Chapter 7

Functions of Dehydrogenases in Health and Disease

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Additional information is available at the end of the chapter

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1. Introduction

Dehydrogenases are a group of biological catalysts (enzymes) that mediate in biochemical reactions removing hydrogen atoms [H] instead of oxygen [O] in its oxido-reduction reactions. It is a versatile enzyme in the respiratory chain pathway or the electron transfer chain. T. Turnberg discovered this group of enzymes between1900-1922. Several dehydrogenases are present in tissues of humans, plants and micro-organisms having enormous biochemical interests. As a result of the polymorphic nature of this enzyme, it is pertinent therefore to limit our interest on the different functions of Lactate dehydrogenase in the diagnosis and treatment of malaria. Lactate dehydrogenase, an oxidoreductase [EC 1.1.1.27] exists in different forms in different tissues possessing different subunits as a multi-enzyme complex called isoenzyme. It is the last enzyme of the glycolytic sequence or pathway essential for ATP generation. The enzyme, 17β-Hydroxysteroid dehydrogenase exists in at least fourteen isoforms in tissues involved in the biosynthesis of estrogenic and androgenic steroids. Lactate dehydrogenase is a tetrameric enzyme, but only two distinct subunits have been found; those designated H for heart (myocardium) and M for muscle. These two subunits are combined in five different ways. The lactate dehydrogenase isoenzymes, subunit compositions and major locations are shown below.

Following myocardial infarction (MI), the serum levels of LDH rise within 24-28 hrs, reaching a peak by 2-3 days and return to normal in 5-10 days. Especially diagnostic is a comparison of the LDH1/LDH2 ratio. Normally, the ratio is less than 1. A reversal of the ratio is referred to as “flipped LDH”. Following an acute myocardial infarction, the flipped ratio will appear in 12-24 hours and it is definitely present by 48 hours in over 80% of cases. Also important, is the fact that, persons suffering from chest pain due to angina only, will not likely have LDH altered levels.

Type	composition	Location
LDH1	HHHH	Found in the heart and red blood cells and Is 17%-27% of the normal serum level.
LDH2	HHHM	Found in the heart and red blood cells and 27%-37% of the normal serum level
LDH3	HHMM	Found in a variety of organs and is 18%-25% of the normal serum level.
LDH4	HMMM	Found in a variety of organs and is 3%-8% of the normal serum level.
LDH5	MMMM	Found in liver and skeletal muscles and is 0%-5% Of the normal serum level.

Table 1. Subunit compositions and major locations of Lactate Dehydrogease Isoforms

2. Measurement of parasite lactate dehydrogenase (pLDH) activity of plasmodium falciparum

Malaria is the most lethal parasitic disease in the world, annually affecting approximately 500 million people and resulting in 800,000 deaths, mostly in Africa and Sub-Saharan countries [1]. Some countries, for example Brazil registered more than 306,000 deaths in 2009, most of which were in the Amazonian region [2]. In Africa, the figures may be higher due to endemic nature of the infection. Transmission occurs through the bite of the female anopheles mosquitoes infected with the parasite of which there are five species affecting humans. Plasmodium falciparum is the most pathogenic species and may cause severe malaria and death in non-immune individuals, especially children under five years [3].Drug resistant malaria parasites have emerged and has resulted in treatment failures. This resistance might be as a result of mutation at the active sites of drug targets or from biochemical changes in the drug receptors [4].

Plasmodium falciparum is a significant cause of morbidity and mortality in travelers to areas where the parasite is endemic. Non –specific manifestations may equally result in failure to recognize malaria until autopsy, when it is often too late to obtain blood for microscopic evaluation, which has been in use for years for the diagnosis of malarial parasitemia. The Plasmodium falciparum lactate enzyme (pLDH) has been considered, a potential molecular target for antimalarials. This is an enzyme assay for the detection of Plasmodium falciparum, employed in the assessment of malarial parasitism. The enzyme assay is based on the observation that Lactate dehydrogenase(LDH) enzyme of *P. falciparum* has the ability to rapidly use 3-acetylpyridine dinucleotide(APAD) as a co-enzyme instead of NAD in the reaction leading to the formation of pyruvate from lactate. Human red blood cells' LDH carries out this reaction at very low rate in the presence of APAD. The measured development of APADH leads to the formation of a product that could establish the basis of an assay that detects the presence of *P. falciparum* from *in vitro* cultures at parasitemic levels of 0.02 %. Lactate dehydrogenase is the most abundant enzyme expressed by *P. falciparum.*

A correlation between levels of parasitemia and the activity of parasite LDH from patients with malaria is worthwhile. The serum assay for PLDH is followed up to monitor the level of PLDH in a patient with cerebral malaria prior to antimalarial therapy and also during recovery period. It is evident that measurement of pLDH has a strong correlation with malarial parasitemia and can follow a method that can be developed into a simple test for the detection of *Plasmodium falciparum* as an assessment of plasmodium parasitemia. In malarial falciparum parasitemia, LDH does not persist in blood, but clears about the time as the parasite, following successful treatment. The lack of antigen persistence after treatment makes the pLDH test useful in predicting treatment failure. In this respect, pLDH is similar to pGluDH. LDH for *P.vivax*, *P.ovale*, and *P. malariae* exhibit 90-92% identity to pLDH *from P. falciparum.*

