Diagnosis of Malaria Parasites *Plasmodium* spp. in Endemic Areas: Current Strategies for an Ancient Disease

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Fast and effective detection of the causative agent of malaria in humans, protozoan *Plasmodium* parasites, is of crucial importance for increasing the effectiveness of treatment and to control a devastating disease that affects millions of people living in endemic areas. The microscopic examination of Giemsa-stained blood films still remains the gold-standard in *Plasmodium* detection today. However, there is a high demand for alternative diagnostic methods that are simple, fast, highly sensitive, ideally do not rely on blood-drawing and can potentially be conducted by the patients themselves. Here, the history of *Plasmodium* detection is discussed, and advantages and disadvantages of diagnostic methods that are currently being applied are assessed.

1. Introduction

1.1. Malaria

Malaria (*mala aria*: “bad air”; a portmanteau word from the 18th century), ague, or swamp fever (frz. *paludisme*) is a mosquito-borne parasitosis that is endemic in 87 countries and causes approximately 219 million clinical cases and 435,000 deaths per year (Figure 1). Morbidity and mortality especially affect children and pregnant women living in the World Health Organization (WHO) African Region.[1,2]

The causative agents of malaria are protozoan parasites of the genus *Plasmodium*. This genus includes more than 200 different species that parasitize various hosts such as reptiles, birds, amphibians, and mammals (53 species total, of which 30 species parasitize primates).[3] To date, six *Plasmodium* species have been identified as human pathogenic, and they cause different types of malaria: *P. malariae* (malaria quartana), *P. ovale* (with two subspecies *P. o. curtisi* and *P. o. wallikeri* causing malaria tertiana), *P. knowlesi* (zoonotic malaria), *P. vivax* (malaria tertiana), and *P. falciparum* (malaria tropica), with the latter two species being responsible for the majority of clinical cases and deaths worldwide.[1,4–6]

1.2. Asexual Replication and Host Erythrocyte Reorganization Can Cause Severe Complications

The life cycle of *Plasmodium* parasites (Figure 2) involves asexual as well as sexual replication that is linked to an obligate host change from a human intermediate host to a female mosquito of the genus *Anopheles* as the final host.[7,8] During this complex life cycle, *Plasmodium* parasites must infect and inhabit multiple cell types to ensure developmental stage progression and progeny production.[7,8] The erythrocytic schizogony, which occurs within the erythrocytes of the human host, involves a sophisticated reorganization of the terminally differentiated and metabolically reduced host cell in the process of securing nutrient supply from hemoglobin digestion as well as blood serum uptake, removal of toxic waste, protection against the host immune response, and life cycle progression.[1,7,9–13]

During schizogony, *P. falciparum* establishes the Maurer’s clefts, a secretory organelle that resides outside the parasite’s cellular boundaries within the erythrocyte cytoplasm, to facilitate host–parasite interaction and sequestration to avoid splenic clearance of the infected erythrocyte.[14] Sequestration is either achieved via cytoadherence of the infected erythrocyte to various receptors presented on the surface of vascular endothelial cells in the postcapillary venules of different organs or via rosetting of infected erythrocytes with uninfected erythrocytes.[15,16] The cytoadherence of *P. falciparum*-parasitized erythrocytes is established by members of the diverse *var* multigene family encoding several different versions of *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which are exclusively expressed in *P. falciparum* and presented on knob-like protrusions on the erythrocyte plasma membrane.[17,18]

The causative agent of zoonotic malaria in Southeast Asia, *P. knowlesi*, also generates a secretory organelle in the erythrocyte cytoplasm called Sinton Mulligan’s clefts that appears to play a crucial role in receiving and harboring proteins produced by the erythrocyte-residing parasite.[19,20] It has been further reported in an in vitro study by Fatih et al. that *P. knowlesi* might be able to establish a cytoadhesive phenotype that would allow attachment of the host erythrocytes to the inducible human endothelial receptors intercellular adhesion molecule-1 (ICAM-1) and vascular cellular adhesion molecule (VCAM).[21] However,
Figure 1. Countries with indigenous cases in 2000 and their status by 2017. Countries with zero indigenous cases over at least the past 3 consecutive years are considered to be malaria free. All countries in the WHO European Region reported zero indigenous cases in 2016 and again in 2017. In 2017, both China and El Salvador reported zero indigenous cases. Source: WHO database. Image published with permission from the WHO.

