Recent Advances:

RECENT ADVANCES IN DIAGNOSIS OF MALARIA

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INTRODUCTION[1-7] :

Malaria is a mosquito-borne infectious disease that affects 3.4 billion people - about half of the world's population. According to the malaria report 2013, there were an estimated 207 million cases of malaria infections in year 2012, causing a toll of 627,000 deaths. The disease is most prevalent in tropical and subtropical countries of Africa, South America and South-East Asia. Obligate intracellular parasites of the genus Plasmodium are the cause of malaria. During its complex life cycle the parasite infects red blood cells (erythrocytes) and replicates within. The parasite directly ingests hemoglobin from the infected erythrocyte as a source of essential metabolites and energy. While there are more than 120 species of Plasmodium, only 5 species are known to infect humans; P. falciparum, P. vivax, P. ovale, P. malariae, and P. knowlesi. P. falciparum is the most common, and is responsible for more than 90% of malaria death. During the maturation in the erythrocyte, three distinct morphological stages can be observed microscopically - the ring, trophozoite, and schizont stage. The cyclical invasion and reinvasion of malaria parasites in red blood cells causes the number of infected red blood cells to increase exponentially, resulting in the symptoms such as fever, headache, rigors, and nausea usually associated with malaria.

The major challenges in current clinical malaria diagnostics are in obtaining a sensitive, robust, fast, and inexpensive measurement from patients' blood samples. One of the leading causes of the high mortality rate in malaria is the delay in medical diagnosis and treatment4. Prompt diagnosis and timely treatment for malaria is often difficult to come by in poverty-stricken countries with limited medical resources, while developed countries, with very few cases, often lack experience and expertise in malaria diagnosis. Most malaria deaths can be prevented if diagnosis and effective treatment were administered within 24 hour after the onset of first symptom. Physicians need the information on the severity of infection, often expressed in parasitemia (the ratio of infected to the total number of red blood cells) to determine the treatment schedule for malaria patients. Furthermore, anti-malarial drugs, such as artemisinin - the current last line of defense against malaria, have become less effective in malaria treatment as malaria parasite have adapted and developed resistance to the drugs.

CLINICAL DIAGNOSIS OF MALARIA[8-10]

A clinical diagnosis of malaria is traditional among medical doctors. This method is least expensive and most widely practiced. Clinical diagnosis is based on the patients' signs and symptoms, and on physical findings at examination. The earliest symptoms of malaria are very nonspecific and variable, and include fever, headache, weakness, myalgia, chills, dizziness, abdominal pain, diarrhea, nausea, vomiting, anorexia, and pruritus . A clinical diagnosis of malaria is still challenging because of the non-specific nature of the signs and symptoms, which overlap considerably with other common, as well

as potentially life-threatening diseases, e.g. common viral or bacterial infections, and other febrile illnesses. The overlapping of malaria symptoms with other tropical diseases impairs diagnostic specificity, which can promote the indiscriminate use of antimalarials and compromise the quality of care for patients with non-malarial fevers in endemic areas . The Integrated Management of Children Illness (IMCI) has provided clinical algorithms for managing and diagnosing common childhood illnesses by minimally trained healthcare providers in the developing world having inappropriate equipment for laboratory diagnosis. A widely utilized clinical algorithm for malaria diagnosis, compared with a fully trained pediatrician with access to laboratory support, showed very low specificity (0-9%) but 100% sensitivity in African settings . This lack of specificity reveals the perils of distinguishing malaria from other causes of fever in children on clinical grounds alone. Recently, another study showed that use of the IMCI clinical algorithm resulted in 30% over-diagnosis of malaria . Therefore, the accuracy of malaria diagnosis can be greatly enhanced by combining clinical-and parasite-based findings.

