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NEW DIAGNOSTIC TOOLS FOR MALARIA- CHALLENGES AND OPPORTUNITIES

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New diagnostic tools for malaria- challenges and opportunities

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Malaria är en av vår tids viktigaste sjukdomar ur ett globalt perspektiv. Malaria är en parasitsjukdom som överförs av myggor. Under de senaste femton åren har det skett en minskning av malariaförekomst och dödlighet tack vare kontrollinsatser i bred skala. Trots detta utgör sjukdomen ett ständigt hot mot liv och hälsa för ca 40 % av jordens befolkning som lever i de dryga 90 länder där malaria förekommer och över 600.000 dör av sjukdomen varje år. Majoriteten av dessa är barn yngre än 5 år och gravida kvinnor i Afrika, söder om Sahara.

Tillgång till en snabb och korrekt parasitbaserad diagnos för att rikta behandling till de med en bekräftad infektion är en mycket viktig hörnsten i malariakontroll. Detta är viktigt för att undvika överdiagnostik, baserat på kliniska observationer, och för att förhindra överanvändning av anti-malarialäkemedel. Detta är viktigt för att minimera risken för att resistens mot läkemedlen utvecklas. Emellertid ställer olika endemiska sammanhang olika krav på dessa diagnostiska hjälpmedel. Nya moderna verktyg såsom snabbtest (RDT), baserade på påvisande av malariaspecifika proteiner i blodprov, har möjliggjort ökad tillgång till en känslig och korrekt diagnos. De nya snabbtesterna har många fördelar; de är enkla att utföra och bedöma, de kräver varken tillgång till utrustning eller elektricitet och lämpar sig därför väl för användande både inom akut- och primärhälsovård världen över. Snabbtester har dock också nackdelar, främst med avseende på känslighet och precision.

I denna avhandling har nyttan av snabbtester jämfört med mikroskopi och molekylära metoder (PCR), både bland feber patienter i ett lågendemiskt/pre-elimineringsområde (Zanzibar), och bland barn under fem år med en bekräftad malariainfektion i ett relativt högendemiskt område (Tanzania), utvärderats. Vårdpersonalens tillit till resultatet på snabbtesten studerades också i Zanzibar. Resultaten visade att snabbtestets känslighet för påvisande av parasiter hade sjunkit sedan en tidigare undersökning då det fanns mycket malaria på Zanzibar, medan vårdpersonalen hade fortsatt högt förtroende för testresultatet, d.v.s. endast de med positivt resultat fick behandling med malarialäkemedel. Barnen i Tanzania följdes upp vid nio tillfällen med mikroskopi, PCR och två olika snabbtester, baserade på två olika malariaspecifika proteiner, upp till dag 42 efter insatt malariabehandling. Detta gjordes för att beräkna hur lång tid de olika diagnostiska metoderna kvarstod positiva samt deras förmåga att upptäckta nya episoder av malaria, något som förekommer ofta i högendemiska områden. En av de utvärderade snabbtesterna visade positivt resultat i medeltal fyra veckor efter den första malariainfektionen och kunde därför inte upptäcka nya episoder.

Hur parasite DNA kan påvisas i snabbtester utvärderades också. Detta kan användas för övervakning av markörer för resistens hos parasiterna och för kvalitetskontroll av snabbtester.

Att utveckla tester som är tillräckligt känsliga för att kunna hitta asymtomatiska bärare av mycket låga parasitnivåer, vilka utgör en risk för fortsatt smitta i områden där malarian närmar sig eliminering, är en utmaning. De nuvarande snabbtesterna är inte tillräckligt känsliga. DNA påvisning med PCR är den metod som har det som krävs avseende diagnostisk känslighet men som genom höga krav på både tid, kunskap och utrustning inte utgör ett alternativ. Under senare år har LAMP (loop-medierad isotermal amplifiering) en snabb, känslig och relativt enkel molekylär metod börjat testas och som i framtiden förhoppningsvis kommer att lämpa sig också för diagnostik och övervakning av malaria i endemiska områden utanför specialutrustade laboratorier. Ett LAMP test utvärderades för påvisande av malariaparasiter på insamlade prov från både feberpatienter och asymptomatiska bärare i Zanzibar. LAMP påvisade parasiter med lika hög känslighet som PCR också hos de asymptomatiska bärarna med mycket låga parasitnivåer, vilket visar att metoden är lovande för framtida användning i endemiska områden.

Resultaten i de fyra delstudierna i denna avhandling utgör viktiga data avseende nyttan av nya diagnostiska verktyg för bättre påvisning och övervakning av malaria i olika endemiska sammanhang.

