Recent Perspectives and Upcoming Directions in Molecular Diagnosis of Malaria: A Systematic Review

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Abstract

Background: Malaria remains a global leading cause of morbidity and mortality. The absence of effective vaccines is still the vital hindrance to the management and elimination of malaria. From that standpoint, accurate laboratory diagnosis could be the right hand for disease management. **Objective:** This review intended to assess the recent perspectives and upcoming directions in molecular diagnosis of malaria. **Methods**: This review was conducted by using internet searching tools where 35 published papers were retrieved from the credible online publishers and among them, 27 papers that satisfy the inclusion criteria were profoundly reviewed.

Results: Among the 27 articles, 22(81.48 %) papers focus on contribution of PCR based-method in malaria diagnosis, 4(14.8%) report on comparison between polymerase chain reaction(PCR) and other molecular techniques, 7(30%) emphasize on advantages and disadvantages of PCR, 4(14.8%) represent relationship among PCR and LAMP, 3(11%) discuss the most promising molecular diagnostic tools, 5(18%) focus on comparative designs of different PCR methods, whereas 1(3.7%) emphasizes on parasite density and 2(7%) on pigment containing monocyte.

Conclusion: This review conclude that microscopy remains the gold standard method for malaria diagnosis and speciation in limited resource settings but also molecular based-methods provide significant alternatives with superior sensitivity and specificity.

Keywords*: Malaria, Molecular Diagnosis, LAMP, PCR, Microscopy,*

Introduction

Malaria is one of the major public health problems hampering developmental efforts in most developing countries [(1)]. Malaria is spread by contaminated mosquito with parasitic protozoans (a group of single-celled microorganisms) belonging to the genus *Plasmodium* [(2)]. Plasmodium genus have five species of parasites that affect vertebrates (humans), these species are *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium knowlesi and Plasmodium ovale* [(1)]. The disease is most frequently transmitted by an infected female Anopheles mosquito. After biting, the mosquito introduces the parasites from its saliva into a person's blood [(2)].

Malaria symptoms typically develop within 10 days to four weeks following the infection. Common symptoms of malaria include: shaking chills that can range from moderate to severe, high fever, profuse sweating, headache, nausea, vomiting, diarrhea, anemia, muscles pain, convulsions, coma and bloody stools [(2)].

In terms of diagnosis, for more than 100 years, microscopy has been the gold standard diagnostic tool for malaria [(3)]. The World Health Organization (WHO) suggests that malaria can be diagnosed by standard

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microscopy or immunochromatographic lateral flow assays (known as rapid diagnostic tests, RDTs) before treatment [4] .The other diagnostic tests for malaria are, serology, fluorescence microscopy and nucleic acid-based amplification techniques like PCR and isothermal amplification where the mostly known is loop mediated isothermal amplification (LAMP) [5].Rapid diagnostic test and quantitative real-time PCR (qPCR), are techniques that are applied to malaria studies and were used to detect Plasmodium species in 10^9 whole-blood samples from patients after their return from malaria endemic areas [6]. Methods of real-time PCR are particularly designed for large scale screening and they can be used in malaria control and elimination programs [7]. Even if WHO recommenced the use of PCR to detect the asymptomatic patients and low parasitemia, PCR requires technical expertise, expensive reagents and infrastructures which make it inaccessible in low- and middle-income countries [5].

Blood smear microscopic examination remains the current gold standard method, because of its availability, low cost and ability for detection of malaria and speciation. PCR assay present an alternative to microscopy which has shown a superior sensitivity and specificity [8] . Microscopy is most frequently used, but at low parasitemia (<20 parasites/µl), the technique becomes less sensitive and time consuming [9] . Rapid diagnostic tests based on Plasmodium antigen detection do frequently not allow discrimination of species as microscopy does, but also become insensitive at <100 parasites/microliter. Judgment concerning malaria treatment depends on the right identification of species causing the disease. Therefore, there is a need for developing new tools of diagnosing malaria that are sensitive, cost effective and capable of performing large scale diagnosis. Here, we review the most promising molecular diagnostics developed so far and give some viewpoints for the near future [9].

