CURRENT AND EMERGING LABORATORY TECHNIQUES FOR MALARIA DIAGNOSIS

ABSTRACT

Effective diagnostic methods must continue to be developed due to the effects of malaria on world health. Malaria is an infectious disease spread by mosquitoes that affects both people and other animals. It is the product of *Plasmodium* parasites which is transmitted through bites from infected female Anopheles mosquitoes. Other sporozoan responsible for malaria infections includes the sporozoan parasites *Plasmodium falciparum, Plasmodium ovale, Plasmodium vivax, Plasmodium malaria, and Plasmodium knowlesi*. Routine diagnosis of malaria is impeded in areas where the disease is endemic by technical and infrastructure issues with laboratories. Since good disease management is one of the primary actions of the Global Malaria Control Strategy, prompt and accurate diagnosis is essential. In order to inform malaria control efforts through epidemiologic screening and surveillance, for research reasons to evaluate the effectiveness of antimalarial medications and vaccines, and for blood bank screening, accurate malaria detection is also crucial. This study focuses on exposing some of the novel and efficient methods of diagnosing malaria that go beyond the light microscopy gold standard, which has several drawbacks.

Key words: ***Malaria, parasite, plasmodium, diagnosis***

INTRODUCTION

Effective diagnostic methods must continue to be developed due to the effects of malaria on world health. Routine diagnosis of malaria is impeded in areas where the disease is endemic by technical and infrastructure issues with laboratories [1]. Since good disease management is one of the primary actions of the Global Malaria Control Strategy, prompt and accurate diagnosis is essential [2]. Thus, it is concerning that ineffective malaria control is still hampered by inadequate diagnosis. This is brought on by a number of elements, including the disease's ambiguous clinical presentation, the high prevalence of asymptomatic infection in some regions, a lack of resources and inadequate access to facilities and trained healthcare professionals, as well as the common practice of self-treating malaria that is clinically suspected. In order to inform malaria control efforts through epidemiologic screening and surveillance, for research reasons to evaluate the effectiveness of antimalarial medications and vaccines, and for blood bank screening, accurate malaria detection is also crucial.

The ability to definitively establish presence or absence of infection, identify the species of malaria present, quantify parasitemia (i.e., parasites per microliter of blood or percent infected red blood cells), detect low-level parasitemia, and enable monitoring of treatment response are all qualities of a useful malaria diagnostic tool (including detection of recrudescence or relapse) [3]. There isn't a single malaria diagnostic technology that satisfies all of these requirements as

of yet. The test properties that matter for diagnosis change depending on the infection epidemiology and control objectives in the area where the test is utilized. To date, however, the laboratory diagnosis of malaria has relied almost entirely on microscopy, a useful tool when used properly but one that is unreliable and inefficient when used incorrectly [4]. Malaria control in rural and urban regions could be significantly enhanced by improving the use of light microscopy and developing alternative diagnostic methods.

Plasmodium parasites, which individuals contract through the bites of infected female Anopheles mosquitoes, are the source of the acute fever sickness known as malaria [5]. Malaria is an infectious disease spread by mosquitoes that affects both people and other animals [6]. The sporozoan parasites *Plasmodium falciparum, Plasmodium ovale, Plasmodium vivax, Plasmodium malaria, and Plasmodium knowlesi* are responsible for causing malaria [6]. In humans, the parasites first develop and multiply in the red blood cells and later in the liver cells. Consecutive parasitic broods develop inside red blood cells in the blood and kill them, producing daughter parasites (merozoites) that continue the cycle by attacking more red blood cells [7]. As a result, the infected mosquito serves as a vector for spreading the disease from one person to another, while infected individuals pass on the parasite to the mosquito. The mosquito vector does not experience any negative effects from the parasites, in contrast to the human host.

Two hosts participate in the malaria parasite life cycle. A female Anopheles mosquito carrying the malaria parasite injects sporozoites into the host during a blood meal [7]. Liver cells are infected by sporozoites, which develop into schizonts and then burst to produce merozoites. (It should be noted that *P. vivax* and *P. ovale* have dormant stages called hypnozoites that, if left untreated, can remain in the liver and trigger relapses by invading the blood stream weeks or even years later.) [7]. The parasites go through asexual multiplication in the erythrocytes after this initial replication in the liver (exo-erythrocytic schizogony) (erythrocytic schizogony). RBCs are infected by merozoites [8]. Trophozoites in the ring stage develop into schizonts, which burst to release merozoites. Certain parasites develop into sexually active erythrocytic stages (gametocytes). Clinical signs of the illness are brought on by blood stage parasites. An Anopheles mosquito eating blood consumes the male and female gametocytes (microgametocytes and macrogametocytes, respectively) [8]. The sporogonic cycle is the process through which the parasites replicate within the mosquito. The microgametes infiltrate the macrogametes in the mosquito's stomach, producing zygotes. As they grow into oocysts, the zygotes convert into elongated, motile ookinetes that infiltrate the mosquito's midgut wall. The oocysts develop, burst, and release sporozoites, which travel to the salivary glands of the mosquito. The malaria life cycle is continued by injecting the sporozoites into a fresh human host.