ABSTRACT

Nearly half of the world's population is at risk for malaria and over 600,000 die from the disease every year. Access to prompt and correct parasite based diagnosis in order to target treatment to those with a confirmed malaria infection and improved malaria surveillance are cornerstones in malaria control. The availability of modern diagnostic tools such as rapid diagnostic test (RDT), polymerase chain reaction (PCR) and loop mediated isothermal amplification (LAMP) represent new opportunities besides microscopy for improved sensitive and accurate parasite-based diagnosis. However, there are different demands on diagnostic tools for different health care settings and endemic contexts.

The performance of RDT was compared to blood smear microscopy and PCR among febrile patients in a low endemic/pre elimination area (Zanzibar). Although the sensitivity of RDT was found to be relatively low (76.5%) the health care workers were highly adherent to test results in prescribing antimalarial drugs.

Parasite clearance and detection of recurrent infections was assessed by different diagnostic methods after malaria treatment in febrile children in a relatively high endemic area of mainland Tanzania. Median clearance time was two days for PCR and microscopy whereas the clearance times were seven and 28 days for the pLDH and HRP2 based RDTs, respectively. pLDH based RDT was a better tool than HRP2 based for treatment follow up and detection of recurrent infection

The usefulness of RDT as a source of parasite DNA was evaluated through different parasite DNA extraction methods. DNA extraction efficacy varied with test device and extraction method. There was no difference in PCR detection rates between RDT and filter paper samples collected from the field. This confirms the usefulness of RDTs stored under field conditions as a modern tool for molecular malaria surveillance and RDT quality control.

A LAMP kit was compared to conventional PCR methods for detection of parasite DNA from dried blood spots collected among both fever patients and asymptomatic individuals in Zanzibar. The LAMP kit had a sensitivity of 98% for detection of Plasmodium(P) falciparum among fever patients and a sensitivity of > 92% and 77% for detection of P. falciparum and P. malariae among asymptomatic individuals. The high diagnostic accuracy of the LAMP kit for detection of low density parasitaemias from minute blood volumes preserved on filter papers supports its role for improved case detection in areas of low density malaria infections.

The results in this thesis provide important data on the usefulness of new diagnostic tools for improved case detection and surveillance of malaria in different endemic contexts.

LIST OF SCIENTIFIC PAPERS

I. Shakely D, Elfving K, **Aydin-Schmidt B**, Msellem MI, Morris U, Omar R, Weiping Xu, Petzold M, Greenhouse B, Baltzell KA, Ali AS, Björkman A, Mårtensson A. The usefulness of rapid diagnostic tests in the new context of low malaria transmission in Zanzibar. PLoS One. 2013 Sep 4;8(9)

II. **Aydin-Schmidt B**, Mubi M, Morris U, Petzold M, Ngasala BE, Premji Z, Björkman A, Mårtensson A. Usefulness of Plasmodium falciparum-specific rapid diagnostic tests for assessment of parasite clearance and detection of recurrent infections after artemisinin-based combination therapy. Malar J. 2013 Oct 1;12:349.

III. Morris U, **Aydin-Schmidt B**, Shakely D, Mårtensson A, Jörnhagen L, Ali AS, Msellem MI, Petzold M, Gil JP, Ferreira PE, Björkman A. Rapid diagnostic tests for molecular surveillance of Plasmodium falciparum malaria -assessment of DNA extraction methods and field applicability. Malar J. 2013 Mar 19;12:106.

IV. **Aydin-Schmidt B**, Xu W, González IJ, Polley SD, Bell D, Shakely D, Msellem MI, Björkman A, Mårtensson A. Loop Mediated Isothermal Amplification (LAMP)Accurately Detects Malaria DNA from Filter Paper Blood Samples of Low Density Parasitaemias. PLoS One. 2014 Aug 8;9(8

CONTENTS

LIST OF ABBREVIATIONS

µL	microliter
ACT	Artemisinin combination therapy
AQ	Amodiaquine
CI	Confidence interval
CNV	Copy number variation
CQ	Chloroquine
Ct	Cycle threshold
Cyt b	Cytochrome b
EIR	Entomological inoculation rat
FIND	Foundation for innovative new diagnostics
G6PD	Glucose-6-phosphate dehydrogenase
glurp	glutamate rich protein gene
Hb	Haemoglobin
HRP2	Histidine rich protein 2
Ig	Immunoglobulin
IMCI	Integrated management of childhood illness
IRS	Indoor residual spraying
ITN	Insecticide treated net
LAMP	Loop mediated isothermal amplification
LLIN	Long lasting insecticide net
Lu	Lumefantrine
MQ	Mefloquine
Msp	merozoite surface protein gene
Mt-DNA	Mitochondrial DNA
NMCP	National malaria control programme
NPV	Negative predictive value
p/ µL	parasites/ microliter
PCR	Polymerase chain reaction

