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Diverse Diagnostic Approaches in Malaria: Review

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Abstract

Malaria is a life-threatening disease, which is transmitted through the bite of a *Plasmodium* parasite infected *Anopheles* mosquito. Malaria Diagnosis is the process of determining the presence of the malaria parasite in the blood and different species of malaria parasite. As different species of malaria has two different treatment protocols, on the basis of diagnosis proper treatment will be given to patient. The bastion of malaria diagnosis has been the microscopic examination of blood, utilizing blood films (thin and thick smear). Although blood is the sample most frequently used to make a diagnosis, but in some cases both saliva and urine has been investigated as alternative, less omnipresent specimens. More recently, in the present we found that modern techniques utilizing antigen tests or polymerase chain reaction have been revealed, though these are not widely implemented in malaria endemic regions. But every technique has its merits and demerits but in present scenario the most important merit is specificity and sensitivity found to be highest in PCR.

Keywords: Malaria, diagnosis, microscopy, QBC technique, PCR, RDT kits, ELISA, Microarray.

Introduction

According to the WHO report 2020, almost 229 million people from 87 malaria endemic countries found to be suffering from malaria with 409 000 malaria deaths. Spanking and accurate diagnosis is very much significant for proper management and control of any epidemiology. At the early-stage of infection, patients show non-specific symptoms or asymptomatic cases especially in non-endemic areas, makes the process difficult for clinical diagnosis (Kasetsirikul *et al.,* 2016). Globally, we required such a diagnostic approach toward malaria that should be effective and handy. There are basically two tactics for malaria diagnosis, clinical or molecular diagnosis. Clinically diagnosis is generally based on the "sign and symptoms" of the patient but for the confirmed diagnosis molecular laboratory test are obligatory (Tangpukdee *et al.,* 2009) (Figure 1). Previously the parasite detection exclusively depends upon the microscope only but with the time, new diagnostic tools open the vast augmentation in proper malaria diagnosis (Ndao, 2012). The proper diagnosis is very important in malaria, as improper diagnosis may lead to high mortality and country like India has two highly prevalent species (*P. falciparum* and *P. vivax*) which have two different treatment protocols. In case misdiagnosis happen in mixed infection and we are unable to diagnose *P. falciparum* then it may result in severe and fatal malaria simultaneously, if *P. vivax* (also in *P. ovale*) remain undiagnosed and only *P. falciparum* treated may lead to relapse fever (Gupta *et al.*, 2010). Misdiagnosis of malaria infection can also lead to recrudescence of parasite that increases the parasite clearance time (Mayxay *et al.*, 2004) and responsible for drug resistance (de Roode *et al.*, 2004). *P. vivax* is generally treated with short half-life drugs, whereas, *P. falciparum* with long half-life drugs that are more proficient (Mayxay *et al.,* 2004). The overdiagnosis of malaria has cause great economic loss to several countries (Stephens *et al.,* 1999; Reyburn *et al.,* 2006). There are several factors which determine the choice of malaria diagnostic practices to be used in a given geographical area. These include (*a*) level of endemicity, (*b*) geographical accessibility, (*c*) social and economic characteristics, (*d*) underlying health infrastructure,

(*e*) available diagnostic tools and (*f*) prevalence and type of drug resistance. Some of the techniques used for malaria diagnosis are elaborated as follows:

Clinical Malaria Diagnosis

The clinical diagnosis of malaria or any other disease based on the sign and symptoms of the patients. Malaria symptoms are very common which can easily overlap with any other viral disease. The methods we generally follow in the clinical trials are very simple, easy to handle and try to distinguish between different species and parasite load in a patient, but for the final confirmation laboratory tests are required. Some of the clinical tests are described here,