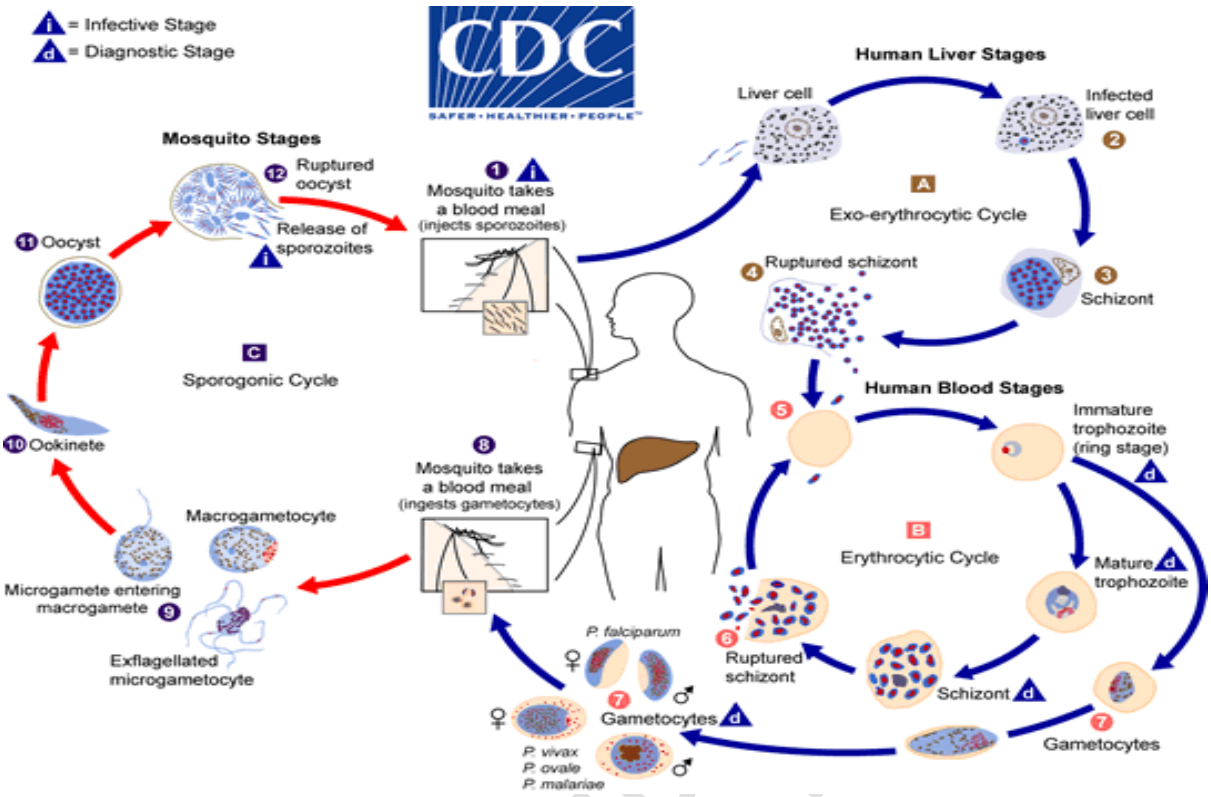


Fig 1 Life cycle of *Plasmodium spp.* Extracted from Centers of Disease Control and Prevention (CDC), [50].

EPIDEMIOLOGY OF MALARIA

The most dangerous *Plasmodium* species are *P. falciparum* and *P. vivax*. The most common and lethal malaria parasite on the continent of Africa is *P. falciparum*. The most common malaria parasite outside of sub-Saharan Africa is *P. vivax* [9]. The initial signs of malaria, including fever, headache, and chills, can be mild and challenging to diagnose. They typically show 10 to 15 days after the infecting insect bite. If P. falciparum malaria is not treated, it can proceed to severe sickness and death in less than 24 hours [10]. Nearly half of the world's population would be at danger from malaria in 2020. Infants, young children, pregnant women, persons with HIV/AIDS, as well as people with low immunity going to places with high malaria transmission rates, such as migrant workers, mobile populations, and travelers, are at significantly increased risk of getting malaria and developing severe disease.

TYPES OF MALARIA DIAGNOSIS

The microscopic study of blood using blood films has been the primary method of diagnosing malaria [11]. While blood is the specimen that is typically used to make a diagnosis, saliva and

urine have also been researched as potential, less invasive alternatives. Antigen tests and polymerase chain reactions have been developed more recently, however these methods are not frequently used in malaria-endemic areas. The main indication to treat for malaria in places that cannot afford laboratory diagnostic procedures is frequently a history of subjective fever. There have historically been three methods used to treat malaria: Clinical (Presumptive) diagnosis, Laboratory (Test-based) diagnosis, and Contemporary diagnosis [12].

CLINICAL DIAGNOSIS OF MALARIA

In the clinical method, a diagnosis and treatment are made primarily based on clinical symptoms and indicators. It also goes by the name "Presumptive diagnosis." It is the method that is most frequently employed in rural regions and at the edges of the healthcare system where there is no laboratory assistance for clinical diagnosis. The most noticeable of the numerous clinical signs and symptoms of malaria is fever, which is frequently accompanied by chills, sweating, anorexia, headaches, vomiting, and malaise. This combination of symptoms is usually recognizable to people living in endemic regions, and they routinely self-diagnose malaria based solely on symptoms. In addition to these straightforward malaria symptoms, severe malaria, which is virtually always caused by Plasmodium falciparum, may also present in different ways. These include severe symptoms such cerebral malaria, severe anemia, and others along with confusion or drowsiness with prostration [13]. Clinical diagnosis is simple to carry out and doesn't need for any specialized tools or materials. The signs of malaria, however, are very vague and resemble those of other fever disorders. Therefore, a clinical diagnosis of malaria is dubious and should, whenever feasible, be supported by laboratory tests. Despite this lack of specificity, there are situations where disease therapy based solely on a clinical diagnosis is acceptable.

LABORATORY DIAGNOSIS OF MALARIA

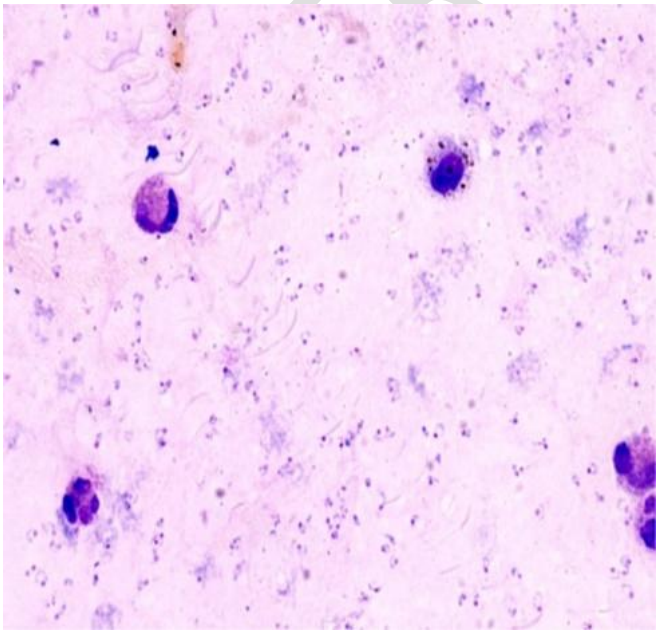
The presumption method was advocated for many years by the World Health Organization (WHO), and it was adopted by all nations in malaria-endemic sub-Saharan Africa. However, in the beginning of 2010, WHO released updated treatment recommendations that urge a switch from a presumptive to a test-based approach. The practices of several decades are effectively ended by this amendment to the standards [5]. Over the years, a number of methods for diagnosing malaria have been used. Each strategy has qualities like cost, performance ease, and accuracy that will decide its suitability in various circumstances. Giemsa-stained blood films and rapid diagnostic tests are part of the outdated conventional approach for diagnosing malaria (RDTs).

