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Review Article

Recently Developed New, Sensitive, Time-Effective and Cost-Effective Diagnostic Tests of Malaria

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Malaria is world's predominant morbidity and mortality causing infectious disease. Diagnosis of *Plasmodium* infections in febrile persons, residing in malaria endemic areas, is a pre-requisite for proper malarial treatment. The presently available dipstick antigen detection (RDT), microscopic (Giemsa stained smear on slide) and polymerase chain reaction (PCR) tests of malaria are poor in sensitivity, time consuming, and expensive. Recently developed rolling circle enhanced enzyme activity detection (REEAD)- and micromagnetic resonance reflaxometric (MMR)- test are amenable to deployment in field conditions and are highly accurate; and cost- and time-effective. The properties of all three conventional and two new malaria tests have been compared here and have been shown to be complementary for usage, under hospital laboratory and field conditions.

Key Words : REEAD Test; MMR Test; Rapid-Diagnostic Tests; Blood-Smear Test; PCR-Test; Malaria Tests

WHO (2013) has reported that malaria is endemic in 107 countries inhabited by half of world's population. In the year 2013 it recorded, 219 million cases of malaria and 627,000 consequential deaths. Malaria is caused by infection of five plasmodium parasite species: *Plasmodium falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi*. The malaria caused by *P. falciparum* is the deadliest. Relapsing kind of malaria results from *P. vivax* and *P. ovale* infections. Among scores of species of Anopheles mosquitoes sensitive to Plasmodium, the main vectors of malaria transmission are *A. gambiae* and *A. stephensi.* The effectivity of artemisinin combination therapy in combating malaria disease has been compromised by development of artemisinin resistance in plasmodia. Artemisinin resistance has evolved many times independently in *Plasmodium* in Western Cambodia and has rapidly spread in Southeast Asian region comprised by Mynamar, Thailand, Cambodia and Vietnam. The new challenges in malaria control are two fold: (a) To stop the migration of artemisinin resistant plasmodia to other parts of Asia (especially India, Nepal, Bangladesh, SriLanka and Pakistan), Middle East, Africa and Latin America; and (b) To develop new drugs that will cure malaria and simultaneously stop transmission of malaria by killing the sexual stage of parasite in humans and reproduction in the mosquito vector (Kumar *et al*., 2014). It is proposed to continue mass campaigns in areas where artemisinin resistance is spreading and disease is assuming epidemic proportions to (a) identify malaria infected persons and treat them with drug(s) to which parasite is sensitive and (b) administer antimalarial prophylactic drug(s) to persons who do not show febrile symptom of malarial parasitemia. An important requirement for malaria

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control is the availability of rapid, sensitive and cost effective diagnostic test for the detection of malarial infection. Recently, two new diagnostic tests of malaria have been described which are more sensitive and/or cost effective than the prevalent tests.

Table 1 compares some important properties of the conventional and new malaria diagnostic tests. The newly developed tests will complement and not replace the malaria tests already in practice. Among the tests in vogue, the microchromatographic tests in which monoclonal antibodies, that will specifically bind to their *Plasmodium* antigens, are coated on dipsticks. These easy to conduct antigen detection tests give results in 15 minutes. On account of their low sensitivity, these Rapid Diagnostic Tests (RDT) often give false negative results. The febrile persons tested negative, in regions of malaria epidemics, need to undergo further confirmatory microscopic- and/or PCR (polymerase chain reaction)-test(s). In the microscopy test, blood smears on slides, stained with Giemsa, are scored for the frequency of erythrocytes infested with *Plasmodium*. Since the sensitivity of this test is also low, the test is repeated every 12 h for several days to decide on presence or absence of infection. In recent years, the PCR test, which detects presence of *Plasmodium* DNA in blood, is also used as a confirmatory test. The PCR detects *Plasmodium* at all stages of its infestation of erythrocytes with some certainty. Whereas RDT tests can be conducted in field conditions, the microscopic and PCR tests require skilled conductors and hospital laboratory infrastructure and equipment. Presently, the most reliable malaria diagnosis depends on PCR tests which also identify the *Plasmodium* species causal of malaria. Recently, Juul *et al*. (2012) and Peng *et al*. (2014) have respectively developed the quantitative Plasmodium detection procedures. Rolling- circle Enhanced Enzyme Activity Detection (REEAD)-on-chip test and the Micromagnetic resonance Relaxometry (MMR) malaria test. The essential features of the techniques are diagrammed in Fig. 1 and explained below.

