Chapter

Malaria Diagnostics

Nikiwe Mhlanga and Hendriëtte Van der Walt

Abstract

The imminent scenario of malaria burden on endemic regions burdens healthcare and is a threat to non-endemic regions. Microscopy and rapid diagnostic tests (RDTs) remain the gold standard for malaria detection in resource-constrained regions. They still present low sensitivity at low parasite density, however, with microscopy also requiring trained personnel, expensive and time consuming. Affordable, rapid, specific, sensitive and simple malaria diagnostics remain elusive. Molecular-based diagnostics, polymerase chain reaction and loop-mediated isothermal amplification, although highly sensitive even at low parasitemia, still have challenges hindering their use in resource-constrained regions. This chapter discusses the conventional microscopy, spectroscopy, RDTs and molecular platforms in malaria detection. It also highlights current interventions on mitigations of their existing hurdles and adaptability to developing regions. Such inventions include the amalgamation of different techniques, nanotechnology and artificial intelligence.

Keywords: microscopy, SERS, RDTs, LAMP, PCR

1. Introduction

The malaria protozoan continues to yield despondency in third World Health Organisation (WHO) regions. In 2019, 227 million global cases were estimated, and in 2020, it increased to 241 million cases [1]. **Figure 1** gives an approximation of endemic WHO regions where malaria transmission still occurs. The continuous battle is prevailing even with the availability of state-of-the-art malaria detection techniques, which have evolved from the gold standard microscopy to rapid diagnostics, nucleic-based polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP). Heightening the infections in these regions is the inaccessibility of the existing techniques. Some of them are too expensive and not readily available, yet even with rapid diagnostic tests (RDTs), which are readily available and simpler, and low sensitivity especially for low parasite density and asymptomatic infections remains a hurdle. A controlled disease and prevention plan including early, sensitive, accessible, affordable, user-friendly and rapid detection tools is mandatory in these regions [3].

Malaria is caused by a protozoan parasite of the *Plasmodium* genus with five species *viz*. *falciparum*, *vivax*, *malariae*, *ovale* and *knowlesi*. The *P. falciparum* is the most prevalent, infectious and fatal of the five. Malaria infection is detrimental in young children and pregnant women due to underdeveloped and temporal loss of immunity, respectively. The parasite is transmitted from a female anopheles mosquito to a human *via* feeding when the latter bites the host [4–7].

