



Innovations in *Plasmodium* spp. diagnosis on diverse detection platforms

Nairo Brilhante-da-Silva^{1,3} · Leandro do Nascimento Martinez^{2,4} · Rosa Maria de Oliveira Sousa³ · Soraya dos Santos Pereira^{1,2,3} · Carolina Bioni Garcia Teles^{2,4,5,6}

Received: 29 June 2021 / Accepted: 3 November 2021 / Published online: 22 November 2021
© King Abdulaziz City for Science and Technology 2021

Abstract

In 2019, 229 million cases of malaria were recorded worldwide. For epidemiologic surveillance and proper treatment of persons infected with *Plasmodium* spp., rapid detection of infections by *Plasmodium* spp. is critical. Thus, *Plasmodium* spp. diagnosis is one of the indispensable measures for malaria control. Although microscopy is the gold standard for diagnosis, it has restrictions related mainly to the lack of qualified human resources, which is a problem in many regions. Thus, this review presents major innovations in diagnostic methods as alternatives to or complementary to microscopy. Detection platforms in lateral flow systems, electrochemical immunosensors, molecular biology and, more recently, those integrated with smartphones, are highlighted, among others. The advanced improvement of these tests aims to provide techniques that are sensitive and specific, but also quick, easy to handle and free from the laboratory environment. In this way, the tracking of malaria cases can become increasingly effective and contribute to controlling the disease.

Keywords Malaria · *Plasmodium* · Diagnostic · Biosensor · Point-of-care · Biomarker

Introduction

Malaria is a parasitic disease caused by protozoa of the genus *Plasmodium*. There are more than 200 species of *Plasmodium* (Martinsen Ellen and Perkins Susan L 2013);

of these, six species are capable of infecting humans and causing the clinical presentation of the disease. The six species known to cause malaria in humans are *Plasmodium vivax*, *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale*, *Plasmodium knowlesi* (BRASIL 2020) and *Plasmodium simium* (Brasil et al. 2017). According to the 2019 report from the World Health Organization (WHO), between 2010 and 2018, the number of malaria cases worldwide dropped significantly by 10% and, consequently, mortality was reduced by an estimated 30% (WHO 2019).

Correct diagnoses are crucial for maintaining this scenario. Microscopy remains the gold standard for the diagnosis of malaria, since it permits direct visualization of malaria parasites on a slide with a blood sample stained with Giemsa under a 100X oil immersion objective lens (CDC—Malaria—Diagnostic Tools 2020). In addition to being the gold standard methodology for diagnosing malaria, optical microscopy is the technique that allowed, for the first time in 1880, visualization of the parasites causing the disease (CDC 2017). Despite the methodological simplicity, its analysis requires a specialized microscopic professional capable of performing differential detection of species and analyzing samples with low parasitic density (BRASIL 2020). However, the turnover of professionals, limited diagnostic

✉ Nairo Brilhante-da-Silva
nairosilvabrilhante@hotmail.com

¹ Programa de Pós-Graduação Em Biologia Celular E Molecular, Instituto Oswaldo Cruz, IOC, Rio de Janeiro, Brazil

² Programa de Pós-Graduação Em Biologia Experimental (PGBIOEXP), Fundação Universidade Federal de Rondônia (UNIR), Porto Velho, RO, Brazil

³ Laboratório de Engenharia de Anticorpos, Fundação Oswaldo Cruz, FIOCRUZ, Unidade Rondônia, Porto Velho, RO, Brazil

⁴ Plataforma Técnica de Bioensaios de Malária E Leishmaniose (PBML), FIOCRUZ, Fundação Oswaldo Cruz, FIOCRUZ, Unidade Rondônia, Porto Velho, RO, Brazil

⁵ Instituto Nacional de Ciência E Tecnologia, Epidemiologia da Amazônia Ocidental (INCT-EpiAmO), Porto Velho, RO, Brazil

⁶ Rede de Biodiversidade e Biotecnologia da Amazônia Legal - Bionorte, Rondônia, Brazil

training, and areas of difficult access make it difficult to establish this technique in basic health laboratories.

These factors make it difficult to train microscopists with enough experience to perform the differential detection of parasites. This becomes an aggravating factor, especially in areas that are co-endemic for *P. falciparum* and *P. knowlesi* (Barber et al. 2013), because, in these cases, treatment is different for each species, which demonstrates the importance of differential diagnosis for *Plasmodium* spp. (Barber et al. 2013; WHO 2018a).

The need to provide increasingly sensitive and specific detection techniques has driven the development of Polymerase Chain Reaction (PCR) assays aimed at diagnosing malaria, a process that began in 1990 (Jaureguiberry et al. 1990; Wataya et al. 1993; Kain et al. 1993). In the same period, the advent of lateral flow assays and the push to provide diagnostic tools that are more accessible to the non-laboratory environment led the WHO, in 1999, to hold the first meeting aimed at discussing the benefits that rapid diagnostic tests (RDT) in conjunction with microscopy would bring to the control of malaria (WHO 2000).

In this context, technological advances have promoted the improvement in different diagnostic platforms for infections by *Plasmodium* spp. Among the diversity of diagnostic models developed, Point of care (POC) lateral flow tests are highlighted (CDC—Malaria—Diagnostic Tools 2020). However, the continuous improvement of detection techniques by molecular biology (Reboud et al. 2019) and electrochemical immunosensors (Hemben et al. 2017) has begun to provide simplified prototypes and greater access to the non-laboratory environment. In addition, in this vein of innovation, smartphones have become collaborative tools in the process of simplifying malaria diagnostic methods (Rosado et al. 2017).

Molecular diagnosis

Molecular tests are highly sensitive and specific for the diagnosis of infections by *Plasmodium* spp., in addition to enabling the detection of multiple infections, genotyping to distinguish between cases of recrudescence/reinfection and identification of strains resistant to the usual medications. However, the longer time needed to obtain results and the need for sophisticated infrastructure, are among the factors that limit the wide use of this method (WHO 2020).

A comparative study between the diagnosis of the three most common techniques, microscopy, Rapid Diagnostic Test (RDT) and Polymerase Chain Reaction (PCR), demonstrated that PCR detects up to 12% and 23% more infections compared to POC and microscopy tests, respectively (Mfuh et al. 2019). In the same vein, the results by Berzosa et al. (2018) indicate that ~19% of negative results by microscopy

may be false, whereas for POC testing these values reach 13%, data that confirms the greater sensitivity of molecular assays.

Ribosomal RNA 18S (rRNA 18S) represents one of the most frequently used targets in malaria diagnostic tests, especially for the detection of *P. falciparum*, because in the immature trophozoite phase, each parasite presents 10,000 copies of 18S rRNA (Murphy et al. 2012). The 18S rRNA gene sequence can also be a useful target for molecular diagnosis through loop-mediated isothermal amplification (LAMP). This gene sequence can be searched for using one of the techniques that is being used for molecular diagnosis, that of loop-mediated isothermal amplification (LAMP).