3. Functions of inhibitors of parasite lactate dehydrogenase (pLDH) as potential antimalarial agents

The *plasmodium falciparum* lactate dehydrogenase enzyme (Pf LDH), has been considered a potential molecular target to antimalarials due to this parasite dependence on glycolysis for energy production by catalyzing the reduction of pyruvate to lactate. It has been a routine activity among drug designers for malarial infestation to embark on the screening of analogs to NADH(an essential cofactor) to pLDH.The continued search for new molecular targets for drug design is an endless search, since the introduction of the quinolones in malaria therapy. Chloroquine interacts specifically with PfLDH in the NADH binding pocket, occupying a position similar to that of the adenyl ring cofactor, hence acting as a competitive inhibitor for this critical glycolytic enzyme [5,6,7,8]. Analogs of NADH have been identified as new potential inhibitors to PfLDH[9]. Computational studies have been undertaken to recognize the potential binding of selected compounds to the pLDH active site. This was analyzed using Motegro Virtual Docker Software. The researchers selected fifty (50) compounds based on their similarity to NADH. The compounds with the best bonding energies included: itraconazole, atorvastatin and posaconazole. These were tested against P. falciparum , chloroquine resistant blood parasites. All these compounds proved to be active in two immunoenzymatic assays performed in parallel using monoclonals specific to pfLDH or a histidine rich protein 2 (HRP 2). The IC_{50} values for each drug in both tests are similar; values were lowest for posaconazole (< 5μM) and were 40-and 100-fold less active than chloroquine. The compounds so tested reduced *P. berghi* parasitemia in treated mice, in comparison to untreated control. The drug itraconazole is the least active compound. Posaconazole is an inhibitor of ergosterol biosynthesis [10]. In this study, it was the most active drug against *P. falciparum*. It is also, the most effective compound against murine malaria caused by *P. berghei* and was the most promising agent in vitro and in vivo. Itraconazole is normally acquired as a tablet, causes a strong inhibition of P. falciparum growth in vitro and is partially active against *P. berghei*. The results of these trials according to the authors proved that molecular docking studies are important in the strategy for discovering new antimalarial drugs. This approach is more practical and less expensive than discovering novel compounds that require studies on human toxicology. The parasite enzyme, lactate dehydrogenase has recently received a great deal of attention, since it may constitute a valid therapeutic target for diseases such as malaria

and cancer. Because the LDH enzymes found in *P.vivax*, *P. ovale* and *P.malariae*, all exhibit 90% identity to PfLDH; It would be desirable to have new anti-pLDH drugs particularly, ones that are effective against *P. falciparum*, the most virulent species of human malaria. Most invasive tumor phenotypes show a metabolic switch (Warburg effect) from oxidative phosporylation to an increased anerobic glycolysis by promoting an up-regulation of the human isoform-5 of lactate dehydrogenase (hLDH-5 or LDH-A), which is normally present in muscles and liver. Hence, inhibition of hLDH-5 may constitute an efficient way to interfere with tumor growth and invasiveness.

4. New enzymatic assay using parasite ldh in diagnosis of malaria in Kenya

The biochemical basis for this assay is on the fact that human red blood cells do not utilize APAD in the metabolism of glucose. The study subjects were of three different categories: the healthy non-infected individuals staying out of malaria endemic area(controlled GP-1).The non-symptomatic and parasitemic healthy individuals living in endemic regions (both field study –GP 2).The non-parasitemic and parasitemic symptomatic individuals living in endemic region (both clinical study Group 3).In the clinical studies, thin smear microscopy gave the highest sensitivity as 75.6 % for plasma, while the highest specificity was 71.4 %. For red blood cells, the highest sensitivity was 78.4% while specificity was 80 %. In field trials, the highest sensitivity was 89 %) using thin smear microscopy, where as the specificity was 45% for the plasma cut off, using thick smear. For red blood cells, the highest sensitivity was 79% while the specificity was 66.7%. The variations in sensitivity and specificity of this assay in comparison to microscopy is a strong indication that pLDH may even be measuring sequestered parasites that cannot be visualized by microscopy. The results of the study validates the use of pLDH as an alternative objective test for malarial diagnosis against microscopy.