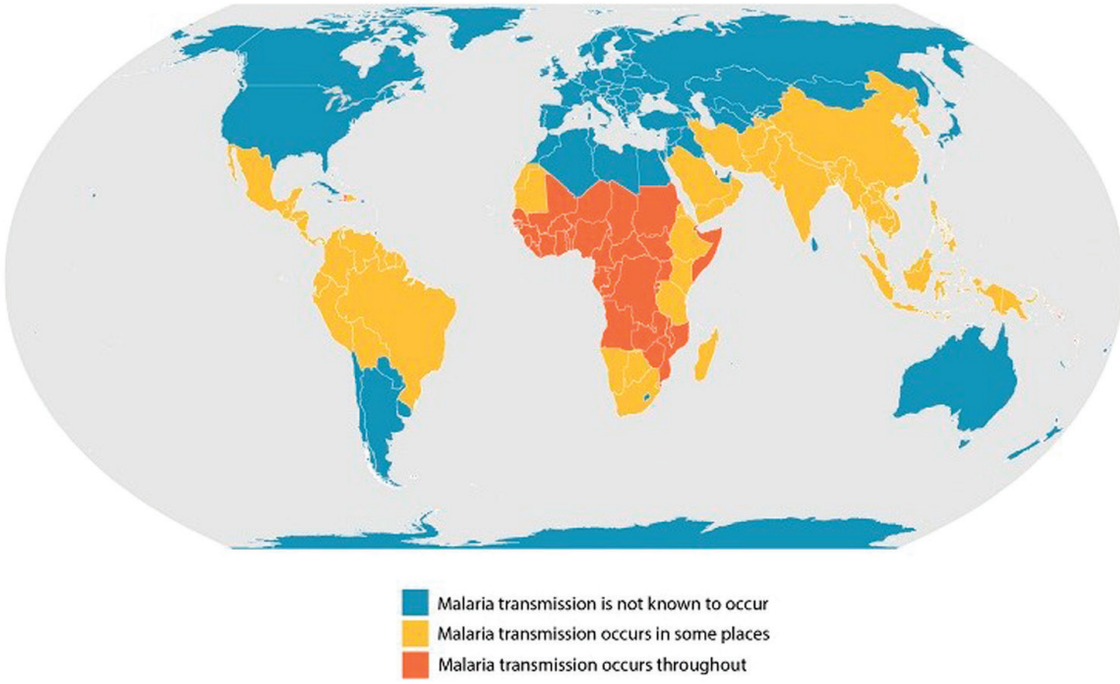

Figure 1.
An approximation of malaria-endemic WHO regions [2].

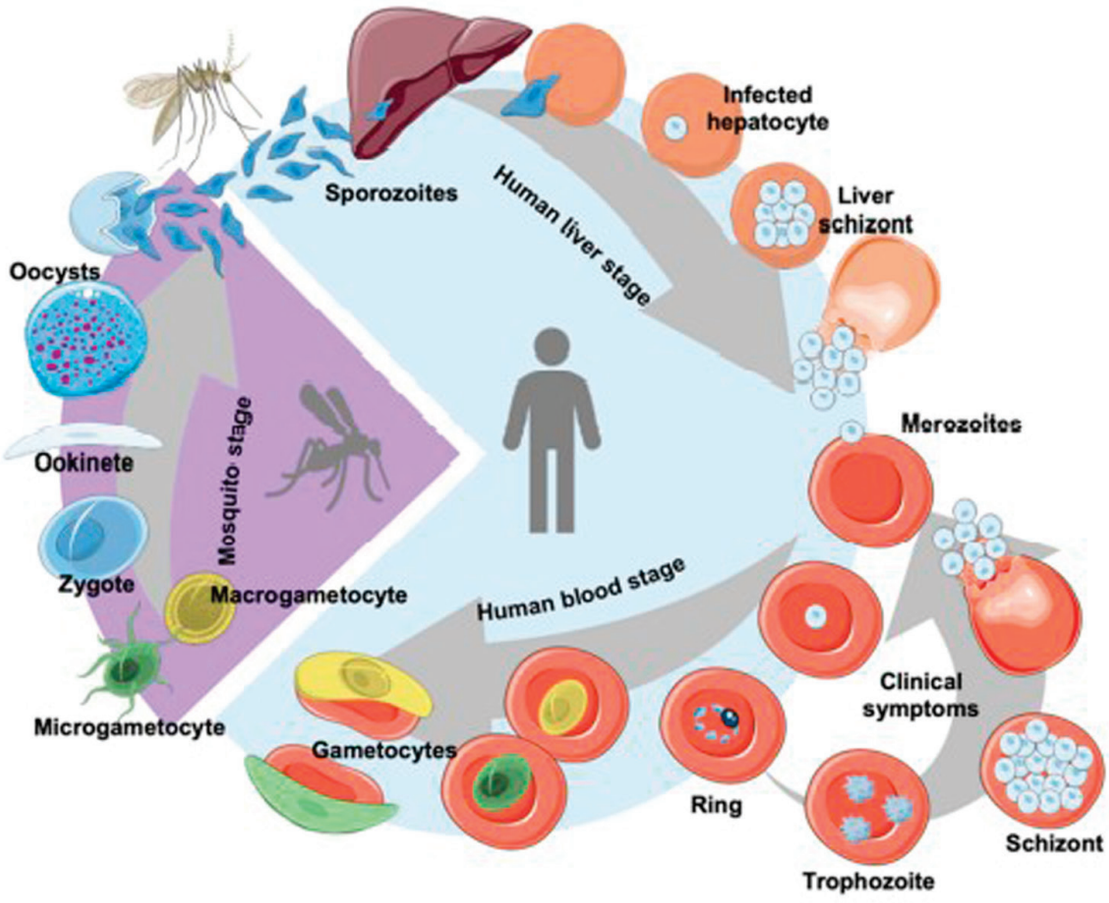

Figure 2.
A malaria parasite life cycle. Reprinted with permission from ACS [3].