1.3. The History of the Malaria Parasite and Vector Discovery Sets the Stage for Today's Gold Standard of Malaria Diagnosis

Malaria is a disease that might already have been documented by the cultures of ancient societies, such as those of ancient China, Egypt, India, Mesopotamia, and Greece (Figure 3). The early Greeks, including Homer, Plato, Empedocles, and Hippocrates, were well aware of the characteristic symptoms of malaria such as distinct fevers, splenomegaly, and generally poor health. In the 17th century, these symptoms were already treated with “Jesuits’ powder,” a component of the cinchona tree bark, which is now known as quinine, and is still in use as an antimalarial drug today. However, another century would pass until the first histopathological discovery of the parasites themselves was made by military physician Charles Louis Alphonse Laveran in 1880. By using direct microscopy, Alphonse Laveran was able to identify granules of black pigment in the erythrocytes of a feverish soldier in a military hospital in Constantine (Algeria). Laveran further suspected that mosquitoes were transmitting the disease, but it was Sir Ronald Ross who verified this hypothesis and solved the life cycle mystery by examining birds that were infected with Plasmodium relictum almost two decades later. In 1898, Italian malarologists, among them Camillo Golgi, identified Anopheles mosquitoes as transmitters of human malaria. Camillo Golgi was further able to connect the distinct fever curves with the rupture of blood schizonts and merozoite release. It took another 50 years until Henry Shottt and
Figure 2. Life cycle of human-pathogenic *Plasmodium* parasites. Schizogony and sporogony of human-pathogenic *Plasmodium* parasites occurring between the intermediate host (*Homo sapiens*) and the final host (*Anopheles*), respectively. An infected female *Anopheles* mosquito inoculates *Plasmodium* sporozoites into the skin of the human host during the blood meal. The sporozoites glide away from the inoculation site to reach a blood vessel that quickly carries them to the liver sinusoids. The sporozoites leave the bloodstream, infect the hepatocytes, and develop into liver schizonts. The liver schizonts produce and release thousands of haploid merozoites into the bloodstream after rupture (liver schizogony). *P. vivax* and *P. ovale* can further form a dormant developmental stage (hypnozoites) that is able to persist in the liver, and can cause a malaria relapse even years after the original *Plasmodium* infection. The freshly released liver merozoites are able to infect the erythrocytes of the human host. Within the erythrocytes, the parasite matures from the juvenile ring developmental stage to the mature schizont developmental stage, which consists of various merozoites (erythrocytic schizogony). These merozoites are released into the bloodstream after schizont rupture and invade new erythrocytes. Eventually, some parasites begin their differentiation into sexual developmental stages (gametocytes: female macrogametocytes and male microgametocytes). The sexual developmental stages are then ingested by the next female *Anopheles* mosquito during the blood meal. Within the midgut of the mosquito, macrogamet and microgamet are mating and form a zygote. This zygote develops into an ookinete that penetrates the midgut wall, where it further develops into an oocyst. The oocyst then produces sporozoites, which are released after oocyst rupture and invade the salivary glands of the mosquito. The sporozoites that have been produced during this sporogony can then again be inoculated into the human intermediate host during the next blood meal via the secretion of the mosquito’s anticoagulant saliva.
Cyril Garnham discovered that a pre-erythrocytic liver schizogony has to occur before the schizogony in the erythrocytes can take place. In 1982, Wojciech Krotoski observed dormant hypnozoite stages that were being produced in the liver of the human host by *P. vivax* (as well as *P. ovale*) and caused relapses of malaria tertiana.

2. Diagnosis of *Plasmodium* Parasites Today: Old but Gold versus New and Fast

The detection of *Plasmodium* parasites replicating in the erythrocytes of the human host is challenging because the parasite density of a patient can range from below 1 parasite/µL to tens of thousands of parasites per microliter. Parasite densities are a result of pronounced age patterns and reflect lifetime exposure as well as naturally acquired immunity at a population level. Any given diagnostic tool must be able to provide a fast and accurate result to allow an appropriate treatment of the patient.

