

Laboratory diagnostics of malaria

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Abstract. Even now, malaria treatment should only be administered after laboratory confirmation. There are several principal methods for diagnosing malaria. All these methods have their disadvantages. Presumptive treatment of malaria is widely practiced where laboratory tests are not readily available. Microscopy of Giemsa-stained thick and thin blood films remains the gold standard for the diagnosis of malaria infection. The technique of slide preparation, staining and reading are well known and standardized, and so is the estimate of the parasite density and parasite stages. Microscopy is not always available or feasible at primary health services in limited resource settings due to cost, lack of skilled manpower, accessories and reagents required. Rapid diagnostic tests (RDTs) are potential tools for parasite-based diagnosis since the tests are accurate in detecting malaria infections and are easy to use. The test is based on the capture of parasite antigen that released from parasitized red blood cells using monoclonal antibodies prepared against malaria antigen target. Polymerase Chain Reaction (PCR), depend on DNA amplification approaches and have higher sensitivity than microscopy. PCR it is not widely used due to the lack of a standardized methodology, high costs, and the need for highly-trained staff.

1. Introduction

Until nowadays, malaria continues to be a major health problem in the tropical and temperate regions of the world. Malaria is caused by *Plasmodium* parasites that infect humans through the bites of an infected female mosquito of the genus *Anopheles* and destroys red blood cells. Of the four known human malaria parasites, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale*. The first two species cause the most infections worldwide¹. Meanwhile, *Plasmodium falciparum* is the most pathogenic and frequently fatal if not promptly given treatment².

The effort to eradicate malaria is based on comprehensive interventions, including strategies to diagnosis the disease. In malaria-endemic regions, many patients are presumptively treated for malaria on the basis of symptoms alone (fever), although malaria is not the only cause of fever³. Presumptive treatment of fever with antimalarials is widely practiced to reduce malaria-attributable morbidity, and mortality, especially at the primary health, cares where microscopy is not available⁴.

Overdiagnosis of malaria implies under-diagnosis and inappropriate treatment of nonmalarial febrile illness while a high proportion of such illnesses are self-limiting diseases and other diseases. The extensive deployment of anti-malarial drugs has provided strong selective pressure on human malaria parasites to evolve mechanisms of resistance and the increased cost of treatments⁵. World Health Organization (WHO) is trying to eliminate presumptive drug treatment of malaria and currently makes the tentative recommendation that parasite-based diagnosis should be used in all cases. All suspected malaria cases are confirmed with a parasite-based diagnostic test prior to therapy



unless parasitologic diagnostic capabilities are not available⁶. Early and accurate diagnosis of malaria is needed in improving the effectiveness of malaria management. There are four principal methods for diagnosing malaria, such as symptomatic (clinical manifestation), microscopy, antigen test (RDTs) and molecular methods (PCR). All of these techniques are reviewed below.

2. Clinical diagnostic

Diagnosing malaria is mostly done by clinical diagnosis when laboratory facilities are not available everywhere in the country. Meanwhile, in other areas, symptomatic diagnosis also often used, but followed by one of the other methods. The clinical diagnosis of malaria depends almost entirely upon the competence of the examiner. A diagnosis of clinical malaria was based on fever or history of fever. The typical symptoms of malaria (fever, chills, headache, nausea, vomiting, muscle aches, malaise, etc.) are not generally typical⁷ and diagnosis by symptoms alone can mislead and even harmful. Even if the examiner happens to be extraordinarily good at identifying malaria, the clinical diagnosis comes with a very important pitfall. The accuracy of the clinical approach to diagnosing malaria is not sufficient for deciding on offering anti-malarial drugs.