Microscopy

Microscopy, using Giemsa, Wright, field or JSB stain examination, has always been considered as a gold standard for malaria diagnosis. Peripheral blood is generally used for slide preparation (finger-prick) as the parasitemia is high (Das *et al.,* 2015). The blood film technique has not been much changed from the time of its discovery. The types of blood films are prepared for malaria parasite analyses thick blood film (for screening the malaria parasite presence) and thin blood film (for species verification) (Bharti *et al.,* 2007). This method is helpful to resolve the magnitude of parasite and due to very little laboratory infrastructure still always generally considered in laboratories for malaria diagnosis in developing countries. This method has number of disadvantages like low sensitivity and in case of mixed infections (two or more parasite invading the single host) conventional microscope generally failed. Like some other techniques it also required trained and supervised technicians (Wilson 2013). The other disadvantage of microscopy is that during the staining process the lysis of erythrocytes makes the technicians difficult to identify malaria parasites (Moody, 2002). With time we are focusing to enhance the microscopy by using the automated imaging method i.e., image formation with the help of computer but the drawback is that, limited to only urban areas (Manescu *et al.,* 2020). Alternative augmentation in the field of microscopy is the use of mobile phones as they are highly potential, easy to handle and cost effective (Pirnstill & Cote, 2015)

Fluorescence Microscopy

The mature erythrocytes are devoid of genetic material but malaria parasite have genetic material which can be labeled by using dyes like acridine orange (AO) (Hanscheid 1999) and benzothiocarboxypurine which stains only intra-erythrocytic parasite (Makler *et al.,* 1991). Rhodamine-123 is another fluorescence dye used assessing the feasible state of parasites; generally, this dye helps to study parasitic membrane (Moody, 2002). Another dye 4′,6-diamidino-2-phenylindole (DAPI) can be used as flurochrome which stains the plasmodial genomic material excited by ultraviolet rays can be visible under microscope (Nchetnkou *et al.,* 2020). The centrifugal quantitative buffy coat (QBC) is another test which is very sensitive technique based on the principle of centrifugal stratification of blood components (Ahmed and Samantaray, 2014). In case of malaria the parasitized erythrocytes get concentrated in a layer which can be visualized with the help of a fluorescence microscopy (Pinto *et al.,* 2001). However, it is more expensive than normal microscopy and required equipment's like centrifuge, ultraviolet rays and filters. The other major downside of this method was the incapability of fluorochrome compounds to distinguish between different species of malaria (Moody, 2002).

Rapid Diagnostics Test Kit (RDT Kit)

According to World health organization (WHO) guidelines, the diagnostic test for determining malaria test should be simple, swift, precise, and cost-effective and RDT tests are quick and do not require electricity and specific equipment (Bell, 2006). This method required a very small amount of infected blood on a test strip by immunochromatographic assay and monoclonal antibodies which give colour result witin 10-15 minutes against antigen (Wongsrichanalai *et al.,* 2007). The bivalent Rapid Diagnostic Test (RDT) kit is based on species-specific antibodies. Some RDTs can detect only single species (either *P. falciparum* or *P. vivax*), while others can detect multiple species (*P. falciparum, P. vivax, P. malariae* and *P. ovale*). The malaria antigen usually targeted for *P. falciparum* is histidine-rich protein-2 (HRP-2) and for other species of *Plasmodium,* lactase dehydrogenase and aldolase are generally considered (Moody, 2002). in recent years an RDT test is also developed for detecting a newly emerged malaria parasite *P. knowlesi* (McCutchan *et al.,* 2008). But contrastingly this method has some demerits such as it gives false negative results for *P. falciparum* due to the deletion of HRP-2 from the population of different countries (Bharti *et al.,* 2016; Parr *et al.,* 2017; Viana *et al.,* 2017) and sometimes gives false positive results as the HRP-2 protein can persist in the human blood up to week even after successful treatment (Bell *et al.,* 2006). The countries and the

nearby countries should conduct a survey from where the deletion of pfhrp2 and pfhrp3 (pfhrp2/3) genes have been reported, so we can reach the threshold for RDT change (WHO report 2020). This method also cannot be used for determining the magnitude of parasitemia like microscopy (Wilson 2012).

