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A Recap on Conventional to Advanced Malaria Diagnosis Trends Used in India

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Abstract: Healthcare is burdened and non-endemic countries are at risk due to the impending malaria burden in endemic regions. In areas with limited resources, microscopy, and rapid diagnostic tests (RDTs) continue to be the gold standards for diagnosing malaria. Even at low parasite densities, they nevertheless exhibit low sensitivity, and microscopy is costly, time-consuming, and requires skilled staff. Malaria diagnostics that are simple, quick, sensitive, specific, and inexpensive are yet unattainable. Molecular-based diagnostics, polymerase chain reaction (PCR) and loop-mediated isothermal amplification, although highly sensitive even at low parasitaemia, still have challenges hindering their use in resource-constrained regions. This review discusses the conventional microscopy, Serology, RDTs and molecular platforms in malaria detection. It also emphasises recent efforts to lessen the obstacles and increase their adaptability to emerging areas. These inventions include artificial intelligence, nanotechnology, and the fusion of many approaches. Microscopy will remain the method of choice for diagnosing malaria due to its high reliability and inexpensive cost. RDTs are inexpensive and easy to use, making them a good option for quick results even though they are less sensitive and specific. ELISA tests are helpful for epidemiological surveys, but they should not be used to diagnose acute malaria. PCR-based assays should be used wherever possible in order to provide more pertinent information (parasite load, species, and resistance). But these tests need well trained staff. PCR can produce exact results, including multiplex, nested, and real-time PCR.

Keywords: Malaria Diagnosis, *Plasmodium*, RDT, Microscopy, Real Time PCR, Serology Based, Hemozoin, CRISPR

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Introduction

Malaria, an illness that has the potential to be fatal and is transmitted by certain species of mosquitoes, primarily affects individuals residing in tropical countries. It is a condition that can be

both prevented and treated. In the year 2022, India constituted approximately 65.7% of the total malaria instances in the WHO South-East Asia region (WHO, 2022) . In the year 2022, the state of Chhattisgarh recorded a total of 24.1 thousand instances of malaria, indicating a reduction in comparison to the preceding year's count of approximately 29.7 thousand occurrences. MalERA, a research agenda for malaria elimination and eradication, is one of several multinational programmes that have been launched with the goal of reducing and eliminating malaria (Rabinovich *et al.*, 2017).

In 2022, there were an estimated 249 million cases of malaria globally across 85 countries and regions where malaria is prevalent, which includes French Guiana's territory, marking a rise of 5 million cases from the previous year. The mortality rates due to *Plasmodium falciparum*, a prominent infectious agent, were notably high on a global scale. While *Plasmodium falciparum* tends to be predominant in tropical regions, *Plasmodium vivax* is commonly found in subtropical zones, with minimal presence in Central Africa where *P. ovale* is more widespread. *Plasmodium malariae* is infrequent, and *Plasmodium knowlesi*, identified as a recently discovered "zoonotic" species, is primarily found in South-East Asia (Davidson *et al*., 2019). The process of certifying the elimination of malaria necessitates the eradication of the four primary species of human parasites, namely *P. falciparum, P. vivax, P. ovale, and P. malariae* (Venkatesan, 2024). After the onset of the new Millennium, *Plasmodium knowlesi*, a parasitic species causing malaria in primates, has been acknowledged as the fifth *Plasmodium* of significant medical relevance (Lee *et al.*, 2022).

Various methods are currently available for malaria diagnosis, including traditional microscopy-based approaches, rapid molecular techniques, immunoassay concepts, enzyme immunoassay, and molecular techniques such as polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP), which have been extensively used in malaria research and diagnosis (Fitri *et al.*, 2022).

Microscopy Methods

The examination of thick and thin blood films is a crucial test for detecting parasitaemia, hence considered as “Gold Standard”. Blood is collected using finger stick or venipuncture. Thick smears are more sensitive and involve placing blood drops on a slide. Thin smears examine parasite morphology with a single layer of cells. Giemsa stain is applied, and slides are examined with a microscope (Mbanefo and Kumar, 2020). Trained personnel conduct this task with high sensitivity and specificity. The method's limit of detection is 50-200 parasites per µl of blood. Skilled personnel can determine parasitaemia level in about 60 min (Mbanefo and Kumar, 2020). One to two droplets of blood must be placed in a circle on a slide to create a thick smear, which is more sensitive. The several blood stages of the malaria parasite, including trophozoites, gametocytes, and schizonts, are released when the red blood cells are lysed. The purpose of thin smears is to identify the morphology of the parasite species. They are made by feathering the edge of a slide with a single layer of cells by spreading a drop of blood across it (Mathison and Pritt, 2017).