Tabitha (2005) used clinical algorithms to reduce the use of drugs that are not on target⁸. However, algorithms for diagnosing malaria are site-specific, and thus the algorithms have to be field tested before recommending their use for these purposes. The poor performance of clinical algorithms in identifying children in need of anti-malarial treatment is in line with the review by Chandramohan (2002), who suggested that their use would lead to drug wastage in areas of low endemicity and an increased failure to treat in those of high endemicity⁹. Presumptive treatment of all fevers with anti-malarial would no more seem a logical and affordable choice. For this reason, the WHO policy changed, recommending restricting antimalarial treatment to laboratory-confirmed cases⁶.

Presumptive treatment of all fevers with anti-malarial would no more seem a logical and affordable choice, not only for economic reasons but also because it is feared that a massive use of the new drugs might induce selection and diffusion of artemisinin-resistant. For this reason, the WHO policy changed, recommending restricting antimalarial treatment to laboratory-confirmed cases⁶. Ideally, the decision to use an anti-malarial drug should be based on a definitive demonstration of parasites in the peripheral blood film.

3. Microscopic diagnostic

The standard method for diagnosing malaria is through microscopy. However, this form of diagnosis is not available or affordable in most of the peripheral health facilities. Simple light microscopy examinations of Giemsa-stained blood films are the most widely practiced method. Microscopic examination has many advantages, including accuracy, availability, low cost, and ability to quantify parasites and monitor parasite clearance. The others advantages are differentiation between species, and ability to distinguish asexual parasite stages from gametocytes¹⁰.

The blood may be prepared in two ways, the so-called thick blood smear, and the thin blood smear. For a thick blood smear, a drop (approximately 6–10 μL) is spread on a clean, dry microscope slide. After drying, the thick blood smear is stained with a Giemsa stain. During the 10–60 minutes of staining in this aqueous medium, the erythrocytes lyse because of osmotic swelling, leaving the leukocytes and parasites, if present, largely (but not entirely) intact¹⁰. The advantage of lysing erythrocytes is that more blood may be examined. The reported limit of detection for a thick blood smear is 4–20 parasites/ μL ¹¹, but this limit is higher (50–100 parasites/ μL) under field conditions with many factors contributing variation and reduced sensitivity as recently summarized¹².

The parasite density is the number of malaria parasites in the circulation, which is usually expressed per μL (or as a percentage of infected RBC) and is usually estimated by microscopy by examining the thick film until we have counted 200 WBC, and all the infected RBC that we detect in the same microscopic fields. This is a rough estimate, of course, moreover in severe infection schizonts tend to be sequestered in the spleen, the liver, the brain and other internal organs and therefore a very high parasite burden may not be mirrored in the peripheral circulation¹³.

The disadvantages of thick blood smears are that the parasites are not viewed in situ within the erythrocyte, are bunched up and less morphologically recognizable, may be hidden behind or above leukocytes, and may be more easily confused with the artifact. If a smaller volume (approximately 2 mL) of blood is spread into a monolayer in the preparation of a traditional blood smear and briefly immersed in methanol, the erythrocytes are fixed and will not subsequently lyse during staining³.

The resulting preparation is commonly referred to as a thin blood smear. Although the thin blood smear limits the examination to less blood, it provides the microscopist the ability to discern morphologic details of the parasite within an erythrocyte and facilitates parasite species identification. Many, if not most, diagnosticians, therefore, combine the use of both smears, thereby optimizing the probability of finding a parasite by using the thick blood smear and identifying it by using the thin blood smear. Species identification and quantification may be clinically important³.

When using blood smears, parasites at lower densities may be missed by microscopy for a variety of reasons. The variation in results among unskilled slide readers can be high, particularly at low parasitemias. Manual leukocyte counts are used as the multiplier in the calculation of parasitemia in thick blood smears, and either leukocyte or erythrocyte counts can be used for thin blood smear quantitation, depending on the method¹⁴.

Despite the advantages, routine microscopy is known to be of low quality due to poor training, the low skill level of laboratory staff, poor infrastructure, the inadequacy of equipment and reagents and unreliable electricity. However, good quality of microscopy is difficult to implement and maintain. It is labor intensive and requires highly skilled personnel and regular quality control. In many malaria-endemic settings, experience alone has often been relied upon to qualify technicians³.