5. Malaria antigen detection tests

There are currently over twenty (20) such tests commercially available (WHO product testing, 2008) and these consist of a group of commercially available tests that allow rapid diagnosis of malaria by unskilled laboratory traditional techniques. The first malaria antigen suitable as target for Rapid Diagnostic Tests (RDTs) was a soluble glycolytic enzyme, Glutamate dehydrogenase. None of the current tests is as sensitive as a thick blood film. A major drawback in the use of all dipstick methods is that the result is essentially qualitative. In many endemic areas of tropical Africa; however, the quantitative assessment of parasitemia is important as a large percentage of the population will test positive. An accurate diagnosis of malaria is becoming more and more important in view of the increasing resistance of *Plasmodium falciparum* and the high price of alternatives to chloroquine. The enzyme pGluDH (parasite Glutamate Dehydrogenase) does not occur in the host red blood cells and was regarded as a marker enzyme for Plasmodium species. The malaria marker enzyme test is suitable for routine work and it's now a standard in most

nations afflicted with malaria. The presence of pGluDH is known to represent parasite viability, and a rapid diagnostic test using pGluDH as antigen would have the ability to differentiate live and dead organisms. It is possible to note that a complete RDT with pGluDH as antigen has been developed in China. Glutamate dehydrogenases are ubiquitous enzymes that occupy an important branch point between carbon and nitrogen metabolisms. Both nicotinamide adenine dinucleotide[(NAD)EC 1.4.1.2] and nicotinamide adenine dinucleotide phosphate (NADP) dependent GluDH [EC1.4.1.4] enzymes are present in plasmodia. The NAD-dependent GluDH is relatively unstable and not useful for diagnostic purposes. Glutamate dehydrogenase provides an oxidizable carbon source used in production of energy as well as a reduced electron carrier, NADH. Glutamate is a principal donor to other amino acids in subsequent transamination reactions. The multiple roles of glutamate in nitrogen balance, make it a gateway between free ammonia and the amino groups of most amino acids. The GluDH activity in *P. vivax*, *P.ovale* and *P. malariae* has been tested, but given the importance of GluDH as a branch point enzyme, every cell must have a high concentration of GluDH. It is well known that enzymes with high molecular weights like GluDH have many isoenzymes which allow strain differentiation (given the right monoclonal antibody).The host produces antibodies against the parasite enzyme, indicating a low sequence identity.

6. New therapeutic approaches for treatment of plasmodium falciparum

The antimalarial treatment so far recommended for P. falciparum consists of drug combinations containing Artemisinin derivatives (ACT) known as artemisinin combination therapy with other antimalarials, including quinolone compounds such as Amodiaquine and Mefloquine. The mechanism of action of the quinolones involve the inhibition of hematin polymerization, thus intoxicating the parasite with the ferriprotoporphyrin groups generated by hemoglobin degradation. Other antimalarials used in the ACT therapy include-Pyrimethamine and proguanil, which inhibit the tetrahydrofolic acid cycle (tetrahydropterate reductase, limiting the formation of folic acid, an important cofactor in DNA biosynthesis. Despite the arsenal of drugs available for malaria treatment, the disease remains a worldwide public health problem. P. falciparum develops resistance under selected drug pressure. Plasmodium vivax is the most prevalent human malaria parasite world over and has been shown to be resistant to chloroquine, including in Brazil and other countries where malaria is endemic. Various efforts have been made to develop new drugs (antimalarials), but resistance to drugs has limited the search. The continued search for new molecular targets for drug design has broadened the therapeutic arsenal and strategies to fight drug resistance in human malarial infestation.

7. Three new parasite ldh (pan-pLDH) tests for diagnosis of uncomplicated malaria

Since Charles Laveran first visualized the malaria parasite in 1880; the mainstay of malaria diagnosis has been the microscopic examination of blood smear. It is the main economic,

most preferred and reliable diagnosis using two types of blood films, which is amenable to the four species of malaria parasite. These two types of films are (a) the thin blood film which is similar to the usual blood films, which allows species to be identified because they can be visualized and the appearance of the parasites are much more distorted.(b)the thick film from which an experienced microscopist can detect parasite levels or parasitemia down to levels as low as 0.0000001% of red blood cells. Diagnosis of species can be difficult because, the early trophozoites ("ring form") of all four species look identical and never possible to diagnose the species on the basis of simple ring form. The success of the method above requires well trained staff, quality equipment and supervision. The scarcity of these facilities within malaria endemic areas becomes limiting. In sub-Saharan Africa and some other areas, clinicians often have to rely on clinical signs and symptoms for diagnosis and in some areas where increasing emphasis is laid on home based management , malaria diagnosis is often equated[11] with fever. It is to be noted that such presumptive treatment without laboratory confirmation could contribute to the development of drug resistance[11]. Today, an alternative method to the blood film diagnosis approach is the rapid diagnostic test (RDTs), recommended by WHO, where reliable microscopy is not reliable or available. Rapid diagnostic tests (RDTs) are antigen detection tests, which are simple to use and interpret, although the tests also use peripheral blood. The most commonly used RDTs, is the histidine-rich protein 2 (HRP 2), produced by trophozoites and young gametocytes of plasmodium falciparum. HRP 2 test has been the most widely evaluated to date test, and has shown consistently high sensitivity. The limitation of this test is that RDTs detect P. falciparum only and can remain positive for several weeks after antimalarial treatment. Besides these, a study to assess the diagnostic capabilities of three parasite lactate dehydrogenase (pan- LDH)-,Vistapan® ,Carestat™ and Parabank® were conducted in Uganda. Similarly, a histidine- rich protein 2 (HPR 2) test, Paracheck-Pf® and a Geimsa-stained blood film were performed with pfLDH tests for outpatients. A total of 460 patients were recruited for the exercise, 248 had positive blood films and 212 with negative blood films. *Plasmodium falciparum* was present in 95% of infections. Sensitivity of the tests above 90% was shown by two pLDH tests-Carestart (96.5%) and Vistapan (91.9%) and sensitivity above 90% by Parabank (94.3%) and Carestart (91.5%). The benefits of these tests when compared with the previous gold standard for laboratory confirmation of malaria diagnosis which is a peripheral blood film examined microscopically shows the high specificity and validity of the tests.