2.1. Clinical Diagnosis

Clinical diagnosis is solely based on the symptoms that a patient displays. It is used as a diagnostic method when laboratory facilities are not available or when the patient is performing self-diagnosis at home. Several tropical diseases cause symptoms that are similar to malaria. Self-diagnosis as well as self-treatment are error-prone procedures that can lead to overdiagnosis and overtreatment. Clinical suspicion of malaria should always be tested at a hospital by applying the diagnostic tools discussed in the following paragraphs.

2.2. Microscopy of Thick and Thin Blood Films

The microscopic examination of Giemsa-stained blood films remains the gold standard method to detect *Plasmodium* parasites in the erythrocytes of patients, although devices that automatically analyze the blood of the patients are currently being developed and tested. Automatic slide reading approaches or vision-based devices have been developed and tested as well, but the manual microscopic examination conducted by trained personnel can still be seen as the preferred approach when it comes to thin or thick blood film analysis.

Regardless, proper diagnosis of malaria and the examination of *Plasmodium* parasites are still challenging almost over 140 years after the discovery of the parasites by Alphonse Laveran. In 1880, Laveran was relying on direct microscopy while he examined the unstained samples of his patients until he discovered parasite-derived changes in the infected erythrocytes. The examination of the blood samples was simplified after the development of various methylene blue-based stains by Paul Ehrlich. With these newly developed stains, it was discovered that human malaria is not caused by one *Plasmodium* species, which was originally hypothesized by Alphonse Laveran, but by various *Plasmodium* species.

In 1891, Dimitry Romanowsky further extended and modified the original staining protocol. By adding eosin to methylene blue, he was able to differentially stain the nucleus and...
the cytoplasm of the Plasmodium parasites replicating within the erythrocytes.\(^{[52]}\) However, preparation and reproducibility of the staining was difficult because of the required aging process of the methylene blue.\(^{[53]}\) After the proposal and testing of various additional modifications by numerous contemporary scientists during that time, Bernhard Nocht and Gustav Giemsa strove to improve the Romanowsky staining.\(^{[53]}\) Bernhard Nocht had discovered that aging of methylene blue leads to the formation of a new dye (“red from methylene blue”) that could stain the chromatin of Plasmodium parasites.\(^{[53]}\) Gustav Giemsa was further able to improve this new staining by identifying the newly discovered dye as azure B and determining that both eosin and an excess of Azure B needed to be dissolved in a mixture of glycerol and methanol to achieve the desired stability as well as reproducibility.\(^{[54]}\) Gustav Giemsa’s stain remains, 115 years after its first publication in 1904, the method of choice for the staining of Plasmodium parasites.

The gold standard for malaria diagnosis is the routinely used microscopic examination of thick and thin blood films stained with Giemsa’s stain.\(^{[55,56]}\) To create a thick blood film, a drop of peripheral blood is removed from the patient’s finger, applied on a glass slide, and laked before or during the subsequent staining with Giemsa’s stain, which causes the rupture of the erythrocytes and allows only the visualization of leukocytes, platelets, and parasites. For the thin blood film, the patient’s peripheral blood is spread on a larger area of the glass slide. The smear is then briefly fixed in methanol and, after a brief drying period, stained in Giemsa’s stain. The additional fixation step performed during the preparation of the thin blood film leaves the infected erythrocytes intact. While thick blood films are generated to detect the presence of Plasmodium parasites and to determine the parasitemia, thin blood smears are used to determine the Plasmodium species responsible for the infection and the developmental stages that are currently circulating within the blood of the patient. Thick and thin blood films allow a comparatively fast preparation and examination of the patient’s blood.