Serological Test

Immunofluorescence antibody testing (IFA) and Enzyme linked immunosorbent assay (ELISA) are some serological tests reliable for malaria diagnosis (Doderer *et al.,* 2007). IFA and ELISA both are based on the antibody's detection in the blood against pathogen, so sometimes they also detect past exposure if antibodies still persist in the blood (Thongdee *et al.,* 2014) IFA test is based on specific antigen coated and stored at -30˚C until used and this test quantifies the two antibodies IgM and IgG. The IgG antibodies response is rapid in malaria patient and starts just after the week of infection (Toble *et al.,* 1966). ELISA based on pLDH (lactose dehydrogenase) are generally used for the identification of plasmodium species (Atchade *et al.,* 2013). New ELISA kits are available which combine with soluble *P. falciparum* and recombinant *P. vivax* but as far as sensitivity is a concern, from previous studies it was found that *P. vivax* sensitivity is low as compared to its counterpart *P. falciparum* (Doderer *et al.,* 2007). Beside this, there is no recombinant antigen available for *P. ovale* and *P. malariae* parasites (Doderer *et al.,* 2007). There are no doubt serological tests are simple and sensitive for malaria diagnosis but they are time consuming. Lack of IFA reagent and requirement of a fluorescence microscope and trained technician are some other drawbacks of this method.

Laboratory or Molecular Malaria Diagnosis

The laboratory tests are highly prescribed and are more consistent than the any clinical test but this is also the fact which can't be ignored that molecular tests are more time consuming and pricier, as they required highly trained person with infrastructure to perform those tests. There are some molecular tests which are performed in labs for malaria diagnosis are as follows,

Flow cytometer (FCM)

The flow cytometry is another approach for malaria diagnosis, to identify parasites and evaluate malaria-infected red cells (van Vianen *et al.,* 1993). The basic principle of this technique is based on the detection of hemozoin, which is produced when the intra-erythrocytic malaria parasites after the digestion of host hemoglobin and released toxic heme get converted into a insoluable crystallized form known as hemozoin (Tangpukdee *et al.,* 2009). The hemozoin can be detected in the host blood by using some staining solution by using scatter light which can detect different fluorescence dye, as cells pass through a flow-cytometer channel (Wongsrichanalai *et al.,* 2007). In recent time, one dimensional photonic crystal is used as biosensor which can detect change in concentration in the blood sample of malaria patients (Suthar & Bhargava, 2021). The shortcomings of this technique are its labor intensiveness, trained technicians required, costly diagnostic equipment like fluorescence microscope and that false positives may occur with other bacterial or viral infections (Tangpukdee *et al.,* 2009).

Automated blood cell counters (ACC)

An automated blood cell counter (ACC) is a practical tool for malaria diagnosis (de Langen *et al.,* 2006) with three different diagnostic approaches reported previously. In the first study Cell-Dyn@ 3500 (CD3500) were used an instrument (based on different scatter characteristics of laser-light) is to detect malaria pigment (hemozoin) in monocytes, and showed a sensitivity of 95% and specificity of 88%, compared with the gold-standard blood smear (Hanscheid *et al.,* 2001). In the second method Cell-Dyn@ 3500 was used again but analyzed depolarized laser light (DLL) to detect malaria parasite, having low sensitivity of 72% and high specificity around 96% (Mendelow *et al.,* 1999). In the last technique Beckman Coulter ACC was used to detect increases in activated monocytes by volume, conductivity, and scatter (VCS), with high sensitivity and specificity (98% and 94% respectively) (Briggs *et al.,* 2006). But none of these techniques is routinely available in the clinical laboratory because more studies are required to progress and validate the instrument and its software. The limitation of this method is high-cost equipment required for this type of diagnosis (Hanscheid *et al.,* 2001).

Mass Spectrophotometer

In recent years a novel method for malaria diagnosis has been developed with a high sensitivity of identifying 10µl/parasite. In this technique cleanup of the blood sample is the first process followed by direct ultraviolet laser desorption mass spectrometry (LDMS). The principle of mass spectrophotometer involved a biomarker and in malaria parasite its heme from hemozoin (Sullivan *et al.,* 1990). Later on, the hemozoin crystals formed efficiently absorb UV photons from a laser pulse, which liberates an intact iron protoporphyrin IX (heme) molecules and heme ions from the deposited blood sample. The LDMS of the heme is structure-specific in case of malaria and the intensity of the signal reflects the parasitemia (number of parasites per unit volume of blood). A low-cost device was designed as light spectrometer prototype which capture electromagnetic spectrum from blood serum and helpful in malaria diagnosis (Maity *et al.,* 2019).