This review is intended to describe the recent and upcoming directions in molecular diagnosis of malaria for the detection of law parasitemia in asymptomatic patients from the perspective of high-throughput and practicability in constrained resource environments for the sake of malaria elimination. This review also emphasizes on the advantages and disadvantages of most promising molecular diagnostics and present recommendations on possible future directions in molecular diagnosis of malaria

Material and Methods

This review was carried out by using important internet searching tool: PubMed. It is actually consisted by the analysis of specific segment of published papers to a given research topic related to malaria diagnostic tools. In other words, it describes, summarizes, evaluates and report on the retrieved papers. This study context provides an update on what is going on in malaria molecular diagnostics and what has been done so far whether it is beneficial to Malaria patients or not. The published articles were carefully searched through PubMed search board in order to obtain different papers which could be perfectly related to the review topic for reviewing. PubMed have been chosen to be the best searching tool due papers accessibility (regularly uploaded and updated research papers), reliability and specificity to medical domain and contains a collection of most of medicine and biomedical sciences that fulfill medical evidence-based guidelines

PubMed covers most papers in medicine domain and allows a quick and advanced search. A computer-based papers' search was done on two different dates (12th and 28thFebruary, 2020) to avoid any search bias that may result; as day after day, in the same database more newly published papers get uploaded. This data was retried from searching engine based on different research keywords related to the study title. The keywords used were malaria, malaria diagnosis and molecular diagnosis of malaria. The targeted reviewed papers were those related to malaria, malaria diagnosis and molecular diagnosis of malaria. This research reviewed most of the published papers on current and future direction in molecular diagnosis of malaria. It considered and extracted different published papers examining factors associated with either malaria diagnosis or molecular

diagnosis of malaria. All the current and updated papers that are straightly relevant to the topic were considered and reviewed in this work

During this study, different inclusion and exclusion criteria were set. For a paper to be included in in this study had to be published in English, giving information on development of molecular diagnosis of malaria, and being published in 2000 to 2020. The papers that were published before 2000, published in a different language other than English, published as a book, papers having a title which is not related to our topic or having insufficient information were rejected

After considering the inclusion and exclusion criteria and other described research considerations, a detailed review strategy can be demonstrated on the following flowchart which shows the reviewed 35 Published papers which yielded 27 papers that were used in this current evaluation.

Reliability and Validity

The results of the 27 reviewed studies are reliable because all these papers considered for our review were retrieved from PubMed which hosts reliable data. PubMed database uploads health related papers with standard quality, from highly cited journals. For instance, our retrieved papers were published in different good journals as follows: Malaria Journal (impact factor: 3.109); Journal of infectious disease (impact factor: 5.997); Journal of Clinical Microbiology (impact factor : 3.993); A Peer- reviewed Open Access Journal (impact factor: 3.234); Korean Journal of Parasitology (impact factor: 1.151); American Journal of Tropical Medicine and Hygiene (impact factor: 2.699); Tropical Medicine International Health (impact factor : 2.329) ;Journal of tropical medicine (impact factor: 0.926); Clinical Microbiology and infection (impact factor: 5.768); Southeast Asian Journal of Tropical Medicine public health (impact factor: 0.719); Pathogen Global Health (impact factor: 1.656). The final reviewed paper's validity is extremely ensured as well, as their content falls within the broad aim of our review.

Results

Research results were obtained by using the identification for analysis where common themes used are PCR based- assay, comparison between PCR and other molecular techniques in malaria diagnosis, advantages and disadvantages of PCR based- method, relationship among PCR based assays, most promising molecular diagnosis of malaria, comparative designs of PCR, pigment containing monocyte based- assay which are presented within the selected twenty-seven articles.

Theme 1: PCR based- assay

PCR based- assay in malaria diagnosis was reported within by 22 of the 27 selected studies. PCR will be used in the future to screen samples from clinically suspected foci to increase the proportion of detected malaria cases. PCR products can also be analyzed using gel-electrophoresis. For instance, the samples that were found to be negative by db-PCR-NALFIA but positive for *Plasmodium* in their initial diagnosis were determined to be negative by gel analysis as well [(10)].

Nested-PCR belongs to PCR based-method and was performed in samples from 30 non-human primates nine samples (30%) of them were found to be positive for *Plasmodium* [(11)]. Different PCR forms have been reported including: two single-PCR reactions which are nested 1PCR and nested 2PCR designed to detect Plasmodium falciparum infections, one single PCR to detect *Plasmodium vivax* infections, and one multiplex one-step PCR reaction to detect both parasite species[(12)].