LABORATORY DIAGNOSIS OF MALARIA

Microscopic diagnosis using stained Thin and Thick Peripheral Blood Smears(11-15)

Malaria is conventionally diagnosed by microscopic examination of stained blood films using Giemsa, Wright's, or Field's stains . This method has changed very little since Laverran's original discovery of the malaria parasite, and improvements in staining techniques by Romanowsky in the late 1,800s. More than a century later, microscopic detection and identification of Plasmodium species in Giemsa-stained thick blood films (for screening the presenting malaria parasite), and thin blood films (for species' confirmation) remains the gold standard for laboratory diagnosis . Malaria is diagnosed microscopically by staining thick and thin blood films on a glass slide, to visualize malaria parasites. Briefly, the patient's finger is cleaned with 70% ethyl alcohol, allowed to dry and then the side of fingertip is picked with a sharp sterile lancet and two drops of blood are placed on a glass slide. To prepare a thick blood film, a blood spot is stirred in a circular motion with the corner of the slide, taking care not make the preparation too thick, and allowed to dry without fixative. After drying, the spot is stained with diluted Giemsa (1 : 20, vol/vol) for 20 min, and washed by placing the film in buffered water for 3 min. The slide is allowed to air-dry in a vertical position and examination using a light microscope. As they are unfixed, the red cells lyse when a water-based stain is applied. A thin blood film is prepared by immediately placing the smooth edge of a spreader slide in a drop of blood, adjusting the angle between slide and spreader to 45° and then smearing the blood with a swift and steady sweep along the surface. The film is then allowed to air-dry and is fixed with absolute methanol. After drying, the sample is stained with diluted Giemsa (1 : 20, vol/vol) for 20 min and washed by briefly dipping the slide in and out of a jar of buffered water (excessive washing will decolorize the film). The slide is then allowed to air-dry in a vertical position and examined under a light microscope . The wide acceptance of this technique by laboratories all around the world can be attributed to its simplicity, low cost, its ability to identify the presence of parasites, the infecting species, and assess parasite density-all parameters useful for the management of malaria. However, the staining and interpretation processes are labor intensive, time consuming, and require considerable expertise and trained healthcare workers, particularly for identifying species accurately at low parasitemia or in mixed malarial infections. The most important shortcoming of microscopic examination is its relatively low sensitivity, particularly at low parasite levels. Although the expert microscopist can detect up to 5 parasites/µl, the average microscopist detects only 50-100 parasites/µl. This has probably resulted in underestimating malaria

infection rates, especially cases with low parasitemia and asymptomatic malaria. The ability to maintain required levels of in malaria diagnostics expertise is problematic, especially in remote medical centers in countries where the disease is rarely seen . Microscopy is laborious and ill-suited for high-throughput use, and species determination at low parasite density is still challenging. Therefore, in remote rural settings, e.g. peripheral medical clinics with no electricity and no health-facility resources, microscopy is often unavailable .

QBC Technique [15-19]

The QBC technique was designed to enhance microscopic detection of parasites and simplify malaria diagnosis . This method involves staining parasite deoxyribonucleic acid (DNA) in micro-hematocrit tubes with fluorescent dyes, e.g. acridine orange, and its subsequent detection by epi-fluorescent microscopy. Briefly, finger-prick blood is collected in a hematocrit tube containing acridine orange and anticoagulant. The tube is centrifuged at 12,000 g for 5 min and immediately examined using an epi-fluorescent microscope. Parasite nuclei fluoresces bright green, while cytoplasm appears yellow-orange. The QBC technique has been shown to be a rapid and sensitive test for diagnosing malaria in numerous laboratories settings . While it enhances sensitivity for P. falciparum, it reduces sensitivity for non-falciparum species and decreases specificity due to staining of leukocyte DNA. Recently, it has been shown that acridine orange is the preferred diagnostic method (over light microscopy and immunochromatographic tests) in the context of epidemiologic studies in asymptomatic populations in endemic areas, probably because of increased sensitivity at low parasitemia . Nowadays, portable fluorescent microscopes using light emitting diode (LED) technology, and pre-prepared glass slides with fluorescent reagent to label parasites, are available commercially [. Although the QBC technique is simple, reliable, and user-friendly, it requires specialized instrumentation, is more costly than conventional light microscopy, and is poor at determining species and numbers of parasites.