The parasites target the lungs and red blood cells (RBCs) and the former has a latent phase that can reoccur after years of initial infection [5]. Sporozoites are transferred from the mosquitoes' salivary glands into the human bloodstream, where after they migrate to the human liver. In the liver, an exoerythrocytic infection stage initiates, that is, asexual reproduction in the liver hepatocyte of sporozoites transformation and multiplication into thousands of schizonts/merozoites. Within a couple of days (6–8), the matured merozoites rapture the host hepatocyte and are released into the bloodstream, where they invade the RBCs. Internalised in the RBCs, they undergo asexual reproduction. This infection stage is named the erythrocytic cycle [4]. The erythrocytic stage is initiated with tiny ring forms that turn into a larger amoeboid forms (trophozoite) and finally merozoites. The host RCBs rupture to release the matured merozoites, which will invade new RBCs. The continuous invasion of the RBCs burdens the RCBs and triggers severe pathological and patient sicknesses such as anaemia [4]. **Figure 2** illustrates the lifecycle of the *Plasmodium* species. Some of the merozoites develop into female and male gametocytes that circulate in the bloodstream to be re-ingested by a mosquito where they start a new cycle. Male and female gametocytes are fertilised into zygotes that transform into sporozoites that flow in the mosquito's salivary glands [4].

2. Microscopic detection of malaria

2.1 Conventional microscopy

Light microscopy, a gold standard conventional malaria diagnostic, uses Giemsa-stained thick and thin blood smears of peripheral blood from the intraerythrocytic malaria cycle to detect the *Plasmodium* species [3, 4, 7, 8]. The thick and thin blood smears are used to deduce the parasite and parasite species, respectively. **Figure 3** shows the *P. falciparum* light microscopy morphology from the smears. Microscopy, however, also presents challenges: low sensitivity at low parasitemia is laborious and costly, and requires a trained microscopist and it is not standardised; uses a bulky visual/light microscope and is not suitable for point-of-care testing (POCT) [3–5]. Also because microscopy is a morphological diagnostic tool, misdiagnosis is possible between species with close morphological resemblance [4]. It has an average detection limit of about 50 parasites/μL.

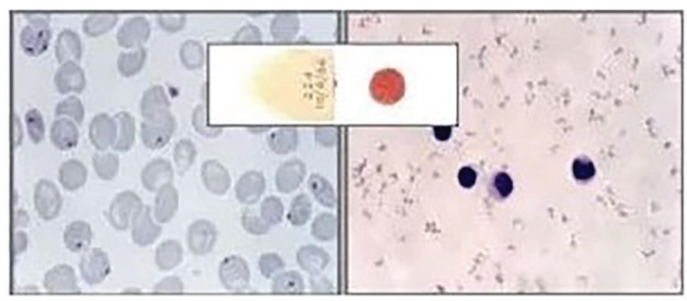

Figure 3.
Microscopy images of P. falciparum *from thick (right) and thin (left) blood smear [9].*

2.2 Modern microscopic interventions

As alluded to in the previous section, although microscopy is the malaria diagnosis gold standard, its bulkiness, expense and lab basis are some of the challenges that require mitigation. The automated microscopy platform is merited by improved and standardised malaria detection. It inherits the benefits of a light microscope and also mitigates its challenges and hence yields rapidity, improved scanning area and enhanced consistency [10]. Human microscopists are eliminated by the automated system. It inherits three reliant elements: sample preparation, digital microscopy with automated scanning and a computer vision algorithm that analyses captured images [10].