An alternative diagnostic method to the rapid diagnostic test (RTD), recommended by WHO, where reliable microscopy is not available. RTDs are antigen detection tests, which are simple to use and interpret and also use peripheral blood. The most commonly used RTD detects histidine -rich protein 2 (HRP2), produced by trophozoites and young gametocytes of *Plasmodium falciparum*. HRP 2 tests have been the most widely evaluated to date and show high sensitivity. However, they are limited in that they detect P. falciparum only and can remain positive for several weeks after successful treatment[12,13].

The second type of RTD detects the malaria antigen parasite lactate dehydrogenase (pLDH), an enzyme produced in the glycolytic cycle of the asexual stage of all species of Plasmodium. Parasite lactate dehydrogenase(pLDH) are produced only by viable parasites,

thus being cleared from the blood stream more rapidly after treatment, resulting in test becoming negative more quickly. There is no doubt that these characteristics suggest that pLDH tests could be used with more confidence for malaria diagnosis at the peripheral level. The development of several new pLDH monoclonal antibodies by Flow Inc. has enabled the production of a new generation of pLDH tests. These characteristics suggest that pLDH tests could be used with more confidence for malaria diagnosis at the peripheral level [14]. For confident diagnosis of malaria in routine outpatient department conditions, a sensitivity of more than 90% is crucial and this has been achieved by both Carestart and Vistapan. The pLDH tests have also demonstrated desirable qualities that could reduce the possibility of patients without malaria being given antimalarials, which may therefore reduce drug pressure, a major concern at a time when Artemesinin combination therapy (ACTs) are being introduced in Africa. The validation of these tests for malaria diagnosis involves many stages from the selection of site, enrolment of patients, sample size, study procedures, laboratory procedures, study outcomes, analysis and results. The validity of the tests after all these procedures must be rated from 90% and above. From the validity tests carried out by some researchers, Carestart had estimates for all validity parameters greater than 90%. Vistapan and Carestart were also sensitive as Paracheck Pf ($p=0.14$ and $p=0.38$) respectively. Parabank was less sensitive than all other tests ($P<0.001$ for each comparison). There was no significant difference between the three pLDH tests, but Parabank had a higher specificity compared with Paracheck Pf ($P=0.02$) for P. falciparum detection. In the study, the ages of patients were taken into consideration. Sensitivity decreased with older age for both Vistapan (97.4%) for the under fives versus 85.7%, $P<0.011$ and Parabank (95.4%) for the under fives versus 73.1%, $P<0.001$. Three tables were used to summarize the results of a study conducted in Mbarara Regional Hospital in Uganda, in a mesoendemic area of malaria transmission. These results are shown in tables 2, 3 and 4 respectively.

7.1. Materials and methods

The first approach is to select a site, which should be a highly malarial infested zone.

7.2. Enrolment for the study.

Patients from the outpatient department were systematically screened for symptoms suspected to be malaria and referred to the research clinic. Inclusion criteria were a clinical suspicion of malaria; weight≥ 5kg; resident in Mbarara Municipality available for two weeks follow up period; and signed informed consent from the study subjects or their legal guardians. Exclusion criteria were signs of severe or complicated malaria [15b], signs of severe disease; and women with visible pregnancy or suspicion of pregnancy based on the assessment of the last normal menstrual period.

7.3. Sample size

The required number of patients with positive blood film was calculated using an estimated sensitivity of the RDTs of 90%, an alpha error of 0.05 and a precision of 6%. This number (n-

96) was doubled to permit a stratified analysis by age group (0--4 and ≥5 years. The same parameters were used to calculate the required number of patients with a negative blood film, thus giving a final minimum sample size of 200 blood-film-positive and 200 blood – film –negative patients.

