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Malaria diagnostic update: From conventional to advanced method

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Abstract

Background: Update diagnostic methods play essential roles in dealing with the current global malaria situation and decreasing malaria incidence.

Aim: Global malaria control programs require the availability of adequate laboratory tests in the quick and convenient field.

Results: There are several methods to find out the existence of parasites within the blood. The oldest one is by microscopy, which is still a gold standard, although rapid diagnostic tests (RDTs) have rapidly become a primary diagnostic test in many endemic areas. Because of microscopy and RDTs limitation, novel serological and molecular methods have been developed. Many kinds of polymerase chain reaction (PCR) provide rapid results and higher specificity and sensitivity. The loop-mediated isothermal amplification (LAMP) and biosensing-based molecular techniques as point of care tests (POCT) will become a cost-effective approach to advance diagnostic testing.

Conclusion: Despite conventional techniques are still being used in the field, the exploration and field implementation of advanced techniques for the diagnosis of malaria are still being developed rapidly.

KEYWORDS
diagnostic, LAMP, Malaria, PCR, POCT, RDT

1 | BACKGROUND

Malaria is still a health problem in the world. Five species can infect humans, namely *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malaria*, *Plasmodium ovale*, and *Plasmodium knowlesi*. Four species are considered true parasites of humans, while *P. knowlesi* is still considered a zoonotic malaria. Among these species, *P. falciparum* and *P. vivax* are the most prevalent worldwide, in which the most common complications of severe malaria occur in *P. falciparum* infection.[1]

The most frequent clinical manifestation of malaria is fever or recent history of fever. However, because many diseases also have fever as primary clinical manifestation even in the endemic area, accurate laboratory parameter is crucial. False-positive will lead to improper use of antimalarial therapy, and obviously, underdiagnosed cases cause an increase in morbidity, mortality, and antimalaria resistance.[2] The most frequently used method for early detection of malaria infection and remains the gold standard for laboratory confirmation of malaria is microscopic because it is easy to use and cheap. However, there are still many weaknesses in its application.[3]

An appropriate and accurate diagnosis for the detection of malaria parasites has been a longstanding challenge for epidemiological screening and surveillance to provide information on malaria control strategies to reduce morbidity and mortality. To answer the challenge, a reliable malaria diagnostic test is highly required. It has to pass some criteria such as the diagnostic test should be able to identify parasites down to species level, has the capability to quantify the level of parasitemia, has a high sensitivity to detect low-level parasitemia, it can confirm the presence or absence of infection and allowing monitoring of response to antimalarial therapy.[4]

Various techniques now are available for malaria diagnosis from the earliest conventional method using microscopy until the novel technique using the rapid molecular method. In the last decade, there has been an interest in the basic immunoassay concept of malaria rapid diagnostic test (RDT) kits for detecting *Plasmodium* species[5,6] and flow cytometry and enzyme immunosorbent assay. Molecular methods such as polymerase chain reaction/PCR (real-time PCR, nested PCR, and Multiplex PCR), and loop-mediated isothermal amplification (LAMP) have begun to be widely used in research and diagnosis of malaria. Here, we will discuss the challenges and prospects of each method.

2 | CONVENTIONAL METHODS

2.1 | Light microscopy for malaria diagnosis

Microscopy has been used for over 100 years and is inexpensive, rapid, and relatively sensitive when used appropriately.[5] World Health Organization (WHO) has recommended that all suspected malaria cases must be confirmed with either microscopy for parasitemia counting or RDT in all clinical settings since 2010. In the case of malaria, the *Plasmodium* parasite within the erythrocyte can be observed using microscopic observation. Nowadays, the widespread reference method to diagnose malaria under microscope is thin and thick blood smear using Giemsa stain. This method remains the gold standard used to quantify and stage malaria parasites.[2]

Light microscopy is one of diagnostic method which is highly available even in a remote laboratory setting, easily used, yet need a skillful and competent microscopist. Some advantages of light microscopy in diagnosing malaria include (a) low direct costs in a high-volume sample; (b) good sensitivity for clinical malaria; (c) parasite's species and stage can be differentiated; (d) parasite density can be measured; (e) drug-induced morphological changes can be observed; and (f) screening for other related blood abnormality at once.[2] In addition, using microscopy, we can quickly determine the parasite density because parasitemia can be manually observed with naked eyes or using software analysis. The beneficial impact of parasite density is the determination of malaria severity and prognosis. Parasite density of more than 5% is classified as severe malaria. Especially on malaria falciparum, the presence of an asexual late development stage within peripheral erythrocyte indicates a severe outcome and poor prognosis. Parasite density counting as a follow-up to measure treatment response and should be continued until parasites are cleared. Parasite density may rise in the early treatment before starting to fall because some drugs are effective in the early parasite development.[7] So far, the objectives of acquiring parasite density can be summarized into (a) to measure malaria infection severity; (b) to compare parasite density before and after treating the patient as an effective antimalarial indicator; (c) as a report to the district health officer in term of eradication policy; and (d) to observe parasite sensitivity toward antimalarial drugs.[8]

Unfortunately, the microscopic method also has many limitations. Observation under the microscope could not distinguish the morphology of mature trophozoite, schizont, and gametocytes between *P. knowlesi* and *P. malariae*, as well as the early trophozoite stage between *P. knowlesi* and *P. falciparum*. These limitations often cause misdiagnosis.[9,10] The other disadvantage of this method is the inability to detect a small number of parasites below the microscopic threshold[11] as the detection threshold at 50 parasites/μl.[12] Low-level parasitemia is common in the hyperendemic area and is usually found in people with adequate host immunity or after receiving of antimalarial agent.[13,14] It is important to note that artifacts in low-quality blood smears can lead to false-positive readings by untrained technicians. The light microscope also has difficulty diagnosing mixed infections, which is a reason for the misdiagnosis of mixed infections as a single infection.[14,15] Giemsa-stained thin and thick blood smears may be sometimes challenging to perform as a gold standard of malaria diagnosis. This technique is tedious and requires a clinical setting equipped with light microscopy and must be observed by experienced and well-trained technicians to accurately interpret data, particularly in a rural or remote area. Microscopy is also time-consuming and labor-intensive and cannot detect sequestered *P. falciparum* parasites.[16]