Faizullah et al. [11] proposed an automated convolutional neutral network (CNN) model for malaria detection from microscopy blood smear images. Knowledge distillation, data augmentation, autoencoder, feature extraction CNN model and classification support vector machine or K-nearest neighbours (KNN) were used to develop the CNN algorithm. The algorithm detected malaria from images with 99.23% accuracy. To enable both clinical and field use, it was integrated into a smartphone and backed up on server-back-up web, to support offline and online applications. Hasan et al. [12] tried different models: Adaboost, KNN, decision tree, random forest, support vector machine and multinomial naive Bayes on a database of 27,558 cell images. Adaboost, random forest, support vector machine and multinomial naive Bayes achieved good results (91%). Yoon et al. [8] developed a fully automated microscopic system characterised by a plastic chip, fluorescent dye for malarial staining and an image analysing program for detection and parasitemia determination. Use of the fluorescent dye as opposed to the Giemsa stain afforded rapidity, sensitivity and accuracy. The automated system showed a higher degree of linearity and precision in the detection of *P. falciparum* culture with a limit of detection of 0.00066112% (30 parasites/μL) [8].

The bulkiness of the traditional microscopy also negates its field application. Cyboloki et al. [13] combined optical principles with origami and fabricated two-dimensional microscopy, a cheaper alternative to the bulky light microscope. The foldscope is assembled from a flat sheet in a few minutes and provides over 2000 x magnification with submicron resolution. It alleviates conventional microscopy and can be used in the field since it does not use external power.

Microscopy under polarised light is also used to diagnose malaria *via* hemozoin (Hz). Hz is an end product of the malaria proliferation stage. Owing to its interesting properties of magnetism and acoustic and optical nature, it is explored as a biomarker [3]. Due to its birefringent nature, it is viewed with polarised light microscopy. This approach detects 30 parasites/ μ L but is negated by the use of an expensive polarised light, complexity and bulkiness [3]. An affordable alternative invention by Pirnstill et al. [14] uses optical smartphone-based transmission polarised light microscopy.

3. Spectroscopic malaria detection

3.1 Ultraviolet visible spectrometer

An ultraviolet-visible (UV-Vis) spectrometer is used in the detection of malaria, that is, confirming malaria cases *via* changes in excitation peaks. This system utilises nanoparticles such as gold (Au) and silver (Ag) that inherently have strong optical absorption, which signal aggregation by red-shift. The nanoparticles in the vicinity

of the biomolecules aggregate and hence trigger a measurable change in the optical signal [5]. Recently, Adegoke et al. [15] advanced this platform by coupling UV-Vis with near-infrared spectroscopy to detect and quantify low parasitemia (1-1x10^{-6}%) of ring-stage malaria-infected blood under physiological conditions using a multiclass classification.

3.2 Vibrational optical spectroscopies

Vibrational optical spectroscopies study the characteristic vibrational modes of molecules. The vibrational modes have specific vibrational frequencies, which are a molecule's characteristic fingerprint. These systems are used to study the molecular attributes of biological molecules in biosensing. Fourier transform infrared (FTIR) and Raman spectroscopy are complementary vibrational spectroscopic systems [16]. Raman spectroscopy has been tried in the detection of malaria focused on plasma [17].

An advanced variant of Raman, surface-enhanced Raman spectroscopy (SERS), uses plasmonic metallic nanoparticles such as Au and Ag as substrates to enhance traditionally weak vibrational peaks. Funing et al. [17] applied SERS to differentiate between healthy and *P. falciparum*-infected RBCs. Ag nanorod arrays were used for the SERS effect and direct detection of the parasite was confirmed *via* SERS spectra. The ring stage characteristic peak was at 1599 cm^{-1}, while trophozoite and schizoid stages had the same peak and an additional stretching vibration peak at 723 cm^{-1}. Thus, the Ag-based SERS platform effectively detected *P. falciparum* from three stages: ring, trophozoite and schizoid.

