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

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Research Article

Keywords: Malaria, Novaplex, Species-specific, qPCR, Sensitivity, Specificity

Posted Date: November 8th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3566326/v1

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Additional Declarations: No competing interests reported.
Abstract

Background

Accurate and rapid diagnosis of malaria is crucial for effective treatment and control. More so, is the accurate species identification during treatment as it is essential 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 NovaplexTM 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 NovaplexTM kit against the rest of the techniques.

Results

Our analyses demonstrated that the NovaplexTM malaria assay yields a superior outcome compared to microscopy and mRDTs in terms of sensitivity, accuracy and NPV. The assay also showed an overall diagnostic agreement with qPCR. 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 NovaplexTM assay was 95.5% and 87.5% respectively, while the sensitivity of microscopy and RDT was 63.7% and 61.5% respectively. The positive and negative predictive values were 99.2% and 53.9% respectively, for the NovaplexTM assay. This was in contrast to NPV values for microscopy and RDT which were 12.5% and 11.9% respectively. The accuracy of the NovaplexTM assay was recorded at 95.1% having a substantial agreement with qPCR at k = 0.642 [0.398–0.885]. For Microscopy and RDT, the level of accuracy was determined to be 65.5% and 63.4% respectively with a slight agreement to qPCR at k = 0.148 [0.047–0.248] and k = 0.136 [0.042–0.230] respectively.

Conclusion

The findings of this study demonstrate that the Novaplex assay outperformed microscopy and 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 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.

Introduction

Malaria remains a health burden in many developing countries in spite of enormous investments in control efforts. Globally, there was an estimated 247 million malaria cases in 2021, with 228 million (95%) in the African region according to the world malaria report 2022. Malaria has claimed the lives of over 619,000 in the year 2021, mostly in sub-Saharan Africa with children below the age of 5 years being especially vulnerable to the disease (WHO, 2022). In Kenya, there are an estimated 3.5 million new malaria cases annually, with 10,700 deaths and an estimated 25 million people at risk of contracting the disease (KMIS 2020). 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 (Ministry of Health, 2015). This was also set out in the WHO guidelines for malaria treatment which stipulate that parasitological confirmation of infection is required prior to treatment (WHO, 2015). 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 (Ohrt et al. 2007). 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 (Fong Amaris et al. 2022). 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 (Ochwedo et al. 2021). 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 (Nderu et al. 2019). 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, circumventing the need for highly trained lab personnel. However, there have been 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 (Rogier et al., 2022). 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 (Marquart et al. 2022). 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 (Jimenez et al. 2017). 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 (PMI, 2011). 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 (Boyce et al. 2018; Kojom and Singh 2020). 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 (Kamau, et. al., 2011). 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-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 Pf364 and Pvr47 as defined by Demas, et. al., 2011. They concluded that the non-ribosomal targets showed a higher sensitivity for detecting mixed infections at 3P/ul (Amarall, et. al, 2019; Demas, et. al., 2011). Since then, highly sensitive qPCR methods have been developed that can detect 0.002P/ul (Wongsrichanalai et. al., 2007). 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 (Yasri & Wiwanitkit 2021). 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 (Oduya 2017). 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 00° 01’
and 00° 47’ north of the Equator. A sample size of 245 participants was determined for this study based on the prevalence of malaria in the region which was estimated to be 19% at the time of sampling.

Sample Collection:

All individuals presenting with suspected malaria (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 were negative using either of the two diagnostic techniques. For all the patients recruited into the study, 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 -80°C. 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 (WHO 2022) 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 (Plowe *et al.*, 1995). 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 primers(Table 1) with modified fluorophores.
### Table 1
Primers and probes used for qPCR amplification