To get the accurate result of light microscopy, the blood smear procedure needs to be standardized, following the standard procedure published by WHO, CDC, or some national guideline. Either vein puncture or finger-tip blood sample will be needed in a small amount (around 2–6 μl). There are several thick and thin blood smear errors commonly made. Too much blood in thin blood smear will cause erythrocytes overlap and difficulties in *Plasmodium* species and stage observation. Similarly, over-dropped blood in the thick smear will cause difficulties in observing malaria parasites due to many leukocytes observed. Meanwhile, too little amount of blood will cause underdiagnose. Besides that, the object-glass used should be clean with a flat edge. Otherwise, a hollow and sharp-edged blood smear may be formed.[17]

Thin and thick blood smear methods can also be used to count the number of parasites. There are two methods in establishing the parasite count: (a) parasite count per microliter of blood and (b) the grade of the number of parasites in thick smears or the plus system. The parasite count per microliter (μl) of blood is more reasonable and acceptable than the plus system, which is less satisfactory. The count can be acquired in the correlation between the number of parasites and the number of leukocytes. The detailed procedure refers to WHO standard methods published in 1991.[8] What microscopists

should notice when practicing parasite count is to count all the species observed under the microscope. Besides that, they must record sexual and asexual parasites separately, particularly when monitoring the response of schizonticide drugs because it does not affect the gametocytes.[8]

2.2 | Immunochromatography-based rapid diagnostic test

Immunodiagnostic methods such as immunochromatography-based RDT, enzyme-linked immunosorbent assay (ELISA), and flow cytometry have been widely used to determine *Plasmodium* parasitic infection. RDT is the most popular method to detect specific antigens or antibodies that correlates to the desired pathogen. In the last decade, RDT was used as an alternative diagnostic for malaria diagnosis when good microscopy quality cannot be provided or is unreliable.[18]

Rapid diagnostic test is a lateral flow immune-chromatographic test on nitrocellulose strip which detects specific antigens in case of malaria, ranging from single species (*P. falciparum*) to multiple species (*P. vivax*, *P. malariae*, and *P. ovale*). The RDT principal diagnosis is based on interaction dye-labeled antibody with target antigen in the blood and will appear as a visible band on the strip. The mode of action of RDTs starts when the drop of blood in the sample area and mixed with buffer move to the channel. If the antigen is present in the sample, free dye-labeled Ab will bind into the antigen then this complex will be bound into the bound Ab on the test line. The excess complexes are trapped and accumulate on the control line. The line color intensity may reflect the number of parasite antigens.[19]

P. falciparum histidine-rich protein-2 (PfHRP2), *P. falciparum* parasite lactate dehydrogenase (*Pf*-pLDH), *P. vivax* specific pLDH (*Pv*-pLDH), common human *Plasmodium* LDH (pan-pLDH), and aldolase are protein targets in malaria RDT. *Pf*HRP2 is synthesized by both the asexual and early sexual stages of all human *Plasmodium* species and released in the extracellular plasma.[20] *Pf*HRP2 antigen is produced in 2–8 h after ring development and has a long half-life up to 3 weeks in the plasma or serum.[21] pLDH is a soluble enzyme and essential for energy production and parasite development.[22–25] pLDH structure differs from human LDHs[22] and rapidly declines when the parasites die.[24] The other prospective protein target is *P. falciparum* Glyceraldehyde-3-phosphate dehydrogenase (*Pf*GAPDH). *Pf*GAPDH levels are around four to six times higher than *Pf*LDH levels.[26]

P. falciparum histidine-rich protein-2 rapid diagnostic test (RDT-*Pf*HRP2) are more sensitive for the detection of *P. falciparum* than *Pf*LDH and aldolase-detecting tests.[27] *Pf*HRP2-detecting RDTs have higher sensitivity but lower specificity than *Pf*-pLDH-detecting RDTs (95.0% vs. 93.2% and 95.2% vs. 98.5% respectively). In *P. vivax*, pan-pLDH versus *Pv*-pLDH-detecting RDT has been proved to have no difference with high sensitivity (>99.0%). However, aldolase-detecting RDTs still give a lower sensitivity number (80.0%–81.4%). RDT, which detects *P. ovale* and *P. malariae*, gives low and variable sensitivities besides their low prevalence.[28]

The sensitivity and specificity of RDT depend on each RDT's manufactures, operator, supply chain, host, parasite and may change in different epidemiological situations. RDT sensitivity may decline due to low parasite density[29] and prozone phenomenon (hyperparasitemia/severe malaria/excess antigen). This phenomenon will cause the exhaustion of antibodies, leading to the loss of signal (false negative).[30] The accuracy of the RDT test (Malaria Antigen P.f®) compared to thick smear and PCR was 98.2% and 97.1%, respectively[31] but studies in Cameron[32] and Nigeria[33] showed weak accuracy. The false-positive result in RDTs related to the antigen concentration available from the blood sample to the peripheral parasite density varies highly due to an internal factor of host and parasite. Therefore, diagnosis of malaria using RDTs should be supported by other diagnostic methods to determine *Plasmodium* infection.[34,35]