GIEMSA-STAINED BLOOD FILMS (LIGHT MICROSCOPY)

Microscopical analysis of blood films stained with Giemsa is the most affordable, favored, and accurate method for diagnosing malaria since each of the four primary parasite species has a

distinctive trait [14]. The "gold standard" for finding and classifying malaria parasites continues to be the meticulous analysis of a well-prepared and well-stained blood film by a skilled microscopist. Giemsa-stained blood films are examined with a 100x oil objective.

Principle: The ingredients in Giemsa solution include eosin and methylene blue (azure). The parasite nucleus is stained red by the eosin component and blue by the methylene blue component. Methanol is used to fix the thin film. The thick film's dehaemoglobination and staining happen simultaneously. Traditional blood films come in two different types: thin films and thick films [15]. Thin films resemble standard blood films and enable species identification because this treatment best preserves the parasite's outward appearance. Blood is spread out in thin smears in a layer whose thickness gradually declines toward the feathery edge. The cells of the feathery edge must be in a monolayer and separate from one another [15]. Thick films are about eleven times more sensitive than thin films, allowing microscopists to screen a larger volume of blood. While this makes it easier to detect low levels of infection, the appearance of the parasite is much more distorted, making it challenging to distinguish between different species [15]. Red blood cells that have been dehemoglobinized or lysed form a thick layer on thick smears (RBCs). The blood components—including any parasites—are concentrated (by about 30 times) in a smaller area than in a thin smear. As a result, thick smears facilitate the more accurate detection of parasites (increased sensitivity). They do not, however, allow for the best picture of parasite morphology. They are frequently insufficient for identifying the species of malaria parasites, for instance: if the thick smear is positive for malaria parasites, the thin smear should be utilized to determine the species. Leishman's stain and Field's stain are more options [15].



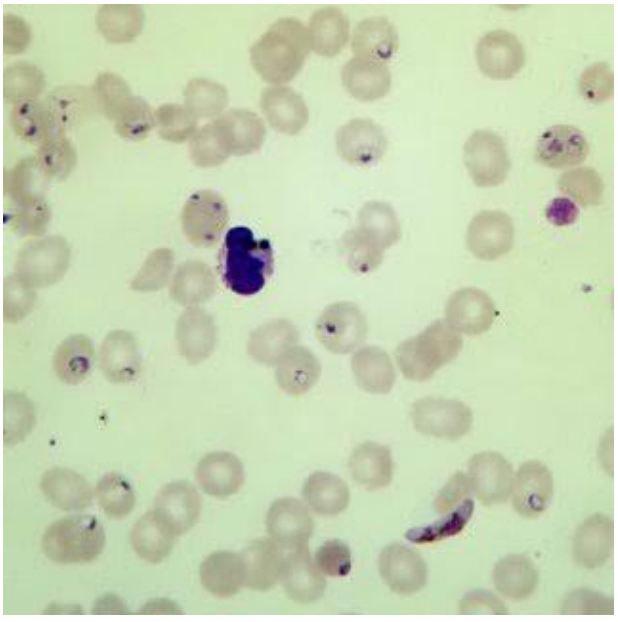


Fig 2. Trophozoite and gametocyte stages of *P. falciparum* on thick and thin film [51, 52].

CONCENTRATION TECHNIQUE FOR MALARIA PARASITE

A whole blood sample can be fractionated as a pretreatment to separate erythrocytes (45%) and plasma from buffy coat, which is made up of white blood cells and platelets (<1% of total blood) (55 %). The buffy coat layer, which is composed primarily of platelets and white blood cells, lies between the plasma and the erythrocytes [16]. In order to prepare the buffy coat, two small narrow-bore EDTA plastic or glass test tubes must be filled with EDTA anticoagulated blood using a narrow stem plastic bulb pipette or Pasteur pipette. This is centrifuged for 15 minutes at RCF 1000g. Buffy coat layer and red cells below it are instantly transferred to one end of a slide (depth of about 1mm) and combined with the end of the pipette. Supernatant plasma above the buffy coat layer is then discarded. To create a thin preparation, a spreader with smooth edges is employed. After being fixed with pure methanol for two minutes, this is allowed to air dry; and additionally Giemsa stain. A 40x and 100x objective examination of the preparation is conducted first.

ANTIGEN DETECTION ASSAY – RAPID DIAGNOSTIC TESTS (RDTs)

These tests work by employing immunochromatographic techniques to find malaria parasite antigens in lysed blood.They often use a dipstick or test strip that has monoclonal antibodies that are specifically directed against the target parasite antigens. Approximately 15 minutes are needed to complete the exams. There are numerous commercial test kits on the market right now. Rapid advancements in technology are being made in the field, and they will surely improve RDTs' capacity for diagnosing malaria [17]. Based on the immunochromatographic concept, the test creates antigen-antibody complexes with the specific malaria antigen released from blood that have been lysed.

ANTIGENS TARGETED BY CURRENTLY AVAILABLE RDTs

A water-soluble protein called histidine-rich protein II (HRP-II) is made by *P. falciparum* trophozoites and immature gametocytes. Only *P. falciparum*-specific commercial kits are currently available to detect HRP-II [18]. The malaria parasite's sexual and asexual stages (gametocytes) both generate parasite lactate dehydrogenase (pLDH). The four *Plasmodium species* that infect people are all detected by the test kits that are now on the market. They are able to distinguish *P. falciparum* from non-*falciparum species*, however they are unable to tell *P. vivax* from *P. ovale* from *P. malariae* apart [18]. An enzyme found in both the *P. falciparum* parasite stage and the non-falciparum malaria parasites is called *Plasmodium aldolase*. The response of monoclonal antibodies to *Plasmodium aldolase* is pan-specific. In kits that combine

detection of the HRP-II antigen of *P. falciparum* with that of an as of yet unnamed "pan-malarial" antigen of the other species, additional antigen(s) that are present in all four species are also targeted [18].

