarial drugs, such as chloroquine, not to mention emerging resistance to the current Artemisinin based antimalarials across different malaria endemic regions. More so, this species is associated with more severe forms of malaria, including cerebral malaria and severe anemia, requiring immediate and aggressive treatment. By accurately determining the infecting species, healthcare providers can better assess the potential severity of the disease and implement appropriate management strategies. Accurate species identification also plays a vital role in monitoring the prevalence and distribution of different Plasmodium species in a particular region. Understanding the species-specific epidemiology helps public health authorities allocate resources effectively, implement targeted control measures, and develop appropriate prevention and treatment policies. It also aids in tracking the emergence of drug resistance and evaluating the effectiveness of malaria control programs. As such, a gap still remains in ensuring a quick, species-specific diagnosis of patients presenting with malaria symptoms. Parasite specific diagnosis will help in prognosis as it will inform on treatment strategies including expected drug susceptibility of the infecting parasite based on response to treatment for previous cases in the specific location of infection. The Novaplex™ malaria assay is a qPCR-based kit that seeks to narrow this gap by providing an optimized diagnostic solution capable of species-specific diagnosis by distinguishing the plasmodium species present in malaria infection. It uses real time PCR equipment to perform a multiplex PCR reaction on extracted parasite DNA to detect the five common Plasmodium species (*P.falciparum, P.malariae, P.ovale, P.vivax and P.knowlesi*) in a single reaction tube. This ensures swift detection of Plasmodium infections. This study therefore aimed at evaluating the performance of the Novaplex™ Malaria Assay**,** compared to routine diagnostic techniques currently in use, including microscopy, rapid diagnostic tests (RDTs), and quantitative polymerase chain reaction (qPCR) for diagnosis of malaria in an endemic zone within Kenya.

Materials and Methods

Study Design

This was a cross-sectional validation study. Samples were collected, between November 2022 and February 2023, from patients presenting with malaria symptoms visiting health facilities within the malaria endemic zone of Busia Kenya, known to have high cases of mixed Plasmodium infections in the population. This region is a lake-endemic malaria transmission zone in Kenya, with malaria accounting for the leading cause of mortality. Rainy seasons, high temperature and humidity conditions in this region favor breeding of malaria transmission vectors especially *Anopheles gambiae spp.* and a subsequent increase in transmission rates. The region borders a large water body, Lake Victoria and lies between latitude 00º 01’ and 00º 47’ north of the Equator.

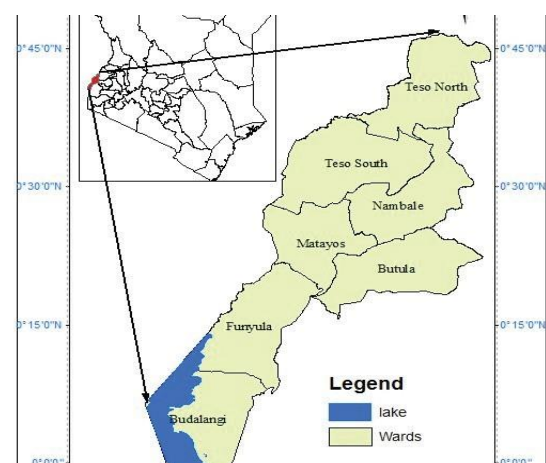

Figure 1: Map showing the location of the study area, Busia County, in Kenya.

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Sample collection was done in Matayos Sub-County. Available from: https://www.researchgate.net/figure/Area-of-study-Busia-County-12-GIS-And-Malaria-Risk-Mapping-Studies-and-developments- on_fig1_303633783.

Sample Collection

All individuals presenting with suspected malaria infections (febrile illness) were screened using microscopy and RDTs as potential participants in the study. After clinical assessment at the healthcare facilities based on routine care, patients who were 6 months and older were consented into the study. The participants included those who tested positive using either RDT and/or microscopy, and those who screened negative using either of the two diagnostic techniques. A total of 142 participants were recruited into the study. For all the patients recruited, up to 2ml of venous blood was collected in an EDTA tube which was labelled with the patient's screening number. 10µl of the collected blood was blotted per spot for a total of 5 spots on a Whatman 503 filter paper for a Dried Blood Spot. The remaining whole blood sample was shipped to the lab for storage at -80ºc. The screening number was also recorded on the frosted edge of the microscopy slide and on the side of the RDT test kit used to screen the participants for follow up confirmation. Samples from consenting participants that tested positive using both RDT and microscopy were enrolled as positive study samples while those that tested RDT negative and showed no malaria parasites on slide microscopy were enrolled as negatives pending PCR confirmation. The malaria positive patients were treated based on the existing malaria treatment guidelines while the negative patients went back into the facility routine care for further clinical management.