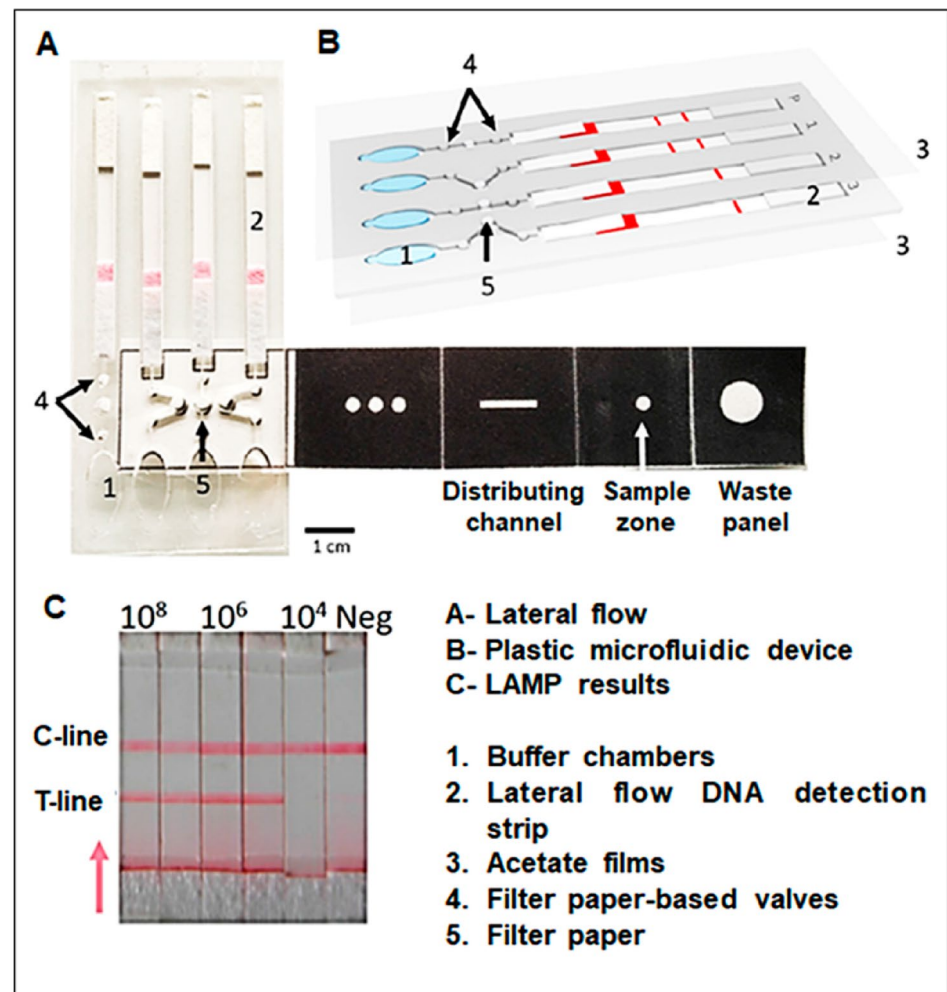
Mohon et al. (2019) developed a LAMP assay that detects between 50 and 100 parasites per mL in samples that were stored on filter paper for several months, a result that reveals the high stability of 18S rRNA. The association of the LAMP technique with microfluidic technology incorporates greater ease of interpretation and speed in the assay, since it eliminates temperature variation cycles, a common process in conventional PCR, and promotes visual interpretation integrated with the lateral flow system (Fig. 1). Through this complex of methods, the result is released within 50 min and interpreted by visually (Reboud et al. 2019).

Real-time quantitative PCR (qPCR) is another method that is part of the methodologies applicable to the diagnosis of malaria. Recently, it was demonstrated how the portable platform, called Q3-plus, favors the expansion of qPCR for non-laboratory environments. This proof of concept is based on the miniaturization of the qPCR instrument and gelation of the reagent mixture to generate a portable device with all reagents ready for the reaction. In addition, as has been shown, the compactness of the method does not interfere with the performance of the assay, as compared to conventional qPCR aimed at diagnosing malaria (Rampazzo et al. 2019). Thus, when compared to conventional LAMP and qPCR assays, the main benefits are the simplification of the preparation steps of the reagent mixture for detection of molecular targets, the stability of reagents at non-freezing temperatures and the feasibility of performing this method in non-laboratory environments. Compared to RDT, specificity and sensitivity are the most prominent advantages of the assay.

Lateral flow assays

Lateral flow assays, commonly called Point of care tests (POCTs), are alternatives to diagnostic methods that require more time and resources, including both professionals and infrastructure, for the detection of infectious agents. The rapid result, ease of handling the test along with simple result interpretation are among the differentials of POC

Fig. 1 LAMP assay associated with microfluidic technology. Image C shows the results of validation of what is outlined in A and B. Each test strip was exposed to different concentrations (10^8 , 10^6 , 10^4) of *Plasmodium* spp. Line T and C correspond to the test and control, respectively. Neg represents the negative control. Adapted from Rebould et al. (2019) and within the recommendations of the Creative Commons Attribution (CC BY) (<http://creativecommons.org/licenses/by/4.0/>)



tests (Anfossi et al. 2018; Tran et al. 2019), benefits that favor their implementation for the diagnosis of infections that mainly affect people in developing countries.

In 2007, the FDA (Food and Drug Administration) approved the first POC test to assist in the diagnosis of malaria; the test is able to indicate when infections are caused by *P. falciparum*, but it does not allow for the distinction of malaria caused by *P. vivax*, *P. malariae* and *P. ovale*. One of the reasons for this approval was the low availability of professionals with experience in microscopy diagnosis, which makes clinical diagnosis difficult (CDC—Malaria—Diagnostic Tools 2020).

The sensitivity of POC tests, such as that of BinaxNow® used in the USA, varies according to parasitic density, as was demonstrated by Wanja et al. (2016). In the study, the POC assay, compared to the method of detection by optical microscopy, showed a sensitivity of up to 100% when there were > 5000 parasites per microliter, but declined to 70% in the presence of samples with < 200 parasites per microliter.

According to WHO-FIND, a study that evaluated the performance of POCs for malaria, the sensitivity of most tests

is similar for samples with parasitic densities of up to 2000 parasites per microliter. However, sensitivity should also remain high in cases of lower parasitemia, since in countries with a low prevalence of malaria, it is common for individuals to have a lower density of parasites. In addition to the criteria of sensitivity, specificity and stability which must be evaluated to choose the tests, one must take into consideration which antigens are researched, since not all parasites express the target antigens. Thus, despite the diversity of existing POCT, numerous research groups are looking for methods to improve lateral flow assays for the diagnosis of malaria (WHO 2018b).