CURRENT DIAGNOSTIC TECHNIQUES

A quick lateral flow test for the detection of PSSP17, a female gametocyte-specific protein, in child saliva was created in a recent work by Tao and team [19]. Early subclinical diagnosis of malaria is essential for its eradication because people who have the parasite but don't show any symptoms serve as the illness's reservoir, causing mosquitoes to become infected and spread the disease. Although saliva-based PCR detection and quantification remain effective and more reliable, this RDT approach is not currently in use. Malaria can currently be diagnosed using the following techniques: Urine malaria test (UMT)-RDTs, Fluorescent DNA-RNA stains (TBF and THBF films (e.g. acridine orange) After centrifugation (QBC), Flow cytometry, and Luminex xMax Technology), Molecular methods (DNA-RNA hybridization, PCR: use of saliva, DNA stored with OMNIgene ORAL kit at RT, Loop-mediated isothermal amplification (LAMP), Real-time PCR (QT-PCR), Real-time nucleic acid sequence-based amplification [(QT-NASBA), and Isothermal thermophilic helicase-dependent amplification (tHDA)], Malaria pigment detection (Dark field microscopy, Automated blood cell analyzers, Depolarizing monocytes containing malarial pigment, Pseudoreticulocytosis, and Transdermal haemozoin detection) [19].

URINE MALARIA TEST (UMT)

The Fyodor Urine Malaria Test (UMT) uses in vitro immunochromatography to detect the protein that the malaria parasite produces in a qualitative manner [20]. Different RDTs respond to various proteins in different ways, which allows them to only detect one or several forms of malaria. Histidine-rich protein 2 (HRP2), which is created by *Plasmodium falciparum*, is the protein to which the UMT reacts. Antigen-antibody reaction serves as the foundation for this test's methodology [20]. The interaction between a recombinant monoclonal antibody placed on the dipstick and the antigen, which are protein fragments in the patient's urine. The first study was conducted in Enugu State, Nigeria, between June and December of 2012, and the findings were published in a report in October of 2014. In 2016, the second investigation was carried out [21]. The malaria parasite's merozoite and gametocyte types create HRP2.

The HRP2 is then carried by the blood to the kidneys, where it is then sent to the bladder as a component of the urine. Normal urine contains very little protein, but fever and malaria both cause this amount to rise. A urine sample is taken as part of the process. The lines on the dipstick are counted after being soaked in the urine for 25 minutes. One line indicates that the patient

does not have malaria, while two lines indicate malaria (positive) (negative). The test needs to be redone if there is no line or only one line in the control (Invalid) [21].

UMT has the advantage of being cheaper and faster, the dipsticks used in RDTs present a result within a few minutes. It is also ideal for home usage as UMT is non-invasive, does not require phlebotomy. It minimizes the risk of bacterial contamination and infection. Pricking the skin for blood collection can be painful and may introduce bacteria into the body, whereas urine is collected with ease. On the other hand, it can only detect types of malaria that produce HRP2 which is *Plasmodium falciparum*, it is not quantitative and some other infections such as schistosomiasis and trypanosomiasis caused by *Trypanosoma brucei gambiense* can give false positive result in endemic regions. All of this pose a disadvantage to the UTM method [21].

FLUORESCENT DNA-RNA STAINS

For routine diagnostics, Light Emission Diode Fluorescence Microscopy (LED FM) offers a trustworthy substitute [22]. Given that LED microscopes utilize less energy, are durable, and have a brighter field of view, they are an extremely valuable tool in field situations. In addition, it provides battery functioning in the absence of electricity. This technique uses the fluorochrome dye acridine orange to stain DNA and RNA immediately. The following fluorescent DNA-RNA stains: thick blood films and thin blood films (e.g. acridine orange), After centrifugation (Quantitative buffy coat) and Flow cytometry.

PROCEDURE FOR THICK AND THIN BLOOD FILMS

The center of a 22x22mm cover slip is placed with a 15-µl droplet of acridine orange (AO) solution (or an 18-µl droplet for a 24x24mm slip). The thin film tip is gently adhered to the AO droplet as the microscope slide is progressively placed onto the cover slip. In order to encourage the spread of the AO solution toward the middle of the thin film, which led to a diminishing concentration gradient of AO stain, the slide and cover slip were kept upside-down and on a little slope (from 5° to 20° from the horizontal) for 1 minute. Thick blood film is treated using the same method [23].

Prior to the staining solution under the cover slip drying out, the AO-stained films need to be viewed right away. At the tip of the thin film, where high AO caused white blood cells (WBCs) to appear as cells with red nuclei and red cytoplasm, each thin film is studied at a magnification of 100x. WBCs can be seen toward the middle of the film as having red cytoplasm and yellow nuclei (or green in the event of UV excitation). A meandering, snake-like search for Plasmodium parasites is conducted in this small, 5mm-wide region using a 400x magnification. In a dim image of a red blood cell, a malaria parasite is seen as a dichromic image (yellow nucleus and red cytoplasm). The ideal viewing zone occasionally extends farther into the thin film's center, where local AO concentrations may be adequate for malaria parasites but too low for WBCs, which have yellow nuclei without red cytoplasm. Paraistaemia is calculated as the number of

parasitized red blood cells (pRBCs) per WBC by counting the number of pRBCs against 100 WBCs. This value is then converted to the number of pRBCs per lμ by assuming 8000 WBCs per 1 μl. The WBC counts in high parasitaemia cases were halted after 5–10 pRBCs were found. After examination, the cover slips will be removed.

QUANTITATIVE BUFFY COAT

Quantitative buffy coat (QBC), is a laboratory test for the detection of parasites that cause malaria and other blood parasites, is based on the centrifugal stratification of blood components [24]. The whole blood separates into plasma, buffy coat, and packed red cell layer during high-speed centrifugation, which is the basis for the QBC technique. The float is automatically positioned inside the buffy coat layer after being buoyed by the packed blood cells. Platelets stay at the top of the buffy coat layer, followed by lymphocytes and monocytes in the center, and granulocytes at the bottom. These blood cells divide into distinct bands based on their densities. The blood is drawn into a QBC capillary tube coated with the fluorescent dye acridine orange, which is then centrifuged to concentrate the fluorescing parasitized erythrocytes into a layer that can be seen under ultraviolet light by fluorescence microscopy at the interface between red blood cells and buffy coat [24].

It could be challenging to manually differentiate between the various types of white blood cells in cases of extremely low white blood cell counts, and it might be nearly impossible to establish an automated differential. In these circumstances, a buffy coat may be obtained by the medical technologist in order to make a blood smear. Compared to whole blood, this smear has a significantly larger concentration of white blood cells.