Malaria microscopy (Table 1) has been the focus of research and development for automated diagnosis of malaria. Traditional microscopy methods are time-consuming and require skilled microscopist (Hang Yu *et al.*, 2023). Portable microscopes, combined with consumer electronics and affordable optics, have shown potential for remote diagnosis of malaria (Gordon *et al.*, 2022).These advancements in malaria microscopy aim to improve the speed, accuracy, and accessibility of malaria diagnosis, particularly in resource-limited areas. Individuals who are asymptomatic and have low levels of parasitaemia that cannot be detected and treated, contribute to the perpetuation of the transmission cycle within the community (Mbanefo and Kumar, 2020). It enables the determination of the percentage of parasitaemia, the morphology of the parasites, and their speciation. It requires a significant amount of

Table 1: Sensitivity, Specificity, PPV, NPV, Efficiency percentage of Malarial Microscopy Methods

SENSITIVITY	SPECIFICITY	POSITIVE PREDICTIVE VALUE	NEGATIVE PREDICTIVE VALUE	EFFICIENCY	REFERENCE
99.70 %	100 %	100%	99.88%	99.91%	Mallepaddi *et al.* (2019)

Table 2: Sensitivity, Specificity, PPV, NPV, Efficiency percentage of Malarial Serological Assays

CATEGORY	SENSITIVITY	SPECIFICITY	POSITIVE PREDICTIVE VALUE	NEGATIVE PREDICTIVE ALUE	EFFICIENCY	REFERENCE
Ag based (IFAT, IFA, ELISA)	99.70 %	100 %	100%	99.88%	99.91%	Mallepaddi *et al.* (2019)
Ab based (EIA, RDT)	92.81%	94.13%	84.02%	97.52%	93.80%	Mallepaddi *et al.* (2019)

time for the detection and quantification of parasitaemia (Rubio *et al.*, 2022).

Serological Assays

Serological tests for malaria (Table 2) rely on the identification of antibodies generated by the human immune system in reaction to infection caused by the malaria parasite. These tests possess significant utility in the context of epidemiological investigations, surveillance, and population screening.

Malaria Rapid Diagnostic assays (RDTs) are straightforward immunochromatographic assays that detect particular antigens of malaria parasites in whole or peripheral blood. They are also commonly referred to as dipsticks or Malaria Rapid Diagnostic Devices (MRDDS) (Obeagu *et al.*, 2018). RDT presently targets three malaria antigens: parasite lactate dehydrogenase (pLDH), *Plasmodium* aldolase, and histidine rich protein II of *Plasmodium falciparum* (PfHRPII). Reports show that HRP2 gene deletion in endemic areas, lead to absence of HRP2 antigens causing negative result with RDT (Orish *et al.*, 2018).

The Enzyme-Linked Immunosorbent Assay (ELISA) is a widely used serological technique employed to identify malaria antibodies in the serum or plasma of patients (Kwenti *et al.*, 2017). ELISA assays employ specific malaria antigens, such as recombinant proteins or synthetic peptides, that are coated onto microplates. The patient samples are then incubated with these antigens, and if antibodies against malaria are present in the sample, they will bind to the antigens. Subsequently, the bound antibodies are detected by employing enzyme-linked secondary antibodies and a chromogenic substrate, resulting in a measurable colour change (Kartal *et al*., 2023).

The Indirect Fluorescent Antibody Test (IFAT) is another serological method utilized for the detection of malaria antibodies. In this, patient samples are incubated with slides coated with malaria antigens, and if antibodies are present, they will bind to these antigens. Following the removal of unbound antibodies through washing, fluorescein-labelled secondary antibodies are introduced, which then bind to the antibodies attached to the antigens. The presence of fluorescence indicates the presence of malaria antibodies (Wu *et al.*, 2015).