However, besides their obvious advantages, both techniques also have distinct disadvantages. A trained expert is required for both techniques to perform the entire procedure from the finger prick to the final microscopic analyses. An untrained examiner might, for example, confuse the ring stage Plasmodium parasites with the ring stages of another protozoan parasite, Babesia, or vice versa.\(^{[57]}\) The correct identification of the Plasmodium species and the determination of the parasitemia are therefore crucial to define the appropriate treatment, to exclude the occurrence of complications due to a severe infection, and to ensure the survival of the patient. Thin blood films are further less sensitive than thick blood films if the parasitemia is low, but in vitro work showed that even in the hands of a trained expert, thick blood films prepared from malaria cultures at known parasitemia consistently underestimate parasite densities.\(^{[56]}\) This might be due to a large number of parasites being lost during staining, which limits the sensitivity of the method and leads to wrong estimates of parasite density.\(^{[56]}\) Another problem is the detection of the low-density asymptomatic submicroscopic malaria (SMM) infections during the dry season in the endemic areas.\(^{[58-60]}\) A lack of breeding sites for the Anopheles mosquitoes can lead to less frequent transmission occurrences. However, many patients can still be infected without displaying any symptoms, and they can act as silent reservoirs by maintaining low-level residual malaria transmission in the community.\(^{[59]}\) Detecting SMM infections is therefore just as important as detecting infections that cause a high parasitemia.

Although light microscopy of Giemsa-stained thick and thin blood films remains the gold standard in the detection of Plasmodium parasites, there is a high demand for more sensitive and automated microscopy techniques to provide the proper determination of the parasitemia of the parasite in the patient’s blood and the Plasmodium species that is causing the infection.

The world health technology (WHT) autoanalyzer is an automated malaria slide scanning system and was one of the first devices that was tested and performed at a level comparable to many human slide readers in a study conducted in 2012.\(^{[61]}\) Prescott et al. acknowledged that minimal additional equipment is needed to examine blood films that have been stained with standard procedures and that the device is able to perform with an estimated limit of detection of 140 parasites µL\(^{-1}.\)\(^{[61,62]}\)

A Global Good Fund prototype was tested in 2017 and showed an estimated limit of detection of 100 parasites µL\(^{-1}\) for P. falciparum while scanning 0.2 µL of blood.\(^{[62]}\) A more recent approach uses a prototype digital microscope device, the Autoscope, that employs an automated microscopy algorithm based on machine-learning to simplify the Plasmodium species detection as well as the parasitemia determination.\(^{[63]}\) The performance of the device was comparable to routine microscopy when the slides contained an adequate volume of blood to meet the preset design assumptions.\(^{[64]}\) However, Autoscope and trained personnel both missed the same low-parasitemia slides that were determined positive via polymerase chain reaction (PCR).\(^{[63]}\)

CellaVision DM96 is another system that digitally determines the cell morphology and is able to automatically classify leukocytes, as well as different forms of erythrocytes such as parasitized erythrocytes, on blood films using its advanced red blood cell application (ARBCA).\(^{[64]}\) Florin et al. examined the specificity of the CellaVision DM96 and compared it to standard light microscopy.\(^{[64]}\) The device generally displayed a low sensitivity, but showed a good correlation with microscopy paired with short turnaround times, which might be applicable in follow-up examinations to determine the parasitemia after initial diagnosis and treatment.\(^{[64]}\)

Another commercialized system, the X-rapid system, has been exclusively developed for smartphone microscopy and provides a lens attachment as well as a light-emitting diode (LED) attachment to allow the examination of erythrocytes.\(^{[64]}\) Future studies still need to be conducted to see whether this system can be used for routine malaria diagnostic procedures.

### 2.3. Microscopic Examination of Fluorescently Stained Plasmodium Parasites

Fluorescent labeling followed by microscopic assessment of Plasmodium parasites is another applied technique in diagnostic procedures. Usually, the blood of the patients is incubated with acridine orange that immediately stains DNA and RNA of the different developmental stages of Plasmodium parasites.\(^{[65,66]}\) The fluorescent parasites are then imaged using either a conventional fluorescence microscope, a halogen lamp plus interference filter...
system or a fluorescence microscope based on LED.[66,67] The latter approach has the advantage that it can be used in field applications because of decreased energy consumption, stronger brightness, and lower costs.[66,67] A recent study by Kimura et al. showed that this approach produces superior results relative to the Giemsa method with a correctly performed acridine orange staining protocol.[66,67] Although this is a feasible method leading to fast diagnostic results, trained personnel are needed to correctly label the blood sample of the patient and to perform the analysis properly with the fluorescent microscope.