Nested PCR assay was also successful in detecting mixed infections that are not detected by microscopy [(13)] . The study concluded that nested PCR and real-time PCR may be highly suitable for asymptomatic malaria detection in large numbers of clinical samples from areas of endemicity[(14)]. The mixed infections detected by nested-PCR assay were 6.5, 22 and 23.5 percent in samples collected from Afghanistan, Iran and Pakistan respectively [(15)]. Nested PCR, multiplex and real-time PCR were investigated for their sensitivity and specificity for the overall detection of Plasmodium

species. They were reported to be 96.6% and was 89.4%, respectively while microscopy is still considered as the gold standard for malaria detection and identification[8]. PCR based diagnostic tools are capable of detecting very low parasitemia due to high sensitivity. *Plasmodium falciparum, P. ovale, and P. vivax* which are the species of malaria parasite were detected by a TaqMan-based real-time PCR qualitative assay[16] ; and Polymerase Chain Reaction (PCR) is a popular nucleic acid-based tool that is used in the diagnosis of various infectious diseases[17].

Molecular methods, including nested polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) and histidine-rich protein 2 (HRP-2) based rapid diagnostic tests (HRP-2-RDTs) were used for the malaria diagnosis [18]. LAMP presents a high diagnostic accuracy for parasite detection among both fever patients and asymptomatic individuals[19]. The same technique is claimed to be a molecular diagnostic test for malaria which is simple and inexpensive to detect the conserved 18S ribosome RNA gene of *P. falciparum* [20] . Malaria LAMP displayed diagnostic accuracy similar to that of nested PCR, by using pan–*Plasmodium* genus primers, LAMP did not differ in performance from nested PCR (P = .3447) [21] . The method which was found to be the most efficient for the detection of mixed infections is the PCR-based method because of its high sensitivity and specificity [22]. The three molecular assays (real-time multiplex polymerase chain reaction , merozoite surface antigen gene [MSP]-multiplex PCR and the PlasmoNex Multiplex PCR Kit) were reported to be involved in detection of plasmodium species, for the real-time multiplex PCR was more sensitive (81%) in detecting *P. vivax, P. falciparum and P. knowlesi* infections compared with the PlasmoNex Multiplex PCR Kit (62%) and MSP-multiplex PCR (50%) and they displayed 100% specificity for detecting malaria samples[23].

Identification of specific malaria parasite infection through PCR often is followed by DNA sequencing of the 18S rRNA gene for confirmatory purpose[24]. For instance, quantitative real-time PCR (qPCR) is commonly used as a confirmatory method for malaria diagnosis; it was performed with high efficiencies of more than 94%. The multiplex qPCR assay detects simultaneously Plasmodium spp., *P. falciparum*, *P. vivax* and human RNaseP gene[25].

The PET-PCR is a new molecular diagnostic tool with similar performance characteristics as commonly used PCR methods that is less expensive and easy to use [26]. The RFLP-dHPLC method detected many more mixed species infections than did microscopy. Their use revealed a high prevalence of sub-microscopic infections. The overall prevalence detected by the RFLP-dHPLC method and microscopy were (68.4% and 30.7%) respectively [27].

Theme 2: Comparison between PCR and other molecular techniques in malaria diagnosis

Four out of 27 reviewed studies (14.8%) report the comparison between PCR and other molecular techniques in malaria diagnosis. The sensitivities of the two nested PCR and real-time PCR techniques were higher than that of microscopy examination (sensitivity, 100% versus 26.4%; kappa values, 0.2 to 0.5). PCR-based molecular methods for malaria parasite detection are relatively simple and provide improved sensitivity and various advantages compared to microscopy and RDTs [14].

Malaria LAMP also demonstrated diagnostic sensitivity significantly superior to that of expert microscopy [21] . Comparison of nested PCR to HRP-2-RDTs show that the poor sensitivity of HRP-2-RDTs indicates that low parasitemia may not be detected after treatment, whereas the low specificity of HRP-2-RDTs indicates that it cannot be applied for treatment follow-up. However, HRP-2-RDTs have similar sensitivity as microscopy but less specific. The db-PCR-NALFIA is much more sensitive than RDT, especially for the detection of *P. vivax*[17].

Theme 3: Advantages and disadvantages of PCR based- method

The advantages and disadvantages of PCR based-method in diagnosis of malaria were reported within 7 out of 27 reviewed papers (30%). PCR-based molecular methods for malaria parasite detection are relatively simple and they are highly specific and capable of reporting highly sensitive test values [13]. PCR is known to be highly sensitive and specific as an advantage but it presents some barriers including its high cost, and the amount of infrastructure required in terms of equipment and a sophisticated laboratory setup with stable power and refrigerators for reagent storage [16] .PCR based- methods have demonstrated high sensitivity and specificity in detection of plasmodium infection and have the ability to quantify parasitemia when used in a quantitative real-time PCR format. The ease and speed of PCR make it an important tool in malaria elimination programs [27]. LAMP is simpler and faster as compared to nested PCR with the advantage of detecting low parasitemia becoming a potential point-of-care test for treatment follow-up. The low sensitivity and the high false positive rate of HRP-2-RDTs is the major limitation of its application for monitoring of the therapeutic response; and PCR tools are too sophisticated and expensive as a disadvantage to apply them in most malaria endemic countries [17]. Real- time PCR does not require post- PCR processing; this reduces sample handling and minimizes the risk of contamination [15]. PCR can be used for precise parasite quantification through qPCR methods[25]. Nested PCR, multiplex and real-time PCR are capable of detecting very low parasitaemia [7].