pfcrt	P. falciparum chloroquine transporter gene
pfdhps	P.falciparum dihydroptereroate synthetase gene
pfmdr1	P. falciparum multidrug resistance 1 gene
*Pf*PR	P.falciparum parasite rate
pLDH	Plasmodium lactate dehydrogenase
PPV	Positive predictive value
PQ	Piperaquine
QBC	Quantitative buffy coat
qPCR	real-time PCR
RBC	Red blood cell
RBM	Roll back malaria
RDT	Rapid diagnostic test
RFL	Restriction fragment length polymorphism
SNP	Single nucleotide polymorphism
SP	SP
USD	US dollar
WBC	White blood cell
WHO	World health organization
ZMCP	Zanzibar malaria control programme

1 INTRODUCTION

1.1 GENERAL BACKGROUND TO MALARIA

Malaria has had a major impact on the development of life and health of mankind historically. Malaria is not just a disease commonly associated with poverty, evidence suggests that it is also a cause of poverty and a major hindrance to economic development [1, 2]. Throughout history, the contraction of malaria has played a prominent role in the fates of government rulers, nation-states, military personnel, and military actions [3].

Malaria is a parasitic disease caused by a protozoan of the genus *Plasmodium (P*). There are more than 100 species of *Plasmodiae* infecting a wide range of vertebrates including reptiles, birds and humans. Prehistoric man in the old world was subjected to malaria [4] and Hippocrates (5 century BC) was the first physician who described the clinical picture of malaria (fever and enlarged spleen) and its relation to season of the year and area where patients lived. The awareness of the association of fever with stagnant water and swamps, the breeding places for the mosquito vector, led to various methods of drainage already at that time. The name malaria comes from the Italian *mal aria* meaning bad air, derived from the belief that the disease was caused by the malodorous air surrounding marshy areas.

In 1880 Alphonse Laveran, a French army surgeon stationed in Algeria, was the first who described a malaria parasite in the human blood and shortly after that in 1897 Roland Ross (Scottish physician working in India) found a developing form of a malaria parasite in the body of a mosquito which had feed on a patient with plasmodia in the blood. Ross later described the complete life cycle of malaria [5]. Both these findings as well as the discovery of therapeutic effect of malaria infection on neurosyphilis by Julius Wagner-Jarregg in 1917 generated Nobel prizes [6].

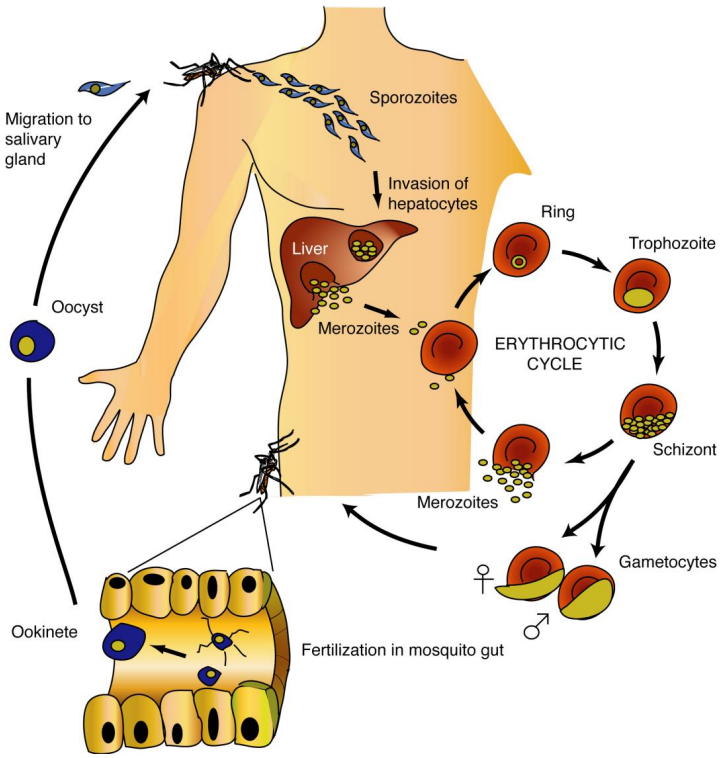
1.1.1 The life cycle

The life cycle of malaria parasites is complex and requires that the parasite goes through a number of highly specialized intra- and extracellular stages both in the human host and in the mosquito vector (definite host). There are 5 species of malaria infecting humans (*P. falciparum*, *P. vivax, P. ovale, P. malariae* and *P. knowlesi*).