Immunofluorescence Assays (IFA) are similar to IFAT but find more frequent application in research and reference laboratories. They employ fluorescently labelled antibodies to identify malaria antibodies bound to antigen-coated slides

Table 3: Sensitivity, Specificity, PPV, NPV, Efficiency percentage of Malarial Molecular Methods

CATEGORY	SENSITIVITY	SPECIFICITY	POSITIVE PREDICTIVE VALUE	NEGATIVE PREDICTIVE VALUE	EFFICIENCY	REFERENCE
PCR based Assays	100 %	100 %	100%	100%	100%	Mallepaddi *et al.* (2019)
Isothermal Based	100%	100%	100%	100%	100%	Antinori *et al.* (2021)

or plates. It can detect various classes of antibodies, such as IgG, IgM, and IgA, thereby providing valuable information regarding the timing and stage of infection (Wu *et al.*, 2015).

Molecular Methods

An alternative technique for diagnosing malaria is polymerase chain reaction (PCR) analysis (Table 3), which enables the sensitive and precise identifi cation of *Plasmodium* species DNA from peripheral blood. Nowadays, real time PCR, reverse transcriptase PCR, nucleic acid sequence based amplification (NASBA), and loop mediated isothermal amplification (LAMP) are among the molecular techniques that have developed (Bousema *et al*., 2014).

(i) *PCR-Based Assays*

Conventional PCR is a technique that allows for the amplification of DNA sequences through repetitive cycles of denaturation, annealing, and extension. It requires a pair of primers that are complementary to the target sequence of interest. The primers are extended by a DNA polymerase, resulting in the synthesis of amplicons. These amplicons are then re-amplified with the same primers, leading to exponential amplification of the DNA molecules. After amplification, gel electrophoresis is typically used to analyse the PCR products (Fitri *et al.*, 2022).

The efficiency and sensitivity of the polymerase chain reaction significantly increased when the nested PCR strategy was implemented (Lima *et al.*, 2011). This approach involves two stages of amplification, with the initial reaction product serving as the template for the subsequent reaction. The second reaction incorporates a primer containing the hybridization sequence oligonucleotide, which is present in the first product. Unlike the primary PCR, the secondary PCR utilizes a different set of primers, known as "nested" or internal primers, which do not bind to primer dimers or non-specific artifacts generated in the primary PCR. In the first PCR amplification (nest-1), *Plasmodium* genus-specific primers were utilized. The amplification product from nest-1 then served as the DNA template for the secondary PCR (nest-2), using primers specifically designed for all human *Plasmodium* species (Fitri *et al.*, 2022).

Multiplex PCR, a modified form of PCR, enables the concurrent amplification of several target sequences in a single reaction well by utilizing distinct primer pairs for each target. Two major processes that introduce bias in multiplex PCR have been identified: PCR drift and PCR selection.The bias caused by variations in the PCR reagent interactions, especially in the early cycles when the concentration of the template is relatively low, is known as PCR drift. On the other hand, PCR selection is a mechanism that favors the amplification of specific templates due to the unique properties of the target's flanking sequences or the entire genome. These properties can include differences in GC content between regions, resulting in preferential accessibility of targets within genomes due to secondary structures, as well as variations in the number of gene copies within a genome (Belachew *et al*., 2022).

A PCR assay that has been validated is available for the real-time detection and identification of *Plasmodium spp*. In a single

Table 4: Comparative Study of Different diagnostic methods for Malarial Parasite Detection