Theme 4: Relationship among PCR based assays

This theme was presented by 4 out of 27 selected studies (14.8%), giving an explanation on relationship among PCR based assays. Nested PCR-2 and real-time PCR displayed similar results with higher sensitivities of 100% reported [13]. LAMP has similar sensitivity and specificity to nested PCR. With high PPV and NPV [17]. Malaria LAMP had a diagnostic accuracy similar to that of nested PCR among collected positive blood samples, one sample characterized as *P. vivax* monospecies infection by nested PCR was found to be positive for *P. falciparum* [20]. Specificity of all three assays (real-time multiplex polymerase chain reaction [PCR], merozoite surface antigen gene [MSP]-multiplex PCR, and the PlasmoNex Multiplex PCR Kit) were tested using gDNAs of all five malaria. And in species of human addition the PlasmoNex Multiplex PCR Kit and real-time multiplex PCR showed similar sensitivity for detecting *P. vivax* [22].

Theme 5: Most promising molecular diagnosis of malaria

The findings obtained from 3 out of 27 reviewed papers (11%), reported on the most promising molecular diagnosis techniques for malaria. The malaria LAMP is the most promising molecular diagnosis for malaria because it has advantages over other molecular tests in speed, sensitivity, and minimal need for specialist training [20]. The same advantages of LAMP have been reported as of being cheaper, simpler and faster. The 100% sensitivity and specificity of real-time PCR methodology supports its status as the best PCR methodology to detect *P. falciparum* and *P. vivax* [8] .

Theme 6: Comparative designs of PCR

Comparative designs of PCR were encountered within 5 out of 27 selected studies (18%). Real-time PCR technique is s based on primer and probe sequences for the gene encoding the 18S Plasmodium rRNA genes; whereas for the nested PCR-2, the species-specific nucleotide sequences of the 18S rRNA genes of *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* are targeted and for the nested PCR-1 strategy is based on use of primers [13] . All PCR reactions can be performed in a 20 µL volume containing 250µM each oligonucleotide primer, 10µL of master mix (Promega) (0.3 units of Taq Polymerase, 200µM each deoxyribonucleotide triphosphates and 1.5 mM MgCl2) and 2µl DNA [11] . Nested-PCR assay uses 18S small sub-unit ribosomal RNA (ssrRNA) gene [16]. Nested polymerase chain reaction (PCR) primers were designed from the 3R region of the 28SrRNA gene [13]. A multiplex qPCR assay was designed to simultaneously detect *Plasmodium* species such as *P. falciparum, P. vivax* and human RNaseP gene

as an endogenous control [25].

Theme 7: Parasite density per microliter of blood

Only one reviewed paper out of the twenty-seven, is reporting on parasite density per microliter technique. Parasite density per microliter of blood in a patient is determined using parasite count adjusted by average WBC count (8000/microliter) observed in microscopic fields of the thick film [28].

Theme 8: Pigment containing monocyte based-assay

Pigment containing monocyte based-assay was reported within two out of twenty-seven selected studies (7%). Those studies include[29–31]. At a discrimination level of one or more atypical pigment-containing monocytes (PCM), negative and positive agreement was found to be 95.6% and 91.6% respectively and many analyses showed that the only significant risk factor for the presence of PCM (odds ratio>200) was malaria infection[31] . Malaria PCM as a distinct cluster in scatter diagrams that is well separated from normal leukocytes. From microscopic inspection of sorted cells and PCM has a different average relative frequency according to the patients: for nonimmune patients is 1.5 x 10(-4) (median), for semi-immune patients 8.8 x 10(-4), and for malaria-negative persons 4.4 x10(-6) [29].

Discussion

All the 27 reviewed research and review articles were selected based on molecular diagnosis of malaria. Some of those studies focus on advantages and disadvantages of PCR based essay in diagnosis of malaria. Currently, diagnosis of malaria by use of microscopy or RDT are not sufficient in specificity or sensitivity to detect low-parasite-density infections. Microscopy and RDTs become relatively less sensitive at parasite densities below 100 parasites/μl [11,29–32].