<table>
<thead>
<tr>
<th>Species</th>
<th>Primer/Probe</th>
<th>Sequence 5’-3’</th>
<th>Fluorescent label</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. falciparum</em></td>
<td>VAR_ATS F</td>
<td>CCCATACACAACCAAYTGGA</td>
<td></td>
</tr>
<tr>
<td><em>P. falciparum</em></td>
<td>VAR_ATS R</td>
<td>TTCGCACATATCTCTATGTCTATCT</td>
<td></td>
</tr>
<tr>
<td><em>P. falciparum</em></td>
<td>VAR_ATS Probe</td>
<td>TRTTCCATAAATGGT</td>
<td>FAM-MGB</td>
</tr>
<tr>
<td><em>P. vivax</em></td>
<td>PvVo F</td>
<td>CAAGCGGAAGGATAAATGG</td>
<td></td>
</tr>
<tr>
<td><em>P. vivax</em></td>
<td>PvVo R</td>
<td>CCGCGATGAAGCAGATGTCT</td>
<td></td>
</tr>
<tr>
<td><em>P. vivax</em></td>
<td>PvVo Probe</td>
<td>AAGGGAGAACC</td>
<td>FAM-MGB</td>
</tr>
<tr>
<td><em>P. malariae</em></td>
<td>PmVo F</td>
<td>CTCAAAATCCACCAAATGAAGAA</td>
<td></td>
</tr>
<tr>
<td><em>P. malariae</em></td>
<td>PmVo R</td>
<td>GATTCGTGCTATATCTGACTTCA</td>
<td></td>
</tr>
<tr>
<td><em>P. malariae</em></td>
<td>PmVo Probe</td>
<td>AGTGAGTTGTGTTACAATAA</td>
<td>FAM-MGB</td>
</tr>
<tr>
<td><em>P. ovale</em></td>
<td>PoRBP2 F</td>
<td>CCA CAG ATA AGA AGT CTC AAG TAC GAT ATT</td>
<td></td>
</tr>
<tr>
<td><em>P. ovale</em></td>
<td>PoRBP2 R</td>
<td>TTG GAG CAC TTT TGT TTG CAA</td>
<td></td>
</tr>
<tr>
<td><em>P. ovale</em></td>
<td>PoRBP2 Probe</td>
<td>TGAATTGCTAAGCGATATC</td>
<td>FAM-MGB</td>
</tr>
</tbody>
</table>


Amplification was done on an AriaMx™ (Agilent™ Technologies) real time PCR system, with each 15-µl reaction mix containing 2µl of sample DNA, 7.5µl of GoTaq® Probe qPCR Master 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 40 cycles for 10 sec at 95°C, then 1 min at 60°C, with plate read at the end of each cycle. A Ct value of \( \leq 40 \) 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 *Pv*ivax probe, HEX labelled *Po*vale probe, Cal Red610 labelled *Pf*alciparum probe, Quasar 670 labelled *Pm*alariae probe and Quasar 705 labelled *Pk*nowlesi probe, Fig. 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 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 \( \leq 45 \) 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 (PPV) and negative predictive value (NPV) calculated for the Novaplex™ malaria assays as described (Monaghan 2021), 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 34.3℃ – 39.4℃ with a median of 37.0℃[IQR: 36.6–37.5]. The socio-demographic characteristics are as summarized in Table 2.
Table 2
Socio-demographic characteristics

<table>
<thead>
<tr>
<th>Socio-demographic characteristics</th>
<th>Study area</th>
<th>Both sites, n [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MSCH, n [%]</td>
<td>SSCH, n [%]</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>70 [73.7]</td>
<td>31 [66.0]</td>
</tr>
<tr>
<td>Male</td>
<td>25 [26.3]</td>
<td>16 [34.0]</td>
</tr>
<tr>
<td>Age group [years]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5–14</td>
<td>31 [32.6]</td>
<td>14 [29.8]</td>
</tr>
<tr>
<td>15–30</td>
<td>35 [36.8]</td>
<td>23 [48.9]</td>
</tr>
<tr>
<td>&gt; 30</td>
<td>17 [17.9]</td>
<td>6 [12.8]</td>
</tr>
<tr>
<td>Height [cm]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimum</td>
<td>64</td>
<td>8.93</td>
</tr>
<tr>
<td>Maximum</td>
<td>184</td>
<td>174</td>
</tr>
<tr>
<td>Median</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight [kg]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimum</td>
<td>5.2</td>
<td>8.02</td>
</tr>
<tr>
<td>Maximum</td>
<td>170</td>
<td>74.5</td>
</tr>
<tr>
<td>Median</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimum</td>
<td>34.3</td>
<td>35.6</td>
</tr>
<tr>
<td>Maximum</td>
<td>39.4</td>
<td>39.2</td>
</tr>
<tr>
<td>Median</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

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 center.
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.