Principle of the assay and most thoroughly researched biomarkers

Commercial POCTs for the diagnosis of malaria may consist of antibodies with affinity for metabolic proteins, such as histidine-rich protein II, specific to *P. falciparum* (PfHRP2), as well as antibodies for the detection of lactate dehydrogenase

of the parasite (pLDH) which indicates infection by other species (WHO 2018b; Kim et al. 2019). In this detection system, recognition signal transduction is performed, in most cases, by gold nanoparticles (AuNPs) functionalized with antibodies specific to the researched target (Lee et al. 2011). In addition, Kim et al. (Kim et al. 2019) demonstrated that colored latex particles can be an alternative to the use of AuNPs, because, in addition to presenting similar performance, positivity for different antigens is represented by different colors.

Although *Pf*HRP II is the preferred biomarker for the identification of *P. falciparum* in POC tests, there are cases in which the gene encoding the protein *Pf*HRP II may be absent, a factor that limits the diagnosis of malaria caused by *P. falciparum* (Fontecha et al. 2018). In this sense, the search for conserved biomarkers is continuous. In an analysis of samples positive for *P. falciparum*, coming from endemic regions of India, it was found that Glutamate Dehydrogenase (GDH) can be considered an alternative biomarker to *Pf*HRP II, since the coding gene is highly conserved (Ahmad et al. 2019). In this perspective, recognition of *P. falciparum* GDH promoted by polyclonal antibodies demonstrated high sensitivity and specificity, results that encourage the application of this biomarker in assays based on antibody-mediated recognition (Kori et al. 2020).

Biosensors that constitute devices for rapid diagnosis of malaria can be integrated with antibodies with affinity for aldolase, another biomarker present in patients infected with *Plasmodium* spp. Dzakah et al. (2013) bioconjugated AuNPs to antibodies with specificity for *P. vivax* aldolase and developed a POC test that, compared to microscopy, showed specificity and sensitivity values of 99.23% and 98.33%, respectively. Biosensors resulting from bioconjugation between gold nanoparticles and antibodies with specificity for *P. vivax* aldolase may, compared to microscopy, show high levels of specificity (> 99%) and sensitivity (> 98%) in a POCT (Dzakah et al. 2013).

Unlike previous tests, with a direct detection method, Cho et al. (2016) presented a POCT aimed at the differential detection of antibodies against merozoite surface protein 1 (MSP1). Two recombinant MSP1, one from *P. vivax* and the other from *P. falciparum*, were the main constituents of the biosensors that differentially recognized the antibodies corresponding to the two species. The sensitivity and specificity of the assay were superior to the SD BIOLINE Malaria P.f/P.v commercial test from Alere Inc and, therefore, still presents MSP1 as an additional biomarker for the construction of effective POC tests.

Since most tests for the diagnosis of malaria caused by *P. falciparum* look for the presence of *Pf*HRP II, some studies propose a previous stage of treatment of the sample to concentrate the amount of HRP II. Ricks et al. (2016) developed the technique based on the principle of histidine's affinity

to divalent metallic ions. The use of nickel functionalized beads provided the capture of *Pf*HRP II and, subsequently, the concentration of the protein in a small sample volume. Thus, without promoting any change in the structure of the POCT employed, the sensitivity of the test increased by more than 20 times and was able to detect as little as 1 parasite per microliter.

With an even simpler technique, Bauer et al. (2017) added resin with Zn^{2+} to the bottom of pipette tips and made purification via the mechanical action of aspirating and dispensing the sample feasible. The simplicity of the HRP II enrichment method has increased the sensitivity of rapid tests by up to 20 times, making it elective as a simple, low-cost technique. Most lateral flow assays have a similar detection system, where the diversity of biomarkers and sample pre-treatment steps do not interfere with the basis of the assay. Figure 2 shows the entire recognition flow of a test aimed at detecting HRP II and LDH, two of the main biomarkers for rapid diagnosis. Table 1 presents the main biomarkers investigated for the detection of *Plasmodium* spp.

Electrochemical immunosensors

The development of electrochemical immunosensor technology has made it possible to advance diagnostic platforms with a higher sensitivity than other assay formats. In these cases, in addition to antibodies, other elements of recognition are used. Among them, as demonstrated by Singh et al. (2018), are the aptamers of ssDNA (single-stranded DNA). The detection method developed in the study was based on the use of small nucleotide sequences in association with gold surface electrodes that, in turn, permit the detection of GDH on a picomolar scale.

The functionalization of gold electrodes with antibodies permits the diversification of the immunosensors' detection system. Antibodies with affinity for biomarkers, such as *P. falciparum* HRP II, can be adsorbed to the electrodes and thus act as detection antibodies (Fig. 3). In the same system, the insertion of biosensors can further amplify the recognition signal and make the assay highly sensitive to small concentrations of HRP II (Hemben et al. 2017).

The use of magnetic beads is another technique for the construction of an electrochemical immunosensor. In this strategy, proteins such as HRP II are recognized by anti-HRP II antibodies adsorbed to magnetic beads that, due to their physical properties, are influenced by an electromagnetic field. Thus, the transduction of the electrochemical signal is performed by magnetic sensors that influence the beads and, through this system, indicate the presence of HRP II in concentrations reaching 0.36 ng/mL^{-1} (De Souza Castilho et al. 2011).

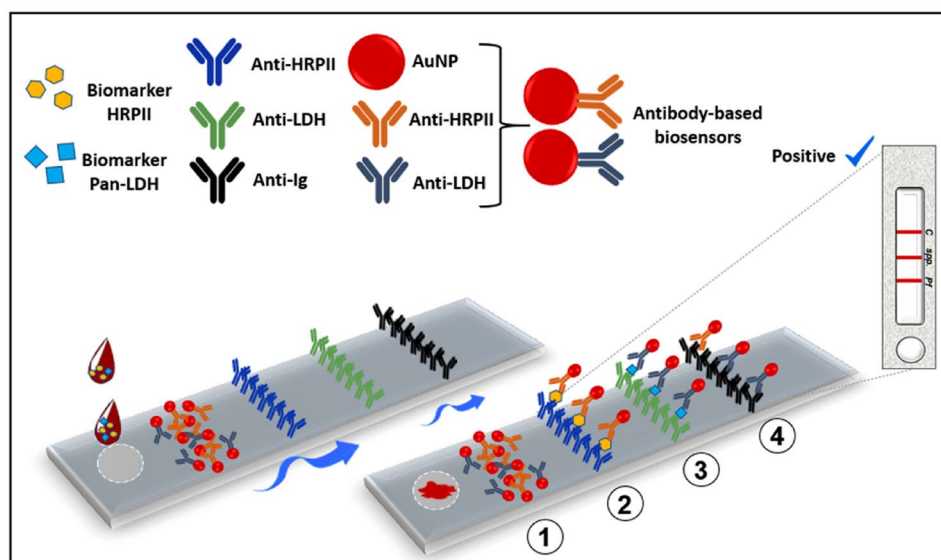


Fig. 2 Layout of a detection system in a POC test for the diagnosis of malaria. In the example, the biomarkers HRPII of *P. falciparum* and LDH of *Plasmodium* spp. are recognized by biosensors functionalized with antibodies specific to these targets (1); When migrating by capillarity, antibodies with affinity for differential epitopes of biomarkers recognize the biosensor+biomarker complex (2), which

promotes the appearance of color in the test region for the first bio-marker (HRPII). In (3), the same process happens for the Pan-LDH biomarker. The control region has Anti-immunoglobulins (Anti-Ig) that recognize the antibodies that make biosensors functional (4); The appearance of the control ensures that the biosensors have migrated through the system and validates the result of the assay