Diagnostic Techniques

Microscopy: Thick and thin blood films for parasite counts were prepared and examined at screening. All the eligible patient's specimens were labeled anonymously. The screening number was recorded on the frosted edge of the slide with a pencil. The Giemsa-stained thick and thin blood films were then examined at a magnification of 100X to identify the parasite species and to determine the parasite density. The parasite density was calculated by counting the number of asexual parasites per 200 white blood cells observed with a hand tally counter, and expressed as the number of asexual parasites per µl of blood. This was done by dividing the number of asexual parasites by the number of white blood cells counted and then multiplying by an estimated white blood cell density of 8000 per µl.

RDT tests protocol: A malaria Pan/HRP2 RDT test was performed using Bioline™ Malaria Ag P.f/Pan mRDT kit [Abbott] as per the manufacturer's instructions. Briefly, the kit test buffer components were mixed well prior to testing. A lancet prick was made at the patient's fingertip where the inverted cup blood transfer device from the test packet was used to transfer the blood specimen from the finger prick to the sample well of the RDT test device. The volume of the specimen was about 5µl. 2 drops of the lysis buffer were added to the test kit at the buffer well and a timer was set for 15 min after which the results were read and interpreted. The RDT results were read and interpreted as per the manufacturer's instructions; a negative result was inferred if only the C line [Control] was present and there was no line presence at the Pf and Pan test lines. If in addition to the C line only the Pan line developed, the test indicated the presence of pLDH antigen which inferred the test was positive for either of the plasmodium species (*P.falciparum, P.vivax, P.malariae, and P. ovale*). If in addition to the C line only the Pf line developed, the test indicated the presence of pHRP2 antigen inferring a positive *Plasmodium falciparum* test. If both Pan and Pf lines developed in addition to the C line the test indicated the presence of both pLDH and pHRP2 showing a positive test for *P.falciparum* and any of the other three plasmodium species(*P.ovale P.malariae, and P.vivax*). No invalid test occurred where no C line developed regardless of the presence or absence of a test line in either Pf or Pan. The mRDT test kits were also labeled with the patients' screening numbers for follow-up of results.

DNA extraction: DNA was extracted from the DBS prepared using the Chelex™ resin method. Briefly, a small piece, 3 mm in diameter, of the DBS sample was cut using a sterile punch and placed into a labeled microfuge tube. 1ml of 0.5% saponin in 1X PBS was added and the mixture incubated overnight at 4°C. The resulting solution was replaced with 1ml of 1X PBS and incubated further for 15 minutes. The solution was discarded after incubation and 150 µL of DNase free water, 50µL of 20% Chelex solution was added. This was then incubated at 100°C for 10 minutes with thorough mixing by vortexing at 2-minute intervals [9]. After centrifugation, the supernatant was transferred into a fresh tube as working DNA leaving the Chelex matrix in the first microfuge tube. The resulting DNA was then used for molecular analysis.

PCR protocol: Plasmodium detection was performed by using TaqMan™ probes specific to each species to detect the plasmodium species present in each sample. This was done using previously published [28] primers(Table 1) with modified fluorophores

FAM – Fluorescein amidites, MGB- Minor groove binder moiety. The listed primers target the following genes: *Pf*var gene acidic terminal sequence[varATS] gene, *Pm* circumsporozoite [cs] gene, *Po* reticulocyte binding protein-2(rbp2) gene, *Pv* enoyl-acyl carrier protein reductase[ecpr] gene.