Mhlanga et al. [18] also explored the SERS platform for the detection and quantification of *P. falciparum*. A labelled SERS detection system that mimics the ELISA sandwich was used and it entailed the following elements: a solid SERS substrate alloyed with plasmonic Ag and Au, a capture P. *falciparum* antibody immobilised on the solid substrate and detection SERS probe conjugate. The detection conjugate was a plasmonic nanoparticle (Au/Ag), conjugated to a SERS tag/label and a detection antibody. The system was tested on the P. *falciparum* antigen and WHO malaria non-infectious blood specimens with varying levels of parasite density. The SERS immunoassays are investigated to be coupled with RDTs [19, 20] and lab-on-chip [21], in preparation for field-based application readiness.

Khoshmanesh et al. [22] explored optical attenuated total reflectance Fourier transform infrared spectroscopy for detection and quantification of the malaria parasite ring and gametocyte forms. A detection limit of 0.00001% parasitemia (1 parasite μL of blood; p 0.008) was reported.

Machine learning algorithms are also the future of spectroscopic malaria detection. Although their application is still in its infancy, it will solve a lot of challenges. Machine learning algorithms could improve SERS data processing for example through calibration multicomponent samples and the identification of interferences in complex biochemical samples, identifying SERS hotspots and analysing complex SERS spectra [23]. Irreproducibility of the SERS substrate is the bottleneck for their commercialisation or clinical application. Artificial intelligence algorithms are envisioned to mitigate this challenge and yield reproducibility and ultimately commercialization.

Nanotechnology is also a platform envisaged to revolutionise diagnostics and alleviate some of the challenges. For instance, the SERS prototype suffers from a lack of reproducibility. Expansion of SERS substrates from traditional plasmonic

nanoparticles to two-dimensional graphitic nanostructures has the potential to mitigate irreproducibility and attain uniformity of the SERS hotspots.

Mwanga et al. [24] mobilised both experimental FTIR and machine learning algorithms to differentiate malaria positive and negative filter papers containing dried blood spots (DBS). For machine learning, the algorithm was trained using a PCR data set of 296 patients with 123 positive results and 173 negative PCR results. The trained model was evaluated *via P. falciparum* detection on the DBS. The logistic regression yielded 92% accuracy on *P. falciparum* and 85% on mixed strains.

4. Nuclei-based malaria diagnostics

The nuclei-acid amplification-based test (NAATs) application in malaria detection surges in high-resourced, clinical care regions. The resourced regions use NAATs in epidemiological studies, and in clinical trials for malaria treatment and vaccine, as a reference standard in the evaluation of malaria diagnostics and general malaria disease management [25]. NAATs in diagnostics offer several advantages: enhanced sensitivity (1–20 parasite/μl), multiplexing potential, parasite quantification, species differentiation and early detection of the parasite [25, 26]. NAATs excellent sensitivity, especially for low parasite density, surpasses those of the gold standards, microscopy and RDTs, although WHO still recommends parasite-based tests for resource-constrained regions due to affordability [25].

4.1 Polymerase chain reaction (PCR) diagnostics

PCR is focused on the amplification of a small quantity of DNA, into manageable and detectable quantities. Performed over three steps, in multiples of 20–40, DNA or RNA is replicated to millions of copies. The steps involve the initial denaturation of the double-stranded DNA at temperatures around 90–96°C, annealing of primers (one each for the 3′ and 5′ ends), specific to the DNA sequence to be replicated, at reduced temperatures of around 50–65°C and the final elongation/extension to complete the DNA strand. The elongation is performed by specialised, high-temperature functioning DNA polymerase. The most commonly used polymerase is extracted from *Thermus aquaticus* (Taq), with optimum elongation performed at 72°C [27–31].

Although highly sensitive and specific, PCR is seldom used for malaria detection in endemic areas due to its high cost and need for skilled personnel. This makes it impractical for use in remote or rural settings [24]. PCR is also able to detect low parasitemia values, undetectable through other methods, and can identify the different species. In addition, real-time PCR can quantify and track infection load [24]. Due to the sensitive nature of PCR, non-invasive testing in other bodily fluids, such as sweat, urine, saliva and faeces, has become possible [24].