Polymerase Chain Reaction (PCR)

PCR assay is comparatively a little bit expensive than other diagnostic methods (Zimmerman *et al*., 2004), but it has very high sensitivity and specificity. PCR diagnoses single infection even with low parasitemia with great efficiency and very efficient to diagnose mixed malaria infections. Nested PCR was generally used for malaria diagnosis as described by Snounou and his co-workers (1993, Jhonston *et al*., 2006), in which the prime targets the 18S small subunit ribosomal RNA gene of *Plasmodium* spp. Before, 1993 several PCR based studies were also performed but focusing on *P. falciparum* and *P. vivax* only (Brown *et al.,* 1992; Barker *et al.,* 1992). This gene is highly conserved across among all the *Plasmodium* spp and has moderate copy number with four to eight copy numbers per parasite (Kasetsirikul *et al.,* 2016). Another promising target for malaria diagnosis is found to be the mitochondrial DNA, due to its greater abundance than nuclear DNA (between 30 and 100 copies per parasite) in the parasite genome (Kasetsirikul *et al.,* 2016). In India and other endemic countries of the world, microscopy technique is generally considered in hospitals, but in most of the cases, they are not able to detect the mixed parasite infections. While microscopic examination in many instances cannot resolve the mixed infection cases, PCR can easily detect the mixed infection in malaria cases (Gupta *et al*., 2010). Later, study from Vietnam (Kawamoto *et al.,* 1996) reported *P. ovale* from that region and for further confirmation sequencing was performed with Dye Terminator kit on 373A Sequencer and this method PCR followed by sequencing was used in several studies from various part of the world. Most of the studies including PCR based diagnosis also include sequencing as enhancement tool for the confirming their results of mixed infection. Additionally, a study conducted in patients infected with *Plasmodium* spp. showed that quantitative PCR may be a promising tool for monitoring the antimalarial therapy (Rougemont *et al.,* 2004). Shokoples *et al.* (2009) were able to identify the four *Plasmodium* spp. in a single reaction that causes infection in humans using the multiplexed RT-PCR assay, even when analyzing a slightly infected sample. No doubt, there is number of PCR assay followed by different scientist but no uniform assay has been established due to laboratory conditions and sometimes parasite parasitemia also effect the method. But the major drawback with this method is the high cost of RT-PCR machine and its reagents.

Microarrays

Microarrays show high potential to provide a platform for the future diagnosis of infectious diseases (Patarakul, 2008). Microarrays can be considered as one of the greatest applications in the field of biomedical sciences as it can solve many pharmacological and toxicological issues related with drugs also (Hardiman, 2006). Microarray is a collection of microscopic spots arranged in a phalanx on grid- like format and attached to a solid surface like glass slide or membrane. Each individual spot is present at a precisely defined location on the substrate. In DNA microarray these spots are single-stranded DNA fragments which are known as probes. These probes hybridize with specific nucleic acid sequence called target which is labeled with a fluorescence dyes and extent of binding between the target and probe is quantified by measuring the signal emitted by labeling dye when scanned under fluorescence microscope (Singh and Bedekar, 2012). Beside diagnosis microarrays can also help to monitor and understand the parasitic resistance to antimalaria drugs (Crameri *et al.,* 2007). The advantage of this technique is that it is highly sensitivity, but it is characterized by a low throughput, time-consuming and expensive equipment like fluorescence microscope (Du and Cheng, 2006).