METHOD	TARGET	MECHANISM BASED	ADVANTAGES	LIMITATION	CHALLENGES	REFERENCE
MICROSCOPY	N/A	Detect the morphology of the parasite species.	Identify Parasitic Morphology and Species Differentiation	Needs Specialist to perform.	asymptomatic individuals with low parasitemia remain undiagnosed	Mbanefo and Kumar (2020)
RDT	pHRP-2, LDH, Aldolase	Detect the parasitic antigens	Less intensive, personnel training, affordable	Unable to detect except *Pf* and *Pv*	unable to keep up with the ever-evolving nature of the malaria parasite	Mbanefo and Kumar (2020)
HAEMOZOIN	Haemozoin	High Magnetic Field	Hz as biomarker is that it appears very early during the life cycle of the parasite and gets cleared from the bloodstream	detect only malaria positive vs malaria negative cases	Gazelle prototype provides qualitative malaria diagnosis without species differentiation	Mbanefo and Kumar (2020)
AG BASED SEROLOGICAL ASSAY	Antigen (Target Macromolecule)	Antibody-Antigen interactions.	low parasite densities were reliably detected in this HRP2 ELISA.	ELISA test kits only for *P. falciparum*	detection of P. falciparum parasitemia when it is superimposed by P. vivax.	Ruiz Moreno *et al.* (2019)
CONVENTIONAL PCR	18S rRNA	Using primers to select a segment of the genome to be amplified	Useful in parasite detection in individuals with low parasite burden.	acquisition of a thermocycler which may be a financial hindrance	does not provide an easy method of estimating parasite burden	Mbanefo and Kumar (2020)
NESTED PCR	18S rRNA and 16S rRNA	involves two sequential amplification reactions	recognized as the most sensitive and specific method of all diagnostic tests for malaria	complex equipment needed to run the PCR assay	Unable to differentiate between colonization and infection	Green and Sambrook (2019)
MULTIPLEX PCR	Multiple genes	Multiple targets sequences can be amplified	higher sensitivity to detect mixed infections	self-inhibition among different sets of primers	Inter-region differences in GC content, resulting in the preferential accessibility of targets within genomes	Lazrek *et al.* (2023)
REAL TIME PCR	Total RNA	amplification of DNA generating thousands to millions of copies of a specific DNA segment	low-cost, portable, and user-friendly instruments suitable for application in remote and resourcelimiting setting	needs specialized bio-containment laboratories, operated by highly trained technicians	critical to allow the effective identification of multiple genes.	Grabias *et al.* (2019)
LAMP	18S rRNA, Mitochondrial DNA	determine the presence of *Plasmodium* parasites in the blood based on the presence or absence of its DNA	detection of low density and sub-microscopic infections with better accuracy and greater ease	performance results reported in the studies are inconsistent	Asymptomatic, low parasite density malaria infections are difficult to detect	Mbanefo and Kumar (2020)

reaction. This assay utilizes a simple collection method. The use of fluorescence-based technology allows for the detection of target amplicons in real-time as they accumulate after each cycle, once a quantitative threshold is reached. Therefore, the detection is not an end-point analysis, but rather an exponential process. This assay operates as a closed system, eliminating the accumulation of hazardous waste and the risk of contamination (Leski *et al.*, 2020).

(ii) *Isothermal Tests*

The LAMP reaction amplifies target DNA by strand displacement and loop production using Bst DNA

polymerase and several primers. This produces amplicons with many loops and concatemers. To amplify the target DNA until all targets are amplified, this process consists of initiation, auto-cycling strand displacement, annealing, DNA amplification, and termination phases (Puri *et al.*, 2022). Without the need for a thermocycler, nucleic acid sequence-based amplification (NASBA) amplifies a target *Plasmodium* sequence using three enzymes: strand displacement polyme rase, reverse transcription, and a thermocycler. Because it does not require a DNA denaturation ph ase, it is less prone to contamination than PCR and can be used for both diagnosis and species identifi cation (Oriero *et al.*, 2014).

Other Advanced Method

CRISPR (Clustered regularly interspaced palindromic repeats)-based diagnostics harness the programmable character of CRISPR-Cas systems for the identification of nucleic acids and the recognition of molecules. Methods based on CRISPR, such as SHERLOCK (Specific High-sensitivity Enzymatic Reporter unLOCKing) and DETECTR (DNA Endonuclease-Targeted CRISPR Trans Reporter), enable accurate detection of malaria DNA or RNA sequences with a high degree of sensitivity and specificity. The adaptability of CRISPR diagnostics for point-of-care testing (POCT) is made possible by the use of portable, battery-operated devices and lateral flow assays, which facilitate the rapid and decentralized diagnosis of malaria in settings with limited resources (Cunningham *et al*., 2021).

Hemozoin is a malaria parasite byproduct and biomarker that has gained attention since it is more stable, readily available, and affordable than pfHRP-II. Researchers have employed β-hematin, a substance akin to hemozoin, in electrochemical sensors to attain minimal detection levels (Baptista *et al*., 2021).

Table 4 illustrates a comparative study of various diagnostic methods for malarial parasite infection.

Conclusion

Conventional microscopic analysis of blood smears is the primary method for diagnosing malaria, offering cost-effectiveness and reliability despite requiring skilled personnel. Rapid diagnostic tests are widely used in remote areas but face challenges with cost and quality control, while serological tests are more suitable for epidemiological studies than acute diagnosis. Molecular techniques are valuable in research settings for tracking drug resistance, species identification, and quantifying parasite levels, with the choice of diagnostic method influenced by factors like endemicity, urgency, healthcare expertise, and budget constraints.

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Conflict of Interest

The authors declare no conflicts of interest.

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