Homann et al. [32] used the 18S rRNA gene for quantitative PCR (qPCR) towards tracking traveller infections over 12 months. In addition, they employed nested PCR towards the genotype of the *msp2* gene of *P. falciparum*. They found a limit of detection of 0.12 and 5 parasites/μl for the qPCR and nested PCR, respectively. Qualitative PCR allows for the quantification of a PCR product, usually through the use of a dye or fluorophore attached to a common piece of DNA sequence. Nested PCR employs two sets of primers, with the first set producing the product to be amplified by the second set of primers. This has greatly improved the sensitivity of PCR [33].

In addition, the use of multiple primers, for various targets, in the form of multiplex PCR, has allowed for the simultaneous amplification of a variety of target DNA strands, making the process quicker, but also allowing for the detection of various species of a *Plasmodium* for example [34]. Allowing this technique is the preference for the diagnosis of multiple malaria species within a single sample [33]. Fitri et al. [33] summarised some of the primers used in nested, semi-nested, hemi-nested, multiplex PCR and LAMP towards the detection of various *Plasmodium* species.

Costa et al. [35] developed a droplet digital PCR (ddPCR) method for the detection of malaria DNA in saliva. They employed a similar technique to qPCR, using the same probes and primers and a droplet generator. When compared with other sample types (blood and mouth swabs), the ddPCR showed a sensitivity of 73% for saliva samples and 99 and 59% for blood and swabs, respectively. Although these results show promise, the authors warn that this might be misleading due to the small number of DNA samples tested.

4.2 Loop-mediated isothermal amplification

Loop-mediated isothermal amplification (LAMP) is a variant of NAATs that proffers simplicity and rapidity as opposed to PCR [26]. It uses the *Plasmodium* species DNA from blood samples to diagnose malaria. The LAMP DNA amplification set-up uses four primers: inner (forward and backward), forward and backward outer primers that bind on six unique sequences on the target sense and antisense strands. The reaction can be catalysed by additional primers, looping forward and backward [36]. A loopam™ malaria Pan/pf kit pioneered in Europe, Uganda and Zanzibar showed higher sensitivity to infections compared with PCR. Although the loopam™ malaria Pan/pf is simple and user-friendly in addition to enhanced sensitivity, it is currently expensive and not as simple as RDTs [37].

LAMP can potentially be extended to field testing because its reagents are stable at 25 and 37°C [26]. For field applications, LAMP is coupled with other diagnostic platforms such as lab-on-a-chip. Safavich et al. [38] comprehensively reviewed the techniques towards the development of LAMP-microchip/microdevice.

5. Malaria rapid diagnostic testing

To decrease the burden on traditional diagnostic techniques, the use of rapid diagnostic tests (RDTs) for the detection of Malaria (predominantly *P. falciparum*) has become increasingly popular. Its popularity stems from its ease of use, short turnaround time and no need for electricity or specialised equipment [39].

Most RDTs function on the same base, where malaria antigen is detected in a blood sample that flows through a membrane, to be captured by specific anti-malaria antibodies [39], depicted by coloured lines on the membrane surface [40]. These products mainly target *P.falciparum*-specific proteins, such as lactase dehydrogenase (LDH), aldolase or histidine-rich protein II (HRP-II) [39]. The *pHRP-II* protein is specific towards the detection of *P. falciparum*, while the pLDH and *Plasmodium* aldolase are found in all species [40].

Histidine-rich protein II of *P. falciparum* is water-soluble and produced by *P.falciparum* gametocytes and the asexual stages. It is expressed on the membrane surface of red blood cells and remains in the blood after the treatment onset. *Plasmodium* aldolase is an enzyme expressed during the parasite glycolytic pathway of the blood

stages of *P. falciparum* and non-falciparum malaria parasites. Monoclonal antibodies produced against *P. aldolase* are pan-specific. They have been used in conjunction with *pfHRP-II*, for the detection of pan malarial antigen (PMA) in combined immunochromatographic assays [41].