Loop-mediated isothermal Amplification (LAMP)

Loop-mediated isothermal Amplification (LAMP) emerged as a molecular technique in recent years used for nucleic acid amplification in isothermal conditions (Ocker *et al.,* 2016). This technique can detect highly conserved 18s ribosomal RNA gene of the four malaria species including *P. falciparum, P. vivax, P. malariae* and *P. ovale* (Han *et al.,* 2007). In this technique, DNA polymerase and a set of four specifically designed primers that recognize six distinct regions of the target DNA are required. The procedure for diagnosis is quick in this assay as amplification and detection of the target gene can be completed in only in one isothermal step (Notomi *et al.,* 2000). In some tests mitochondrial genome was also target in malaria diagnosis (Piccot *et al.,* 2020). Even study on frozen blood with LAMP technique for malaria diagnosis and has shown a great sensitivity and specificity comparable with that of nested PCR (Han *et al.,* 2007). LAMP is being simpler, faster, sensitive and lower in cost as compare to other molecular diagnostic assays like PCR (Dhama *et al.,* 2014). In recent years large number of studies were performed using LAMP as diagnostic tool from various parts of the globe (Han *et al.,* 2007; Sirichaisinthop *et al.,* 2011; Tegegne *et al.,* 2017) but still more clinical trials are required to validate the viability and clinical utility of LAMP and other drawback of this technique is that like PCR, LAMP reagents also require cold storage (Erdman *et al.,* 2008).

Conclusion

A comparative summary of various diagnostic assays for the clinical diagnosis of malaria are provided in table 1. The traditional diagnosis of malaria includes microscopic observation of thick and thin Giemsa-stained blood slides which are always considered as 'gold standard' for its simplicity, low cost, real visualization of the parasite as well as its density (Fleisher, 2004). But its limitation including low sensitivity at low parasitemia and unable to diagnose mixed malaria infections, has often led to misdiagnosis of the malaria disease and hence there is chance for either delay/misuse of malaria treatment resulting in patient's suffering (Anthony *et al.,* 2013). Rapid diagnostic tests (RDTs) based on *Plasmodium* spp. specific antibodies are useful and feasible for field surveys of malaria diagnosis. RDTs are used to detect the parasite antigens in blood using nitrocellulose strips that are coated with monoclonal antibody (IgM) against *P. falciparum* antigens, such as horseradish peroxidase-2 (HRP-2) and lactate dehydrogenase (LDH). RDT kits are designed to diagnose mixed-species of malaria infections using antigens like aldolase or specific lactate dehydrogenase (pLDH) (Ehtesham *et al.,* 2015). *P. vivax* normally exhibits a lesser parasite density (on average 10 times lower) than *P. falciparum*, and thus making it more complicated to identify using RDTs and microscopy. The deletion of HRP-2 gene is being reported from different regions of the world, and thereby threatening the RDTs ability to diagnose malaria infection rapidly in the fields (WHO report, 2017). Beside all these methods of malaria diagnosis some other techniques are also available which are in there very nascent stage like latex agglutination assay (Polpanich *et al.,* 2007), cultivation of live malaria parasites (Udomsangpetch *et al.,* 2008) and mobile-device-based tool for malaria diagnosis (Oliveira *et al.,* 2017). In the present scenario, we required a diagnostic assay which is portable equipment (Fraser et al., 2018) and which should be fast with high accuracy (Krampa et al., 2020) or we can say that a diagnostic tool which can be accurate and helpful in the utility of malaria point-of-care (POC) tests (Choi *et al.,* 2016; Dutta, 2020; Malekjahani *et al.,* 2019). . A range of point-of-care diagnostics in future is therefore under development, including simplifications of sample preparation, and infrastructure for lab development in field level (Roth *et al.,* 2016). There is no doubt every method has its own merits and demerits but on the comparative basis nested PCR and LAMP are the two techniques which proves to be better than other methods but LAMP is still in very early stage of malaria diagnosis. The most extensively used molecular technique for malaria diagnosis is the nested PCR assay using 18s-rRNA and sequencing (Mixson-Hayden *et al.,* 2010). PCR followed by sequencing may be a suitable alternative over traditional malaria diagnosis assays, although costs and technical requirements currently obstructed their implementation in malaria endemic regions The comparative study of PCR over other traditional diagnostic assays revealed the very high sensitivity for detecting malaria parasite (Roth *et al.,* 2016). In recent years number of scientists are working on to improve minimum time utilize to do diagnosis and making equipment's which will less costly and side by side easy to handle, may be in future we have better options but in the present conditions, nested PCR based on 18s rRNA found to be the most promising

diagnostic assay.