FLOW CYTOMETRY

A technique called flow cytometry (FCM) provides quick examination of a large number of statistically relevant cells at the single cell level. The usage of flow cytometry has grown throughout time as a result of its ability to quickly analyze a variety of cell properties (both qualitative and quantitative). A particle's size, granularity or internal complexity, and fluorescence intensity can all be determined using this method [25]. An optical-to-electronic coupling method that detects the cells based on laser scattered by the cells is used to ascertain these features. It enables the quick measurement of light scattered and fluorescence emission generated by appropriately illuminated cells (fluorochromes) that bind to particular cell compounds like proteins, nucleic acids, and lipids. They can also be conjugated to antibodies, depend on enzymatic activity, or depend on physiological factors in the cell like pH and membrane potential. In order to combat infection brought on by bacteria, viruses, fungi, and parasites, flow cytometry has been suggested. It can be used to directly detect vital components including nucleic acids and proteins in clinical specimens as well as to isolate bacteria, identify

them, and assess antibodies to a specific parasite or pathogen at various illness stages. It is numerical [26].

It can distinguish between different P. falciparum developmental stages based just on nucleic acid content, without using morphological alterations. High efficiency and precision are provided. The fundamental idea behind this method is based on the light scattering and fluorescence emission that take place when a laser beam strikes cells that are moving through a controlled fluid stream. When a particle deflects incident laser light, light scattering is the result. The degree to which this occurs is determined by a particle's physical characteristics, specifically its size and internal complexity. Hoechst 333258, Acridine orange, Thiazole orange, Hydroethidine, YOYO-1, SYBR Green I, and CD235A are just a few of the stains that are employed. These DNA-targeting dyes can be used simultaneously, and their various target specificities and fluorescence intensities enable improved cellular population separation.

Antibody Staining

Following sample preparation, fluorochrome-conjugated antibodies specific for the surface markers found on various cell types are coated onto the cells. Either direct, indirect, or intracellular staining can be used to accomplish this. In indirect staining, cells are treated with an antibody that has been fluorophore-directly conjugated at 37°C. For instance, 503µl of the staining solution—which contains 1 g/l of dodecyl methyl ammonium chloride and 3 mg/l of acridine orange in 10 mM tricine and 120 mM NaH_2PO_4 at pH 9—are added and allowed to sit for 15 seconds. Following incubation, the stained cell suspension will be picked up by FCM. Green (GF) and red (RF) fluorescence are concurrently measured, and the fluorophore-conjugated secondary antibody recognizes the primary antibody. Antigens that are present inside the cytoplasm or nucleus of a cell can be directly measured using the intracellular staining method. By analyzing scatter grams from the offered computer software, the parasite regions, ring form, trophozoites, and schizont are found [27].

LIMITATIONS

The distribution or location of proteins within cells are not disclosed by this procedure. Also debris accumulates over time, which could lead to inaccurate results. Another limitation is that pre-treatment required for sample preparation and staining takes a lot of time. The pricey procedure of flow cytometry requires personnel who have advanced degrees.

LUMINEX XMAX TECHNOLOGY

Bead-based flow cytometry is a technique used by Luminex technology. It makes use of microsphere beads that, when activated by a laser, give distinctive fluorescent signals that enable the simultaneous detection of several targets [28]. There are up to 100 microspheres that can

form covalent bonds with probes like oligonucleotides, antibodies, or antigens. Using a relatively little volume of reaction, the assay could detect various genotypes of a certain organism or multiple species simultaneously. The diagnosis of parasite illnesses can be done extremely effectively with this method. All four human plasmodium species' blood stages could be detected. Because it does not require gel electrophoresis, and because samples can be handled concurrently and continuously using a 96-well plate format from DNA extraction through automated, uniform data analysis, Luminex technology can increase the speed, accuracy, and reliability of existing PCR procedures [28].

MOLECULAR METHODS FOR MALARIA DIAGNOSIS

The analytical sensitivity of tests for human diseases, including malaria parasites, has grown thanks to molecular diagnostic assays. It uses more than 65 primer sets that can be used to test up to five different species of human malaria, including *P. falciparum*, *P. ovale*, *P. vivax*, *P. malariae*, and *P. knowlesi* [29]. Malaria molecular diagnosis refers to novel laboratory diagnostic methods designed to identify, demonstrate, and characterize the malaria parasite with high sensitivity, high specificity, and minimal subjectivity. Since the publication of the first molecular diagnostic detection methods in the 1980s, there has been significant advancement in the field, including DNA extraction, streamlined diagnostic protocols, specie-specific identification, quantification of parasite loads, and quick processing of large numbers of samples. Among these molecular biological methods are Polymerase Chain Reaction (PCR) (single-step, nested, and real-time), Loop-mediated Isothermal Amplification (LAMP), Microarray, Mass Spectrometry (MS), Real-time SYBR, and Nucleic Acid-based Sequence Amplification technology (SDA).

This test significantly increased parasite detection, the capacity to identify species using species-specific primers or probes, and the ability to precisely quantify parasites in compared to materials with a standard curve [29].

POLYMERASE CHAIN REACTION (PCR) TECHNIQUES

DNA and RNA analysis is done using the advanced polymerase chain reaction method [24]. From a complicated genome, it accomplishes selective amplification [30]. Moreover, the parasite's small subunit 18s ribosomal RNA (rRNA) gene is the target [24].

DETECTION OF *PLASMODIUM FALCIPARUM* DNA IN SALIVA

The OMNIgene® ORAL kit is successful at transporting and preserving malaria parasite DNA in saliva at room temperature, making saliva a potential alternative non-invasive sample for the diagnosis of malaria [31]. Human saliva is easily accessible and is becoming more and more valued as a crucial diagnostic sample. The salivary fluid contains several constitutive and infiltrating electrolytes, proteins, and DNA that have been investigated for the diagnosis and

monitoring of a wide range of disorders, even though more than 98 percent of human saliva is made up of water. For instance, saliva of people with oral cancer has been found to include molecular cancer indicators such p53 mutations and carcinoembryonic antigen [32]. Additionally, saliva-based diagnostic tools for the detection of the HIV and Human Papillomavirus have been created [33]. For the purpose of finding parasites, mobile analytical laboratories and field hospitals have recently created field PCR units and portable real-time PCR systems using freeze-dried reagents.