Amplification was done on a CFX96 ™ real time PCR machine (Biorad™), with each 15-µl reaction mix containing 2µl of sample DNA, 7.5µl of GoTaq® Probe qPCR Master

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Table 1: Primers and probes used for qPCR amplification

Species	Primer/ probe	Sequence 5'-3'	Fluorescent label
P. falciparum	VAR_ ATS F	CCCATACACAACCAAYTGGA	
P. falciparum	VAR_ATS R	TTCGCACATATCTCTATGTCTATCT	
P. falciparum	VAR_ATS Probe	TRTTCCATAAATGGT	FAM-MGB
P. vivax	PvVo F	CAAGCGGAAGGGATAAATGG	
P. vivax	PvVo R	CCGCGATGAAGCAGATGTCT	
P. vivax	PvVo Probe	AAGGGAGAACCCC	FAM-MGB
P.malariae	PmVo F	CTCAAATTCCACCAAGTCAAGAAA	
P.malariae	PmVo R	GATTCGTGCTATATCTGACTTCTAACTCA	
P.malariae	PmVo Probe	AGTGAGTTGTGTTACAATAA	FAM-MGB
P. ovale	PoRBP2 F	CCA CAG ATA AGA AGT CTC AAG TAC GAT ATT	
P. ovale	PoRBP2 R	TTG GAG CAC TTT TGT TTG CAA	
P. ovale	PoRBP2 Probe	TGAATTGCTAAGCGATATC	FAM-MGB

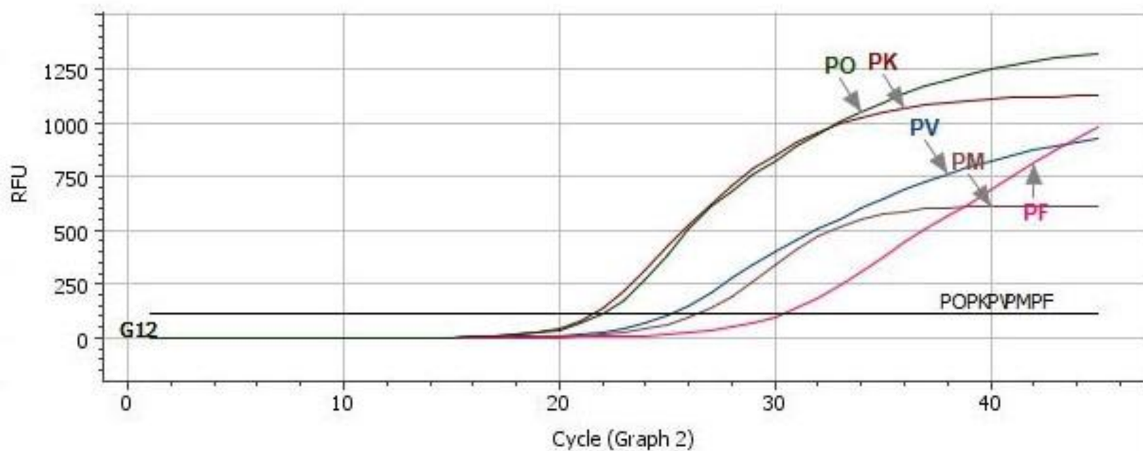
Mix (Promega™), and the different primer and probes for each target analyte. This amplification was not multiplexed. The PCR conditions included an initial denaturation at 95°C for 15 min, followed by an amplification of 45 cycles for 10 sec at 95°C, then 1min s at 60°C, with plate read at the end of each cycle. A Ct value of ≤40 indicated a positive test for the target analyte

Novaplex™ Malaria Assay

The Novaplex™ assay is a multiplex real time PCR kit that uses species specific primers with different fluorophores for the different plasmodium species target probes; FAM labelled *P.vivax* probe, HEX labelled *P.ovale* probe, Cal Red610 labelled *P.falciparum* probe, Quasar 670 labelled *P.malariae* probe and Quasar 705 labelled *P.knowlesi* probe, Figure 2 below. qPCR amplification was done according to the manufacturer's instructions. Briefly, a 20µl reaction tube contained 5µl of the kit PCR mix, 5µl of the kit primer mix, 5µl of RNase free water, and 5µl of the sample. Amplification was done on a CFX96 ™ real time PCR machine (Biorad™) with the protocol set for initial denaturation at 95°C for 15 min, followed by amplification for 45 cycles of 10 sec at 95°C, 15sec s at 60°C annealing, and 10 secs at 72°C extension with fluorescence acquisition at the end of the annealing and extension steps. A Ct value of ≤40 indicated a positive test for the target analyte. An Internal Control was included in every reaction to confirm amplification.