Rapid Diagnostic Tests (RDTs) [20-28]

Since the World Health Organization (WHO) recognized the urgent need for new, simple, quick, accurate, and cost-effective diagnostic tests for determining the presence of malaria parasites, to overcome the deficiencies of light microscopy, numerous new malaria-diagnostic techniques have been developed . This, in turn, has led to an increase in the use of RDTs for malaria, which are fast and easy to perform, and do not require electricity or specific equipment . Currently, 86 malaria RDTs are available from 28 different manufacturers . Unlike conventional microscopic diagnosis by staining thin and thick peripheral blood smears, and QBC technique, RDTs are all based on the same principle and detect malaria antigen in blood flowing along a membrane containing specific anti-malaria antibodies; they do not require laboratory equipment. Most products target a P. falciparum-specific protein, e.g. histidine-rich protein II (HRP-II) or lactate dehydrogenase (LDH). Some tests detect P. falciparum specific and pan-specific antigens (aldolase or pan-malaria pLDH), and distinguish non-P. falciparum infections from mixed malaria infections. Although most RDT products are suitable for P. falciparum malaria diagnosis, some also claim that they can effectively and rapidly diagnose P. vivax malaria . Recently, a new RDT method has been developed for detecting P. knowlesi . RDTs provide an opportunity to extend the benefits of parasite-based diagnosis of malaria beyond the confines of light microscopy, with potentially significant advantages in the management of febrile illnesses in remote malaria-endemic areas. RDT performance for diagnosis of malaria has been reported as excellent ; however, some reports from remote malaria-endemic areas have shown wide variations in sensitivity . Murray and co-authors recently discussed the reliability of RDTs in an "update on rapid diagnostic testing for malaria" in their excellent paper . Overall, RDTs

appears a highly valuable, rapid malaria-diagnostic tool for healthcare workers; however it must currently be used in conjunction with other methods to confirm the results, characterize infection, and monitor treatment. In malaria-endemic areas where no light microscopy facility exists that may benefit from RDTs, improvements are required for ease of use, sensitivity for non-falciparum infection, stability, and affordability. The WHO is now developing guidelines to ensure lot-to-lot quality control, which is essential for the community's confidence in this new diagnostic tool . Because the simplicity and reliability of RDTs have been improved for use in rural endemic areas, RDT diagnosis in non-endemic regions is becoming more feasible, which may reduce time-to-treatment for cases of imported malaria.

Serological Tests[29-31]

Diagnosis of malaria using serological methods is usually based on the detection of antibodies against asexual blood stage malaria parasites. Immunofluorescence antibody testing (IFA) has been a reliable serologic test for malaria in recent decades . Although IFA is time-consuming and subjective, it is highly sensitive and specific . The literature clearly illustrates the reliability of IFA, so that it was usually regarded as the gold standard for malarial serology testing . IFA is useful in epidemiological surveys, for screening potential blood donors, and occasionally for providing evidence of recent infection in non-immunes. Until recently, it was a validated method for detecting Plasmodium-specific antibodies in various blood bank units, which was useful for screening prospective blood donors, so avoiding transfusion-transmitted malaria.. In France, for example, IFA is used as a part of a targeted screening strategy, combined with a donor questionnaire . The principle of IFA is that, following infection with any Plasmodium species, specific antibodies are produced within 2 wk of initial infection, and persist for 3-6 months after parasite clearance. IFA uses specific antigen or crude antigen prepared on a slide, coated and kept at -30℃ until used, and quantifies both IgG and IgM antibodies in patient serum samples. Titers > 1 : 20 are usually deemed positive, and < 1 : 20 unconfirmed. Titers > 1 : 200 can be classified as recent infections . In conclusion, IFA is simple and sensitive, but time-consuming. It cannot be automated, which limits the number of sera that can be studied daily. It also requires fluorescence microscopy and trained technicians; readings can be influenced by the level of training of the technician, particularly for serum samples with low antibody titers. Moreover, the lack of IFA reagent standardization makes it impractical for routine use in blood-transfusion centers, and for harmonizing inter-laboratory results.

MOLECULAR DIAGNOSTIC METHODS

As mentioned above, traditional malaria diagnostic methods remain problematic. New laboratory diagnostic techniques that display high sensitivity and high specificity, without subjective variation, are urgently needed in various laboratories. Recent developments in molecular biological technologies, e.g. PCR, loop-mediated isothermal amplification (LAMP), microarray, mass spectrometry (MS), and flow cytometric (FCM) assay techniques, have permitted extensive characterization of the malaria parasite and are generating new strategies for malaria diagnosis.