There are several issues of RDTs importance in malaria control: (i) there should be a clear benefit when the result of positive parasitemia is revealed in the clinical setting as sensitivity ranges from 85% to 94.8% and specificity usually above 95%; (ii) RDTs can effectively monitor 50–100 parasites per μl of blood; (iii) a "cold chain" for transport and storage can be maintained; (iv) trained-health workers can be provided; and (v) supported by the local policy.[18] Despite there are some RDTs-related issues, RDTs give some advantages over microscopy in the rural/remote clinical setting. RDTs offer low direct cost, do not need special equipment, have fast results, and are simple.[31,36–39]

3 | DEVELOPED METHOD

3.1 | Enzyme-linked immunosorbent assay

An enzyme-linked immunosorbent assay (ELISA) has been developed as a validated method for detecting PfHRP-2 in blood, serum, and plasma of malaria patients.[40] Very high sensitivity of the DELI assay, a double site pLDH ELISA for drug susceptibility determination, also has been appointed. The assay reagents are not available in a commercial kit. Compared to commercialized rapid tests, this assay has a much lower detection threshold as its capability to detect one parasite per μl, which is the lowest detectability reported in the scientific literature to date.[41]

An antibody-based ELISA method has been used to determine immune response against *Plasmodium* infection. Most studies on human immunity to malaria have focused on the roles of immunoglobulin G (IgG). However, recent studies showed that immunoglobulin M (IgM) also plays an essential role in malaria immunity.[42,43] A long-lived antibody response, merozoite-specific IgM plays an important role in the immune response to parasites at the blood stage and contributes to immunity against malaria. Merozoite-specific IgM persisted for extended periods, with no difference in decay compared to IgG, suggest that there is induction and maintenance of long-lived IgM-secreting cells or memory IgM+ B cells after clinical malaria. This IgM blocked merozoite invasion of red blood cells in a complement-dependent manner and was also associated with a

significantly reduced risk of clinical malaria in a longitudinal cohort study.[42] In the future, ELISA-based immunodiagnostic tests targeting merozoite-specific IgM should also be considered to give a new insight in malaria immunity.

3.2 | Flow cytometry

Firstly, an automated method for detecting malaria parasites in blood samples using flow cytometry is presented by van Vianen, 1993.[44] The blood sample was mixed with a solution containing formaldehyde, causing red blood cells to lyse while parasites and white blood cells were preserved, then flow cytometric analysis was performed in a single-step procedure. The cells were stained with the fluorescent dye Hoechst 33258 and a FACStar flow cytometer subsequently analyzed the DNA. Parasites and white blood cells were distinguished and counted based on blue Hoechst fluorescence and forward scattering. Wongchotigul et al., in 2004 developed a flow cytometric assay (FCM) using reagents from Sysmex Corporation, Japan. In this study, the FCM gave a sensitivity of 91.26%, specificity of 86.28%, and accuracy of 87.42% with a greatly simplified and accelerated method. The parasite counts by flow cytometric measurement correlated well with the parasitemia measured by Giemsa stain with regression coefficient 0.9409. The detection limit was 0.05%–0.1% parasitemia.[45] It has also been reported that using propidium iodine, the density of parasite DNA in *P. falciparum* culture could be analyzed using flow cytometry.[46]

In 2011, Malleret et al. developed a fast and simple flow cytometry known as the tri-color method (TCM) for quantifying and grading various malaria parasites in red blood cells of whole blood or in vitro cultured *P. falciparum*. Parasites were stained with dihydroethidium and Hoechst 33342 or SYBR Green I, and leukocytes were stained with CD45 monoclonal antibody. This protocol distinguishes infected red blood cells from white blood cells and allows the selection of uninfected reticulocytes and normocytes. It can also assess the proportion of parasites at different stages of development. This finding demonstrates how this technique can be applied to detect *Plasmodium* infection and antimalarial drug testing. TCM requires the use of a UV laser for Hoechst detection which is only available in large, sophisticated instruments thus are not suited for utilization in the field. SYBR Green I could be used to replace Hoechst dye, that allowing detection with two laser cytometers available in a portable format. In this study, the parasitemia of *Plasmodium berghei*-infected mice that calculated by SYBR Green and Hoechst showed similar values. A more extensive comparison between the dyes was performed again using malaria mice model, and the calculated parasitemia was also found to be comparable. In summary, they stated that SYBR Green I can substitute Hoechst staining with the limitation that the parasite staging is less well defined.[47]

One of the well-known flow cytometry markers used to detect malaria infection is the malaria pigment, known as hemozoin (Hz), produced when the intra-erythrocytic malaria parasites digest host hemoglobin into the Hz. Hemozoin within phagocytes can be detected by depolarization of laser light as cells pass through a flow-cytometer channel. This method could be used for malarial diagnosis and is potentially helpful for diagnosing clinically unsuspected malaria as this method may provide a sensitivity of 49%–98% and a specificity of 82%–97%.[48] Hz-containing leukocytes in the circulation indicate *Plasmodium* spp., which may have prognostic relevance in malaria.[49] However, even only a single pigmented leukocyte was identified, it is highly indicated of malaria infection. The question arose whether the Hz-containing leukocyte count might hold useful clinical information. Most studies established a highly significant, positive correlation with disease severity, although the results from different study sites were highly variable.[50–53] The methodology of how pigmented leukocytes are counted is very different, as has been shown previously. The disadvantages are its labor intensiveness, the need for trained technicians, expensive diagnostic equipment, and false positives with other bacterial or viral infections. Therefore, this method should be considered as malaria screening tool.[48]

4 | ADVANCED MOLECULAR TECHNIQUE

The discovery of the structure of DNA leads the development of crucial molecular biology techniques called nucleic acid amplification technologies (NAATs). NAATs are very sensitive because of their ability to amplify extremely small of the target DNA or RNA.[54] The overall category of NAATs is PCR (real-time PCR, multiplex PCR, and nested PCR), LAMP, and molecular-based point of care test (POCT). One of the most significant scientific developments in molecular genetics in the last decade has been the invention of PCR.[55]

Polymerase chain reaction is the most frequent genotyping technique used in the laboratory because of its robust thermal cycling methodology, specific, simple, rapid, and sensitive.[56,57] PCR has provided the opportunity to devise highly sensitive methods of parasite detection and the specificity inherent to this method allows the unequivocal identification of the parasite genome at the species level. Table 1 describes the comparison between the conventional and molecular methods for malaria diagnosis.