Data Analysis

Data from the CRFs and laboratory analysis were coded and recorded on Microsoft Excel 2019. This was then analyzed using both STATA 15.1 and Prism 10.0.0(GraphPad, San Diego, CA), all at a p value of < 0.05. STATA was used to create summary tables for the analysis results after which contingency tables were drawn in Prism and the accuracy, diagnostic sensitivity and specificity, positive predictive value



PO-*Plasmodium ovale*, PK-*Plasmodium knowlesi*, PV- *Plasmodium vivax*, PM-*Plasmodium malariae*, PF- *Plasmodium falciparum*

Figure 2: Amplification curves for a control sample containing all five plasmodium targets using the Novaplex™ assay.

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(PPV) and negative predictive value (NPV) calculated for the Novaplex™ malaria assays as described [18], using real-time TaqMan probe-based qPCR as the reference test. Measures of distribution, mean and median (with Inter Quartile Ranges) were used to describe the socio-demographic features of the sample population. Agreement between the diagnostic tests was calculated using Cohen's kappa where kappa < 0 indicated no agreement, kappa between 0.00 and 0.20 showed slight agreement, kappa between 0.21 and 0.40 highlighted fair agreement, kappa between 0.41 and 0.60 translated to moderate agreement, kappa between 0.61 and 0.80 inferred substantial agreement and kappa between 0.81 and 1.00 was an almost perfect agreement.

Results

Socio-demographic characteristics

This study recruited a total of 142 patients. Ninety-five of the patients [66.9%] were recruited from Matayos Sub-County hospital and the remaining 47 [33.1%] recruited from Sioport Sub-County hospital. The total population consisted of 101 [71.1%] females and 41 [28.9%] males. Most of the participants were between the ages of 15-30, with a median age of 16 years [IQR:9.35-22.5]. The axillary temperature at recruitment ranged from 34.3ºc – 39.4ºc with a median of 37.0ºc [IQR: 36.6 – 37.5]. The socio-demographic characteristics are as summarized in Table 2.

Malaria diagnosis by different diagnostic methods

The study samples were analyzed using different methods. Microscopy and RDT were used at the field sites during sample collection by the health center staff, after which PCR analysis and analysis using the Novaplex™ kit were done at the lab. Only 60.6%[86/142] were diagnosed with clinical malaria at the health centers, which is highly contrasted with a positivity of 90.8%[129/142] by Novaplex® assay, and 95.1%[135/142] by qPCR. There was a single case of an RDT positive diagnosis which was negative by microscopy, and 4 cases of RDT negative diagnoses which were positive by microscopy. All of these were observed from Matayos field site, with estimated sample parasite densities of 143, 200, 360, and 400 parasites/ul.

Socio-demographic characteristics	Study area		Both sites, n [%]
	MSCH, n [%]	SSCH, n [%]	
Gender			
Female	70 [73.7]	31 [66.0]	101 [71.1]
Male	25 [26.3]	16 [34.0]	41 [28.9]
Age group [years]			
<5	12 [12.6]	4 [8.5]	16 [11.3]
5–14	31 [32.6]	14 [29.8]	45 [31.7]
15–30	35 [36.8]	23 [48.9]	58 [40.8]
>30	17 [17.9]	6 [12.8]	23 [16.2]
Height[cm]			
Minimum	64	8.93	8.93
Maximum	184	174	184
Median			155
Weight[kg]			
Minimum	5.2	8.02	5.2
Maximum	170	74.5	170
Median			50.1
Temperature			
Minimum	34.3	35.6	34.3
Maximum	39.4	39.2	39.4
Median			37

The table highlights the population characteristics of the study participants. MSCH was Matayos SubCounty Hospital where 95 participants were recruited while SSCH was Sioport SubCounty hospital where 47 participants were enrolled. It summarizes the gender distribution, age characteristics, height weight and temperature of the participants at recruitment

Table 2: Socio-demographic characteristics.

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Table 3: Positivity across different test methods.

Test method	Study sites		
	Matayos [n = 95]	Sioport [n = 47]	Total [n = 142]
	MP+ [%]	MP+ [%]	MP+ [%]
Microscopy	52 [54.7]	34 [72.3]	86 [60.6]
RDT	49 [51.6]	34 [72.3]	83 [58.5]
qPCR	90 [94.7]	45 [95.7]	135 [95.1]
Novaplex®	88 [92.6]	41 [87.2]	129 [90.8]

Table 3 highlights the proportion of positive sample from the two field sites Matayos and Sioport as determined by different test methods. qPCR methods were able to detect more positive samples as compared to Microscopy and RDT.