There have been reports of the malaria parasite HRP-2, lactate dehydrogenase, and P. falciparum DNA being found in infected people's saliva in the field of malaria diagnostics [42]. Although some researchers have also found malaria parasite DNA in urine samples, saliva-based detection of malaria parasite DNA has a much higher sensitivity than pee-based detection [43]. Storage condition of saliva samples from the point of collection can affect DNA stability and PCR sensitivity [44]. Saliva samples can be kept chilled up until DNA extraction from the moment of collection [45]. Although this strategy is sound, it is expensive to maintain and impractical in many isolated and resource-constrained regions. The development of kits for the collection and preservation of microbial nucleic acid has been facilitated by advancements in basic science research. One such kit is the OMNIgene® ORAL saliva collection and microbial DNA stabilization kit, which is intended to preserve the stability of total DNA in saliva at room temperature [46].

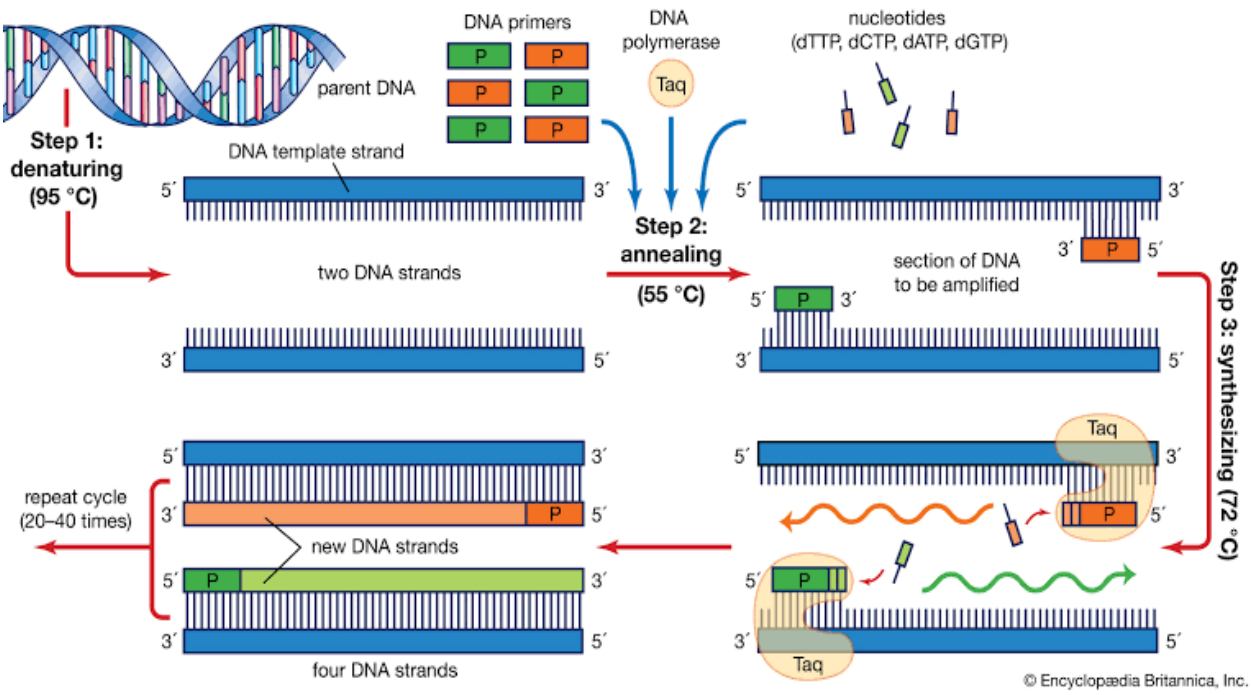


Fig. 3. Schematic diagram of the steps in polymerase chain reaction [53].

LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) TECHNIQUE

LAMP was initially introduced in 2000 [34]. Although previous investigations suggested that *P. vivax, P. ovale, P. malariae,* and *P. knowlesi* were also detectable using this technique, it only detects the conserved 18s RNA gene of *P. falciparum*. This method uses DNA polymerase with strand-displacing activity to achieve DNA amplification under isothermal conditions. It requires four to six different primers that are designed to recognize six to eight specific gene sequences, and during the process, pyrophosphates are formed, causing turbidity that makes visualization easier than with PCR [35]. A Loop-amp real-time turbidimeter can also be used for real-time detection [36]. The fluorescence of LAMP results can also be seen when illuminated by a blue light-emitting diode [37]. Alternatively, by using a microcrystalline wax dye capsule that contains the SYBR green 1 DDNA fluorescent dye [38]. As a molecular diagnostic tool, LAMP has the potential to combine both the effect of high sensitivity with the possibility of performing the test under field condition with limited technical resources and it can be used for Point-of-care testing in both developing and developed countries and also be used as confirmatory assay in place of a PCR-based assay [36]. LAMP has some advantages over PCR in the diagnosis of malaria because it can run the reaction and read the results without opening the test tubes. It also has the potential to be used for clinical diagnosis and infectious disease surveillance in developing countries without the need for specialized equipment or trained personnel. Furthermore, utilizing heat-treated clinical samples, a genus-specific and species-specific diagnostic approach was created, simplifying the DNA extraction procedure and making it suitable for use in the field.

NUCLEIC ACID SEQUENCE BASED AMPLIFICATION (NASBA)

This is a brand-new nucleic acid amplification technique for RNA target detection (mRNA, rRNA and genomic RNA). This area is uniform [34], sensitive, isothermal and transcription-based amplification system that uses three specific enzymes and do not require expensive thermal-cycling equipment. Reverse transcriptase from the avian myeloblastosis virus (AVM-RT), RNase H, and T7 DNA dependent RNA polymerase are some of these enzymes (DdRp) [39]. It makes a lot of RNA copies every cycle, making it possible to produce observable products faster than other amplification methods. The final product is a single-stranded RNA antisense to the original RNA template. NASBA enables for exact determination of the parasite load throughout a range of 20-10^8 parasites per milliliter of blood and has a detection limit of 0.02 parasites per microliter of blood. NASBA is sometimes referred to as Transcription Mediated Amplification and Self-Sustained Sequence Replication (3SR) (TMA).

Compared to microscopy, which has a sensitivity of 100% and a specificity of 94%, NASBA has a sensitivity of 97.5% and a specificity of 81.5% for the detection and semi-quantification of malaria parasites. Quantitate-NASBA (QT-NASBA), which has been used to measure the prevalence and density of plasmodium gametocytes, has the capacity to detect as few as 0.02-0.1

gametocytes per microliter of blood [40], and it is also used increasingly to detect both *P. falciparum* and *P. vivax* gametocytes [41].