RDTs are inexpensive, simple to perform, and provide results in 15-20 minutes. Despite high sensitivity and specificity for *Plasmodium falciparum* infections, RDTs have several limitations that may reduce their utility in low-transmission settings: they do not reliably detect low-density parasitemia (≤200 parasites/μL), many are less sensitive for *Plasmodium vivax* infections, and their ability to detect *Plasmodium ovale* and *Plasmodium malariae* is unknown [33].Microscopic examination of blood smears remains the current gold standard for malaria detection and speciation. However, PCR assays present an alternative option to microscopy which has been shown to have superior sensitivity and specificity [8].

PCR based molecular method when compare to thick blood smear this test displayed a sensitivity ranging from 65% to 81% and specificity was close to 100%; and this method of assay detects all five Plasmodium species[22]. The highly sensitive and specificity of PCR makes it an advantageous technique, but the same technique presents some barriers including its high cost, and the amount of infrastructure required in terms of equipment and a sophisticated laboratory setup with stable power and refrigerators for reagent storage[17]. In a different study, the prevalence of asymptomatic parasitemia was reported at 17% by microscopy and 47% by PCR [34].The easiness and speed of PCR makes it an important tool in malaria elimination programs and should be improved to detect asymptomatic infections[34]. The diagnosis of malaria parasites by one of the PCR based assays, nested PCR, in the present assessment is at medium level with an accuracy of 84.21% (48/57). In comparison of microscopy to nested PCR, microscopy showed the sensitivity and specificity of 85.39% and 100% respectively, whereas the sensitivity and specificity of the nested PCR assay was found to be 99.08% and 100% respectively. Nested PCR assay was also successful in detecting mixed infections that are not detected by microscopy[13].

The PCR-based method was used as the reference standard because of its high sensitivity and specificity over microscopy, particularly in cases with low-level parasitemia. When the three PCR-based methods (nested PCR-1, nested PCR-2 and real- time PCR) were examined for detecting malaria infection in residents of regions of Myanmar where malaria is endemic, two

nested PCR and real-time PCR assays showed that asymptomatic infection was detected in about 1.0% to 9.4% of residents from the surveyed areas; and they showed similar sensitivity in genus-specific tests. However, it was observed that nested PCR-1 showed a lower sensitivity in detecting *Plasmodium spp*, especially *P. falciparum* and *P. malariae*, compared to nested PCR-2 and real-time PCR. The limited detection by nested PCR-1 resulted in 14 samples being positive in a genus-specific assay, while reported negative in a species-specific assay. The results suggest that nested PCR-2 and real-time PCR may be highly suitable for asymptomatic malaria detection in large numbers of clinical samples from areas of endemicity[6,14].

All three PCR methodologies (nested, multiplex and real-time PCR) investigated, were sensitive, specific and capable of detecting very low parasitemia. Although economies of scale can be applied to PCR methods to reduce the time and cost involved in processing each sample, the results from the three molecular speciation techniques (nested PCR, multiplex PCR, and real-time PCR) were used to develop a molecular consensus (two or more identical PCR results) as an alternative gold standard. According to the molecular consensus, 9.6% (13/136) of microscopic diagnoses yielded false negative results. Multiplex PCR failed to detect *P. vivax* in three mixed isolates, and the nested PCR gave a false positive *P. falciparum* result in one case. Although the real-time PCR melting curve analysis was the most expensive method, it was 100% sensitive and specific and least time consuming of the three molecular techniques investigated [8].

The sensitivity and specificity results for the four speciation techniques differed depending upon whether microscopy or molecular consensus was used as the gold standard. Using microscopy as gold standard, the sensitivity of all three molecular techniques for the overall detection of *Plasmodium spp*. was 96.6% (86/86+3) and the specificity was 89.4% (42/42+5). However,

When Molecular Consensus Was Used As The Gold standard the sensitivity and specificity of microscopy was 94.5% (86/86+5) and 93.3% (42/42+3) respectively[8]. By evaluating A TaqMan-based real-time PCR, qualitative assay was performed on 122 whole blood samples from patients who presented with malaria-like symptoms and fever, real-time PCR assay showed a detection limit with analytical sensitivity of 0.7, 4, and 1.5 parasites/µl for *P. falciparum, P. vivax,* and *P. ovale* respectively. Real-time PCR can yield results within 2 hours and does not require post-PCR processing, which reduces sample handling and minimizes the risks of contamination[16].