7.4. Study procedures

On the day of inclusion, demographic and clinical information were recorded, and a thick/thin blood film and the four rapid tests (Vistapan, Carestart, Parabank , and Paracheck-Pf) were performed. Women with positive pregnancy test and hyperparasitemic patients (P. falciparum›250,000 parasites/µl) were given quinine and excluded from further follow up. All other patients with positive blood film received an artemether-lumefantrine(Coartem®, Norvatis Pharma AG,Bassel,Switzerland), six –dose regimen under directly observed therapy. This treatment modality have been shown to be very efficacious with a prompt reversion to a negative [16] blood film after treatment. Patients receiving Coartem were asked to return to the Clinic on the third, seventh and 14th day after inclusion to repeat the blood film and all RDTs

7.5. Laboratory procedures

Blood films and rapid tests were performed from the same finger-prick blood. Blood films were dried, thin films fixed in methanol, and both films stained with 3% Giemsa for 45 minutes. Smears were read by experienced technicians, counting parasites against 200 or 500 white blood cells (WBC) or 200 high power fields before declaring a blood slide negative. The parasite density per micro-liter was calculated by multiplying the asexual parasite count by 8000 and dividing by the number of WBC counted [17].Plasmodium species were confirmed on the thin film and slides with mixed infections had only P.falciparum monoinfection, had the asexual density per microliter calculated as for P.falciparum. Gametophytes were recorded with species identification where possible. All inclusion slides were blinded and double read, with a third reading performed in case of discordance, ie; positive/negative discordance for asexual stages; asexualdensity discordance (difference in parasitemia≥50%);positive/negative gametocyte discordance. Twenty percent of the follow-up visit slides were also blinded and double read. External quality control of 290 inclusion slides was performed by Shokia Malaria Research Unit, Thailand, giving Mbarara laboratory, a sensitivity of 95.5% and a specificity of 100%. All RDTs were performed and interpreted according to the manufacture's instructions. Each test result was interpreted by two independent health care providers blind to the result of the blood film and reading according to a rota to avoid observer bias. The first reading was performed at the time specified by the manufacturer (15 min after preparation for Paracheck Pf and Parabank and 20 mins for Carestart and Vistapan. The second reading was performed within 15 min of the first one. Discordant results were read by the laboratory supervisor for a definitive result. Each reader also classified the test as either invalid or doubtful. A doubtful test was defined as a test for which the reader was not sure if there was any indication of a line present. At

the end of the study, two test readers and two laboratory technicians involved in preparing the tests completed a questionnaire concerning the ease of use and interpretation of each test.

Analysis. All data were either recorded directly or transcribed from source data forms to an individually numbered case report form (CRF). Data were double entered and validated using EpiData version 3.1(EpiData Association,Odense, Denmark)and analysed using Stata 9.1 (Stata Corp. college station,TX,USA).The study profile and base-line characteristics were summarized, including comparative tests between age groups (x 2 test ,Ma-Whitey U test). The validity for each test was calculated overall and then stratified by age group, level of parasitemia(parasites/μl 1---99,≥100,≥200,≥500), presence/absence of fever, duration of illness(0—2vs.3 days and above) and a history of taking antimalarials, using comparative tests(x 2 test, Mann-Whitney U test) to compare differences between groups. Kappa statistics were calculated for iter-reader reliability for each test on the day of diagnosis. A test was considered as reliable if k ≥ 0.8. Univariable and multivariate analyses were performed to investigate the association between explanatory factors and the test remaining positive at each follow- up visit.

Results. Demographical and Parasitological characteristics of study subjects.

Between 26 April and 27th July, 2005, 485 patients from the out-patient department were screened. Nine were ineligible (three had severe illness, five were non-residents and one was not in the appropriate age group after completion of recruitment in the under fives. Sixteen patients did not consent to participate in the study; 239 under fives and 221 aged 5 years and above. The mean age was 12 years(SD 13years;Table 2). There were 248 positive blood films with P. falciparum monoinfections(93.6%), P. malariae monoinfections(2.4%), P. falciparum+ P. malariae mixed infections(0.8%) and P. falciparum+ P. vivax mixed infections(0.8%). Of the 212 negative films, nine had gametocytes present. Parasitological characteristics of positive subjects are given in Table 2. Slides positive with P. falciparum had higher parasite densities than those of the other two species.

Validity of RDTs.

Only Carestart had estimates for all validity parameters greater than 90% (Table 3). Vistapan and Carestart were as sensitive as Paracheck-Pf(P=0.14 and P=0.38 respectively. Parabank was less sensitive than all other tests (P‹0.001 for each comparison). There was no significant difference in specificity between the three pLDH tests, but Parabank had a higher specificity compared with Paracheck.Pf (P= 0.02) for P. falciparum detection. Sensitivity decreased with older age for both Vistapan [97.7% (under fives) vs 85.7%, P‹0.01] and Parabank[95.4%(under fives)vs73.1%, P‹0.001]. Sensitivity increased with axillary temperature≥ 37.5 ^{0}C at inclusion for Paracheck.Pf (98.8 vs91.4%, P=0.04), Vistapan(97.6 vs 89.0%, P=0.03) and Parabank(91.8vs81.0%, P=0.04) compared with patients with axillary temperature ‹37.5 ^{0}C. Although, the small number of non-falciparum monoinfections does not permit reliable calculations of validity of non-falciparum mutants, all tests detected 100 % (n=6) of the P. malariae monoinfections. Plasmodium vivax was detected in 4/6 infections by Carestart, 2/6 by Vistapan and 1/6 by Parabank.