PCR Technique[32-38]

PCR-based techniques are a recent development in the molecular diagnosis of malaria, and have proven to be one of the most specific and sensitive diagnostic methods, particularly for malaria cases with low parasitemia or mixed infection . The PCR technique continues to be used extensively to confirm malaria infection, follow-up therapeutic response, and identify drug resistance . It was found to be more sensitive than QBC and some RDTs . Concerning with the gold standard method for malaria diagnosis, PCR has shown higher sensitivity and specificity than conventional microscopic examination of stained peripheral blood smears, and now seems the best method for

malaria diagnosis . PCR can detect as few as 1-5 parasites/µl of blood (≤ 0.0001% of infected red blood cells) compared with around 50-100 parasites/µl of blood by microscopy or RDT. Moreover, PCR can help detect drug-resistant parasites, mixed infections, and may be automated to process large numbers of samples . Some modified PCR methods are proving reliable, e.g., nested PCR, real-time PCR, and reverse transcription PCR, and appear to be useful second-line techniques . Recently, the PCR method has become widely accepted for identifying P. knowlesi infections. Although PCR appears to have overcome the two major problems of malaria diagnosis-sensitivity and specificity- the utility of PCR is limited by complex methodologies, high cost, and the need for specially trained technicians. PCR, therefore, is not routinely implemented in developing countries because of the complexity of the testing and the lack of resources to perform these tests adequately and routinely . Quality control and equipment maintenance are also essential for the PCR technique, so that it may not be suitable for malaria diagnosis in remote rural areas or even in routine clinical diagnostic settings .

LAMP technique[39-41]

The LAMP technique is claimed to be a simple and inexpensive molecular malaria-diagnostic test that detects the conserved 18S ribosome RNA gene of P. falciparum [68]. Other studies have shown high sensitivity and specificity, not only for P. falciparum, but also P. vivax, P. ovale and P. malariae [69,70]. These observations suggest that LAMP is more reliable and useful for routine screening for malaria parasites in regions where vector-borne diseases, such as malaria, are endemic. LAMP appears to be easy, sensitive, quick and lower in cost than PCR. However, reagents require cold storage, and further clinical trials are needed to validate the feasibility and clinical utility of LAMP .

Microarrays[42-45]

Publication of the Plasmodium genome offers many malaria-diagnostic opportunities . Microarrays may play an important role in the future diagnosis of infectious diseases . The principle of the microarrays technique parallels traditional Southern hybridization. Hybridization of labeled targets divided from nucleic acids in the test sample to probes on the array enables the probing of multiple gene targets in a single experiment. Ideally, this technique would be miniaturized and automated for point-of-care diagnostics .A pan-microbial oligonucleotide microarray has been developed for infectious disease diagnosis and has identified P. falciparum accurately in clinical specimens . This diagnostic technique, however, is still in the early stages of development .

FCM assay[46-50]

Flow cytometry has reportedly been used for malaria diagnosis . Briefly, the principle of this technique is based on detection of hemozoin, which is produced when the intra-erythrocytic malaria parasites digest host hemoglobin and crystallize the released toxic heme into hemozoin in the acidic food vacuole. Hemozoin within phagocytotes can be detected by depolarization of laser light, as cells pass through a flow-cytometer channel. This method may provide a sensitivity of 49-98%, and a specificity of 82-97%, for malarial diagnosis , and is potentially useful for diagnosing clinically unsuspected malaria. The disadvantages are its labor intensiveness, the need for trained technicians, costly diagnostic equipment, and that false-positives may occur with other bacterial or viral infections. Therefore, this method should be considered a screening tool for malaria.

Automated blood cell counters (ACC)[51-53]

An ACC is a practical tool for malaria diagnosis , with 3 reported approaches. The first used a Cell-Dyn® 3500 apparatus to detect malaria pigment (hemozoin) in monocytes, and showed a sensitivity of 95% and specificity of 88%, compared with the gold-standard blood smear . The second method also used a Cell-Dyn® 3500, and

analyzed depolarized laser light (DLL) to detect malaria infection, with an overall sensitivity of 72% and specificity of 96% . The third technique used a Beckman Coulter ACC to detect increases in activated monocytes by volume, conductivity, and scatter (VCS), with 98% sensitivity and 94% specificity . Although promising, none of the 3 techniques is routinely available in the clinical laboratory; further studies are required to improve and validate the instrument and its software. The accuracy these methods promise, for detecting malaria parasites, mean ACC could become a valuable and routine malaria-diagnostic laboratory method.