The malaria parasite is injected into the human host from the bite of an infected female *Anopheles* mosquito. The sporozoite stage is injected with the saliva and transported to the liver within an hour, where they develop into exoerythrocytic schizonts in the parenchyma cells (hepatocytes) of the liver. This development takes 7-15 days depending on malaria species. With *P. vivax* and *P. ovale* some of these sporozoites differentiate into liver hypnozoites, which can remain dormant for many months before they develop further into schizonts and cause relapses of malaria disease. The mature liver schizonts contain up to 30,000 merozoites which are released into the blood stream through the burst of infected liver

cells. There they invade the erythrocytes (RBC) within a few minutes and the disease-causing asexual stage is initiated. The merozoite is transformed to an early trophozoite, the ring form, with a nucleus and cytoplasm surrounding a vacuole. The trophozoite matures into an erythrocytic schizont within 24 (*P. knowlesi*) to 72 (*P. malariae*) hours. During maturation the trophozoite feeds on the erythrocytic hemoglobin (Hb) forming microscopically visible pigment (hemozoin). The mature schizont contains between 8 (*P. malariae*) and 32 (*P. falciparum*) merozoites, which cause cell rupture and the merozoites are released into the blood stream where they rapidly re-invade new RBCs. All of the clinical symptoms of malaria, including fever, chills and anemia, are caused by the asexual cycle in RBCs. During the asexual cycle, some of the parasite cells develop into male and female gametocytes. Gametocytes circulate in the blood stream up to several weeks, being available to be taken up by a feeding mosquito. The male and female gametes fuse in the mosquito gut to form a zygote that develops into an ookinete. The ookinete crosses the gut wall to form an oocyst, where division (sporongy) occurs generating thousands of sporozoites. After oocyst rupture, sporozoites migrate to the salivary glands of the insect, from which they eventually can be injected into the human host during subsequent feedings [7].

Figure 1. The *P. falciparum* life cycle



(With kind permission from Dr. Cristine Sisowath)

1.1.2 The malaria parasite

There are 5 species of malaria infecting humans. Their characteristics are presented in Table 1.

P. falciparum

P. falciparum is found in tropical areas i.e. in most parts of Africa, Asia and Latin America. It is the most disease causing human malaria parasite. *P. falciparum* is responsible for over 90% of the malaria morbidity worldwide [8]. *P. falciparum* was in year 2010 estimated to be the cause of approximately 660,000 deaths, especially in Africa among children and pregnant woman [6, 8]. This is a decrease from over 1 million/year before year 2000 (6).

The *P. falciparum* parasite belongs to the lineage Laverania subgenus and has been suggested to be of more recent origin compared to other malaria species due to the low level of polymorphism within the *P. falciparum* genome [9, 10]. However, other researchers have found that the *P. falciparum* parasites co-evolved with its human host [11, 12] and recent reports have shown that *P. falciparum* can infect both monkeys and gorillas, which may constitute a reservoir complicating efforts to eliminate the parasite [12, 13].

P. falciparum is the species causing the highest parasite load because each schizonts can harbor up to 32 merozoites that are able to infect RBCs of all ages. This enables the infection to become hyper parasitemic with parasite densities of more than 5% causing massive lysis of RBCs and subsequent anemia, a common cause of severe malaria in children. *P. falciparum* has a tertian (48 hour) cycle even though fever paroxysms generally do not show a distinct periodicity.

Maturing stages of *P. falciparum* are expressing cyto-adherent proteins, forming knobs on the RBC surface. The *P. falciparum* Erythrocyte Membrane Protein1 (PfEMP1), encoded by the *var* gene family, plays a major role in cytoadherence [14]. The knobs make the infected cells "sticky", binding uninfected RBCs to their surface forming rosettes [15, 16]. These proteins also mediate binding of the infected RBC to the endothelial of the deep vessels, known as sequestration. This prevents the infected RBCs from being cleared from the circulation by the spleen [17]. The sequestered parasites clog in the vessels hampering the blood circulation. When this occurs in the brain it can result in cerebral malaria [18], a complication that stands for a large proportion of malaria deaths.