### 2.4. Molecular Approaches to Diagnose Malaria via Plasmodium-Specific Genes

The PCR was developed in 1983 by Kary Mullis, who won the Nobel Prize for this work 10 years later (Figure 2).[68,69] The PCR uses the properties of thermostable DNA polymerases of bacterial origin to amplify even small DNA fragments of interest by using distinct temperature changes and exposure.

To detect different species of Plasmodium parasites replicating in the erythrocytes of the patients, multiple PCR approaches such as nested PCR (nPCR) or semi-nested multiplex PCR (SnM-PCR) can be applied.[70–73] nPCR and SnM-PCR are defined by two consecutive amplification rounds with a second set of primer and the previously amplified DNA fragment or fragments representing the new template. The primer selection depends on the copy number of the targeted gene, the amount of expected Plasmodium-infected erythrocytes, and the body fluid that is being examined.[72,74,75] For example, the small-subunit 18S rRNA gene and the highly conserved pfk13 encoding the Kelch13 protein, can be targeted when fresh or even dried patient blood samples are examined.[71,76–78] A recent publication by Lloyd et al. probed for cox3 and varATS genes (encoding for the cytochrome c oxidase subunit 3 and the var gene acidic terminal sequence, respectively) when looking for *P. falciparum* parasites in the saliva of the patients.[72]

The PCR approach represents the most sensitive method for the diagnosis of Plasmodium parasites because it is able to detect submicroscopic parasitemias that are usually missed by other diagnostic approaches.[79]

The application of various PCR approaches is further of particular importance when epidemiological data are needed for monitoring malaria control and transmission during different seasons, general mass screening and elimination interventions.[79]

Despite the clear advantages of the PCR approaches, the experimental setup can be expensive because of the required material. Another factor is the time necessary for the preparation of the samples, setup of the reaction (storage of the reagents), time until the thermocycler has completed the reaction, and the subsequent analyses of the results. A trained expert is further needed to setup and to conduct the experiment, to interpret the results, and to perform troubleshooting in case the chosen PCR approach does not work. A PCR-based diagnosis can fail when parasites have genetically diverse sequences at the target region of the primers or when the copy number of the target gene is low, which results in a lower amplification efficiency that will reduce the sensitivity.[80,81]

However, various quantitative PCR (qPCR) assays have been developed to reliably detect *Plasmodium* parasites in clinical patients and even in asymptomatic samples.[84] Recently evaluated diagnostic real-time qPCR, comprising a portable device and the Q3-Plus silicone chip, has been developed to avoid the use of bulky thermocyclers, temperature-controlled transportation, and storage of the necessary reagents.[82] However, absolute quantification of parasites by qPCR is challenging because a standard curve must be established, which can vary between laboratories.[84] Droplet digital PCR (ddPCR) allows accurate and absolute quantification by counting DNA molecules encapsulated in approximately 15 000 discrete, volumetrically defined, water-in-oil droplet partitions that are subjected to endpoint PCR.[84,85]

Loop-mediated isothermal amplification (LAMP) might be an alternative to the various PCR approaches, because the reaction can simply be conducted in one tube at a constant temperature, and it does not need a thermal cycler, which is particularly important for a fast malaria diagnosis in remote areas.[85] LAMP is also a sensitive method that is able to detect placental malaria in the peripheral blood of patients.[86]

### 2.5. Rapid Diagnostic Tests: An Alternative for Fast Malaria Diagnosis

In areas where microscopy of thin and thick blood films or other approaches to detect *Plasmodium* parasites in the blood of patients cannot be provided, antigen-based rapid diagnostic tests (RDTs) can be an important alternative for an easy and fast diagnosis that should not take longer than 15 min.[66,86] In the last decade, the amount of available RDTs and the frequency of their use in epidemic investigations, as well as surveys, have rapidly increased (Figure 2).[86] RDTs can also be a useful tool of malaria self-diagnosis when applied by tourists traveling in endemic areas.[87]