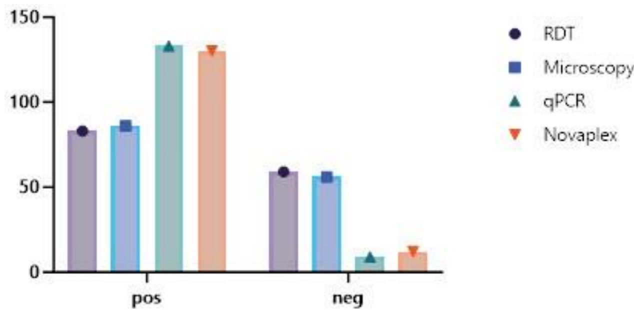

Figure 3 summarizes the diagnosis of the results of positivity analysis using RDT, Microscopy, qPCR, and Novaplex™ assay. It shows the number of positives and negatives from the sample population of 142 as determined by each of these methods.

Figure 3: Comparison of diagnostic performance of the different test methods.

Submicroscopic malaria infections

These infections were defined as Plasmodium infections that were either negative by microscopy but positive by qPCR, or negative by microscopy and positive by RDT. The data showed that there was only one case of a positive RDT test that was negative by microscopy but 47 cases of PCR positive samples which were negative on microscopy. This shows that qPCR detected 33.1% more cases of malaria compared to microscopy.

Diagnostic accuracy using qPCR as a reference

The sample speciation analysis was compared for microscopy and the Novaplex™ malaria assay using the results obtained from qPCR as the “gold standard”. The results of this comparison are summarized in Table 4.

The positive qPCR samples for *P.ovale, P.malariae*, and a select population of *P.falciparum* 20% (23/119)were reanalyzed to affirm reproducibility.

Performance of the Novaplex™ malaria assay

The Novaplex™ assay was able to detect 129 positive samples from the 142-sample population. This is highly comparable to 135 positive samples which were detected by qPCR. The two assays showed a significant level of agreement in the results at k=0.679 (95% CI: 0.442 to 0.917). The sensitivity of the Novaplex™ assay was 94.8% and its accuracy was 95.8% with a PPV of 100% and an NPV of >53.9%. This is a far better performance compared to RDT and microscopy which recorded sensitivities of 63.4% and 65.5% respectively in comparison to qPCR. Their agreement, albeit comparable at k=0.136(95% CI: 0.042 to 0.230) and

	Microscopy			qPCR			Novaplex® assay		
	N	%	95% CI	N	%	95% CI	N	%	95% CI
Negative samples	56	39.4	31.8-47.7	7	4.9	2.4–9.8	13	9.2	5.4-15.0
P. falciparum	50	35.2	27.8-43.4	119	83.8	76.9–90.0	111	78.2	70.7-84.2
P. falciparum/.P.malariae	23	16.2	11.0-23.1	8	5.6	2.9-10.7	11	7.7	4.3-13.3
P. malariae	13	9.2	5.4-15.0	–	–	0.0-2.6	1	0.7	0.0-3.9
P. ovale	–	–	0.0-2.6	–	–	0.0-2.6	1	0.7	0.0-3.9
P.falciparum/P.ovale	–	–	0.0-2.6	6	4.2	2.0-8.9	4	2.8	1.1-7.0
P.vivax	–	–	0.0-2.6	–	–	0.0-2.6	–	–	0.0-2.6
P.falciparum/P.malariae/P.ovale	–	–	0.0-2.6	2	1.4	0.3-5.0	1	0.7	0.0-3.9

Results of the diagnosis by microscopy, q-PCR, and Novaplex™ assay. N=142 samples in all the cases. The percentage of every species was calculated in relation to the total positive samples in each case at a 95% Confidence Interval.

Table 4: Speciation results with different methods.