P. vivax

This species is found mostly in Asia, Latin America, and in some parts of East Africa. *P. vivax* normally requires the Duffy blood group antigen, expressed on the RBC surface to invade the cell and is therefore seldom seen in West Africa where Duffy negativity is common [19]. However, a recent report has shown *P. vivax* infections among some Duffy negative individuals in Africa [20*]*. *P. vivax* is generally seen as a benign form of malaria even though there are several reports on severe manifestations in the last years [21]. *P. vivax*

has a tertian cycle with more distinct intervals between fever paroxysms. *P. vivax* has a dormant liver stage, hypnozoites that can activate and invade the blood (relapse) several months or years after the initial infection. Strains of *P. vivax* can develop in the mosquito in temperate climates where they often do not produce primary attacks shortly after the infective bite, instead the clinical symptoms arise up to nine months later [22].

P. ovale

P. ovale is found mostly in Africa (especially West Africa) and on the islands of the western Pacific. It is biologically and morphologically very similar to *P. vivax*. However, and in contrast to *P. vivax*, it can infect individuals who are negative for the Duffy blood group. Genetic studies have shown that *P. ovale* actually comprises two non-recombining species that are sympatric in Africa and Asia and these are morphologically identical [23]. Mixed infections with *P. falciparum* are common. Like *P. vivax, P. ovale* has long been considered to have a dormant hypnozoite stage that can persist in the liver and cause relapses, but Richter et al. have questioned whether such a stage actually exists for *P. ovale* [24]. *P. ovale* also has a tertian cycle. Both *P. vivax* and *P. ovale* preferably invades young RBC, i.e., reticulocytes, which make parasite densities therefore seldom exceed 1%.

P. malariae

P. malariae is distributed over most of the malaria endemic area and is the only human malaria species that has a quartian (72 hours) cycle [22, 25]. If untreated, *P. malariae* often causes a long-lasting, chronic infection which often remains latent and can in some cases probably last a lifetime (23). Infections with *P. malariae* can in chronically infected patients cause serious complications such as nephrotic syndrome [26]. In Africa mixed infections with *P. falciparum* and *P. ovale* are common, and all three species can even occur simultaneously. *P. malariae* preferably invades RBCs older than 100 days making parasite densities seldom exceed 1%.

P. knowlesi

P. knowlesi is found in Southeast Asia as a natural pathogen of long and pig-tailed macaques. It has recently been shown to be a significant cause of zoonotic human malaria in that region, particularly in Malaysia where it accounts for up to 70% of human malaria cases [27]. So far there is no evidence that *P. knowlesi* can develop gametocytes in humans and be transmitted from human to human [28]. *P. knowlesi* has a 24-hour replication cycle and can rapidly progress from an uncomplicated to a severe infection; fatal cases have been reported [29].

Table 1.

	P. falciparum	***P. vivax***	***P. ovale***	***P. malariae***	***P. knowlesi***
Temperature for mosquito sporogony	min. 18 °C	min. 16 °C			27 °C
Incubation period (days)	9-14	12-16	16-18	18-40 or more	10-12
Hypnozoites	no	yes	yes	no	no
Erythrocytic cycle (hours)	48	48	50	72	24
Erythrocyte preference (age)	all	young	young	old	
Merozoites per schizont	8 - 32	12 - 16	6 - 10	6 - 12	10- 16
Sequestration	yes	no	no	no	no
Parasite densities % erythrocytes infected (range)	<0.1 - 40 %	<0.1-2%	<0.1-1%	<0.1-1%	<0.1 – 25%
Fever pattern	irregular	tertian	tertian	quartian	daily
Clinical severity	+ - +++	+ +	+	+	+ - +++
Duration of untreated infection (years)	1 - 1 1/2	3 - 4	3 - 4	3 - 50	
Relapses	no	yes	yes	no	no
Drug resistance	+ + +	+	-	-	-

min.= minimum [22, 25, 28-31]

1.1.3 The vector

Natural transmission of malaria occurs through the bite of a female mosquito of the genus *Anopheles (A*). Gametocyte stages of the parasite are ingested with the blood meal. Although there are more than 400 species described, only around 40 are considered of importance for malaria transmission [32]. Their effectiveness to transmit malaria is highly dependent on the feeding behavior, in terms of night or day, in or outdoor, human or animal preference. The main vectors for transmission of *P. falciparum* in Africa are *A. gambiae* and *A. funetus* which both are very efficient. In south of Sweden the *A. messeae* is present, probably being the mosquito spreading malaria in Sweden at the time when Carl von Linnaeus in 1735 described a malaria like disease and its association with water [33].

The malaria incidence is highly dependent on the mosquito numbers. In most areas endemic for malaria there are transmission peaks in association to rainy seasons, when breeding places for mosquitos are plentiful.