Parameters	Group A (<5yrs)	Group B (≥5yrs)	Overall	p-value
Baseline characteristics	n=239	n=221	n=460	
Gender ratio (M:F)	0.98 (118:121)	0.52 (76:145)	0.72 (194:266)	0.001(X 2)
Mean age(SD)	2yrs (14 months)	22yrs (12yrs)	12yrs (13 yrs)	N/A
Median duration of illness in days (range)	3(1-14)	3(1-30)	3(1-30)	0.2(Kruskai-Wallis)
Previously taken antimalarial (n,%)	81(33.9)	60(27.3)	141(30.7)	0.13(X 2)
Fever on presentation (axillary temp≥ 37.5 ^{0}C)	99(41.4)	31(14.0)	130(28.3)	<0.001(X 2)
Parasitological characteristic	n=129	n=119	n=248	----------
Asexual parasitemia range (parasites/μl)	16-703411	16-233241	16-703411	0.001(Kruskal-Wallis)
Geometric mean of asexual parasitemia(95%Cl)	7433(4869-11346)	1524(975-2384)	3475(2521-4790)	0.001(t test)
Interquartile range (Interquartile value)	1682-45748(44066)	166-11070(10904)	641-23827(23186)	----------
Gametocyte carriage(n,%)	36(27.9)	22(18.5)	58(23.4)	0.11(X 2)

Table 2. Baseline characteristics of all study subjects and Parasitological characteristics of Slide-positive subjects attending Mbarara Regional Referal Hospital, Outpatient department, South -Western Uganda

Reliability

The k statistic for the inter-reader reliability for all tests was above 0.90(very good agreement) [Carestart,k=0.96(95.0%, Cl 0.94—0.99); Vistapan, k=0.94(95% Cl 0.91- t 0.97); Parabank, k=0.96(95% Cl 0.94-0.99); Paracheck.Pf, k=0.97(95% Cl 0.95-1.0)]

Time to Negativity of RDTs.

There were no positive blood films on follow up visits, and therefore, every positive RDT result on day 3,7 or 14 was considered a false positive result (Table 4). All three RDTs tested had significantly fewer false positive results on every day of follow-up compared with Paracheck.Pf(P‹0.001 for all tests on day 3,7 and 14). There was o difference between the pLDH tests by day 14, with the percentage of positive tests ranging from 4.6 to 9.5%.

Younger age group and higher parasite level at inclusion were related to positive Paracheck. Pf on all follow-up days (logistic regression, P‹0.01), for all. Age group, fever at diagnosis and presence of gametocytes on day 3 were all related to a positive pLDH test on day 3 (except age group for Parabank)(age group:Vistapan P=0.026, Carestart P‹0.001.

Parameters	Carestart % [95 Cl]	Vistapan% [95%Cl)	Parabank% [95% cl)	Paracheck =Pf%(95%cl)
Sensitivity	95.6(237/248) [90.2-96.6]	91.9(228/248) [87.8-95]	84.7(210/248) [79.6-88.9]	94(233/248) [90.2-96.6]
Specificity	91.5(194/212) [86.9-94.9]	89.6(190/212) [84.7-93.4]	94.3(200/212) [90.3-97.0]	87.3(185/212) [82.0-91.4]
PPV	92.9(237/255) [89.1-95.8]	91.2(228/250) [87-94.4]	94.6(210/222) [90.7-97.2]	89.6(233/260) [85.3-93]
NPV	94.6(194/205) [90.6-97.3]	90.5(190/210) [85.7-94.1]	84.0(200/238) [78.7-88.4]	92.5(185/200) [87.9-95.7]

PPV: Positive Predictive Value; NPV: Negative Predictive Value

Table 3. Validity of four rapid diagnostic tests for the detection P. falciiparum species in patients attending Mbarara Regional Hospital, out-patient department, southwestern Uganda

RDT	Day 0 n[a]	Day 3% [95% Cl]	Day 7 %[95Cl]	Day 14 % [95% Cl]
Paracheck - Pf	226	86.2(193/224) [81.7-90.7]	80.8(181/224) [75.6-86.0]	69.7(152/218) [63.1-75.7]
Vistapan	221	36.1(79/219)[29.7-42.5]	3.4(51/218)[17.8-29.0]	8.9(19/213) [5.1-12.7]
Carestart	230	42.5(97/228) [36.1-48.9]	27.6(63/228) [21.8-33.4]	9.5(21/221) [5.6-13.4]
Parabank	204	17.8(36/202) [12.5-23.1]	8.9(18/202) [5.0-12.8]	4.6(9/196) [1.7-7.5]

a n is the number of positive tests for each RDT on day 0 in patients who were followed up.