Mass Spectrophotometry[54]

A novel method for in vitro detection of malaria parasites, with a sensitivity of 10 parasites/μl of blood, has been reported recently. It comprises a protocol for cleanup of whole blood samples, followed by direct ultraviolet laser desorption mass spectrometry (LDMS). For malaria diagnosis, the principle of LDMS is to identify a specific biomarker in clinical samples. In malaria, heme from hemozoin is the parasite-specific biomarker of interest. LDMS is rapid, high throughput, and automated. Compared with the microscopic method, which requires a skilled microscopist and up to 30-60 min to examine each peripheral blood smear, LDMS can analyze a sample in < 1 min . However, the remote rural areas without electricity are inhospitable for existing high-tech mass spectrometers. Future improvements in equipment and techniques should make this method more practicable.

Microfluidic Cell Enrichment and Magnetic Rresonance Relaxometry (MRR) Detection[55]

The miniaturization of magnetic resonance relaxometry (MRR) systems has been attracting considerable interest in recent years due to the promising applications in disease biomarker detection, point-of-care diagnosis, and cancer screening by measuring the transverse relaxation rate, R2 of proton present in the bio-samples14,15,16,17. There are two main benefits to the miniaturization of MRR systems; it offers higher mass sensitivity and requires smaller sample volume than conventional nuclear magnetic resonance systems, which require high-strength, spatially uniform magnetic field18,19. Furthermore, each R2 measurement only takes approximately 1 minute. Recently, we have demonstrated that the presence of paramagnetic malaria pigment or hemozoin crystallites in malaria infected red blood cells (iRBCs) serves as the natural biomarker for malaria detection20. The bulk magnetic susceptibility of the iRBCs is significantly higher than the healthy red blood cells (hRBCs), which in turn induces a substantial change in the transverse relaxation rate. Nonetheless, MRR detection has a major drawback in that it measures the absolute value of a blood sample instead of detecting a relative change in the R2 value.

Plasmodium Specific Phospholipases A2 Levels in Malaria[56-59]

Currently lot of research is goin on on levels of cytosolic and scretory Phospholiapse A2 levels in the children with malaria.Cytosolic PLA2 is acute phase reactant produced by host macrophage activation and is a nonspecific marker of the inflammotory process amd its levels corresponds to the severity of inflammation.

However Plasmodium specific Secretory PLA2 release occurs only by the specific parasite like vivax and falciparum..Vivax PLA2 is low mol wt protein(13-19kDa).Falciparum PLA2 is hmw protein (110-130 kDa) also known as RhopH3 PROTEIN. Whether Detection of these plasmodium specific PLA2 by Rapid zymogram will help early detection of malarial parasite is experimental.

Recently, other reliable malaria-diagnostic tests have been developed and introduced, and some tests are commercially available, for example, enzyme linked immunosorbent assay (ELISA)/enzyme immunoassay (EIA) , latex

agglutination assay, and cultivation of live malaria parasites . Post-mortem organ diagnoses, by investigating malaria parasites in tissue autopsy, e.g. liver and spleen , kidney and brain. have also been described. However, parasite culture, molecular techniques, serology techniques and pathobiological diagnostic techniques, although sometimes useful in research laboratories, are not practical or appropriate for the routine clinical diagnosis of malaria.

CONCLUSION :

Conventional microscopic examination of peripheral thick and thin blood smears remains the gold standard for malaria diagnosis. Although this method requires a trained microscopist, and sensitivity and specificity vary compared with recent technical advances, it is inexpensive and reliable. Quick and convenient RDTs are currently implemented in many remote settings, but are costly and need improved quality control. Serological tests are useful for epidemiological surveys, but not suitable for the diagnosis of acute malaria. Molecular-biological techniques are appropriate for research laboratories; they can be used to identify the development of drug-resistance, are useful for species identification, and also for quantifying parasite density with low parasitemia. Finally, the level of malaria endemicity, the urgency of diagnosis, the experience of the physician, the effectiveness of healthcare workers, and budget resources, are all factors influencing the choice of malaria-diagnostic method.

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