1.1.4 The endemic situation

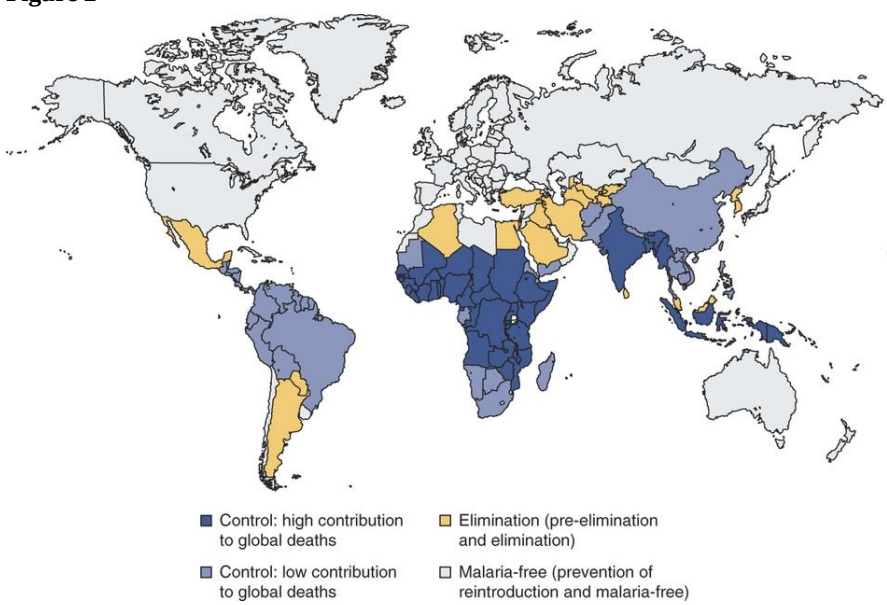
In 2013, 97 countries had ongoing malaria transmission (Figure 2 map).

There were an estimated 207 million cases of malaria in 2012 and 627,000 deaths. 90% of all malaria deaths occured in sub-Saharan Africa. In 2012, malaria killed an estimated 482, 000 children under five years of age, equivalent to 1300 children every day, or one child almost every minute.

Between 2000 and 2012, the scale-up of interventions helped to reduce malaria incidence rates by 31% and mortality rate by 49% in the WHO African Region, the corresponding global reduction was 25% and 42%, respectively [34].

The goals set by the World Health Assembly and the Roll Back Malaria (RBM) partnership to reduce the numbers of malaria cases and deaths recorded in 2000 by 50% or more by the end of 2010 and by 75% or more by 2015 have and will not be achieved. Several factors like weak public health systems in some low-income countries, poverty and political instability, drug-resistant parasites, increasing insecticide-resistance and outdoor biting habits of mosquitos, are all contributing to that these goals will not be achieved [35].

Figure 2



1.1.5 Measurement of malaria transmission

Measurement of malaria transmission has traditionally been based on the sporozoite infection rate (SR), i.e. fraction of infectious mosquito in the population. It is also used to determine the entomological inoculation rate (EIR), i.e., the number of bites by infectious mosquitoes per person per unit time. The EIR estimates the level of exposure to *P. falciparum*-infected mosquitoes [36]. Also spleen rate, i.e. number of palpable enlarged spleens /100 individuals of similar ages (usually children 2-9 years), has been used to measure endemicity [37]. SR, EIR and spleen rate are useful surveillance tools in areas with high and stable transmission, but less useful in low endemic areas with unstable transmission. Nowadays parasite rate *(Pf*PR), i.e. *P. falciparum* parasite carriers /100 individuals, as determined by microscopy, rapid diagnostic test (RDT) or polymerase chain reaction (PCR), and annual parasite incidence (API), i.e. cases/year/1000 inhabitants, are the most commonly used tools.

The level of malaria transmission/endemicity can be divided into the following:

- Hypoendemic: Low intermittent transmission, spleen rate 0-10%, *Pf*PR < 10%
- Mesoendemic: Regular seasonal transmission, spleen rate 11-50%, *Pf*PR 11-50%
- Hyperendemic: Seasonally high malaria transmission, spleen rate > 50%, in adults > 25%, *Pf*PR > 50%
- Holoendemic: Perennial high transmission, spleen rate > 75%, in adults low spleen rate, *Pf*PR > 75% in infants 0-11 months

(spleen rate in children 2-9 years, *Pf*PR in children 2-9 years)

Transmission is considered stable in hyper- and holo- (high) endemic areas and unstable in hypo- and meso- (low) endemic areas [37, 38].