The soluble, glycolytic parasite lactate dehydrogenase enzyme (pLDH) is produced by both sexual and asexual stages of the living parasite. The enzyme is present and gets released from the erythrocytes infected by the parasite. The enzyme is present in all four species of human malaria, with different pLDH isomers for each [41].

Although various reports have mentioned the excellent performance of these RDTs, reports of large variation in sensitivity and reliability in malaria-endemic countries have led to guidelines developed by the WHO for establishing lot-to-lot quality control measures [39]. Although this rapid diagnostic tool seems to be of great use to the healthcare worker, it still relies on other methods for disease confirmation, infection characterisation and treatment monitoring [39].

As RDTs are immune-chromatographic lateral flow antigen-detection tests, they rely on capturing antibodies labelled with a dye to visualise the detection and control lines on a nitrocellulose membrane encased in a plastic casing, cassette. For the detection of malaria, the dye-labelled antibodies detect the *Plasmodium* antigen in the testing sample. The formed complex is captured by a secondary antibody immobilised on the membrane, resulting in a visible test line. The control line confirms the dye-labelled antibody's integrity, although not its ability to detect the parasitic antigen [42].

The test is usually conducted from a finger prick with only around 5–15 μl of blood and a total test time of only 5–20 min. As HRP II persists for up to 30 days post-eradication of parasitic infection, it is not suggested for monitoring disease progression. Disease progression is best monitored by pLDH or aldolase that is eliminated quickly, although plasmodial gametocytes also produce pLDH that may result in false positives [43, 44].

As the pHRP-II protein detects *P. falciparum*, pLDH specifically detects either *P. falciparum* or *P. vivax* or is pan-specific (common to all species). *P. aldolase* is pan-specific. The development of an RDT with all these variants allows for the detection of only *P. falciparum*, only *P. vivax* or any combination [45].

The RDTs do have various diagnostic limitations: 1) the before mentioned proteins cannot distinguish or detect *P. ovale*, *P. malariae* or *Pheidole knowlesi*, 2) some *P. falciparum* species (South America) do not produce the common HRP-II proteins, excluding the use of RDTs based on these proteins in those areas, 3) assays containing *P. falciparum HRP-II* (*pfHRP-II*) proteins, but not pHRP-II, show cross-reactivity with *Schistosoma mekongi* infections, 4) rheumatoid factor and other circulating auto-antibodies can cross-react with some assays, 5) high parasitemia for *P. falciparum* could give false-positive results for a *pHRP-II* assay meant to detect *P. vivax*, 6) the RDTs cannot determine parasitemia magnitude, while microscopy can, and 7) as *HRP-II* is not cleared from the blood, it cannot be used to monitor therapy effectiveness [45]. A list of the commercially available RDTs is available from The Global Fund [46].

To combat these limitations, various groups are working towards the inclusion of nanomaterials into the RDTs. Here, the nanomaterials with tuneable size, shape and plasmonics increase the selectivity and sensitivity of these point-of-care devices. The incorporation of metallic gold, silver, copper and platinum-palladium nanoparticles, as well as magnetic iron oxide, silica, ceria nanoparticles and luminescent quantum dots, is summarised by Nishat et al. [47]. Au nanoparticles (40 nm) were coupled with *pfHRP-II* towards the development of a biosensor for malaria detection. In

another study, Ag nanoparticles were catalysed by Au nanoparticles to enhance *HRP-II* detection [47].

As previously mentioned, the paramagnetic nanoparticle, Hemozoin, is a by-product and biomarker for the malaria parasite, similar chemically and structurally to β-hematin. As hemozoin is more stable, available and cheaper than *pfHRP-II*, it is recommended for use in the RDT development [48].