Table 1 Main biomarkers investigated for the detection of *Plasmodium* spp

<i>Plasmodium</i> spp.	Target	Source
<i>P. falciparum</i>	<i>Pf</i> HRPII	Kim et al. (2019)
	<i>Pf</i> GDH	Ahmad et al. (2019)
	<i>Pf</i> LDH	Lee et al. (2011)
	Anti- <i>Pf</i> MSP1 antibody	Cho et al. (2016)
	18S rRNA (MAL5_18S, MAL7_18Sa)	Murphy et al. (2012)
<i>P. vivax</i>	<i>Pv</i> LDH	Lee et al. (2011)
	<i>Pv</i> Aldolase	Dzakah et al. (2013)
	Anti- <i>Pv</i> MSP1 antibody	Cho et al. (2016)
	Non-ribosomal Pvr47	Amaral et al. (2019)
	18S rRNA gene of <i>P. vivax</i>	Rougemont et al. (2004)
<i>P. malarie, P. ovale, P. knowlesi</i>	Species-specific	Rougemont et al. (2004)
	18S rRNA	Komaki-Yasuda et al. (2018)

rRNA 18S, Ribosomal RNA 18S; *Pf*HRPII, *Plasmodium falciparum* histidine-rich proteins II; *Pf*LDH, *Plasmodium falciparum* lactate dehydrogenase; *Pf*GDH, *Plasmodium falciparum* glutamate Dehydrogenase; MSP1, merozoite surface protein 1

Like HRPII, LDH is part of the group of biomarkers eligible for diagnosis using aptamers (Figueroa-Miranda et al. 2018) and anti-LDH antibodies associated with immunosensors (Low et al. 2019). As a consequence of the importance of the correct diagnosis of malaria caused by *P. falciparum*, most of the electrochemical assays being developed, as well as those of the POC type, are focused on the detection of HRPII (Sharma et al. 2008; Dutta et al. 2017; Dutta and Lillehoj 2018).

Despite the variety of biomarkers used in diagnostics, there are research fronts aiming to establish a recognition system based on the conformational changes of erythrocytes infected by the parasites. Quantitative detection of parasitized cells can be performed with electrochemical impedance spectroscopy, since the use of specific monoclonal antibody for infected erythrocytes permits the distinction between normal and infected cell types (Kumar et al. 2016). These morphological changes in erythrocytes may be

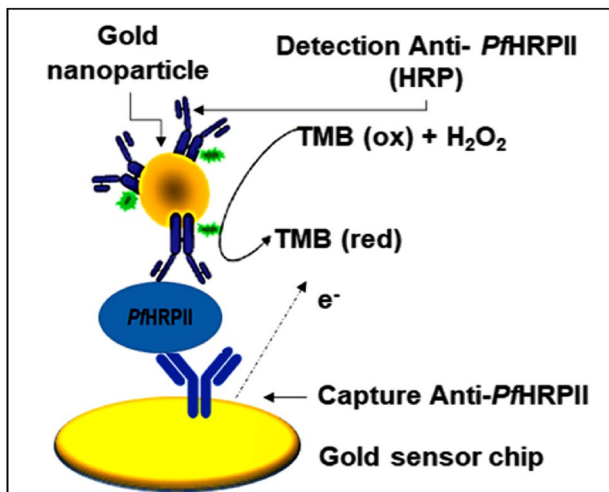


Fig. 3 Electrochemical sensor for the detection of *P. falciparum* HRP II (*PfHRP II*). In this sandwich format, *PfHRP II* is recognized by anti-*PfHRP II* capture antibodies adsorbed on gold chips. Anti-*PfHRP II* detection antibodies are conjugated to gold nanoparticles and associated with horseradish peroxidase enzyme (HRP). TMB represents the substrate for HRP. Adapted from Hemben et al. (Hemben et al. 2017) and within the recommendations of the Creative Commons Attribution (CC BY) (<http://creativecommons.org/licenses/by/4.0/>)

a useful diagnostic indicator in cases of *P. falciparum* infections (Birch et al. 2015; Paul et al. 2017), but is limited to the recognition of *P. vivax* due to its preference for parasitizing reticulocytes. The combination of these alternative techniques may lead to advances in diagnostic tests for malaria.

Automatic image processor

Cellular devices have become useful tools for improving diagnostics based on microscopic visualization of the parasite. These devices permit, for example, the integration of a profound learning model based on the recognition of imaging patterns, which facilitates the distinction between cells that have been parasitized by *Plasmodium* spp. and those that have not been parasitized. With accuracy close to 100%, this method eliminates the use of the internet and works like a common cell phone application (Fuhad et al. 2020). This image processing methodology can also distinguish the species and the morphological stage of the parasites in a blood smear slide through a fully automated process (Rosado et al. 2017) (Fig. 4).

Hemozoin crystals are also indicators of the presence of malaria parasites and can be detected with the aid of cellular devices. These portable platforms can act as a polarized light microscope which, coupled with a microscopic visualization system, contributes to the facilitated detection of hemozoin crystals. This idealized formatting presents a level of performance comparable to the conventional polarized light microscope, without greater complexity and costs for the detection of this pigment (Pirnstill and Coté 2015).

The most commonly used biomarkers in the diagnosis of malaria using POC tests, such as GDH, HRP II and aldolase, are susceptible to detection on platforms developed in association with smartphones (Stemple et al.

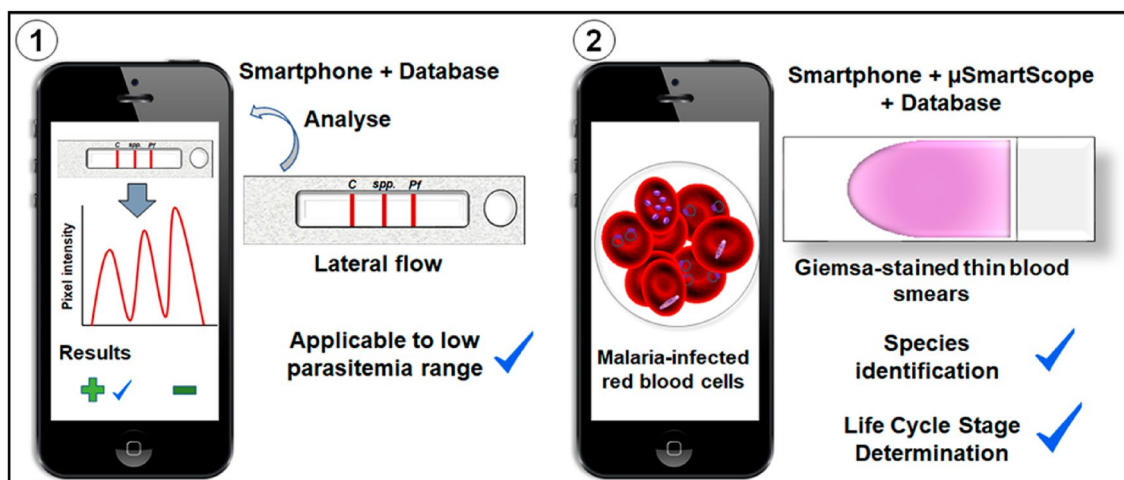


Fig. 4 Exemplification of the operation of two diagnostic systems with the aid of smartphones. In 1, the smartphone is used to photograph the result of a lateral flow assay. From the image, the number of pixels of the test lines is measured and the correlation is performed to determine the presence or absence of the parasites. In image 2, the smartphone is used along with μ SmartScope equipment to analyze a blood smear slide and determine the species and morphological