Polymerase chain reaction-based assays have been developed to overcome the limitations and the weakness of malaria conventional diagnostic methods.[14] All samples found to be positive for malaria by other methods were subjected to diagnostic PCR.[66] In the recent decade, PCR has been used not only for the identification of disease but also for gene assignation, the discovery of the importance of single nucleotide polymorphisms and methylated DNA[14]

Nested PCR methods have been widely used to detect *Plasmodium* spp. They are continuing the advanced techniques such as multiplex PCR and real-time PCR (qPCR). Nested and multiplex PCR results can give accurate determination and differentiation when complex morphological problems arise during attempts to identify parasites at the species level.[14,67] However, these techniques need a reliable power supply, are expensive, time-consuming, and challenging to use in low-resource settings or at the point-of-need. To solve this problem, isothermal DNA amplification methods

TABLE 1 Comparison among microscopy method, RDT, and PCR

Parameters	Microscopy	RDT	PCR
Principle techniques	Morphologic interpretation	Antigen and antibody binding	DNA amplification
Target diagnosti c[11,20,22–25,58–62]	All stages of the parasite (early trophozoite, mature trophozoite, schizont, and gametocyte)	*Pf*HRP2, *Pf*-pLDH, *Pv*-pLDH, pan-pLDH, Aldolase and *Pf*GAPDH	Small subunit rRNA/ssrRNA, SICAvar gene
Sensitivity[35,48,63,64]	Up to 5 parasites/μl (the expert microscopist) 50–100 parasites/μl (the average microscopist)	50–250 parasites/μl	Below five parasites/μl
Specificity[14,15]	High (unless for *P. knowlesi*), difficult to distinguish mix and single infection	Moderate (limited to *P. falciparum and P. vivax)*, cannot identify *P. ovale*, *P. malariae*, and *P. knowlesi*	High, can identify and differentiate among species
Time consume[48,57,63,65]	Up to 60 min	10–20 min	2–8 h
Interpretation	Quantitative	Qualitative	Quantitative and qualitative
Advantages[31,36–38,57]	Low direct cost. can be stored for a long time	Simple, fast, more practical, and applicable method	Requiring only a small sample
Disadvantages[16,57]	Need special equipment and well-trained technicians	Cannot use for drug monitoring, more expensive	Supply costs, machinery fees, and training expenses

have been developed, such as nucleic acid sequence-based amplification, rolling circle amplification, LAMP, recombinase polymerase amplification (RPA), and clustered regularly interspaced short palindromic repeats (CRISPR). RPA and CRISPR are simple, high sensitivity, selectivity, compatibility for multiplexing, rapid amplification, and done at room temperature.

4.1 | Real-time PCR for Detection and Identification of *Plasmodium* spp.

A validated real-time PCR assay to detect and identify *Plasmodium* spp. in a single reaction by using a simple collection method had been developed. Using fluorescence-based technology provides the target amplicons are detected in real-time as they accumulate after each cycle once a threshold is reached as quantitative data. Thus, the detection is exponential rather than an end-point analysis. It is a closed system with no accumulation of hazardous waste, no contamination, no post-amplification processing, and the imaging system is a part of the real-time instrumentation. It is cost-effective, with high sensitivity and specificity.[68]

Real-time PCR assays have the potential capacity to detect mixed infections and detect low levels of parasitemia.[69] Real-time PCR detection of *Plasmodium* species by melting curve analysis is faster and accurate compared to other molecular methods. Melting curve analysis based on nucleotide variations within the amplicon provided a basis for precise differentiation of *Plasmodium* species.[70] The SYBR Green I-based real-time PCR assay for *Plasmodium* species detection is more sensitive than microscopy in a previous study. The prevalence of malaria parasite infection from Giemsa staining and reassessment by SYBR Green I-based real-time PCR show a high discrepancy. The study results indicated that the diagnosis efficiency of microscopy is very low for species-specific and mixed infection detection. Therefore, real-time PCR-based species diagnosis should be applied for clinical diagnosis and quality control purposes to prevent the advent of drug-resistant strains due to misdiagnosis and mistreatment.[71]

Real-time PCR assay that can detect *Plasmodium* spp. accurately could be done in a single reaction by using a simple method for sample collection consisting of blood spotted on treated filter paper. It can be used to validate microscopic findings and can be used without the need for multiple blood specimens or even microscopic examination in specific settings for the primary identification of an infected patient. The sensitivity, precision, positive predictive value, negative predictive value, and accuracy of real-time PCR tests related to microscopic analysis were validated from morphological evaluation results of an experienced technician.[72]

4.2 | Multiplex PCR

Despite being considered highly sensitive and specific for malaria diagnosis, rt-PCR has disadvantages. It involves five to six separate PCR reactions to detect the five species of *Plasmodium*, which consumes time, labor, and materials and has a high potential to produce cross-contamination.[73] Multiplex PCR is a variant of PCR in which multiple targets sequences can be amplified simultaneously in a single reaction well, with a different pair of primers for each target. Multiplex PCR can generate considerable time and labor savings compared to microscopic methods, especially in field surveys in endemic areas.[74,75]

The optimization of multiplex PCR can present several difficulties, including non-specific amplicon formation, imbalances the

amplification of different targets, reduces sensitivity and specificity. It is well-known phenomenon in multiplex PCR that one target sequence is amplified in preference to another. Multiplex PCR also suffers from the following unique problems such as referred target amplification and non-specific amplification, including primer dimers and mispriming artifacts. One of the most important factors is the optimal primary-template ratio. Primer dimers are formed when the proportions are too high, such as very dilute template conditions or excessive primers. If the ratio of primer–template is too low, the product will no longer accumulate exponentially because the newly synthesized target strands will regenerate after denaturation and inhibit the formation of PCR products. Therefore, the primer dimers may be amplified more successfully than the favored target, consuming reaction components and affecting annealing and extension rates.[75,76] The standard primers used to detect malaria are described in Table 2.