The db-PCR-NALFIA is also used for the detection of Plasmodium species directly from blood samples and is much more sensitive than RDT, especially for the detection of *P. vivax* as well as for P. *falciparum.* There were differences in results between the db-PCR-NALFIA and microscopy and RDT. Although in both field settings there were false-negative db-PCR-NALFIA samples that were determined to be positive by microscopy and/or RDT, there was a greater number of false-positive db-PCR-NALFIA samples that were negative with the other methods. The db-PCR-NALFIA is a relatively easy-to-use method that is robust, sensitive, and specific and could have great potential in locations where malaria is endemic; especially in areas where there is low transmission of malaria, and thus a very sensitive technology is warranted [10].

Microscopy remains the gold standard method for malaria diagnosis because of its simplicity, affordability and the ability to quantify the parasite density. As molecular techniques become much more widely used and acceptable as alternate or as confirmatory assays to microscopy, they must meet and exceed qualities and characteristics that have made microscopy popular. Absolute quantitative multiplex qPCR assay simultaneously detects three *Plasmodium* targets (*P. falciparum* and *P. vivax* parasites) and the human RNaseP as an endogenous control. If qPCR assay is going to replace microscopy as the gold standard diagnostic method, the absolute quantification is

reported as parasites/µl, the same units as those used in microscopy. Currently, qPCR assays use relative standard quantification methods to quantify parasite density in a sample where cultures or clinical samples with known parasite density are used [25].

PCR-based molecular methods are good for both their sensitivity and specificity values, but too sophisticated and expensive to be applied in most malaria-endemic countries[28]. LAMP has similar sensitivity and specificity to nested PCR, with high PPV and NPV. LAMP is a cheap, simpler and faster method as compared to nested PCR; with its advantage of detecting low parasitemia, it is becoming a potential point-of-care test for treatment follow-up[18]. In a 128 febrile children study, a complete set of four malaria tests was carried out. Positive results for HRP-2-RDTs, microscopy, nested PCR, and LAMP, were 68(53%), 47(37%), 64(50%), and 65(51%) respectively. When nested PCR was used as a reference standard, only LAMP was comparable and both HRP-2-RDTs and microscopy had moderate sensitivity. HRP-2-RDTs had poor positive predictive value (PPV) and a moderate negative predictive value (NPV) for the treatment follow-up. HRP-2-RDTs have similar sensitivity as microscopy but less specificity. However, as compared to nested PCR, the poor sensitivity of HRP-2 and RDTs indicates that low parasitemia may not be detected after treatment; and the low specificity of HRP-2-RDTs indicates also that it cannot be applied for treatment follow-up[18].

Malaria LAMP had a diagnostic accuracy similar to that of nested PCR, with a greatly reduced time to result. Malaria LAMP demonstrated diagnostic sensitivity significantly superior to that of expert microscopy. In the analysis, using pan–*Plasmodium* genus primers, LAMP did not differ in performance from nested PCR (P = 0.3447). LAMP with *P. falciparum* primers was found to have significantly different diagnostic accuracy than PCR (P = 0.004), with a positive predictive value of 83.5% and a negative predictive value of 99.8%. The current format of pan-Plasmodium genus and *P. falciparum*–specific LAMP is able to identify all *P. falciparum*–infected individuals. The costs of LAMP reagents are close to those used for nested PCR, but comparing both techniques in terms of equipment and labor costs, reveals that LAMP would be more affordable for laboratories in malaria-endemic countries. The malaria LAMP test, evaluated for the primary diagnosis of malaria has advantages over other molecular tests in speed, and minimal need for specialist training; and it is a suitable test for diagnosing imported cases of malaria in minimally equipped clinical laboratories[21].

Conclusion

In this review on malaria diagnosis, microscopy remains the current gold standard method due to its availability, low cost and its ability to detect and identify various *Plasmodium* species. PCR assay is a molecular diagnostic method presents an alternative tool to microscopy and has shown a superior sensitivity and specificity. Molecular tests are valuable tools for the confirmation of *Plasmodium* species and in detecting mixed infections in malaria endemic regions. However, most of molecular techniques used in malaria diagnosis present some bottlenecks including the high cost of labor and less access to reagents, compared to the examination of blood smears. Among PCR based- assay methods, real-time PCR is ranked the best due to its high sensitivity and specificity (100%) to detect both *P. falciparum* and *P. vivax.* As a major outcome of our review, the LAMP technique is claimed to be a simple and inexpensive malaria- molecular diagnostic test that detects the conserved 18S ribosome RNA gene of *P. falciparum*. In other studies, the same technique has shown high sensitivity and specificity, not only for *P. falciparum*, but also for *P. vivax, P. ovale* and *P. malariae.* LAMP appears to be easy to conduct, sensitive, quick and lower in cost than PCR.