Currently available RDTs are, for example, able to detect histidine-rich protein 2 (HRP2), *Plasmodium* lactate dehydrogenase (pLDH), and aldolase and provide the patient with a qualitative, but not a quantitative, result.[33,88,89] It has previously been suggested that cytoadherent biomass sequestered in the vascular bed of the inner organs can be estimated from HRP2 concentrations in the blood plasma.[33,89] However, a high rate of false positives was recently reported in high-transmission areas on account of the persistent antigenicity of HRP2 even weeks after the infection.[33,89] Aldolase tests have been shown to be less sensitive when the patient is not infected with *P. falciparum* but with another *Plasmodium* species.[15] pLDH test can also vary in performance or even fail, depending on the *Plasmodium* species the patient is infected with.[90] *Plasmodium* glutamate dehydrogenase (pGDH) is another potential RDT candidate, because the homohexameric protein is being produced by the parasites throughout the entire schizogony, while being absent from host erythrocytes. However, there is a high similarity between the glutamate dehydrogenase produced by the different *Plasmodium* species, which can cause cross reaction in areas with mixed *Plasmodium* species infections.[92,93] Some studies further reported the patient serum concentration of the protein lies usually in nanomolar range.[93,94] Therefore, further tests are
needed to determine whether pGDH-based RDTs can be applied to detect *Plasmodium* parasites in endemic areas.\[93\]

Another problematic point is that RDTs might give positive results in febrile patients whose fevers are not caused by *Plasmodium* parasites.\[47\] Quality-assured RDTs can be important to confirm malaria cases, while the cause of fever in the non-malaria patients should be followed up and managed appropriately.\[46\]

A newly developed, highly sensitive RDT (HiS-RDT) that has recently been tested for its ability to diagnose malaria in pregnancy in a low-transmission setting might be a good strategy to better detect *Plasmodium* parasites in the future.\[95\]

2.6. Serological Detection of *Plasmodium* Parasites Using Serum from Malaria Patients

The immunofluorescence antibody test (IFAT) has been a reliable serological test for the presence of *Plasmodium* parasites.\[106\] This approach can be used to detect *Plasmodium*-specific antibodies in epidemiological surveys and in the screening procedures of potential blood donors.\[96,97\] *Plasmodium* antigen is prepared on a slide and stored at −30 °C until patient serum is applied, and the amount of immunoglobulin G and M antibodies can be quantified using fluorescence microscopy.\[96\]

A similar method for the detection of *Plasmodium*-specific antibodies in the blood of patients is the enzyme-linked immunosorbent assay (ELISA) that uses different antigens from different *Plasmodium* species for antibody detection using a 96-well plate and an appropriate plate reader.\[97\] Although both approaches are relatively simple and moderately sensitive, they are very time consuming, and trained personnel are also needed to conduct the experiments and analyze the results.

2.7. Flow Cytometric Approaches to Identify Infected Erythrocytes

Flow cytometry was initially developed in 1950s and is today used for the laser-based sorting and measurement of various parameters of several thousand cells per second in a rapidly flowing fluid stream.\[78\] Various studies reported the successful detection of *Plasmodium* species in different flow cytometry assays using different devices over the years.\[99–104\] The Sysmex hematology analyzers (Sysmex Corporation, Kobe, Japan) are promising adjunctive diagnostic tools to facilitate a fast detection of malaria cases in a fluorescence flow cytometry setup by counting and differentiating between cell types and *Plasmodium* species.\[100\]

Another promising approach in the detection of *Plasmodium* parasites is the noninvasive in vivo photoacoustic flow cytometry (PAFC) that irradiates circulating erythrocytes directly in peripheral vessels through the skin by using focused linear laser beams as well as laser-induced photoacoustic waves or fluorescence light that are detected with ultrasound transducers and photodetectors, respectively.\[105\] PAFC shows greater sensitivity than conventional flow cytometry and allows the examination of an almost entire blood volume.\[105\]

Flow cytometry is a very sophisticated approach to accurately detect *Plasmodium* parasites, but the equipment can be expensive and might need to be operated and carefully maintained by trained personnel to ensure an appropriate diagnosis.