An ideal RDT should be able to detect a minimum of 100 parasites/μL. Armani et al. [49] utilised β-hematin and reported a limit of detection at <8.1 ng/mL in 500 μL blood (26 parasites/μL) [48]. Electrochemical sensors employing β-hematin incorporated CuO, Fe_2O_3 and Al_2O_3 nanoparticles. The catalytic activity with Au electrodes was shown, with CuO affording consistently low levels of detection (0.83 μg/mL) [48]. As mentioned by Noah et al., biosensors have seen little attention, with vast potential in not only detecting malaria parasites but also quantifying parasitemia, allowing for infection tracking [48].

Ditombi et al. [50] compared four RDTs for malaria and found that all RDTs had a sensitivity >95%, with only two tests showing specificity above 85%. With the inclusion of the WHO Methods Manual for product testing of malaria rapid diagnostic tests [51], the quality and trust in RDTs improved. Aidoo et al. [52] reported an increase in the RDT testing rate for malaria from 36 (2010) to 84% (2018) in sub-Saharan Africa, with an increase from 100 million supplied malaria RDTs in 2010 to 348 million in 2019. RDTs have also allowed for increased availability of testing for malaria in remote and rural areas [52].

Feleke et al. [53] conducted an immense study on the mutation of *P. falciparum* to evade detection by *pfHRP-II* RDTs. They found that 28.9% of the samples, positive

	Microscopy	**RDT**	**PCR**
Principle technique	Morphological interpretation	Antibody and antigen-binding	Amplification of DNA
Target diagnostic	All parasite stages (early trophozoite, mature trophozoite, schizont and gametocyte)	*Pf*-HRP-II, *Pf*-pLDH, pan-p-LDH, aldolase and *Pf*-GAPDH	rRNA/ssrRNA small subunits, SCIAvar gene
Sensitivity	≥5 parasites/μl (Expert), 50–100 parasites/μl (Average)	50–250 parasites/μl	< 5 parasites/μl
Specificity	High (difficult to diagnose *Pheidole knowlesi* from single and mixed infection)	Moderate (Can only identify *P. falciparum* and *P. vivax*	High, specie differentiation and identification
Time consumption	≤ 60 minutes	10–20 minutes	2–8 hours
Interpretation	Quantitative	Qualitative	Qualitative and quantitative
Advantages	Direct cost is low, and samples can be stored	Fast, simple, applicable and practical	Requires a tiny sample
Disadvantages	Specialised equipment, well-trained staff	Expensive, cannot perform drug monitoring	Large cost towards supplies, machinery and staff training

Table 1.
Comparison of diagnostic methods for Plasmodium species: microscopy, PCR and RDTs [33].

for *P. falciparum*, had discordant profiles within the RDT. This links to a pfHRP2/3 deletion for *P. falciparum*, making them only detectable through LDH-based RDTs.

6. Conclusion

Malaria remains problematic for both endemic and non-endemic WHO regions. Non-endemics regions due to globalisation and travelling are at risk of importing malaria cases. Malaria disease management is mandatory in the endemic regions and include efficient diagnostics and treatment. The detection assays for malaria should inherently be affordable, rapid, specific, sensitive and simple for low-resourced regions. **Table 1** comprehensively compares the available malaria detection platforms: microscopy, RDTs and PCR. PCR remains superior in its ability to detect sub-5 μL parasite values, with differentiation between all known *Plasmodium* species. It takes longer than microscopy, where 5 μL parasitemia can still be detected by advanced microscopists, but both still require highly skilled personnel and laboratory equipment. Currently, RDT, although with low sensitivity, remains a better option for low-resourced regions merited by detection limit at least 10x higher than microscopy and PCR, and simplicity and POCT usability without specialised equipment [33]. Artificial intelligence, nanotechnology and multimodal systems are envisioned to transform malaria diagnostics, especially in the mitigation of challenges associated with the different diagnostics.

Acknowledgements

Authors acknowledge the Department of Science and Innovation, South Africa and Mintek for funding.

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