In multiplex PCR, two major processes that create bias have been identified: PCR drift and PCR selection. PCR drift is a bias assumed to be due to fluctuation in the interactions of PCR reagents, specifically within the early cycles, that could appear in the condition of very low template concentration. Meanwhile, PCR selection is a mechanism that favors the amplification of certain templates because of the particular properties of the target's flanking sequences or the complete target of the whole genome. These properties include inter-region differences in GC content, resulting in the preferential accessibility of targets within genomes due to secondary structures and the number of gene copies within a genome.[75]

The most challenging multiplex PCR reactions can be significantly improved by increasing the concentration of DNA polymerase and magnesium chloride as well as the use of PCR additives such as DMSO, glycerol, formamide, and betaine. Since most primers are GC-rich sequences, the addition of PCR additives could enhance the amplification by interrupting the secondary structure formation and facilitating the annealing of primers to the DNA template. In the multiplex PCR, DMSO and glycerol gave conflicting results. Therefore, the usefulness of these adjuvants should be tested in each case. At concentrations up to 0.8 mg/ml, bovine serum albumin significantly improved PCR efficiency over DMSO or glycerol.[75,76] In addition, other methodologies have been developed, such as multi-step manipulations of the template DNA before PCR amplification which significantly increase the number of targets able to be amplified. A hot start technology that serves to modify the DNA polymerase to be inactive at the lower temperature setup conditions has been demonstrated to improve multiplex PCR.[76]

4.3 | Nested polymerase chain reaction

Polymerase chain reaction efficiency and sensitivity increased markedly when the nested PCR strategy was adopted. In this method, two steps of amplification are performed, with the product of the first reaction serving as the template for the second reaction in which the hybridization sequence oligonucleotide primer is contained in the first product. This secondary PCR uses a different set of primers, "nested" or internal, to those used in the primary PCR and these primers will not find priming sites on primer dimers or non-specific artifacts generated in the primary PCR. In the first PCR (nest-1) amplification, *Plasmodium* genus-specific primers were used. The amplification product of this nest 1 then served as DNA template for secondary PCR (nest-2) using primary specifically for all human *Plasmodium* species.[10,11,77–80] A single parasite can be detected in a DNA template purified directly from 10 μl of blood (0.000002% parasitemia). When substantially higher blood volumes are used, host genomic DNA may adversely affect amplification efficacy. It is estimated that PCR can detect malaria infection with parasitemia as low as 5–6 parasites/μl. Thus PCR-based methods have proven to be a powerful tool for malaria diagnosis.[11,77] High-quality DNA could be obtained from fresh whole blood as a common, reliable source of DNA, while Giemsa-stained or unstained thick blood smear and, particularly, blood conserved on filter papers could be used as a source of DNA in molecular and epidemiological studies. The following describes the standard procedure in isolating DNA. Oligonucleotide primers for nested PCR can be obtained commercially and all purified using high-performance liquid chromatography.[14,77,78]

Most of the primers designed for nested PCR are based on small subunit ribosomal RNA (ssrRNA) genes, which are frequently used for phylogenetic analysis and characterized from various *Plasmodium* species. Sequence comparisons have revealed the existence of a small part of DNA strands that are conserved and unique to each species of human *Plasmodium* which are not found in the other organisms.[11] The *Plasmodium* spp. has two distinct ssrRNA subunits expressed differently during the parasite's life cycle. These ssrRNA genes are different in their sequence and size, one expressed in the asexual stage and the other in the sexual stage. Although the oligonucleotide primers used for PCR analysis were initially designed to recognize only one of the two types of ssrRNA genes, amplification of other gene types is possible when large amounts of parasite DNA are present in the blood sample. This may explain the slightly higher specific band of molecular size often observed for samples initially containing many parasites.[11,60]

In 2004 Singh developed a nested PCR test to detect *P. knowlesi*, a different species of *Plasmodium* that can infect humans. This research was conducted in Kapit Sarawak, Malaysia. This study used two pairs of primers: for the first amplification using rPLU1 and rPLU5, and for nested use, specific primers Pmk8 and Pmkr9 designed based on the ssrRNA gene sequence of *P. knowlesi* isolates. The Pmk8–Pmkr9 primers target the ssrRNA-S gene expressed during the sexual stages.[10] This study showed that the isolates initially identified as *P. malariae* through microscopic examination were clarified as *P. knowlesi* by nested PCR examination using the primer.[10,79] However, these primers still produced false positives results. *P. knowlesi* target region of the 18S ribosomal RNA (rRNA) can cross-react with *P. vivax*.[58,60–62] When the *P. knowlesi* and *P. vivax* ssrRNA-S gene from GenBank were aligned, the sequences of the region targeted by the Pmkr9 primer were identical. In contrast, in the region corresponding to the Pmk8 primer, the *P. vivax* sequence