MALARIA DIAGNOSIS USING A MOBILE PHONE POLARIZED MICROSCOPE

New techniques for low-cost, highly sensitive diagnosis are crucial, particularly in rural and underdeveloped regions of the world where malaria continues to be a major global health burden.

Here, a low-cost transmission polarized light microscope system based on an optical mobile phone is described for imaging the haemozoin pigment, which is a component of malaria.

Even for experienced microscope workers, distinguishing the pigment from the background and other artifacts can be challenging. Polarized light microscopy makes it considerably simpler to see the pigment. Polarized light microscopy is not yet widely used, however, because the commercially available equipment has complex designs, necessitates extensive maintenance, is frequently big, can be expensive, and would necessitate retraining for current microscopy technicians. In order to do this, a mobile phone-based polarized light microscopy system that is equivalent to bigger bench-top polarized light microscopy systems but significantly less expensive and complex is shown. Both a traditional polarized microscope and a cell-phone based technology are used to demonstrate the identification of malaria in fixed and stained blood smears. In a low-cost, simple-to-use, modular platform, the cell-phone based polarimetric microscopy design demonstrates the ability to have both the resolution and specificity to identify malaria in fixed and stained Giemsa-stained blood smears.

The polarized light cell-phone design that is the subject of this article aims to get around these issues and help doctors in clinics and low-resource settings improve accurate malaria diagnosis with improved detection via a field-based modular polarized microscope. A mobile-optical-polarization imaging device, or MOPID, is an add-on gadget that uses the camera capabilities of smartphones (about 8–20 megapixels) to produce high-resolution photographs of objects 10 times smaller than the thickness of a human hair. The device uses polarized light to take photographs of a blood sample in order to find the malaria parasite haemozoin crystals, which are an accurate indicator of infection and appear as very bright dots in the image. Once the device is connected to the phone, the diagnosis utilizing a phone application only takes a few minutes. In several fields, the amount of parasites and red blood cells is counted to diagnose malaria.

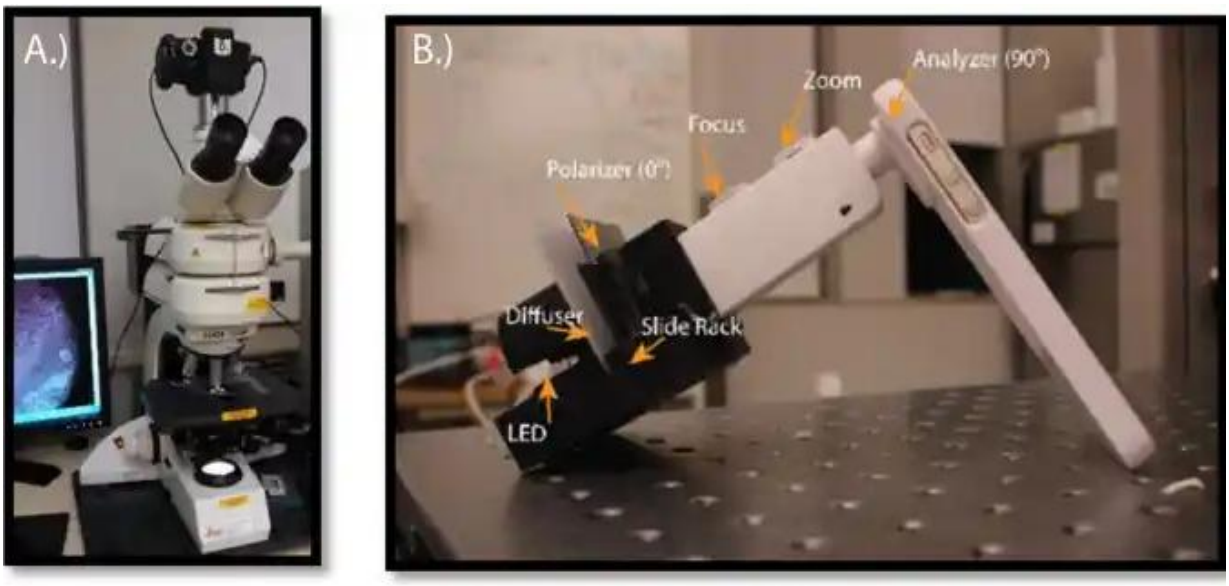


Fig. 4. Pictorial demonstration of malaria diagnosis using a mobile phone polarized microscope. (A) Leica Direct Metal Laser Melting (DMLM) polarized white light microscope used as reference for comparison. And (B) a microscope lens combination implemented into a 3D-printed fitting to allow similar function to a traditional polarized microscope. The Mobile-optical-polarization-imaging device (MOPID) system configured in transmission mode with a magnification designed for 40x when using a mobile phone camera. An iPhone 5s is used with polarizer sheets added and a 3D-printed fitting to hold the light source, diffuser, sample slide and microscope attachment [54].

AUTOMATED BLOOD CELL ANALYZERS

One of the most used laboratory procedures in clinical medicine, the complete blood count (CBC), has a variety of uses, including the assessment of feverish patients who may have malaria.

The majority of these analyzers use radiofrequency conductance, flow cytometry, multiple-angle light scatter, impedance detection (Coulter principle), and nucleic acid fluorescence detection techniques. Anecdotal occurrences of malaria-related changes in the CBC were reported up until the early 1990s. These cases included pseudoreticulocytosis in a Sysmex R-1000 and aberrant additional peaks in the white blood cell (WBC) histograms of an analyzer. This indicated the

possible utility of these so-called "large-unstained cells" for malaria screening with a percentage of >3%. Sadly, changes in WBC populations, reticulocytosis, or an increase in "large-unstained-cells" may also be present with other illnesses, making these changes inaccurate for detecting malaria. The first report demonstrating the ability of a Cell-Dyn® (CD) analyzer to detect malaria pigment in leukocytes with a rather high degree of specificity. This discovery sparked a series of research projects that proved these tools can help with malaria diagnosis.

For WBC analysis, the Cell-Dyn equipment employ so-called multiple-angle polarized scatter separation, which involves laser light scattering at various angles. In addition to identifying haemozoin-containing granulocytes and monocytes, it may distinguish between eosinophils and neutrophils based on the light depolarizing characteristics of their granules.

Detection

Purple-coded signals the presence of monocytes when it appears. Green dots represent granulocytes that carry hemozoin while blue dots represent lymphocytes. *Plasmodium vivax* also detects hemozoin as red spots in infected erythrocytes.