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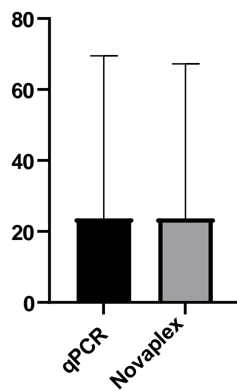
Performance metric [qPCR standard]	Test		
	RDT	Microscopy	Novaplex® assay
TP [qPCR = 135]	83	86	129
FP [qPCR negative]	0	0	0
TN [qPCR =7]	7	7	7
FN [qPCR positive]	52	49	6
Sensitivity % [95% C.I.]	61.5[52.7–69.7]	63.7[55.0 - 71.8]	94.8 [89.6–97.9]
Specificity % [95% C.I.]	100[59.0–100.0]	100[59.0–100.0]	100[59.0–100.0]
PPV % [95% C.I.]	100	100	100
NPV % [95% C.I.]	11.9[9.8–14.3]	12.5[10.3–15.2]	53.9[34.8–71.8]
Accuracy %	63.4[54.9–71.3]	65.5[57.0–73.3]	95.8[91.0-98.4]
kappa value [95% C.I.]	0.136 [0.042–0.230]	0.148 [0.047–0.248]	0.679 [0.442–0.917]

TP true positive, *FP* false positive, *TN* true negative, *FN* false negative, *PPV* positive predictive value, *NPV* negative predictive value

Table 5: Performance of the Novaplex™ malaria assay, microscopy and RDT using qPCR as a reference



A paired t-test comparison between the performance of the Novaplex assay and 18S PCR assay with a 95% CI and a correlation coefficient of 0.9987 shows that the difference between the performance of two tests was not statistically significant(P>0.5).

Figure 4: paired t test between Novaplex™ assay and qPCR

k=0.148(95% CI: 0.047 to 0.248) respectively, showed only a slight agreement with qPCR. The false negatives for microscopy and RDT were 49 and 52 which is highly contrasted to only 6 false negatives when the Novaplex® assay was used. The error rate was 34.5% when microscopy diagnosis was used,35.6% when RDT was used, and 4.2% for the Novaplex™ assay.

A paired t-test comparison between the performance of the Novaplex™ assay and qPCR assay at a 95% CI. Correlation coefficient of 0.9987 shows that the difference between the performance of two tests was not statistically significant ($p > 0.05$).

Discussion

Quick and accurate diagnosis is the first and most crucial step in the treatment of any malaria case. Malaria misdiagnosis could result in delayed and/or inappropriate treatment which could lead to such eventualities like severe illness, recrudescence, drug tolerance or death. In principle, there are four methods for diagnosing malaria; symptomatic, microscopy, mRDTs and molecular methods. Diagnosis relying on clinical symptoms alone is often misleading as there are several other diseases that present symptoms similar to malaria. Microscopy is the most widely used tool in malaria diagnosis as it can give important information such as parasite species, stages, and density. This technique is however labor-intensive requiring highly skilled personnel who are not always readily available especially in non-endemic settings. mRDTs which constitute immunochromatographic methods is recommended by WHO when reliable microscopy is not available. In Kenya, microscopy and RDTs are the most commonly used diagnostic tools for routine care in health centers. Many studies have highlighted the shortcomings of these methods some of which are confirmed by this study, such as low sensitivity. This warrants the need for a quick, accurate, and species-specific diagnostic alternative to Microscopy and mRDTs. Owing to this, we set out to test the performance of the Novaplex™ malaria assay kit against microscopy, mRDTs, and qPCR methods in the diagnosis of malaria. The findings highlighted herein provide a comparative analysis of the two commonly used malaria diagnostic tools and a qPCR-based kit in malaria detection from two sites in an endemic area of Western Kenya. In this study, compared to qPCR, mRDTs did not detect 36.6%[52/142] of PCR-positive malaria infections while microscopy did not detect 34.5%[49/142] of PCR-positive malaria cases. These findings are worrisome as malaria treatment in the study areas is based entirely on results from these two methods. This would then, owing to the misdiagnosis, indicate inadequate treatment contributing to the challenges mentioned in the previous paragraph which pose a serious challenge to malaria control efforts. The failure of microscopy to detect such a substantial proportion of positive infections alludes to submicroscopic

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parasitemia most probably due to low parasite densities and/or operational shortcomings by the laboratory technicians. Nevertheless, these findings concur with a meta-analysis of 42 other similar studies which showed that microscopy failed to detect at least 50% of PCR-positive malaria infections [19]. The study highlights the fact that molecular methods offer better sensitivity and specificity in malaria diagnosis which is consistent with the results of our current study.