1.2 CLINICAL PRESENTATION

Clinical malaria is primarily characterized as a febrile disease. Patients with malaria typically become symptomatic a few weeks after the infective mosquito bite when the parasite has entered the erythrocytic stage, although the host's previous exposure and immunity to malaria affects the symptomatology and incubation period. An uncomplicated malaria infection can include the following clinical symptoms:

- Fever
- Fatigue
- Splenomegaly
- Myalgia
- Cough
- Headache

Paroxysm of fever, chills, and sweats typically occur every 24, 48 or 72 h, depending on species. The periodicity is often not observed in *P. falciparum* infections. Children in endemic areas often develop fever in combination with cough, stomach pain and diarrhea, symptoms that often overlap with other childhood viral or bacterial infections.

Severe manifestations of *P. falciparum* malaria primarily in non-immunes and small children may include the following:

- Severe anemia (Haemoglobin (Hb) <5g/100 mL)
- Nausea and vomiting
- Acute respiratory distress
- Cerebral malaria
- Hyper parasitaemia (>5% infected RBC)
- Hypoglycemia (<2.2 mmol/L)

Severe anaemia and hypoglycemia are features of severe malaria more commonly seen in children than in adults [39]. Among adults cerebral malaria is the most common severe manifestation and cause of death [40, 41].

Clinical episodes with *P. vivax* and *P. ovale* are commonly uncomplicated. However for *P. vivax* severe complications like significant hepatomegaly, thrombocytopenia, acute renal failure, and severe anemia have increasingly been reported e.g. among Indian patients hospitalized for *P. vivax* complications (42).

P. malariae does not have a hypnozoite stage, but patients infected may have a prolonged, asymptomatic erythrocytic infection that becomes symptomatic many years after leaving the endemic area. Chronic infections with *P. malariae* can cause nephrotic syndrome because immune complexes may cause structural glomerular damage in the kidney [25].

P. knowlesi often causes severe disease due to the rapid multiplication with the 24 hour cycle of the parasite. A cerebral malaria-like syndrome has not been reported, but consciousness may be impaired secondary to the severity of illness in the context of multiorgan failure or hypoglycemia. Anemia is seldom reported, whereas thrombocytopenia is the most frequently reported blood abnormality [28].

1.2.1 Malaria in pregnancy

Malaria in pregnancy is associated with an increased risk of maternal anemia, low infant birth weight and premature births with increased risk of infantile death [6].

Pregnant women are more susceptible than non-pregnant women to malaria, and this susceptibility is greatest in first and second pregnancy. Susceptibility to pregnancy-associated malaria probably represents a combination of immunological and hormonal changes

associated with pregnancy, combined with the unique ability of a subset of infected RBC to sequester in the placenta [42].

1.2.2 Immunity

Genetic factors like red cell polymorphism causing sickle-cell trait (carriers of Hb-S) and thalassemia have proven to have protective effect against *P. falciparum* malaria. Also glucose-6-phosfate dehydrogenase (G6PD) deficiency and Duffy blood group negativity have proven to have a protective effect against *P. falciparum* and *P. vivax* infection, respectively. These factors are primarily present in areas endemic for malaria. Malaria selection has played a major role in the distribution of all these polymorphisms [43] and this so called "malaria hypothesis" is thus an example of an interaction between human genetics and infectious diseases [44].

Acquired immunity

Infants are protected during their first months of life through transfer of maternal antibodies and also by fetal Hb [45]. Young children in endemic areas exhibit an "antidisease immunity" after being exposed to multiple episodes of malaria during their first years. The acquisition of immunity against malaria is, however, species and strain specific [46]. The protection is more rapidly acquired in high endemic areas and results in reduced mortality or severe clinical disease already by the age of 5 years. Sterilizing immunity against infection is never fully achieved, and an asymptomatic carriage of relatively low densities of parasites is common among adults. In the absence of continual exposure, the immunity against clinical disease may be relatively short lived [47].

1.2.3 Diagnosis and Treatment

Diagnosis of malaria is mainly based on detection of parasites by microscopy, RDT or PCR and will be further presented in the next section.

The first known remedy for treatment of malaria associated symptoms was already used among Indians in South America in 17th century and known as "fever bark tree". Carl von Linnaeus gave it the name *Cinchona officinalis* and its active compound, quinine, remains an efficient antimalarial drug, today primarily recommended for treatment of severe malaria. During the World War II the devastating effects of malaria among military troops triggered the development of new drugs such as chloroquine (CQ), proguanil, amodiaquine (AQ) and

later sulphadoxine-pyrimethamine(SP). Following the Vietnam War the US army developed mefloquine (MQ) and halofantrine to protect their soldiers from malaria.