cycle of the parasites. The use of image databases is common to both systems. Images 1 and 2 were constructed, respectively, based on the study by Scherr et al. (Scherr et al. 2016) and Rosado et al. (Rosado et al. 2017). Each image corresponds to an adapted and summarized example of the authors' proposal. The studies are licensed by the Creative Commons Attribution (CC BY) (<http://creativecommons.org/licenses/by/4.0/>)

2014; Gopal and Raychaudhuri 2017; Sanjay et al. 2020). This simplification of the diagnosis does not compromise the efficiency of the method, as was observed in the trial by Sanjay et al. (2020), where the *P. falciparum* GDH detection threshold occurred on a picomolar scale. The ease of access to smartphones facilitates another aspect of these detection methods. Low parasitemia, for example, can compromise the correct interpretation of results in POC tests. In these cases, the device's camera can be used to photograph the result and direct the photo to an automatic image processor (Fig. 4). Thus, the software measures the intensity of color present in the test membrane and correlates it with the concentration of parasites (Scherr et al. 2016). Table 2 shows the performance of some of the commercial or non-commercial detection methods for the diagnosis of malaria, and described in Table 3, are the main advantages and limitations of six diagnostic methods: microscopy, PCR, qPCR, LAMP, RDT and electrochemical immunosensor.

Conclusion

The progressive control of infections by *Plasmodium* spp. is strictly linked to quality diagnoses. The method of detection employing optical microscopy is the most usual; however, to achieve accuracy with this technique, it is necessary to work with a microscopist with experience and expertise in the area. The lack of these professionals, both in endemic and non-endemic regions for malaria, becomes an aggravating factor, since infections result in samples with low parasitic density, which makes diagnosis even more difficult. These limitations led to the development of alternative and complementary diagnostic formats to microscopy.

Lateral flow POC-type tests are the most commonly used. HRPII, LDH, GDH and Aldolase make up the group of biomarkers that are detectable in these tests. Due to the severity of *P. falciparum* infections, most tests detect *PfHRPII* and one biomarker that generally indicates the presence of other species. Despite the sensitivity, specificity and ease of interpretation, the method continues to be improved. The use of different nanomaterials, such as latex particles, previous processing of the sample to

Table 2 Performance of different diagnostic formats for malaria. The performances of some commercial tests (POC, qPCR and LAMP) are also included

Method	Sensitivity (%)	Specificity (%)	Limit of detection (LOD)	Target	Minutes	Source/Commercial kit
Microscopy	57	99	-	Parasites	60	Mfuh et al. (2019)
Microscopy	55.3	91.28	-	Parasites	60	Berzosa et al. (2018)
POC	99.7 for <i>P. falciparum</i> 93.5 for <i>P. vivax</i>	94.2 for <i>P. falciparum</i> * 99.8 for <i>P. vivax</i>	-	<i>PfHRPII</i> <i>PvLDH</i>	15	BinaxNow®
POC	96.5 for <i>P. falciparum</i> 95.3 for <i>P. vivax</i>	> 99.4	150 parasites/μL for <i>P. falciparum</i> 250 parasites/μL for <i>P. vivax</i>	<i>PfLDH</i> <i>PvLDH</i>	15–20	Lee et al. (2011)
POC	98	99.7	-	Anti- <i>PfMSP1</i> antibody Anti- <i>PvMSP1</i> antibody	15	Cho et al. (2016)
qPCR	-	-	0.5 copies/μL	18S rRNA	~60	artus Malaria RG PCR Kit (QIAGEN)
qPCR	-	-	1.5 parasites/μL	18S rRNA	80	Rampazzo et al. (2019)
LAMP	> 90	> 99	1 parasite/μL	18S rRNA	60–120	The Loopamp™ Malaria
LAMP	97	99.1	25 parasites/mL from whole blood 50–100 parasites/mL from dried blood spots	18S rRNA	30–60	Mohon et al. (2019)
Electrochemical	~62	-	0.77 pM	<i>PfGDH</i>	< 30	Singh et al. (2018)
Electrochemical	-	-	40 pg·mL ⁻¹	<i>PfHRPII</i>	5	Dutta and Lillehoj (2018)

POC point-of-care, LAMP loop-mediated isothermal amplification, PCR Polymerase Chain Reaction, qPCR Real-time quantitative PCR, rRNA 18S Ribosomal RNA 18S, *PfHRPII* histidine-rich protein II, *pLDH* lactate dehydrogenase of the parasite, AuNP gold nanoparticles, GDH Glutamate Dehydrogenase, *MSP1* merozoite surface protein 1, ssDNA single-stranded DNA

Table 3 Main advantages and limitations of malaria diagnostic methods, namely, microscopy, PCR, qPCR, LAMP, RDT and electrochemical immunosensor