REEAD quantifies the activity of *Plasmodium* specific enzyme topoisomerase 1 (pTOP1) present in the infected cells of blood or saliva; pTOP1 is absent in human cells free of *Plasmodium*. A hairpin structured DNA substrate (S) sensitive towards pTOP1 activity has been designed. S has a loop which extends into a double stranded stem of which one strands is sciccible. The loop has a sequence complementry to a primer (p) and another complementary to a probe/indicator (i). The pTOP1 enzyme is able to cleave a piece of DNA sequence close to the 3´-end of stem and can ligate the new 3´-end to the 5´-end of stem. The product upon denaturation opens into a single stranded circle. Provision of primer and reagents necessary for DNA replication allows the S-circle to replicate as a rolling circle such that each S-molecule produces a DNA product of some length which is microscopically visualizable by hybridization with a fluorescent labelled probe/indicator sequence. To carry out a REEAD reaction, a 50 µL mixture is prepared with substrate, blood or saliva, lysing buffer and DNA replication reagents. A 5 µL droplet of it is placed on a glass slide to which primer sequences are already attached. After the reaction time the DNA product(s) on the slide are hybridized with the red fluorescent labelled i-probe DNA. The signals produced are read microscopically. Equipment suitable for mechanically delivering and mixing the ingredients in micro amounts and placing the mixture aliquots on slide have been designed, manufactured and tested. Work is in progress to bring the cost of equipment down. REEAD is the most sensitive malaria diagnostic procedure available; it can detect parasite presence at a level of 0.1 parasite/µL of blood/saliva and is also suitable to follow the progress of malaria treatment in addition to field identification of malaria infected persons in population screenings.

The MMR measures the presence of paramagnetic Fe^{3+} ions in the hemozoin of the Plasmodium parasite invaded erythrocytes. Hemozoin is the waste product of hemoglobin digested by parasite for its growth. Heme from the digested hemozoin is transformed into hemozoin crystals wherein heme groups are dimerized via iron carboxylate links and the resulting three dimensional structure is stabilized by hydrogen bond formation. Hemozoin crystals interfere with synchrony in the spinning of hydrogen atoms under a magnetic field.

Table 1: Comparative properties/parameters of the conventional microscopic, dipstick and PCR tests and recently developed REEAD and MMR tests for malaria parasite infections

S.No.	Property	Microscopy of thick and thin blood smears stained with Giemsa	Immunochromatographic (antigen detecting) dipstick rapid diagnostic tests based on monoclonal antibodies against		Plasmodium specific nucleic acid amplification or topoisomerase (pTOP1) detection		Micromagnetic resonance reflaxometric (MMR) quantification of paramagnetic Fe^{3+} ions present in hemozoin
			Histone-rich protein 2 (pfHRP-2)	Lactate dehydrogenase (pLD)	Polymerase chain reaction (PCR) and reverse transcriptase PCR	Rolling circle enhanced enzyme activity detection (REEAD)	
	A	B	C	D	E	F	G
1	Nature of sample material taken from the subject and its quantity	A few drops of blood from pricked finger or ear lobe	One drop of blood	One drop of blood	One drop of blood	One drop of blood or saliva	One drop of blood
2	Whether test can be performed in field conditions (outside of hospital laboratory)?	No	Yes	Yes	No	Yes	Yes
3	Whether test can be performed and results interpreted by person(s) with limited training?	No[a]	Yes	Yes	No[a]	Yes	Yes
4	Whether the test can accurately diagnose malaria at the pre-parasitaemic stages of infection?	No	No	No	Yes	Yes	No
5	Whether malaria caused by all of Plasmodium *falciparum, vivax, ovale* and *malariae* is detected?	Yes[b]	No; only that caused by *Plasmodium falciparum* is detected	Yes[c]	Yes[d]	Yes	Yes
6	Whether the test gives malaria positive result even after parasitemia has been cured?	No	Yes[e]	Yes[e]	Yes[e]	Yes[e]	No
7	Whether the test gives quantitative estimate of parasitemia?	Yes	No	No	No	Yes	Yes
8	Sensitivity of the test (number of parasites that should be present in one µL of blood for the test to give malaria positive result)	50-500	100-200	100-200	1-5	<1	10-50
9	Time taken for the result to be known	1h to many days[f]	<30 min	<30 min	= 24 h	2-5 h	5 min
10	Cost of a test in ´[g] Rupees	40	160	160	250	120[h]	6[h]
	References:	Moody (2002); Warhurst and Williams (1996); Mouatcho and Goldring (2013); White *et al*. (2014)	Moody (2002); Mouatcho and Goldring (2013); White *et al*. (2014)	As in column C	Moody (2002); Johnston *et al*. (2006); Rougemont *et al*. (2004); Cordray and Richard Kortum (2012); Mouatcho and Goldring (2013); White *et al*. (2014)	Juul *et al*. (2012)	Peng *et al*. (2014)

Explanation: a= These tests are performed by specially trained/ skilled persons; b= Malaria infections caused by different species of *Plasmodium* can be discriminated from the cytological configurations of parasites in erythrocytes; c= Different species of parasites can be differentiated; d= Plasmodium species are differentiated by use of primers of small subunit of 18S rRNA and circumsporozoite genes etc; e= Presence of gametocytes gives positive results; f = Test is carried out on febrile presons at intervals of 8-12 h for several days, if initial tests give negative results; g= costs mentioned are averages based on expenditure on reagents and do not take into account salaries of staff and expenditure on equipment etc.; h= These costs are thought to come down in future