Conflict of Interest: The authors declare that they have no competing interest related to this study

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References

1. Organisation WH. World Malaria Report 2009. World [Internet]. 2009;12:66. Available from: http://whqlibdoc.who.int/publications/2009/9789241563901_eng.pdf
2. Greenwood BM, Bojang K, Whitty CJM, Targett GAT. Malaria. Lancet. 2014;365(9469):1487–98.
3. Murphy SC, Shott JP, Parikh S, Etter P, Prescott WR, Stewart VA. Review article: Malaria diagnostics in clinical trials. Am J Trop Med Hyg. 2013;89(5):824–39.
4. Fransisca L, Kusnanto JH, Satoto TBT, Sebayang B, Supriyanto, Andriyan E, et al. Comparison of rapid diagnostic test Plasmotec Malaria-3, microscopy, and quantitative real-time PCR for diagnoses of Plasmodium falciparum and Plasmodium vivax infections in Mimika Regency, Papua, Indonesia. Malar J. 2015;14(1):1–11.
5. Britton S, Cheng Q, McCarthy JS. Novel molecular diagnostic tools for malaria elimination: A review of options from the point of view of high-throughput and applicability in resource limited settings. Malar J. 2016;15(1):1–8.
6. Dakić Z, Ivović V, Pavlović M, Lavadinović L, Marković M, Djurković-Djaković O. Clinical significance of molecular methods in the diagnosis of imported malaria in returning travelers in Serbia. Int J Infect Dis. 2014;29(October):e24–30.
7. Lucchi NW, Narayanan J, Karell MA, Xayavong M, Kariuki S, DaSilva AJ, et al. Molecular Diagnosis of Malaria by Photo-Induced Electron Transfer Fluorogenic Primers: PET-PCR. PLoS One. 2013;8(2):1–7.
8. Boonma P, Christensen PR, Suwanarusk R, Price RN, Russell B, Lek-Uthai U. Comparison of three molecular methods for the detection and speciation of Plasmodium vivax and Plasmodium falciparum. Malar J. 2007;6:1–7.
9. Mens PF, Schoone GJ, Kager PA, Schallig HDFH. Detection and identification of human Plasmodium species with real-time quantitative nucleic acid sequence-based amplification. Malar J. 2006;5:1–6.
10. Mens PF, De Bes HM, Sondo P, Laochan N, Keereecharoen L, Van Amerongen A, et al. Direct blood PCR in combination with nucleic acid lateral flow immunoassay for detection of Plasmodium species in settings where malaria is endemic. J Clin Microbiol. 2012;50(11):3520–5.
11. De Alvarenga DAM, De Pina-Costa A, De Sousa TN, Pissinatti A, Zalis MG, Suaréz-Mutis MC, et al. Simian malaria in the Brazilian Atlantic forest: First description of natural infection of capuchin monkeys (Cebinae subfamily) by Plasmodium simium. Malar J. 2015;14(1):1-9.
12. Fontecha GA, Mendoza M, Banegas E, Poorak M, De Oliveira AM, Mancero T, et al. Comparison of molecular tests for the diagnosis of malaria in Honduras. Malar J. 2012;11:2–6.
13. Pakalapati D, Garg S, Middha S, Acharya J, Subudhi AK, Boopathi AP, et al. Development and evaluation of a 28S rRNA gene-based nested PCR assay for P. falciparum and P. vivax. Pathog Glob Health. 2013;107(4):180–8.
14. Wang B, Han SS, Cho C, Han JH, Cheng Y, Lee SK, et al. Comparison of microscopy, Nested-PCR, and real-time-PCR assays using high-throughput screening of pooled samples for diagnosis of malaria in asymptomatic carriers from areas of endemicity in myanmar. J Clin Microbiol. 2014;52(6):1838–45.
15. Zakeri S, Kakar Q, Ghasemi F, Raeisi A, Butt W, Safi N, et al. Detection of mixed plasmodium falciparum & P. vivax infections by nested-PCR in Pakistan, Iran & Afghanistan. Indian J Med Res. 2010;132(7):31–5.
16. Perandin F, Manca N, Calderaro A, Piccolo G, Galati L, Ricci L, et al. Development of a Real-Time PCR Assay for Detection of Plasmodium for Routine Clinical Diagnosis. J Clin Microbiol. 2004;42(3):1214–9.
17. Nair CB, Manjula J, Subramani PA, Nagendrappa PB, Manoj MN, Malpani S, et al. Differential diagnosis of malaria on Truelab Uno®, a portable, real-time, MicroPCR device for point-of-care applications. PLoS One. 2016;11(1):1-14.
18. Lee PW, Ji D Der, Liu CT, Rampao HS, Do Rosario VE, Lin IF, et al. Application of loop-mediated isothermal amplification for malaria diagnosis during a follow-up study in São Tomé. Malar J. 2012;11:1-9.
19. Aydin-Schmidt B, Xu W, González IJ, Polley SD, Bell D, Shakely D, et al. Loop mediated isothermal amplification (LAMP) accurately detects malaria DNA from filter paper blood samples of low density parasitaemias. PLoS One. 2014;9(8):1-9.