2.8. Microfluidic Devices: Small but Powerful Tools for Malaria Diagnosis

Microfluidic devices are portable, easy-to-use, self-contained, and low-cost diagnostic devices that allow the precise manipulation of small sample volumes while reducing reagent consumption, which is crucial for malaria endemic areas with limited access to healthcare.\[106–109\] One of the first microfluidic approaches in an effort to diagnose malaria was conducted by Hou et al. in 2010, who used distinct changes of erythrocyte plasma membrane stiffness to detect *Plasmodium* parasites in the erythrocytes of the human host.\[110\] In 2016, Xu et al. used origami paper folding for sequential steps of DNA extraction followed by LAMP and a fluorescence readout to detect *Plasmodium* parasites in finger prick blood samples within 45 min.\[111\] A recent study conducted in Uganda by Reboud et al. again applied a paper-based microfluidic device that combined the vertical sample-processing steps of the origami device with a microfluidic lateral flow LAMP amplification and a simple visualization system for multiplexed DNA-based malaria diagnosis from finger prick blood samples.\[108\] This approach showed a higher sensitivity than microscopy and RDTs and is currently being evaluated by the Uganda Vector Control Division, Ministry of Health, for potential usage in areas without centralized facilities.\[108\]

2.9. Aptamer-Mediated *Plasmodium*-Specific Diagnosis of Malaria

Aptamers or “chemical antibodies” are short, single-stranded oligonucleotides such as DNA, RNA, or synthetic xeno nucleic acids molecules, obtained by in vitro evolution techniques and defined by high affinity as well as specificity to interact with any desired corresponding target by folding into distinct tertiary structures.\[112–115\] Since the invention of the process of aptamer selection, called “systematic evolution of ligands by exponential enrichment” (SELEX) in the early 1990s by Tuerk and Gold, great efforts have been made to make the application clinically relevant for various diseases such as macular degeneration, cancer, thrombosis, or inflammatory diseases.\[115\] The diagnostic application of aptamers can be advantageous because the oligonucleotides are cheap and easy to synthetize, have a high stability in harsh environmental conditions, and can be stored without functional degradation.\[114\] The selection for the ideal aptamer candidate usually begins with a randomized oligonucleotide library that is incubated with the target molecule followed by affinity selection rounds and PCR amplification steps to determine the sequence with the highest affinity.\[115\] Several aptamers have already been commercialized to date.\[115\] So far, various aptamer target proteins of *Plasmodium* parasites such as pLDH, the high mobility group Box 1 protein (HMGB1), PfEMP1, and pGDH have been tested.\[112,116–122\] The future will tell whether the chemical flexibility of aptamers can further be exploited for binding optimization and stability in blood, plasma, or samples, but...
aptamers represent a very promising approach for malaria diagnosis.\[117\]

### 2.10. Bloodless Malaria Diagnosis: Examining Bodily Fluids and Feces for Parasite Detection

Drawing blood for diagnostic purposes can be challenging in endemic areas. Bodily fluids such as saliva and urine or fecal matter represent a good alternative because they can be noninvasively obtained and tested for the presence of *Plasmodium* parasites using different markers and technical approaches such as PCR, nPCR, lateral flow immunoassay, microfluidics with DNA sensor substrate, and immunochromatography.\[80,132–137\] The successful detection of *Plasmodium* DNA or proteins depends on their concentration in the bodily fluid chosen for detection. A recent study conducted in 2017 by Oyibo et al. reported that the urine malaria test (UMT) developed by Fyodor Biotechnologies, Inc. (Baltimore, MD, USA) is the only nonblood malaria test that has undergone a full-scale premarket evaluation trial.\[138\]

### 2.11. Raman Spectroscopy to Identify *Plasmodium*-Infected Erythrocytes via Different Parasite and Host Erythrocyte Parameters