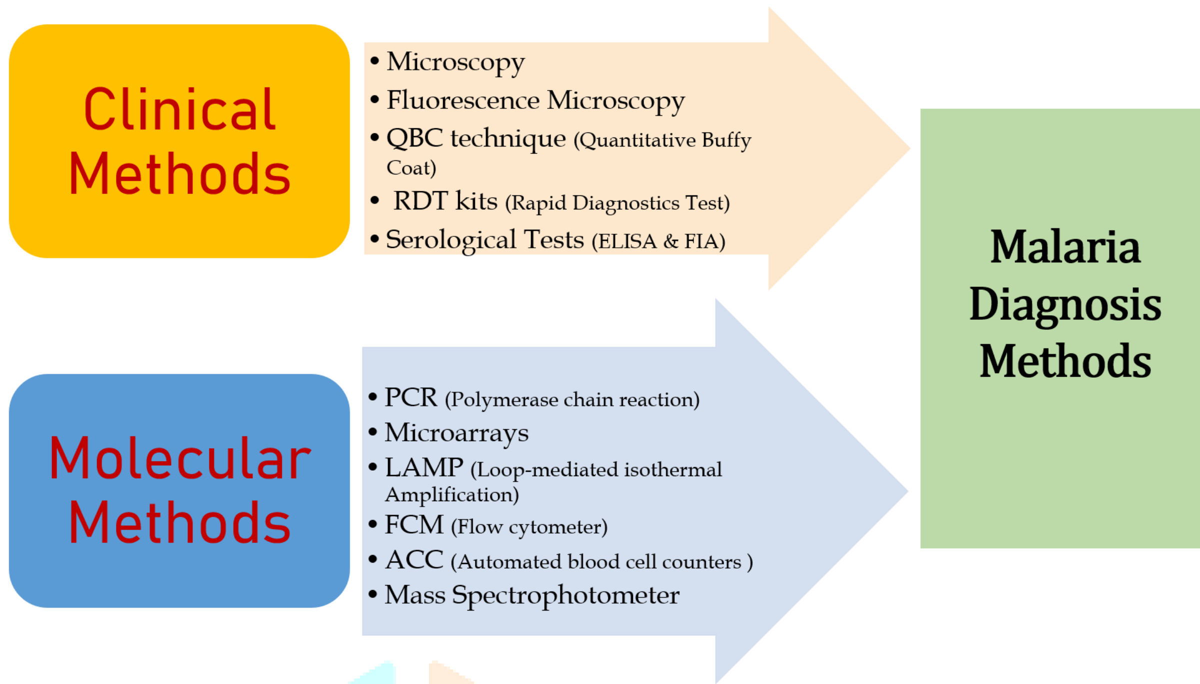

Figure 1. This diagram representing two main different diagnostic approach for malaria diagnosis (Clinical and molecular methods)