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 2 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.

**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.
Table 4

Speciation results with different methods

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

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.

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, one case being a false positive. 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 $k = 0.642$ [0.398–0.885]. The sensitivity of the Novaplex™ assay was 95.5 [90.5–98.3] (128/134) and its accuracy was 95.1 [90.1–98.0] with a PPV of 99.2% [95.3–99.9] and an NPV of > 53.9%. Its specificity was 87.5 [47.4–99.7] (7/8). This is a far better performance compared to RDT and microscopy which recorded sensitivities of 61.5% [52.7–69.7], (83/135) and 63.7% [55.0–71.8], (86/135) respectively in comparison to qPCR. Their agreement, albeit comparable at $k = 0.136$ [0.042 to 0.230] and $k = 0.148$ [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 38.5% [52/135] when microscopy diagnosis was used, 36.3% [49/135] when RDT was used, and 5.2% [7/135] for the Novaplex™ assay.
Table 5
Performance of the Novaplex™ malaria assay, microscopy and RDT using qPCR as a reference

<table>
<thead>
<tr>
<th>Performance metric[qPCR standard]</th>
<th>Test</th>
<th>RDT</th>
<th>Microscopy</th>
<th>Novaplex® assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP [qPCR = 135]</td>
<td></td>
<td>83</td>
<td>86</td>
<td>128</td>
</tr>
<tr>
<td>FP [qPCR negative]</td>
<td></td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>TN [qPCR = 7]</td>
<td></td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>FN [qPCR positive]</td>
<td></td>
<td>52</td>
<td>49</td>
<td>6</td>
</tr>
<tr>
<td>Sensitivity % [95% C.I.]</td>
<td></td>
<td>61.5[52.7–69.7]</td>
<td>63.7[55.0–71.8]</td>
<td>95.5[90.5–98.3]</td>
</tr>
<tr>
<td>Specificity % [95% C.I.]</td>
<td></td>
<td>100[59.0–100.0]</td>
<td>100[59.0–100.0]</td>
<td>87.5[47.4–99.7]</td>
</tr>
<tr>
<td>PPV % [95% C.I.]</td>
<td></td>
<td>100</td>
<td>100</td>
<td>99.2[95.3–99.9]</td>
</tr>
<tr>
<td>Accuracy %</td>
<td></td>
<td>63.4[54.9–71.3]</td>
<td>65.5[57.0–73.3]</td>
<td>95.1[90.1–98.0]</td>
</tr>
<tr>
<td>kappa value [95% C.I.]</td>
<td></td>
<td>0.136[0.042–0.230]</td>
<td>0.148[0.047–0.248]</td>
<td>0.642[0.398–0.885]</td>
</tr>
</tbody>
</table>

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

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 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. In this study, compared to qPCR, mRDTs did not detect 38.5%[52/135] of PCR-positive malaria infections while microscopy did not detect 36.3%[49/135] of PCR-positive malaria cases. This concurs with a meta-analysis of 42 other similar studies which showed that microscopy failed to detect at least 50% of PCR-positive malaria infections [Okell et al.,2009]. The study highlights the fact that molecular methods offer better sensitivity and specificity in malaria diagnosis which is consistent with the results of the current study. Analysis of sub-microscopic parasitemia, defined as Plasmodium infections that were either negative by microscopy but positive by qPCR, or negative by microscopy and positive by RDT, 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 further highlighting the shortcomings of the routine tests.
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 [Mouatcho & Goldring 2013]. The Novaplex™ malaria assay, however, at a sensitivity of 95.5% is well within this recommendation. 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 99.2% 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 95.1%, while for microscopy and RDT, the accuracy was 65.5% and 63.4% respectively. The present study sensitivities at 63.7% and 61.5% 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[Afriyie et al., 2023]. The higher Novaplex™ accuracy indicates that it’s a superior diagnosis method compared to routine tests.