Raman spectroscopy measures the wavelength (or wavelength shift) as well as the intensity of inelastically scattered light (Raman effect) that has been emitted from a molecule in a liquid, solid, or gas due to interactions of the incident light with the molecule’s vibrational energies or signature, which allows substance identification and the observation of molecular changes.\[139–141\] In malaria diagnosis, surface-enhanced Raman spectroscopy (SERS) is, for example, applied to detect the malaria pigment hemozoin, an iron-containing paramagnetic byproduct of the hemoglobin digestion (see below) during asexual replication, by using silver nanoparticles that enhance the hemozoin Raman signal by 10^6 to 10^7 folds.\[142\] Hemozoin has already been the selected molecule for Raman spectroscopy-based *Plasmodium* detection for quite a few years.\[143\]

Another previously published SERS approach has shown that different stages of *Plasmodium*-infected erythrocytes, in particular the SERS spectra from ring stage-infected erythrocytes, differ from those of uninfected erythrocytes and erythrocytes infected with later developmental stages of the parasite.\[144\]

Raman spectroscopy is a sophisticated method that has the potential for a very sensitive detection of *Plasmodium* infection in malaria patients. However, like standard medical instruments, the clinical Raman system should be small in size, easy to transport with a robust calibration and a high sensitivity among other crucial parameters to become a successful tool in malaria diagnosis.\[145\]

### 2.12. Hemozoin-Based Detection of *Plasmodium* Parasites

*Plasmodium* parasites are not able to digest the toxic heme that remains from the digestion of hemoglobin.\[157\] Heme is stored in a metabolically crystallized form called malaria pigment or hemozoin.\[157\] Hemozoin does not only serve as heme storage, but also represents a potent inhibitor of immune cells.\[146\] Therefore, hemozoin is an important molecule that can serve as a marker for malaria diagnosis. Besides the above-mentioned Ra-
man spectroscopy, hemozoin is being investigated as a potential marker for several different malaria diagnosis approaches such as magneto-optical detection, laser desorption-time of flight (LD-TOF) mass spectrometry, hemozoin-catalyzed precipitation, magnetic purification, etc.[147–154]

Interestingly, hemozoin is also being produced by other parasites that feed on blood such as Schistosoma mansoni, Haemoproteus columbae, and the vector of the Chagas parasite, Rhodnius prolixus.[155] These hemozoin molecules also show a similar structural identity, which is advantageous concerning treatment, but might potentially be challenging for diagnosis in case of a co-infection.[155] Hemozoin production is further a hallmark of older developmental stages of Plasmodium parasites that, in case of P. falciparum, are able to sequester in a specific organ and can cause a local hemozoin accumulation there.[15,156] These sequestered parasites do not circulate in the peripheral blood of the patients and remain undetected while juvenile ring stages, that have not yet produced a significant amount of hemozoin, are also not considered during a hemozoin-based diagnostic procedure.[157]

3. Conclusions and Outlook

Malaria is a curable disease: If you know you are infected.

Many efforts are being made to understand the biology of Plasmodium parasites and Anopheles mosquitoes to fight and eradicate a disease that has strongly shaped human evolution and history. Early, fast, and accurate diagnosis of Plasmodium parasites is crucial for disease management and to avoid suboptimal care that can lead to presumptive antimalarial over- or undertreatment, causing the emergence of parasite drug resistance.[46,79] All presented diagnostic methods have advantages and disadvantages and it is hard to determine which method will be the most promising in the future.

The question is whether there will ever be a “perfect” method that consists of a device that is cheap to produce and easy to handle while providing sufficient sensitivity. This ideal device should also be affordable for patients or hospitals, noninvasive, portable, and with a potential to be applied at the patient’s home, given that people living in rural areas might be too sick or do not have time or money to travel to a hospital. The journey towards simplifying malaria diagnosis continues, and for now we have to use the available methods. However, more innovative ideas are coming to the forefront in a bid to improve malaria diagnosis, as we have reviewed here.

Another important point is the documentation of malaria cases worldwide. This could be achieved with a platform that allows tracking of the geographical distribution and evolution of cases to improve general disease surveillance and facilitate result exchange among researchers and physicians in endemic areas.

Time will tell whether the ultimate malaria test can be developed and whether the observation of clinical cases will help physicians and researchers monitor the current situation better and provide adequate help and care.

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Conflict of Interest

Brian Gitta is the C.E.O. of Matibabu and discloses financial interest. Nicole Kilian declares no conflict of interest.

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