4. Rapid Diagnostic Test (RDTs)

The use of malaria rapid diagnostic tests (RDTs) is recommended by WHO when reliable microscopy is not available. RDTs identify *Plasmodium* antigens using immunochromatographic techniques. The test is based on the capture of parasite antigen from the peripheral blood using monoclonal antibodies prepared against malaria antigen target and conjugated to either a liposome containing selenium dye or gold particles in mobile phase². Most tests have a control line, that is the only one that appears in a negative test, while in the positive test a second line appears, usually within 15 minutes or less, making the reading straightforward and reproducible.

Malaria antigens commonly targeted by RDTs are HRP-2, parasite lactate dehydrogenase (pLDH) and Plasmodium aldolase (PL-also). Moody (2002) demonstrated that *Plasmodium* species secrete these proteins thus the accuracy of RDTs are measured based on them. *P. falciparum* has been shown to secrete lots of HRP-2 more than HRP-1 and HRP-3 whereas pLDH and PL-also are found in other species¹⁵.

RDTs based on HRP-2 protein have shown a variable accuracy for malaria infection in field studies, with microscopy taken as the gold standard. Most HRP2-based tests have been shown to have a high accuracy [pooled sensitivity: 92.7% (95% CI 91.0–94.5%) and pooled specificity: 99.2% (95% CI 98.2–99.9%)]¹⁶. A review reported results ranging from 87.5% to 100% for sensitivity and from 52% to 99.5% for specificity. Subsequently, *Plasmodium* lactate dehydrogenase (pLDH), which is conserved across all *Plasmodium* spp., was used as a target antigen. The pLDH-based RDTs not only had lower accuracy estimates [pooled sensitivity: 67.1% (95% CI 62.8–71.3%) and pooled specificity: 98.4% (95% CI 97.5–99.6%)] but were unable to distinguish between the different *Plasmodium* spp.^{17,18}.

RDT's performance is influenced by the target antigen used, the incidence of malaria, the degree of parasitemia and the predominant *Plasmodium* spp. in the region¹⁶. Azikiwe (2012) showed that *P. falciparum*, *P. vivax*, and *P. malariae* showed 94.6%, 92.9% and 94.7% degree of sensitivity using RDTs in malaria concentration of $\geq 1000/\mu\text{L}$ respectively, but, they showed percentages lower than an average of 58% sensitivity in malaria concentration of $\leq 100/\mu\text{L}$ ². Another study showed that several RDTs are available which consistently detect malaria at low parasite densities (200 parasites/ μL), have low false positive rates. In other studies, their sensitivity was lower than 95%, the minimum level

recommended by the WHO^{5,17}. Over 400 parasites/ μ L, the sensitivity was higher than 95% and approached 100% over 4,000 parasites/ μ L. RDTs have a very high negative predictive value¹⁹ and can be used widely in fevercase management to exclude malaria infection in malaria-endemic settings⁶. But vice versa, RDTs can also be used to detect asymptomatic malaria, especially by conducting periodic mass blood surveys²⁰.

However, contrarily to traditional microscopy, RDTs do not provide an estimate of the parasite density, and their specificity for malaria-attributable fever is highly variable, being very low in some epidemiological contexts. Use of RDTs to support diagnosis resulted in a 39% reduction in anti-malarial drugs prescriptions that translates to approximately a 2-fold reduction in anti-malarial drugs prescriptions across all epidemiological settings⁵. But in another study showed that the adherence of health workers to the test result was poor, with many patients being treated for malaria even after a negative test result, causing an obvious waste of resources²².