Table 4. Percentage of positive tests on each follow up visit in patients attending Mbarara Regional Hospital, Outpatient department, SouthWestern Uganda.

In overall, there are not large differences between the tests in terms of ease of use. Some tests have small advantages or disadvantages over others. For example, Vistapan has individual buffer sachets, considered to be an advantage, where as Carestart has a delay time of over 60 seconds between blood application and buffer application, considered to be a disadvantage. All tests results are stable for a minimum of 24hr.The number of invalid tests was ‹0.5% for Parabank and between 0.5 and 2% for Carestart and Vistapan. No test had items requiring refrigeration and all tests have undergone temperature stability studies up to 30 ^{0}C.

This study appears to be a pioneer study [17] to evaluate a new generation of pLDH tests for malaria diagnosis, performed in a mesoendemic African setting with a predominance of P. falciparum infections. The authors showed that several of these tests were validated and should be of great use in malaria endemic countries, where microscopy is not available and well trained microscopists are lacking. For confident diagnosis of malaria in routine outpatient departments, a sensitivity of more than 90% is crucial and this was achieved for Carestart and Vistapan.

The pLDH tests also demonstrated desirable qualities that could reduce the possibility of patients without malaria being treated or given antimalarial drugs, thereby reducing drug pressure and resistance, a major concern at a time when artemisinin combination therapy (ACTs) are being introduced throughout Africa. Their high specificity would reduce the number of patients with false positive results. Secondly, the great reduction in the number of tests remaining positive for a long time after treatment had been effected. This is reminiscent of the test carried out by HRP 2. Thirdly, the ability to detect both P. falciparum and P. vivax, would increase confidence on a negative test result. A variety of factors may contribute towards differing sensitivities of the test, such as patients age and level of parasitemia, which will vary according to endemic nature of P. falciparum in the locality. Lower test sensitivity may be related to low parasitemia in adults in an area of stable transmission. This may be a limitation of the tests; although, such patients are less at risk from severe clinical episodes to perpetuate parasite transmission.

8. Comparison of two rapid field immunochromatographic tests to expert microscopy in malaria diagnosis.

The vast majority of malarial tests adopted and used in the past depended on light microscopy and expertise in attaining results. Currently, the vast majority of malaria cases in the world are diagnosed by the century old standard of light microscopy and stained blood smears. Although the technique is sensitive and very inexpensive, there are several disadvantages. The disadvantages include practical issues such as electrical requirement, the need for experienced staff as well as difficulties in accurate species identification. Efforts have been made to develop malaria rapid diagnostic devices (MRDDs) to facilitate field diagnosis [18]. While the first generation tests diagnosed only Plasmodium falciparum, newer devices were designed to recognize both Plasmodium falciparum and P. falciparum specific antigen as well as Plasmodium genus specific antigen.

The ICT malaria Pf/P.v test is a rapid immunochromatographic assay for the detection of P. falciparum-specific Pf HRP 2 and a pan-malarial antigen, manufactures in test card form. The name is misleading as the pan malarial antigen may be produced by Plasmodium ovale, and Plasmodium malariae as well. The test has been shown to be specific and sensitive. Malaria P.f/P.v test has been shown to be 96% sensitive and 90% specific for P. falciparum and 75% sensitive and 95% specific for P. vivax [19,20].

The OptiMAL dipstick test detects two forms of plasmodium lactate dehydrogenase parasites pLDH1; one P. falciparum-specific and one common to the four plasmodium species which infect humans. Many researchers reported a 95% sensitivity and 100% specificity for P. falciparum, and a 96 % sensitivity and 100% specificity for P.vivax. Sensitivity for P. ovale and P.malariae were significantly lower (57% and 47%) respectively. Using a batch OptiMAL dipstick procedure in 1999, a large scale field evaluation showed the following result: 91% sensitivity and 95% specificity for P.falciparum and 83% sensitivity and 100 % specificity for P.vivax [21].

Methodology. The methodology involved the use of patients both symptomatic and asymptomatic , who are villagers referred to the study and informed consent obtained. For each patient, a finger prick was made and the following were obtained. Fifty microliters of blood in a pre-heparinized Eppendorf tube for dipstick assays and two thick/ thin smears, one for on –site microscopic diagnosis by Acridine orange(AO) technique (for immediate treatment purposes, and the other, for reference Geimsa-microscopist.OptiMAL reader and ICT reader, were all blinded to each other's diagnosis. Patients positive for malaria were treated. Thick and thin blood smears were prepared and stained with Geimsa according to standard procedures. To declare a sample negative, thick smears were read for 200 microscopic fields (1000 X) without finding a parasite.If found positive, the number of asexual malaria parasites were counted per 500 WBC separately for each species. If there are more than 250 parasites/500 WBC, parasites were counted on the corresponding thin film per 10,000RBC. Density calculations were based on approximations of7500 WBC/μl and 5X10 6 RBC/μl. ICT malaria P.f/P.v test kits were used as per manufacturers instructions. Ten microliters (10μl) of whol blood was transferred to a sample pad. A buffer reagent was added to induce cell lysis and allow PfHRP 2 and pan malarial antigens to bind to colloidal gold-labeled antibodies. Additional buffer caused the blood and immune complexes to migrate up the test strip and cross monoclonal (mAb) lines. Finally, more buffer was added to clear blood from the membrane and facilitate reading.