20. Polley SD, González IJ, Mohamed D, Daly R, Bowers K, Watson J, et al. Clinical evaluation of a loop-mediated amplification kit for diagnosis of imported malaria. J Infect Dis. 2013;208(4):637-44.
21. Kim JY, Goo YK, Ji SY, Shin H Il, Han ET, Hong Y, et al. Development and efficacy of real-time PCR in the diagnosis of vivax malaria using field samples in the Republic of Korea. PLoS One. 2014;9(8):1–5.
22. Lau YL, Lai MY, Anthony CN, Chang PY, Palaeya V, Fong MY, et al. Comparison of three molecular methods for the detection and speciation of five human plasmodium species. Am J Trop Med Hyg. 2015;92(1):28–33.
23. Mbenda HGN, Das A. Molecular evidence of plasmodium vivax mono and mixed malaria parasite infections in duffy-negative native cameroonians. PLoS One. 2014;9(8):1–9.
24. Kamau E, Alemayehu S, Feghali KC, Saunders D, Ockenhouse CF. Multiplex qPCR for Detection and Absolute Quantification of Malaria. PLoS One. 2013;8(8).
25. Talundzic E, Maganga M, Masanja IM, Peterson DS, Udhayakumar V, Lucchi NW. Field evaluation of the photo-induced electron transfer fluorogenic primers (PET) real-time PCR for the detection of Plasmodium falciparum in Tanzania. Malar J. 2014;13(1):1–6.
26. Steenkeste N, Rogers WO, Okell L, Jeanne I, Incardona S, Duval L, et al. Sub-microscopic malaria cases and mixed malaria infection in a remote area of high malaria endemicity in Rattanakiri province, Cambodia: Implication for malaria elimination. Malar J. 2010;9(1):1–11.
27. Yin JH, Yan H, Huang F, Li M, Xiao HH, Zhou S Sen, et al. Establishing a China malaria diagnosis reference laboratory network for malaria elimination. Malar J. 2015;14(1):1–7.
28. Dromigny JA, Jambou R, Scott CS, Perrier-Gros-Claude JD. Performance evaluation of automated depolarization analysis for detecting clinically unsuspected malaria in endemic countries. Trans R Soc Trop Med Hyg. 2005;99(6):430–9.
29. McMorrow ML, Aidoo M, Kachur SP. Malaria rapid diagnostic tests in elimination settings-can they find the last parasite? Clin Microbiol Infect. 2011;17(11):1624–31.
30. Gunasekera WMKTDAW, Abeyasinghe RR, Premawansa S, Fernando SD. Usefulness of polymerase chain reaction to supplement field microscopy in a pre-selected population with a high probability of malaria infections. Am J Trop Med Hyg. 2011;85(1):6–11.
31. Krämer B, Grobusch MP, Suttorp N, Neukammer J, Rinneberg H. Relative frequency of malaria pigment-carrying monocytes of nonimmune and semi-immune patients from flow cytometric depolarized side scatter. Cytometry. 2001;45(2):133-40.
32. Thomas H, Martin P. How useful is PCR in the diagnosis of malaria ? Trends Parasitol. 2002;18(9):395–8.
33. Nsobya SL, Parikh S, Kironde F, Lubega G, Kamya MR, Rosenthal PJ, et al. Molecular evaluation of the natural history of asymptomatic parasitemia in Ugandan children. J Infect Dis. 2004;189(12):2220–6.
34. Padilla-Rodríguez JC, Olivera MJ, Guevara-García BD. Parasite density in severe malaria in Colombia. PLoS One. 2020;15(6 June):1-11.