TABLE 2 List of primers used in various nucleic acid amplification tests

Method	Primer name	Sequence (5′ to 3′)	Specificity	Size (bp)
Nested PCR[11,82]	rPLU1	TCAAAGATTAAGCCATGCAAGTGA	*Plasmodium*	1600–1700 bp
	rPLU5	CCTGTTGTTGCCTTAAACTCC		
	rPLU5	CCTGTTGTTGCCTTAAACTCC	*Plasmodium*	1200 bp
	rPLU6	TTAAAATTGTTGCAGTTAAAACG		
	rPLU3	TTTTTATAAGGATAACTACGGAAAAGCTGT	*Plasmodium*	235 bp
	rPLU4	TACCCGTCATAGCCATGTTAGGCCAATACC		
	rVIV1	CGCTTCTAGCTTAATCCACATAACTGATAC	*P. vivax*	120–121 bp
	rVIV2	ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA		
	rFAL1	TTAAACTGGTTTGGGAAAACCAAATATATT	*P. falciparum*	205–206 bp
	rFAL2	ACACAATGAACTCAATCATGACTACCCGTC		
	rMAL1	ATAACATAGTTGTACGTTAAGAATAACCGC	*P. malariae*	144–145 bp
	rMAL2	AAAATTCCCATGCATAAAAAATTATACAAA		
	rOVA1	ATCTCTTTTGCTATTTTTTAGTATTGGAGA	*P. ovale*	approx. 800 bp
	rOVA2	GGAAAAGGACACATTAATTGTATCCTAGTG		
	rOVA1	ATCTCTTTTGCTATTTTTTAGTATTGGAGA	*P. ovale*	226 bp
	rPLU2	AGAATTTCACCTCTGACATCTG		
Nested PCR[10]	rPLU1	TCAAAGATTAAGCCATGCAAGTGA	*Plasmodium*	1600–1700 bp
	rPLU5	CCTGTTGTTGCCTTAAACTCC		
	Pmk8	GTTAGCGAGAGCCACAAAAAAGCGAA	*P. knowlesi*	153 bp
	Pmkr9	ACTCAAAGTAACAAAATCTTC CGTA		
Nested and semi-nested PCR[60,83,84]	PkF1140	GATTCATCTATTAAAAATTGCTTC	*P. knowlesi*	410 bp
	PkR1550	CTTTTCTCTCCGGAGATTAGAACTC		
	PkF1160	GATGCCTCCGCGTATCGAC	*P. knowlesi*	498 bp
	PkR1550	CTTTTCTCTCCGGAGATTAGAACTC		
Heminested PCR[58]	SICAf1	5′-GGTCCTCTTGGTAAAGGAGG-3′	*P. knowlesi*	228–249 bp
	SICAr1	5′-CCCTTTTTGACATTCGTCC-3′;		
	SICAf2	5′-CTTGGTAAAGGAGGACCACG-3′		
	SICAr1	5′-CCCTTTTTGACATTCGTCC-3′;		
Multiplex PCR[85]	UNR	GACGGTATCTGATCGTCTTTC	Universal	Position 1229 on the published sequence X03205 for human 18srRNA
	PLF	AGTGTGTATCAATCGAGTTTC	*Plasmodium*	787 bp
	FAR	AGTTCCCCTAGAATAGTTACA	*P. falciparum*	395 bp
	VIR	AGGACTTCCAAGCCGAAGC	*P. vivax*	499 bp
LAMP[86]	F3	TGTAATTGGAATGATAGGAATTTA	*P. falciparum 18S rRNA*	–
	B3	GAAAACCTTATTTTGAACAAAGC		
	FIP (F1c-F2)	AGCTGGAATTACCGCGGCTGGGTTCCTAGAGAAACAATTGG		
	BIP (B1-B2c)	TGTTGCAGTTAAAACGTTCGTAGCCCAAACCAGTTTAAATGAAAC		
	Loop F	GCACCAGACTTGCCCT		
	Loop B	TTGAATATTAAAGAA		

showed only difference at the 3′ end and two mismatches in the first 19 bases.[60]

Following that problem, Imwong designed three primers suitable for semi-nested PCR amplification of a fragment of the *P. knowlesi* ssrRNA gene expressed during the asexual stages (ssrRNA-A) that arranged into two new primer sets, namely PkF1060-PkR1550 and PkF1040-PkR1550. These primers were designed to target regions that differ in the corresponding related *P. vivax*

gene. The results of this study indicate a specific identification of *P. knowlesi* without any overlap with other species.[60,79] In semi-nested PCR, the process also uses two PCRs running sequentially.[80] The difference is that in the second PCR process, one of the primers used in the first process is used again and the other primer is within the target sequence. This method allows nucleic acid amplification without the need to transfer amplicon from the first PCR result to a second reaction tube with nested primers, thus reducing both the chance of contamination and the time for analysis.[65]

The nested primers will only prime any specific product generated in the primary PCR and help to maintain PCR specificity through the large number of cycles of the combined primary and secondary PCRs. Standard controls, both positive and negative, were included in every PCR reaction. Nested PCR results were analyzed using electrophoresis in gel agarose, visualized under UV light. Interpretation of results was done based on the band size.[80]

A modified nested PCR was developed and is called direct nested PCR. This method allows for PCR amplification without any prior DNA extraction and purification steps. The other advantage of direct PCR is overnight DNA extraction step could be omitted. The Phusion blood DNA polymerase used in the assay had an error rate 25 times lower than that of the typical *Thermus aquaticus* polymerase. A blood spot is punched out of each filter paper sample and washed with double-distilled water at 50°C for 3 min. The water is discarded, and the PCR mixture is brought at once to the rinsed punch. The standard modified Nested-PCR protocol is used to evaluate genus- and species-specific *Plasmodium* DNA within the highly conserved regions of the small-subunit rRNA gene. The rPLU1/rPLU5 is used for the nest one reactions and a short sequence of PLU3/rPLU4 is recommended for the genus-specific nest two amplifications. Whenever the genus-specific nest two PCR revealed positive results, the following species-specific nest two primers were used to determine the *Plasmodium* species: rFAL1/ rFAL2, rVIV1/ rVIV2, rMAL1/rMAL2, rOVA1/rPLU2, and Pmk8/Pmkr9 (Table 2). This new technique provides rapid and highly accurate diagnoses to treat and control malaria adequately.[81]