In the current study, the results show that the routine diagnostic tools used for malaria detection have a lower sensitivity (63.7% and 61.5%, for microscopy and RDT respectively) compared to the WHO recommendation of at least 95% for an effective diagnostic tool [20]. This presents a challenge in that even with mass screening, a significant proportion of the population who are malaria positive will still go undetected. The Novaplex™ malaria assay, however, at a sensitivity of 95.6% is well within this recommendation and could be used as a referral option to supplement routine diagnosis. Albeit relatively expensive, the benefit and impact of such a method in malaria elimination efforts cannot be understated. The high PPV [100%] but low NPV for microscopy and RDT[12.5%, and 11.9% respectively] show that they are both quite useful in predicting malaria but a negative result does not fully imply no presence of malaria parasites. This has been confirmed using the Novaplex™ assay, which gave a PPV of 100% and an NPV of >53.9% detecting more positive cases than the two methods. The accuracy of the Novaplex™ malaria assay was determined to be 95.8 %, while for microscopy and RDT, the accuracy was 65.5% and 63.4% respectively. The present study sensitivities at 65.5% and 63.4% for microscopy and RDT respectively is consistent with a previous study done in Ghana in which the sensitivity of both microscopy and RDT was shown to be as low as 39.3% and 55.7% compared to qPCR methods [21]. The higher Novaplex ™ accuracy indicates that it is a superior diagnosis method compared to routine tests.

Routine diagnostic methods for *P. falciparum* were only able to identify 50 positive cases, whereas Novaplex and qPCR detected 111 and 119 cases, respectively. Studies have highlighted instances where individuals testing negative for *P. falciparum* using conventional methods may actually be positive due to deletions or variabilities in the hrp2/3 antigens, commonly used in most mRDTs as diagnostic markers. QPCR outperformed the Novaplex assay by detecting an additional 8 *P. falciparum*-positive infections. This discrepancy can be attributed to the fact that many P. falciparum strains have a relatively lower number of 18S ribosomal subunits, unlike the multicopy varATS target employed in qPCR, which boasts approximately 59-60 copies per genome [22,23]. Consequently, varATS qPCR demonstrates tenfold greater sensitivity than traditional 18S rRNA PCR, with a lower limit of detection at approximately 0.03-0.15 parasites per microliter [24].

When comparing microscopy, RDT, and qPCR, it is essential to take into account the significantly varying limits of detection among these methods. Enhanced sensitivity is observed when extracting from whole blood as opposed to dried blood spots. The concentration of DNA during extraction plays a crucial role, with multi-copy genes such as varATS demonstrating heightened sensitivity compared to single-copy genes like 18S [25]. Consequently, the limits of detection vary significantly across multiple orders of magnitude. A more sensitive PCR leads to the detection of more low-density infections, resulting in lower sensitivity for microscopy, RDT, and even alternative qPCR methods like the Novaplex ™ assay. In this particular study, DNA extraction from dried blood spots and amplification of a multi-copy target resulted in an exceptionally low limit of detection for the qPCR method.

In summary, the Novaplex™ assay performed significantly better than the routine diagnostics tests showing better agreement with the highly sensitive qPCR at kappa = 0.679 [0.442–0.917] compared to kappa's of k=0.136 [0.042–0.230] and k=0.148 [0.047–0.248] for RDT and microscopy, and also recording a higher accuracy, sensitivity, and Negative predictive value compared to the two.

Conclusion

Taken together, our data demonstrated that Novaplex™ assay yields superior malaria diagnostic outcome compared to conventional diagnostic methods such as RDT and microscopy. The challenges and limitations associated with conventional diagnostic including false positives, false negatives, limit of detection, submicroscopic infections, polymorphisms and deletions of diagnostic target antigens e.g., *hrp2*, require the need for more sensitive molecular tools (qPCR, PCR, LAMP etc.) to complement their usage at clinical centers. Molecular tools have a high detection sensitivity and hence can detect parasite densities even at submicroscopic levels. The major caveat in the use of molecular methods is their technicality and extended turnaround time, which have been addressed with the Novaplex™ malaria assay. Novaplex™ assay provides a quicker alternative in diagnosis owing to its ease of use. For instance, master mix preparation for Novaplex™ assay involves addition of equal volumes of all the three components; a buffer, primer mix, and PCR water; which is contrasted to qPCR where the primers and probes are individually added thus lengthening the preparation time and eventual turnaround time. Data analysis for the Novaplex™ assay is also straightforward as the proprietary software analyzes the results and automatically enlists the Plasmodium species present in any sample. This makes the Novaplex™ assay a better diagnostic option in clinical settings as there are minimal technical capability requirements. The qPCR assay used in this study resulted into a higher sensitivity as it involved individual amplification of each target analyte in a