5. Polymerase Chain Reaction (PCR)

In hypoendemic areas, diagnosis of malaria requires more sensitive tools such as polymerase chain reaction (PCR) capable of identifying as few as 1-10 parasites/ μ L. The proportion of infections missed by microscopy may increase up to 88% where PCR prevalence declines to 10%. Another meta-analysis showed that parasite prevalence was measured by both PCR and microscopy found that microscopy only detects about 50% of the parasite carriers detected by PCR, and points out that this percentage decreases even further with decreasing transmission²³.

Discrepancies have often been found between microscopy and PCR results for the determination of asymptomatic *Plasmodium* carriage, where PCR is always superior. Baliraine (2009) reported 20.7% difference (12.6% by microscopy and 33.3% by PCR), Dal-Bianco (2007) found 25% difference (27% by microscopy and 52% by PCR) and May (1999) showed 19% difference (48.9% by microscopy and 67.9% by PCR)²⁴.

In general, PCR assays are more sensitive than microscopic examination for malaria diagnosis, but they are labor-intensive, time-consuming, requires electric power which cost a fortune, cannot easily be considered for use than others and have amplicon contamination problems². Numerous PCR assays for malaria diagnosis, including conventional and real-time PCR techniques, have been developed on mostly genus- or species-specific sequences of the *Plasmodium* 18S subunit rRNA gene²⁵. Of the three PCR assays tested, the real-time PCR is the least time-consuming method and gives the most sensitive detection limit of the three PCR assays.

6. Conclusion

Malaria management policies cannot be the same everywhere. In highly endemic areas where laboratory support is not available, the policy of offering anti-malarial drugs to all febrile illnesses is appropriate. The consequences of delays in offering appropriate treatment for malaria probably outweigh the benefits of reducing the wasteful use of anti-malarial drugs⁹. RDTs are now replacing microscopy almost everywhere in endemic countries. In where malaria incidence is (or has become) low or very low, RDTs have clearly a key role in limiting unnecessary malaria prescriptions.

Asymptomatic malaria cases are known to be prevalent in endemic areas²⁶ and are generally untreated, resulting in a significant source of gametocytes that may serve as a reservoir of disease transmission²⁷. Efforts to control or to eradicate malaria should take into account asymptomatic *Plasmodium* carriers because elimination of parasites in only symptomatic patients will not be enough as long as the pool of asymptomatic carriers will continue to act as a parasite reservoir²⁸. The reservoir is the cause of fluctuations in malaria cases in many areas²⁹. Asymptomatic malaria could be detected when RDTs were performed in all inhabitants, not only used in symptomatic patients. Periodic mass blood surveys are necessary to identify asymptomatic malaria cases in hypoendemic areas²⁰. Detection of asymptomatic malaria in primary health care facilities can be improved through the optimization of the routine microscopic examination and introducing serial microscopic examination in patients at a higher risk of malaria but presenting with no fever³⁰.

The renewed efforts to eradicate malaria, require surveillance systems that strive to identify the last parasite carriers. New and even more sensitive diagnostic tools, may also be useful to identify pockets of infected people, regardless the presence of fever, and treat them, both to their benefit and that of the community³¹. The usefulness of PCR, RDTs, and serology was, therefore, explored as techniques for surveillance. In peripheral centers, PCR is not available, and lab technicians lack expertise in microscopy. Moreover, malaria treatment should be initiated as soon as possible.

Microscopy has traditionally been considered the standard gold test for malaria diagnosis. Under optimum conditions, microscopy can detect 50 parasites/ μL of blood. Incapable hands it is very sensitive for parasitemia $\leq 50/\mu\text{L}$ ³². Detection of asymptomatic malaria in primary health care facilities can be improved through the optimization of the routine microscopic examination and introducing serial microscopic examination in patients at a higher risk of malaria³⁰. Many health workers consider the microscopic examination of Giemsa-stained thick and thin blood films to be the most suitable diagnostic instrument for malaria control. Not only can it differentiate between species (as many RDTs can do), but it can also provide detailed information about stages present and their counts per unit volume of blood (which no RDTs can do)¹².

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