A novel highly species-specific *P. knowlesi* PCR assay based on a conserved region of 5′ and 3′ end of an exon of the 50–70 members of the gene family encoding *P. knowlesi*-specific schizont-infected cell agglutination variant antigens (SICAvar) has also been developed. This primer provides good sensitivity for *P. knowlesi* identification and is effective in detecting submicroscopic parasites as well as moderate numbers of *P. knowlesi* coinfections with *P. vivax*.[58]

4.4 | DNA isothermal amplification mediated by a loop (LAMP)

Loop-mediated isothermal amplification is a simple molecular diagnostic method based on isothermal amplification, which does not require special equipment in laboratories. LAMP provides high efficiency, with DNA being amplified 10^9–10^{10} times in 15–60 min and allowing monitoring amplification by SYBR Green I dye mediated naked eye visualization or automated detection of results by real-time monitoring using an inexpensive turbid meter according to the situation.[87–89] LAMP method for malaria diagnosis provided on portable microfluidics chip platform by optimizing DNA extraction using the boil and spin method.[90]

Unlike PCR, which requires alternating temperature conditions, LAMP assay has the great advantage of nucleic acids (RNA and DNA) amplification at a constant temperature (isothermal), typically around 62–65°C without initial denaturation. Hence, it requires less time and thus, thermocycler is not needed. To initiate synthesis, LAMP uses strand-displacing DNA polymerase, and two of the four to six primers used shape loop structures to make subsequent amplification rounds easier. Additional advantages of LAMP are its tolerance to inhibitory substances present in blood samples (such as hemoglobin and immunoglobulin) and the possibility of using small amounts of blood on filter papers.[87,88] Unfortunately, the LAMP assay currently lacks sufficient accuracy[91,92] and limited implementation in the field as it still needs cold storage for the reagent, burdensome blood sample preparation and limited multiplexing ability due to the complex sample preparation steps required.[88]

According to the current findings, LAMP-based assays are suitable for detecting low-level malaria parasite infections in the field and maybe useful tools in malaria control and elimination programs. More extensive sample studies on LAMP evaluation in passive malaria surveillance are required in the future.[93] The LAMP primers are described in Table 2.

4.5 | Recombinase polymerase amplification

Recombinase polymerase amplification is accustomed to amplifying single-stranded DNA, double-stranded DNA, methylated DNA,[94] and miRNA.[95] RPA amplification technique has been adopted for bacteria, viruses, protozoa, fungi, animals, and plants, with different samples such as cultured microorganisms, body fluids, surgical biopsy specimens, organ tissues, animal, and plant products. The RPA reaction starts when a recombinase protein (enzymes) binds to primers in the presence of ATP and high molecular polyethylene glycol, forming a recombinase–primer complex. The complex then explores the double-stranded DNA for homologous sequence and promotes strand invasion by primer at the cognate site. After that, the recombinase disassembles, and a strand displacing DNA polymerase binds to the 3′ end of the primer to elongate it in the presence of dNTPs.

Primer for RPA reaction was designed with 30–35 bases in length and standard PCR primers can be used with efficient amplification.[96,97] Primer up to 45 nucleotides could form secondary structures and primer artifacts. It is recommended to use cytidines at the 3′ and avoids guanines at the 5′ ends. A GC content for RPA reaction between 30% and 70%, and target amplicon between 100 and 200 bp are recommended. The designed primer for RPA is the same as PCR and there is no software available to design primers special for RPA, but the most important is avoiding the formation

TABLE 3 List of primers used in RPA methods

Specificity	Primer name	Sequence (5′ to 3′)
Plasmodium genus[99]	Forward	5′-CACGAACTAAAAACGGCCATGCATCACCATCC-3′
	Reverse	5′-biotin-CCTTATGAGAAATCAAAGTCTTTGGGTTCTGGGG-3′
	Probe	5′-FAM-ATCAAGAAAGAGCTATTAATCTGTCAATCCTAC-THF-CTTGTCTTAAACTAGTG-3′
Plasmodium genus[100]	Forward	5′-CATGGCTATGACGGGTAACGGGGAATTAGA-3′
	Reverse	5′ digoxigenin-AATTGGGTAATTTACGCGCCTGCTGCCTTC-3′
	Probe	5′-FAM-CATGGCTATGACGGGTAACGGGGAATTAGA-THF-TTCGATTCCGGAGAG-3′
P. knowlesi[100]	Forward	5′-CCGTTCTCATGATTTCCATGGTCCAGGGTT-3′
	Reverse	5′-biotin-CCTGAACACCTCATGTCGTGGTAGAAATAG-3′
	Probe	5′-FAM-CCGTTCTCATGATTTCCATGGTCCAGGGTT-THF-AGTTTTTTCGGTCCC-3′
P. falciparum[101]	Forward	5′-GTGTTCATAACAGACGGGTAGTCATGATTGAGT-3′
	Reverse	5′-digoxigenin-ACATCTGAATACGAATGCCCCCAAAGATACTCC-3′
	Probe	5′-FAM-GTGTTTGAATACTACAGCATGGAATAACAA-THF-TATGAATAAGCTAATTATT-3′

of primer-dimers.[98] RPA primers were designed for the *Plasmodium* genus, *P. falciparum*, and *P. knowlesi* showed in Table 3.