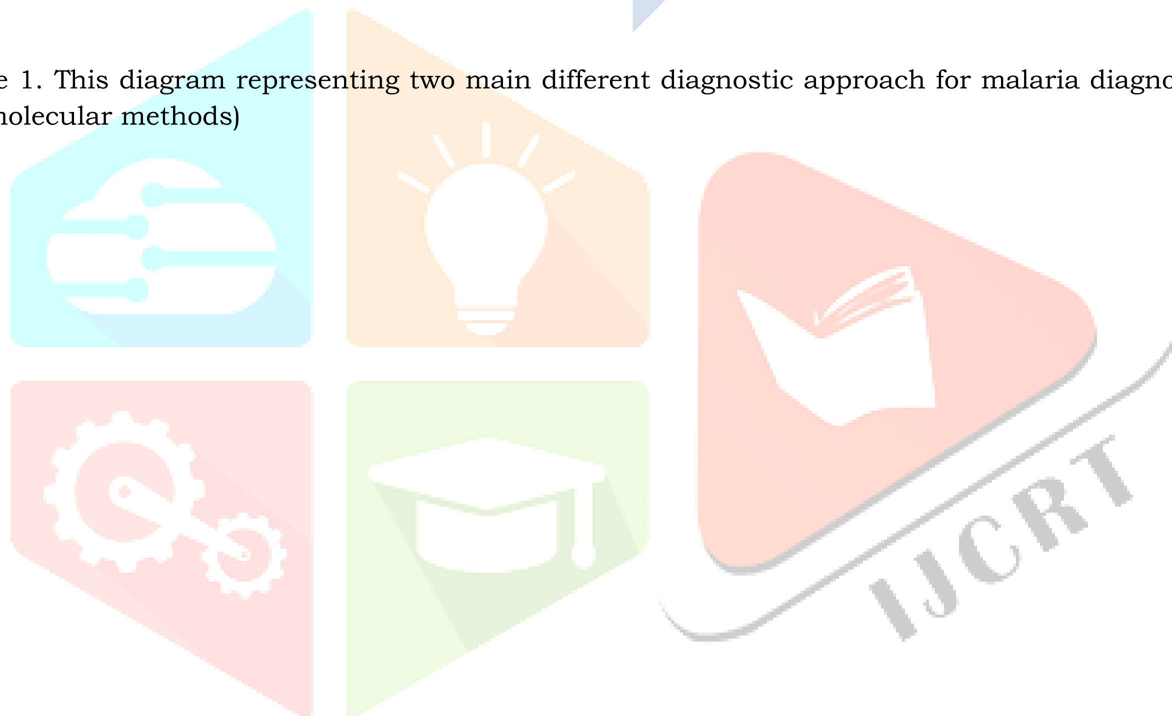

Table 1 Comparison of various diagnostic assays used for malaria diagnosis

Methods	Malaria parasite	Specimen	Target/infected stage	Parasite Density	Sensitivity & Specificity	Cost	Time required (min)	References
Microscopy	All *Plasmodium* spp	Blood	All stages	50-100	Depend on expertise	Cheapest method	30-60	Payne *et al.*, 1989; Moody 2002
QBC technique	*P. falciparum* *P. vivax*	Blood	All stages	Not mentioned	Higher than microscopy	moderate	<15	Pinto *et al.*, 2001
FCM	*P. falciparum*	Blood	Hemozoin	Poor correlation with parasitemia	Variable Sensitivity with high Specificity	expensive	Automated	Tangpukdee *et al.*, 2009
RDT kit	*P. falciparum*	Blood	HRP-2	>100	Moderate, depend upon parasite density	moderate	15-20	Moody 2002
	Other *Plasmodium* spp.	Blood	Aldolase					
IFA	*P. falciparum* *P. vivax* *P. malariae* *P. ovale*	Blood	Antibodies (IgM & IgG)	>100	Relatively high as compare to traditional method	expensive	30-60	She *et al.*, 2007
Cellabs EIA	*P. falciparum* *P. vivax* *P. malariae* *P. ovale*	Blood	Enzyme	>100				
Nested PCR	*P. falciparum* *P. vivax*	Saliva, Urine and Blood	Mitochondrial cytochrome b gene (*cytb*)	>10	Good but less than 18s rRNA PCR	expensive	45-360 (depend upon method used)	Najafabadi *et al.*, 2014
	All *Plasmodium* spp	Saliva, Urine and Blood	18s rRNA gene	>10	Excellent			Tangpukdee *et al.*, 2009
Micoarrays	*P. falciparum*	Blood	DNA probes	undetermined	Relatively High	expensive	<60	Yatsushiro *et al.*, 2010
LAMP	*P. falciparum* *P. vivax* *P. malariae* *P. ovale*	Blood	18s rRNA gene	>5	Excellent	expensive	<60	Han *et al.*, 2004
ACC	*P. falciparum* *P. vivax* *P. malariae* *P. ovale*	Blood	Hemozoin	5-20	Variable	expensive	Automated	Padial *et al.*, 2004; Tangpukdee *et al.*, 2009
Mass Spectophotometry	All *Plasmodium* spp	Blood	Heme from Hemozoin	>100	undetermined	expensive	Automated	Tangpukdee *et al.*, 2009

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