Method	Advantages	Limitations	Source
Microscopy	(I) Differential detection of <i>Plasmodium</i> species; (II) Simplicity in preparing the sample that will be analyzed; (III) Provides rapid results; (IV) Determines parasitemia; (V) Detection of different morphological stages of the parasites	(I) Requires a highly specialized professional microscopist; (II) Samples with low parasite density limit parasite detection; (IV) Professional turnover limits the establishment of reference units for diagnosis; (V) Does not allow for the identification of strains that are resistant to antimalarial drugs	Barber et al. (2013) CDC (2017) CDC - Malaria - Diagnostic Tools, (2020) BRASIL (2020)
POCT	(I) Provides rapid results (~ 30 min); (II) A highly specialized professional is not required to perform the test; (III) The interpretation of the result is visual, which does away with the need for expensive equipment; (IV) Can be performed in non-laboratory environments;	(I) Sensitivity varies according to parasite density; (II) Does not allow for the differential detection of morphological stages of the parasites; (III) Elevated temperature is a factor that can affect the stability of some tests; (IV) Does not provide quantitative results (V) Does not detect strains that are resistant to antimalarial drugs;	Wanja et al. (2016) Tran et al. (2019) WHO (2018b)
PCR	(I) Highly sensitive and specific; (II) Less likely to produce false positive or false negative results; (III) Differential detection of <i>Plasmodium</i> species; (IV) Identification of drug-resistant strains; (V) Distinction between recrudescence and reinfection	(I) Needs laboratory infrastructure; (II) Compared to microscopy and RDTs, the time to provide results is longer; (III) A team of qualified professionals is required to perform and interpret the assays; (IV) Samples need to go through a prior nucleic acid extraction step	Berzosa et al. (2018) Mfuh et al. (2019) WHO (2020)
qPCR	(I) Quantitative result; (II) Unlike conventional PCR, the result is shown in real time; (III) Differential detection of <i>Plasmodium</i> species; (IV) Highly sensitive and specific; (V) Identification of drug-resistant strains; (VI) Distinction between recrudescence and reinfection	(I) As with PCR, it needs laboratory infrastructure; (II) Compared to microscopy and RDTs, the time to provide results is longer; (III) Previous nucleic acid extraction step (IV) A team of qualified professionals is required to perform and interpret the assays	WHO (2020) Grignard et al. (2020) Sazed (2021)
qPCR by Q3 plus	(I) Sensitive and specific quantitative result; (II) It is performed on a portable diagnostic platform; (II) Increased stability of assay reagents at non-freezing temperatures; (III) Minimizes the need for the assay to be performed in a sophisticated laboratory infrastructure	(I) Requires previous nucleic acid extraction steps; (II) Requires a control unit (laptop/tablet); (III) Due to the format of the assay, samples with two or more species of <i>Plasmodium</i> are not compatible; (IV) Despite the assay being simplified, the presence of a qualified professional is still required to interpret the results	Rampazzo et al. (2019)
LAMP	(I) Highly sensitive and specific; (II) Provides rapid results; (III) Methodological steps are more simplified than those of qPCR	(I) Most tests require a previous nucleic acid extraction step, which takes longer; (II) Indirect detection methods, such as evaluation of results through turbidity (III) Some tests recommend the use of additional equipment such as a Loopamp Real-time Turbidimeter to confirm the results	Sattabongkot et al. (2014) Reboud et al. (2019) Mohon et al. (2019)
Electrochemical	(I) Highly sensitive and specific; (II) Qualitative and quantitative results; (III) Has the possibility of detecting multiple protein targets	(I) Complexity of the assays may be elevated; (II) Information on stability of the assays is not always available; (III) Lack of studies evaluating the effectiveness of these tests in a large number of clinical samples	Hemben et al. (2017) Singh et al. (2018) Dutta and Lillehoj (2018) Krampa et al. (2020)

POCT point-of-care test, LAMP loop-mediated isothermal amplification, PCR Polymerase Chain Reaction, qPCR Real-time quantitative PCR

concentrate the biomarker, and automated interpretation on smartphones demonstrate the quest for innovation in this detection system.

The progressive miniaturization and simplification of immunosensors also establish yet another research front for facilitated detection of malaria parasites. The possibility of using DNA aptamers, antibodies, biosensors and differentiated signal amplification systems, incorporates greater sensitivity to the test, which is essential in cases of low parasitemia. Despite the complexity of molecular techniques, studies demonstrate the feasibility of adapting them to the non-laboratory environment, where more simplified methods are desirable. The validation of LAMP and qPCR assays, structured in a compact way, prove that the improvement of these detection platforms can be performed without compromising the effectiveness of the assay and, thus, providing a robust diagnostic tool to the non-laboratory environment. The integration of smartphone technology also serves this purpose, as it is applicable both in the interpretation of a POC assay and in image processing to determine the *Plasmodium* spp. species and stage of infection.

The global technical strategy for malaria 2016–2030 highlights that the development of diagnostic methods that are increasingly accessible and that have sensitivity and specificity comparable to that of more sophisticated tests, should be part of the set of strategies that aim to reduce cases of malaria by 90% before 2030 (WHO 2021). Since the tests may have limitations at different levels, the combination of at least two diagnostic techniques is recommended, as is being used in the Malaria Control and Elimination Strategy implemented in Thailand. In this plan, it is evident that microscopy and RDTs are essential to promote the rapid detection of infections, an essential step for the control of malaria cases (Lertpiriyasuwat et al. 2021). In these strategies, molecular biology assays are mainly applied to detect cases of resistance or to detect infections with low parasitemia. However, advances in studies increasingly demonstrate the feasibility of these tests in the non-laboratory environment (Rampazzo et al. 2019; Selvarajah et al. 2020). As a result, it is hoped that these diagnostic methods will begin to be implemented more broadly in malaria control strategies.

The objective of facilitating the diagnosis of malaria is a factor common to the diagnostic formats presented in this study, since they represent the most recent advances in the area. Although the aforementioned diagnostic methodologies represent the most recent advances in different diagnostic platforms, they do not correspond to all of the methodologies that are being developed to improve the diagnosis of malaria.

Acknowledgements We thank Amy Grabner for the English edition of the manuscript.

Author contributions NBdS: conceptualization, data curation, formal analysis, writing—original draft. LdNM: conceptualization, investigation, methodology. RMdOS: data curation. Sd SP: data curation, writing - review & editing. CBGT: data curation, writing—review & editing, visualization.

Funding The authors express their gratitude to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado de Rondônia (FAPERO), Instituto Nacional de Ciência e Tecnologia, Epidemiologia da Amazônia Ocidental (INCT-EpiAmO) and Fundação Oswaldo Cruz (Fiocruz) for the financial support.

Data availability Not applicable.

Code availability Not applicable.

Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

References

- Ahmad A, Verma AK, Krishna S et al (2019) *Plasmodium falciparum* glutamate dehydrogenase is genetically conserved across eight malaria endemic states of India: exploring new avenues of malaria elimination. PLoS ONE 14:1–13. <https://doi.org/10.1371/journal.pone.0218210>
- Amaral LC, Robortella DR, Guimarães LFF et al (2019) Ribosomal and non-ribosomal PCR targets for the detection of low-density and mixed malaria infections. Malar J 18:154. <https://doi.org/10.1186/s12936-019-2781-3>
- Anfossi L, Di Nardo F, Profitti M et al (2018) A versatile and sensitive lateral flow immunoassay for the rapid diagnosis of visceral leishmaniasis. Anal Bioanal Chem 410:4123–4134. <https://doi.org/10.1007/s00216-018-1067-x>
- Barber BE, William T, Grigg MJ et al (2013) Limitations of microscopy to differentiate *Plasmodium* species in a region co-endemic for *Plasmodium falciparum*, *Plasmodium vivax* and *Plasmodium knowlesi*. Malar J 12:1–6. <https://doi.org/10.1186/1475-2875-12-8>
- Bauer WS, Richardson KA, Adams NM, et al (2017) Rapid concentration and elution of malarial antigen histidine-rich protein II using solid phase Zn(II) resin in a simple flow-through pipette tip format. Biomicrofluidics 11:1. <https://doi.org/10.1063/1.4984788>
- Berzosa P, De Lucio A, Romay-Barja M et al (2018) Comparison of three diagnostic methods (microscopy, RDT, and PCR) for the detection of malaria parasites in representative samples from Equatorial Guinea 11 Medical and Health Sciences 1108 Medical Microbiology. Malar J 17:1–12. <https://doi.org/10.1186/s12936-018-2481-4>
- Birch CM, Hou HW, Han J, Niles JC (2015) Identification of malaria parasite-infected red blood cell surface aptamers by inertial microfluidic SELEX (I-SELEX). Nat Publ Gr 1–16. <https://doi.org/10.1038/srep11347>