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single reaction tube. Multi-gene copy primers were also used qPCR assay increasing the primer targets thus contributing to its better sensitivity. This is however not feasible in a routine setting as the amount of time it would take to make a diagnosis in a multiplicity of infections is relatively longer compared to the Novaplex™ assay. Also optimizing the primer and probes concentration would require technical expertise, unlike the already optimized Novaplex™ Malaria Assay. This study therefore demonstrates that the Novaplex ™ assay performs well in species-specific diagnosis of clinical samples and would provide a better option at point of care, thus improving case detection, better patient care, management and optimal treatment in all malaria prone regions. Incorporation of standards and cut off values is however critical to distinguish between clinically active and residual infections owing to the highly sensitive nature of the assay.

Limitations

This specific study was done during a relatively low malaria transmission season at the study location. A similar study would be recommended for during peak transmission season to evaluate the effect of higher parasite densities and expected infection multiplicities on the Novaplex ™ assay as a point of care diagnostic tool.

Roles and responsibilities

This project was done by the KEMRI-CBRD Malaria lab team. The team developed the protocol (LK, FK, KT), implemented the study (LK,FK,MN,MA,LW,MO,NM), did the analysis (LK, MO, MA), compiled (FK, KT, LK, MN) and reviewed the report (FK, KT, LK, MN, NM, MO, LW). This manuscript has been submitted with the approval of the Director General KEMRI.

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Ethical consideration

Patients were enrolled in the study according to the KEMRI-SERU (Scientific Ethics Review Unit) procedures which follow the Helsinki declaration and the ICH-GCP guidelines. The study protocol was approved by the SERU at Kenya Medical Research Institute study approval number KEMRI/CBRD/235/SERU4522. It was also approved by the Kenyan National Commission for Science and Technology (NACOSTI) License number: P/22/20841. The Busia County health directorate approved implementation of this study in the field health facilities, approval reference: CG/BSA/H/PRT/5/12/VOL1(15) and CG/BSA/ADM/1/56/VOL11.82.

Conflict of Interest

The authors declare no conflicts of interest. The authors are neither employees nor receiving any gain, financial or otherwise, from the funding agency.

Study test items

The Novaplex™ assay kit used in this study is a product patented by the funding agency.

Source of funding

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Appendices

Well	Name	Type	FAM		HEX		Cal Red 610		Quasar 670		Quasar 705		Quasar 670		Auto Interpretation
			PV	C(t)	PO	C(t)	PF	C(t)	PM	C(t)	PK	C(t)	IC	C(t)	
B05		SAMPLE	-	N/A	-	N/A	+	37.53	+	40.68	-	N/A	+	26.52	PF,PM
A06		SAMPLE	-	N/A	-	N/A	+	28.34	+	39.93	-	N/A	+	28.59	PF,PM
G06		SAMPLE	-	N/A	-	N/A	+	32.42	+	40.28	-	N/A	+	27.65	PF,PM
A07		SAMPLE	-	N/A	-	N/A	+	34.21	+	26.60	-	N/A	+	29.88	PF,PM
E11		SAMPLE	-	N/A	-	N/A	+	32.31	+	38.53	-	N/A	+	29.30	PF,PM
G11		SAMPLE	-	N/A	-	N/A	+	36.12	+	37.09	-	N/A	+	28.26	PF,PM
E12		NC	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	Negative Control(-)
F12		NC	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	Negative Control(-)
G12		PC	+	25.14	+	22.01	+	30.33	+	26.44	+	21.60	+	25.28	Positive Control(+)
H12		PC	+	26.43	+	22.32	+	30.64	+	27.55	+	22.46	+	26.39	Positive Control(+)

The Auto Interpretation feature lists all the present plasmodium species in a sample. PO-*Plasmodium ovale*, PK-*Plasmodium knowlesi*, PV- *Plasmodium vivax*, PM-*Plasmodium malariae*, PF- *Plasmodium falciparum.* C(t)- Cycle threshold, IC- Internal Control

Figure 5: Analysis report for the NovaplexTM assay using the proprietary Seegene ® reporting software.

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