The malaria detection sample is usually blood and needs DNA extraction before use to RPA reaction.[100,101] High genomic DNA concentrations inhibit RPA reaction in blood samples (20–100 ng/ml). This problem can solve with enrichment of target DNA prior to amplification[102] and heating with AVL buffer and Trizol, followed by centrifugation.[103]

The optimum temperature for RPA reaction is at 37–42°C.[101,104,105] Incubator, heating block, chemical heater, and body heat can be used to control the temperature reaction.[104–106] The time required to amplify the DNA target is 3–20 min depends on the number of starting DNA copies[100,107] and no longer than 25 min incubation because the recombinase enzymes consume all the available ATP within 25 min.[108] The crowding agent is critical in RPA to prevents the spontaneous recombinase primer disassembly. However, it will be increasing time amplification on RPA reaction because of the viscosity effect. A mixing 5 min after initiation of the RPA reaction for 5 sec[109] and reducing the volume of the reaction mixture to 5 μl[110] can minimize this effect.

Multiplexing in RPA reaction is possible but depends highly on target sequences, amplicon size, and primer design.[111] The primer, probe ratios, and concentrations also need to be optimized for each multiplexing assay. Optimization is essential because primers can compete for the recombinase proteins and may be one of the reactions being suppressed.[112] In malaria detection multiplexing, there are two type primers from the *Plasmodium* genus and *P. knowlesi*.[100]

Most of the RPA detection methods for malaria usually using a visual read-out format relies on lateral flow assay.[100,101,109] In this format, three different oligonucleotides are required (two primers and one probe) and the Twist-Amp®nfo kit is used for assay designs compatible with strip detection.[113] Many choices for the antigenic label are used in this format, such as carboxyfluorescein group (FAM), Alexa fluor 488, and digoxigenin.[114] The two labels on one DNA amplicon will interact with antibodies or antibody/streptavidin (sandwich format) and can observe with visual detection. SYBR Green I also was employed for end-point detection in malaria diagnosis.[109] SYBR Green I will bind to double-stranded DNA and results in a DNA-dye complex. This complex emits green light and can be observed by the naked eye.[115] RPA detection also can be combined with several detection techniques such as bridge flocculation assay,[116–118] gel agarose, colorimetric fluorescence,[101] quantum dots,[119,120] electrochemical,[121–124] and surface-enhanced Raman scattering detection.[125,126]

4.6 | Molecular-based point of care test

Point of care tests are widely used in remote areas and have insufficient laboratory infrastructure. The POCT should be affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and delivered (ASSURED). In addition, POCT should be concordant with an established laboratory method, devices together with associated reagents, and consumables are safe to use.[127]

Nowadays, molecular approaches based on nucleic acid have gained interest as the promising tools for advanced POCT. NAATs have the ability to detect malaria significantly in low-level infections, identify the species in the presence of mixed infection, allows for quantification, and able to detect polymorphisms associated with drug resistance gained interest as a candidate for POCT. Integrated NAATs POCT should have efficient nucleic acid extraction technologies for diverse and complex sample types and high sensitivity to amplify and detect the nucleic acid in the sample.[128] There are many strategies to decrease the cost, provide a user-friendly method, and perform a detection tool with high sensitivity and specificity and low detection limit.[99,129]

Although many studies have been described for detection of *Plasmodium* spp. based on nucleic acid testing as the point of care for diagnostic malaria, no commercial product is yet available due to several technical barriers such as thermocycler availability, optimization of each reaction with proper materials, and handling of NAATs reagents. The most promising techniques to be further developed as

molecular-based POCT candidates for malaria are isothermal amplification techniques such as LAMP and RPA since they do not require a complex and sensitive instrument, using less energy and time to achieve a sensitive target detection. Another method to consider is microfluidic (either conventional or paper-based assays), which can overcome most of the obstacles of sample preparation and adequate amplification and detection of genomic targets. Collaboration of microfluidic with any NAATs to make feasible a robust, sensitive, and specific malaria POC diagnostic assay.[130–132]

4.7 | Other advanced molecular techniques in malaria diagnosis

Several advance techniques such as droplet digital PCR (ddPCR) and next generation sequencing (NGS) were used in the various field of malaria. ddPCR is a digital PCR method based on water–oil emulsion technology providing absolute and direct quantification of DNA target without the need of standard curve.[133,134] This techniques was used to detect almost all *Plasmodium* species with better sensitivity than qPCR in laboratory setting.[133–135] In contrast, another sophisticated method like NGS is vastly developed for better understanding of malaria transmission pattern and parasites movement[136] and also for identifying multidrug resistance genes in *Plasmodium* as the major therapeutic obstacles nowadays.[137,138]

5 | CONCLUSION

Microscopy will remain the method of choice for malaria diagnosis due to its high reliability and low cost. The RDTs are helpful for the rapid results due to offering simplicity, low cost, and potentially used in a remote area but are less sensitive and specific. ELISA tests are helpful for epidemiological surveys but not suitable for the diagnosis of acute malaria. Flow cytometry can be used for species identification and quantifying the parasite density with low parasitemia but requires well-trained technicians and expensive equipment. PCR-based tests should be the choice of testing wherever possible to provide more relevant information (parasite load, species, and resistance) but still need well-trained professionals and electric power supplies. PCR (real-time PCR, multiplex PCR, and nested PCR) can give accurate determination, differentiation, and excellence in sensitivity and specificity to detect low levels of infections in the blood samples of malaria patients. However, these techniques need a reliable power supply, are expensive, time-consuming, and difficult to use in low-resource settings or at the point-of-need. On the other hand, isothermal DNA amplification methods such as LAMP and RPA are promising methods for diagnosing malaria in the field. LAMP and RPA assay is fast, simple, and only need affordable equipment. Combining LAMP and RPA with biosensing technology is very potential for point-of-care diagnostic due to its simplicity, inexpensive, sensitivity, and specificity for malaria diagnosis in a remote area.

CONFLICT OF INTEREST

Authors declare no conflict of interest in constructing this manuscript.

DATA AVAILABILITY STATEMENT

There is no other data resulted from the process of constructing this review paper except those cited in the references.

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