- BRASIL (2020) Guia de tratamento da malária no Brasil/Ministério da Saúde. Secretária de Vigilância em Saúde. Departamento de imunização e Doenças Transmissíveis - Brasília; Ministério da Saúde, 2020., 1ª. Brasília
- Brasil P, Zalis MG, de Pina-Costa A et al (2017) Outbreak of human malaria caused by *Plasmodium simium* in the Atlantic Forest in Rio de Janeiro: a molecular epidemiological investigation. *Lancet Glob Heal* 5:e1038–e1046. [https://doi.org/10.1016/S2214-109X\(17\)30333-9](https://doi.org/10.1016/S2214-109X(17)30333-9)
- CDC (2017) CDC—Malaria—About Malaria—History—Laveran and the Discovery of the Malaria Parasite. <https://www.cdc.gov/malaria/about/history/laveran.html>. Accessed 4 Sep 2021
- CDC—Malaria—Diagnostic Tools (2020) CDC—Malaria—Diagnostic Tools. https://www.cdc.gov/malaria/diagnosis_treatment/diagnostic_tools.html. Accessed 23 Aug 2021
- Cho SJ, Lee J, Lee HJ et al (2016) A novel malaria Pf/Pv ab rapid diagnostic test using a differential diagnostic marker identified by network biology. *Int J Biol Sci* 12:824–835. <https://doi.org/10.7150/ijbs.14408>
- De Souza CM, Laube T, Yamanaka H et al (2011) Magneto immunoassays for *Plasmodium falciparum* histidine-rich protein 2 related to malaria based on magnetic nanoparticles. *Anal Chem* 83:5570–5577. <https://doi.org/10.1021/ac200573s>
- Dutta G, Lillehoj PB (2018) Wash-free, label-free immunoassay for rapid electrochemical detection of PfHRP2 in whole blood samples. *Sci Rep* 8:17129. <https://doi.org/10.1038/s41598-018-35471-8>
- Dutta G, Nagarajan S, Lapidus LJ, Lillehoj PB (2017) Enzyme-free electrochemical immunosensor based on methylene blue and the electro-oxidation of hydrazine on Pt nanoparticles. *Biosens Bioelectron* 92:372–377. <https://doi.org/10.1016/j.bios.2016.10.094>
- Dzakah EE, Kang K, Ni C et al (2013) *Plasmodium vivax* aldolase-specific monoclonal antibodies and its application in clinical diagnosis of malaria infections in China. *Malar J* 12:1. <https://doi.org/10.1186/1475-2875-12-199>
- Figuerola-Miranda G, Feng L, Shiu SC et al (2018) Aptamer-based electrochemical biosensor for highly sensitive and selective malaria detection with adjustable dynamic response range and reusability. *Sensors Actuators B Chem* 255:235–243. <https://doi.org/10.1016/j.snb.2017.07.117>
- Fontecha G, Mejía RE, Banegas E et al (2018) Deletions of pfhrp2 and pfhrp3 genes of *Plasmodium falciparum* from Honduras, Guatemala and Nicaragua. *Malar J* 17:1–10. <https://doi.org/10.1186/s12936-018-2470-7>
- Fuhad KMF, Tuba JF, Sarker MRA et al (2020) Deep learning based automatic malaria parasite detection from blood smear and its smartphone based application. *Diagnostics* 10:329. <https://doi.org/10.3390/diagnostics10050329>
- Gopal NS, Raychaudhuri R (2017) Detection of *Plasmodium* aldolase using a smartphone and microfluidic enzyme linked immunosorbent assay. *Malar Res Treat* 2017:1–4. <https://doi.org/10.1155/2017/9062514>
- Grignard L, Nolder D, Sepúlveda N et al (2020) A novel multiplex qPCR assay for detection of *Plasmodium falciparum* with histidine-rich protein 2 and 3 (pfhrp2 and pfhrp3) deletions in polyclonal infections. *EBioMedicine* 55:102757. <https://doi.org/10.1016/j.ebiom.2020.102757>
- Hemben A, Ashley J, Tohill IE (2017) Development of an Immunosensor for Pf HRP 2 as a biomarker for malaria detection. *Biosensors* 7:28. <https://doi.org/10.3390/bios7030028>
- Jaureguiberry G, Hatin I, D'Auriol L, Galibert G (1990) PCR detection of *Plasmodium falciparum* by oligonucleotide probes. *Mol Cell Probes* 4:409–414. [https://doi.org/10.1016/0890-8508\(90\)90031-T](https://doi.org/10.1016/0890-8508(90)90031-T)
- Kain KC, Brown AE, Mirabelli L, Webster HK (1993) Detection of *Plasmodium vivax* by polymerase chain reaction in a field study. *J Infect Dis* 168:1323–1326. <https://doi.org/10.1093/infdis/168.5.1323>
- Kim J, Cao XE, Finkelstein JL et al (2019) A two-colour multiplexed lateral flow immunoassay system to differentially detect human malaria species on a single test line. *Malar J* 18:1–10. <https://doi.org/10.1186/s12936-019-2957-x>
- Komaki-Yasuda K, Vincent JP, Nakatsu M et al (2018) A novel PCR-based system for the detection of four species of human malaria parasites and *Plasmodium knowlesi*. *PLoS ONE* 13:e0191886. <https://doi.org/10.1371/journal.pone.0191886>
- Kori LD, Valecha N, Anvikar AR (2020) Glutamate dehydrogenase: a novel candidate to diagnose *Plasmodium falciparum* through rapid diagnostic test in blood specimen from fever patients. *Sci Rep* 10:6307. <https://doi.org/10.1038/s41598-020-62850-x>
- Krampa FD, Aniweh Y, Kanyong P, Awandare GA (2020) Recent advances in the development of biosensors for malaria diagnosis. *Sensors* 20:799. <https://doi.org/10.3390/s20030799>
- Kumar B, Bhalla V, Bhadoriya RPS, et al (2016) Label-free electrochemical detection of malaria-infected red blood cells. *ROYAL Soc Chem* 1–3. <https://doi.org/10.1039/C6RA07665C>
- Lee GC, Jeon ES, Le DT et al (2011) Development and evaluation of a rapid diagnostic test for *Plasmodium falciparum*, *P. vivax*, and mixed-species malaria antigens. *Am J Trop Med Hyg* 85:989–993. <https://doi.org/10.4269/ajtmh.2011.11-0265>
- Low YK, Chan J, Soraya G V, et al (2019) Development of an Ultrasensitive Impedimetric Immunosensor Platform for Detection of *Plasmodium Lactate* Dehydrogenase. *Sensors* 19:. <https://doi.org/10.3390/s19112446>
- Martinsen Ellen, Perkins Susan L (2013) The diversity of *Plasmodium* and other haemosporidians: The intersection of taxonomy, phylogenetics and genomics | Request PDF. *Malar Parasites Comp Genomics, Evol Mol Biol* 1–15
- Mfuh KO, Achonduh-Atijegbe OA, Bekindaka ON et al (2019) A comparison of thick-film microscopy, rapid diagnostic test, and polymerase chain reaction for accurate diagnosis of *Plasmodium falciparum* malaria. *Malar J* 18:1–8. <https://doi.org/10.1186/s12936-019-2711-4>
- Mohon AN, Getie S, Jahan N et al (2019) Ultrasensitive loop mediated isothermal amplification (US-LAMP) to detect malaria for elimination. *Malar J* 18:1–10. <https://doi.org/10.1186/s12936-019-2979-4>
- Murphy SC, Prentice JL, Williamson K et al (2012) Real-time quantitative reverse transcription PCR for monitoring of blood-stage *Plasmodium falciparum* infections in malaria human challenge trials. *Am J Trop Med Hyg* 86:383–394. <https://doi.org/10.4269/ajtmh.2012.10-0658>
- Paul A, Padmapriya P, Natarajan V (2017) ScienceDirect Diagnosis of malarial infection using change in properties of optically trapped red blood cells. *Biomed J* 40:101–105. <https://doi.org/10.1016/j.bj.2016.10.001>
- Pirnstill CW, Coté GL (2015) Malaria diagnosis using a mobile phone polarized microscope. *Sci Rep* 5:13368. <https://doi.org/10.1038/srep13368>
- Rampazzo RCP, Graziani AC, Leite KK et al (2019) Proof of concept for a portable platform for molecular diagnosis of tropical diseases: on-chip ready-to-use real-time quantitative pcr for detection of *Trypanosoma cruzi* or *Plasmodium* spp. *J Mol Diagnostics* 21:839–851. <https://doi.org/10.1016/j.jmoldx.2019.04.008>
- Reboud J, Xu G, Garrett A et al (2019) Paper-based microfluidics for DNA diagnostics of malaria in low resource underserved rural communities. *Proc Natl Acad Sci USA* 116:4834–4842. <https://doi.org/10.1073/pnas.1812296116>
- Ricks KM, Adams NM, Scherr TF et al (2016) Direct transfer of HRPII-magnetic bead complexes to malaria rapid diagnostic tests significantly improves test sensitivity. *Malar J* 15:1–8. <https://doi.org/10.1186/s12936-016-1448-6>