In this study, qPCR was able to detect 8 more *P.falciparum*-positive infections compared to the Novaplex™ assay. This is because most *P. falciparum* strains possess relatively fewer 18S ribosomal subunits unlike the multicopy *varATS* target, which were used as the qPCR target and have approximately 59–60 copies /genome (Dimonte et al., 2020; Russell et al., 2021). This makes *varATS* qPCR tenfold more sensitive than traditional 18S rRNA PCR with a lower limit of detection at ~0.03–0.15 parasites/ul (Hoffman et al., 2015).

When comparing microscopy, RDT, and qPCR, we need to consider vastly different limits of detection between the methods. Extraction from whole blood results in better sensitivity compared to dried blood spots. When DNA is concentrated during extraction, multi-copy genes such as *varATS* offer superior sensitivity compared to single-copy genes like 18S (Oyedeji et al., 2007). As a result, the limit of detection differs across several orders of magnitude. A more sensitive PCR will result in more low-density infections being detected, and thus a lower sensitivity for microscopy, RDT, and even other qPCR methods like the Novaplex™ assay. In the current study, DNA was extracted from Dried blood spots and a multi-copy target was amplified, resulting in a very 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 = 0.642 [0.398–0.885] 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**

The challenges and limitations associated with conventional diagnostic methods like RDT and microscopy including false positives, false negatives, limit of detection, submicroscopic infections, polymorphisms and deletions of diagnostic target antigens e.g., *hrp2*, will 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. Master mix preparation with the same involves adding equal volumes of three components, a buffer, primer mix, and PCR water; this is contrasted to qPCR in which the primers and probes are added individually lengthening the preparation time and eventual turnaround time. Data analysis is also straightforward as the proprietary software analyzes the results and lists the *Plasmodium* species present in any sample, Fig. 3. This makes it a better diagnostic option in clinical settings as the technical capabilities required to use this assay effectively are minimal. The qPCR assay used in this study has a higher sensitivity as it involved individual amplification of each target analyte in a single reaction tube. Multi-gene copy primers were also used 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 an excellent option at point of care, helping in improved case detection, better patient care, management and optimal treatment in all malaria prone regions.

**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.

**Declarations**

**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.

**Acknowledgments:**

The authors would like to express gratitude towards the Seegene® Institute Clinical Research team for their financial support towards this research. Special thanks to the Busia County Department of Health field research team, Agnetta Asembo, Leah Ouma, James Elung’ata of Matayos Sub County Hospital; Orodi N. Nancy, Stephen K. Emerikwa, and Hezron Menego of Siopori Sub County Hospital who consented and screened the patients at the clinical facilities. Many thanks to the patients, the children and their guardians,
for their participation in this study. Inqaba biotech Africa for their facilitation during the research. The laboratory staff and students at the Center for Biotechnology Research and Development and the Director general KEMRI for approving this publication.

**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:**

The study was funded by the Seegene™ Clinical research team (Seoul, Korea), under a collaborative malaria diagnostics validation clinical project.

**References**


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**Figures**

![Figure 1](image-url)
Map showing the location of the study area, Busia County, in Kenya.

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 [accessed 26Jul, 2023]].

Figure 2

Amplification curves for a control sample containing all five *Plasmodium* targets using the Novaplex™ assay.

Figure 3

Figure 2 Comparison of diagnostic performance of the different test methods

![Bar chart comparing qPCR and Novaplex assay performance](image)

Figure 4

Figure 2 paired t test between Novaplex™ assay and qPCR

A paired t-test comparison between the performance of the Novaplex™ assay and qPCR assay at a 95% CI. A correlation coefficient of 0.9987 shows that the difference between the performance of two tests was not statistically significant (P>0.9999).
Fig 5

**Fig 3. Analysis report for the Novaplex™ assay using the proprietary Seegene ® reporting software.**

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 Contro