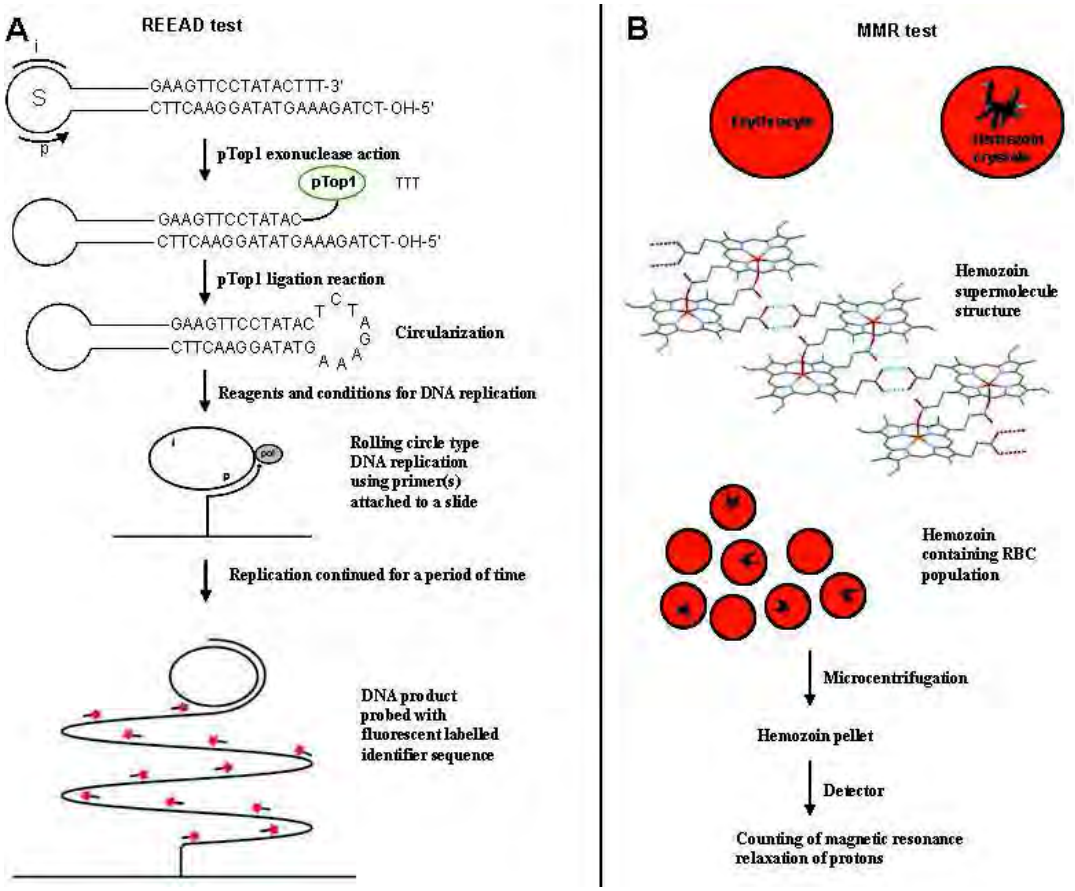

Fig. 1: A= Diagram of the Rolling Circle Enhanced Enzyme Activity Detection (REEAD); and B= Paramagnetic Fe^{3+} ion containing hemozoin produced from hemoglobin by *Plasmodium* in infected erythrocytes. REEAD measures the activity of the topoisomerase 1 (pTOP1) enzyme synthesized by *Plasmodium* in infected cells. Topoisomerase produces a single stranded circular DNA from a synthetic DNA sequence (S) which has a loop and double stranded stem. In the loop are encoded a primer annealing sequence (p) and a probing sequence (i). pTOP1 cleaves a small sequence at a site close to the 3´ end in S. the truncated 3´-end gets ligated with the protruding 5´ end to produce a covalently closed single stranded circle. The circle is amplified by use of a primer sequence complementary to the *p* site. The rolling circle amplified product is hybridized to a short red fluorescent nucleotide sequence complementary to *i* site. The signals are counted. The DNA replication reaction is carried out on a slide.

Presence of paramagnetic Fe^{3+} ions in hemozoin disrupts the synchrony of hydrogen atoms under a powerful magnetic field, by a relaxation process such that more of hemozoin is present more quickly the synchrony is disrupted. In the structure of hemozoin provided, the hydrogen bonds between hematin units are shown in dotted lines and red lines denote the coordinate bonds between iron atoms and carboxylate side chains (http://en.m.wikipedia.org/wiki/Hemozoin). The MMR procedure is outlined

The disruption of the synchrony in the spinning of protons or relaxation is proportional to the amount of hemozoin present or concentration of Fe^{3+} present in hemozoin. The method uses intact blood cells and equipment that pellets the cells and exposes them to nuclear magnetic spectroscopy and has been designed in such a manner that assays can be carried our using portable bench-top level field programmable equipment. The results of MMR become available in a few minutes at a very low cost.

MMR and REEAD together with microscopic, RDT and PCR tests are going to facilitate surveillance of malaria in remote areas as well as in hospitals in the process of control of malaria transmission, treatment of disease and development of new antimalarial drugs. MMR and REEAD have increased the accuracy of malaria testing and made malaria diagnosis really cost effective.

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