- Rosado L, da Costa J, Elias D, Cardoso J (2017) Mobile-based analysis of malaria-infected thin blood smears: automated species and life cycle stage determination. *Sensors* 17:2167. <https://doi.org/10.3390/s17102167>
- Rougemont M, Van Saanen M, Sahli R et al (2004) Detection of Four *Plasmodium* species in blood from humans by 18S rRNA gene subunit-based and species-specific real-time PCR assays. *J Clin Microbiol* 42:5636–5643. <https://doi.org/10.1128/JCM.42.12.5636-5643.2004>
- Sanjay M, Singh NK, Ngashangva L, Goswami P (2020) A smartphone-based fiber-optic aptasensor for label-free detection of *Plasmodium falciparum* glutamate dehydrogenase. *Anal Methods* 12:1333–1341. <https://doi.org/10.1039/C9AY02406A>
- Sattabongkot J, Tsuboi T, Han E-T et al (2014) Loop-mediated isothermal amplification assay for rapid diagnosis of Malaria infections in an area of endemicity in Thailand. *J Clin Microbiol* 52:1471–1477. <https://doi.org/10.1128/JCM.03313-13>
- Sazed SA, Kibria MG, Alam MS (2021) An optimized real-time qPCR method for the effective detection of human malaria infections. *Diagnostics* 11:736. <https://doi.org/10.3390/diagnostics11050736>
- Scherr TF, Gupta S, Wright DW, Haselton FR (2016) Mobile phone imaging and cloud-based analysis for standardized malaria detection and reporting. *Sci Rep* 6:28645. <https://doi.org/10.1038/srep28645>
- Selvarajah D, Naing C, Htet NH, Mak JW (2020) Loop-mediated isothermal amplification (LAMP) test for diagnosis of uncomplicated malaria in endemic areas: a meta-analysis of diagnostic test accuracy. *Malar J* 19:211. <https://doi.org/10.1186/s12936-020-03283-9>
- Sharma MK, Rao VK, Agarwal GS et al (2008) Highly sensitive Amperometric Immunosensor for detection of *Plasmodium falciparum* histidine-rich protein 2 in serum of humans with Malaria: comparison with a commercial kit. *J Clin Microbiol* 46:3759–3765. <https://doi.org/10.1128/JCM.01022-08>
- Singh NK, Arya SK, Estrela P, Goswami P (2018) Capacitive malaria aptasensor using *Plasmodium falciparum* glutamate dehydrogenase as target antigen in undiluted human serum. *Biosens Bioelectron* 117:246–252. <https://doi.org/10.1016/j.bios.2018.06.022>
- Stemple CC, Angus SV, Park TS, Yoon J (2014) Smartphone-based optofluidic lab-on-a-chip for detecting pathogens from blood. *J Lab Autom* 19:35–41. <https://doi.org/10.1177/2211068213498241>
- Tran TV, Nguyen BV, Nguyen TTP et al (2019) Development of a highly sensitive magneto-enzyme lateral flow immunoassay for dengue NS1 detection. *PeerJ* 2019:1–18. <https://doi.org/10.7717/peerj.7779>
- Wanja EW, Kuya N, Moranga C et al (2016) Field evaluation of diagnostic performance of malaria rapid diagnostic tests in western Kenya. *Malar J* 15:1–10. <https://doi.org/10.1186/s12936-016-1508-y>
- Wataya Y, Arai M, Kubochi F et al (1993) DNA diagnosis of falciparum malaria using a double PCR technique: a field trial in the Solomon Islands. *Mol Biochem Parasitol* 58:165–167. [https://doi.org/10.1016/0166-6851\(93\)90101-3](https://doi.org/10.1016/0166-6851(93)90101-3)
- WHO (2019) World Malaria Report. <https://www.who.int/malaria>
- WHO (2018a) WHO | The World malaria report 2018
- WHO (2000) New perspectives: malaria diagnosis: report of a joint WHO/USAID INFORMAL CONSULTATION
- WHO (2020) Global Malaria Programme. <https://www.who.int/teams/global-malaria-programme/case-management/diagnosis/nucleic-acid-amplification-based-diagnostics>. Accessed 26 Aug 2021
- WHO (2018) Malaria rapid diagnostic test performance: results of WHO product testing of malaria RDTs: round 8 (2016–2018). World Health Organization, Geneva, p 2018
- WHO (2021) Global technical strategy for malaria 2016–2030, 2021 update. Geneva