Tests are counted as valid, if control lines are observed. They were counted P. falciparum positive, if Pf HRP 2 specific and pan-malarial antigen lines were visible or if only PfHRP 2-specific lines were seen only. If the control and pan-malarial antigen lines were observed, the sample was counted as positive for a malaria parasite other than P. falciparum. The test result was assigned a value of +0, if no line was seen; +1, if test line intensity was less than control line intensity; +2,if it was equal and +3, if it was greater in intensity. Other factors can exacerbate drug resistance. The second type of RDT detects the malarial antigen parasite-lactate dehydrogenase (pLDH), an enzyme produced in the glycolytic pathway or cycle of the asexual stage of all species of plasmodium. Parasite lactate dehydrogenase is produced only by viable parasites, being cleared from the blood stream more quickly after treatment, resulting in the test becoming negative more quickly [22].

9. Clinical and epidemological findings based on histopathology and immunohistochromatographic detection of p.falciparum antigens

This new technique for determination of P.falciparum is not adopted during autopsy when actual diagnosis of P.falciparum infestation is missed mostly during improper diagnosis. Many organs and tissues manifest the severity of this type of malaria before death. The heart, lung, liver tissues are always available for post mortem analysis. Some other tissues used include: spleen, kidney, and brain. The following tissues were equally used in a case of five travelers suspected to have died from other chronic diseases not related to malaria. These other tissues include: tongue, trachea, thyroid and adrenal glands, gall bladder and testis. Viral and or bacterial hemorrhagic fever pathogens were suspected at death [23, 24,

25]. In the study, three novel IHC assays targeting HRP 2, aldolase, and pLDH were developed and confirmed on severe P. falciparum infection in five travelers whose deaths were wrongly suspected.

Malaria is the most common cause of fever in travelers returning to industrialized cities or countries from malaria endemic countries. However, the clinical features of the disease are not specific and in some areas where P.falciparum is not endemic, fatal malaria is often not suspected. The pathological features of malaria resemble many other viral, rikettsial and bacterial infections. An unequivocal diagnosis can be made only by laboratory testing. IHC assays and histopathologic review confirmed P. falciparum infections in many study cases as being responsible for unsuspected deaths. Abundant hemozoin pigment, a by-product of parasite metabolism was distributed diffusely throughout peripheral tissues and in the blood vessels in the central nervous system. The findings are consistent with reports of hemozoin localization. The density of hemozoin increases in proportion to the duration of falciparum infestation and decreases with adequate and appropriate therapy. It has been discovered that the hallmark of P.falciparum infection is sequestration, characterized by the adherence of mature stage falciparum pRBCs(trophozoites and schizonts) to endothelial cells of capillaries and venules.

Many studies in humans and other animals have described sequestration of trophozoites and schizonts in a variety of tissues, including the brain, heart, lung, skeletal muscles and subcutaneous tissues. As a result of sequestration, peripheral blood parasitemia, traditionally evaluated using Geimsa bood smear, may not give substantive correlative result in the pathogenesis of the severity of P. falciparum, hence,the severity of the infection may be under-estimated. Plasmodium falciparum infection caused respiratory symptoms that resemble influenza like-illness in correlation with results from studies. There is pulmonary edema with intra-aveolar hyaline membranes and proteinaceous debri, associated with malarial antigens. Pulmonary edema is associated with high parasitemias and often leads to respiratory distress syndrome. Rust tinged urine described for several patients afflicted with this malaria, is associated with hyperbilirubinemia caused by erythrocyte destruction. In many cases of P. falciparum, malarial antigens can be detected in tubular epithelial cells in association with erythrocyte casts. The HRP 2 antibody used in this study was specific for P.falciparum, whereas the aldolase and the pLDH antibodies reacted with both P. falciparum and P. vivax.

In conclusion, the current approach in the diagnosis and development of new drugs for the treatment of Plasmodium falciparum infections is quite novel and holds promise for the future. The ubiquitous nature of the enzyme, Lactate dehydrogenase especially, parasite lactate dehydrogease (pLDH) as malaria antigen, is indicative of the vital biochemical process of metabolism of pyruvate and lactate in microbial cells. The endemic nature of malaria in Africa and some Mediterranean countries poses a great challenge to humanity. There should be a more radical approach especially in Africa and other countries afflicted to tackle this problem which tend to decimate world population. Other frontline drugs which are designed to inhibit the enzyme should be developed to add to the success of this protocol. In as much as mosquitoes have developed resistance against chloroquine, the drug in some places remains the only option for radical cure of malaria.

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