MAGNETO-OPTICAL DETECTION

This requires the use of the Gazelle malaria diagnostic tool, which uses magneto-optical detection to detect the presence of haemozoin crystals (Hz), which are formed by all species of human malaria parasites [47].

Under the influence of strong (~55T) and weak magnetic fields, a beam of polarized light is transmitted through the lysed diluted blood sample. The existence of Hz implies the presence of magnetic fields with high and low fields, which suggests the possibility of malarial infection. It is an in vitro diagnostic equipment with a reader that may be utilized in difficult tropical situations that may be heated (operating temperature range 5°C to 45°C) or high in humidity and runs on either electric or lithium battery power. Using a typical Micro-USB charger, like those for Android phones, the reader may be charged. To save a record of the tests, the reader contains internal storage.

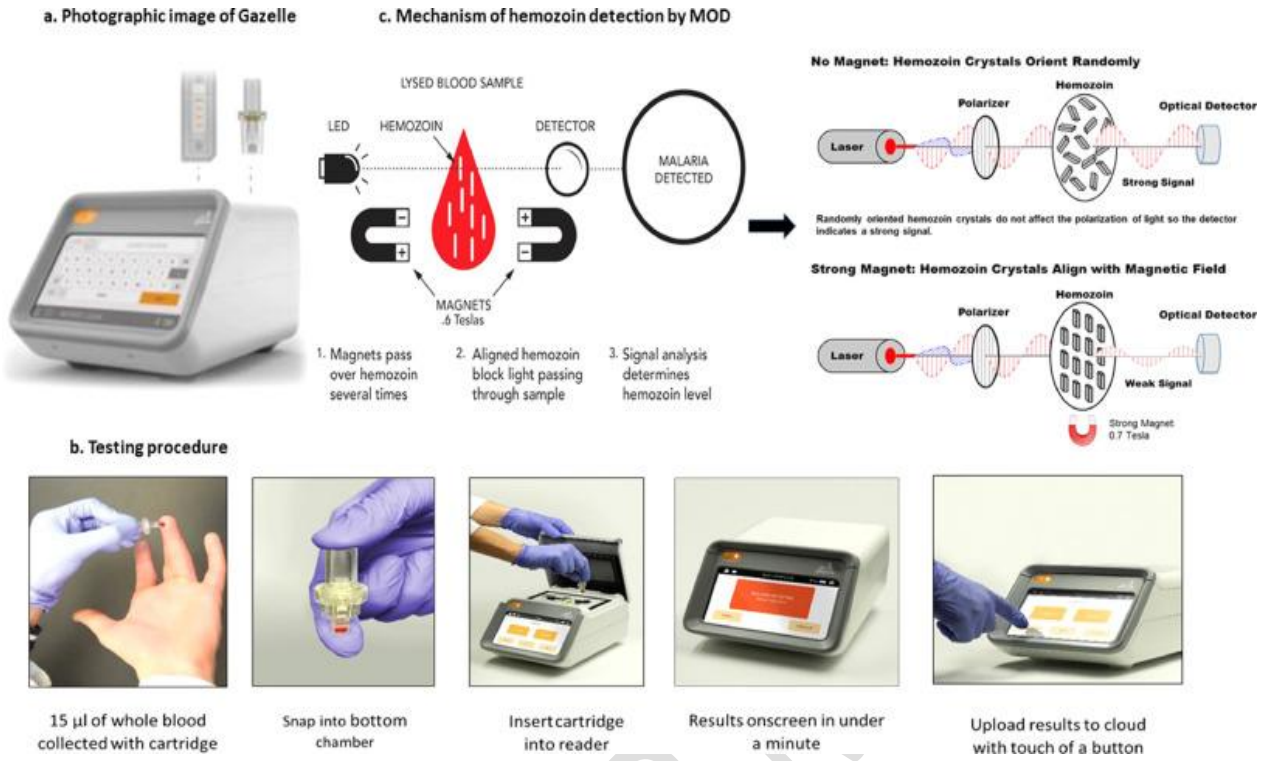


Fig. 5. Pictorial representation of (a) the Gazelle device (b) testing procedure and (c) mechanism of haemozoin detection by magneto detector, [47].

CONTEMPORARY DIAGNOSIS

Malaria screener and MALIVA are two modern techniques utilized in diagnosis.

MALARIA SCREENER

Using a smartphone and a segmented blood smear image, a group of six engineers from the North South University in Bangladesh, India, has created a program that can automatically detect malaria. In situations with low resources, this method may be able to replace the requirement for costly equipment and highly skilled individuals for the detection of malaria. The program (app) may examine the photographs for the presence of malaria parasites if patient samples, or "blood smears," are imaged using a mobile phone and microscope [48]. While maintaining extremely high classification accuracy, the new model developed by Faizullah Fuhad and his coworkers (Faizullah, 2020) uses nearly 4 million times less processing power than the prior model.

To train the model, the researchers used a publicly available dataset containing 27,558 pictures of red blood cells from 150 infected and 50 healthy patients. The pictures were captured using a

smartphone mounted on a regular light microscope. The model's robust performance was then validated on a different public dataset by the developers, who were able to confirm it. The mobile application screens both thin and thick blood smear images for *P. falciparum* parasites using high-resolution cameras and the processing capabilities of contemporary smartphones. Malaria Screener's slide screening procedure involves picture acquisition, smear image analysis, and result visualization. It also has a database to make it simple to access the data that has been acquired. The program can automatically take images of and analyze a patient's thick blood smear by connecting the phone's camera to the microscope's eyepiece.

MALIVA

Maliva is a chewing gum that helps identify certain malaria antigens in saliva. It was developed by UCLA researcher Andrew Fung and his team [49]. The stages in utilizing Maliva are as follows: The user begins by chewing on flavored gum. Gum's sweetness encourages salivation, which contains a variety of antigens and malaria parasite-produced compounds. The saliva will then enter the gum's interior chambers, which contain polystyrene beads and magnetic nanoparticles. Antigens and biomarkers from the saliva will then adhere to the magnetic nanoparticles. Chewing gum is removed and placed on a magnet after a few minutes. A tiny line will appear where the nanoparticles have attached themselves to the malaria biomarkers and antigens.

CONCLUSION

Finally, it can be said that due to the endemic nature of malaria in Nigeria and other developing nations, researchers have been challenged to create novel and efficient methods of diagnosing malaria that go beyond the light microscopy gold standard, which has several drawbacks.

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