Today the most widely used drug against *P. falciparum* comes from the Chinese traditional use (already some 2000 years ago) of artemisinin (Qinghaosu) (ART), the Sweet Wormwood (*Artemisia annua*). Due to the fast and effective action of artemisinin, and its derivatives, it is now recommended for first line treatment in combination with slower acting partner drugs (artemisinin combination therapy-ACT) against uncomplicated *P. falciparum* malaria [48]. The commonly used partner drugs are AQ, lumefantrine (Lu), MQ, SP and piperaquine (PQ). Parenteral artemisinin has been shown to be the most effective drug for treatment of severe malaria [49].

CQ is used for treatment of the *non- falciparum* malaria infections and remains effective against most *P. vivax*, all *P. ovale*, all *P. malariae* [50] and *P. knowlesi* [28].

Malarone (atovaquone-proguanil), MQ and doxycycline are today commonly used as malaria chemoprophylaxis in travelers visiting malaria endemic areas.

1.2.4 Antimalarial drug resistance in *P. falciparum*

Development of drug resistance is probably a step-wise process from first showing increased tolerance against the drug action until being able to survive a full dose of treatment.

Spread of CQ resistance against *P. falciparum* developed from Southeast Asia and Colombia in the late 1950s and spread over most endemic areas within 20 years. CQ has remained effective only in some areas of Central America [51] but it appears that CQ-sensitive *P. falciparum* parasites may re-emerge after cessation of CQ use in some areas [52, 53]. CQ-resistance in Africa led to major increase in mortality and morbidity in the 1990s [54]. More recently developed antimalarials such as SP and MQ have had shorter life span from introduction to development of resistance [55]. Other drugs such as AQ has shown to be effective in Africa, whereas resistance is widespread in South America were it has been extensively used since 1950s.

The use of artemisinin in combination with a long-lasting partner drug with a different mode of action has been widely recommended to avoid the development of artemisinin resistance, but mono-therapy with artemisinin should be avoided. There are recent reports of development of resistance against artemisinin mono-therapy in four countries of the Greater Mekong region [56, 57] and also against both components of ACTs in Cambodia [58]. Despite this, the treatment recommendations are strongly emphasizing ACT, since there are no better alternatives.

Resistance to antimalarial drugs has been associated with certain genetic polymorphisms. The most well investigated are presented below:

- *Plasmodium falciparum chloroquine transporter (Pfcrt)* gene where the K76T exchange of a lysine in the wilde type to a threonine is the main molecular marker for CQ-resistance, but has also been associated with AQ resistance [59, 60]. *Pfcrt* K76 (wild type) has also been associated with reduced susceptibility to Lumefantrine [61, 62].

- *Plasmodium falciparum multidrug resistance 1(Pfmdr1)* gene which belongs to the ATP binding cassette (ABC) transporters. Single Nucleotide Polymorphism (SNPs) at several amino acid positions have shown to be associated with resistance against a variety of antimalarials [63]. The most frequently studied SNPs are N86Y, Y184F and D1246Y [64]. Resistance against MQ and Lu has also been associated with copy number variations (CNV) in the *Pfmdr1*-gene [65].

- Mutations in the *dihydrofolate reductase (DHFR)* gene and *dihydropteroate synthase (DHPS*) gene have a strong correlation with resistance to SP [66].

- Artemisinin resistance has recently shown a strong association with single point mutations in the "propeller" region of the *P. falciparum* kelch protein gene on chromosome 13 (kelch13). These mutations are now detected throughout mainland Southeast Asia from southern Vietnam to central Myanmar [67].

1.2.5 Therapeutic efficacy studies

The standard way to access the efficacy of antimalarial drugs for treatment of *P. falciparum* is through clinical trials following parasite clearance by microscopy during a follow up of between 28 and 42 days after initiation of treatment. Usually patients are followed daily (or more frequently) up to day three and thereafter on day 7 followed by weekly, for clinical and parasitological assessments. Prolonged initial clearance time can be an early warning sign of increased tolerance of the parasites [68, 69].

During follow up, re-appearing parasites have to be genotyped to distinguish a new infection (reinfection) from treatment failure (recrudescence). The PCR genotyping involves stepwise analysis of highly polymorphic markers in the parasite genes, merozoite surface proteins 1 and 2 (*msp*-1, *msp*-2) and glutamate- rich protein (*glurp*). WHO and Medicines for Malaria Venture (MfMV) have developed a standard for interpretation of these genotyping data. For each marker, recrudescence is defined as the presence of at least one matching allelic band and re-infections is defined as the absence of any matching allelic band in samples at enrolment (day 0) and at day of recurrent infection [70, 71].

The outcome of these efficacy studies are, however, influenced not only by the true susceptibility of the parasite to the test drug but also several factors such as immune status of study participants, the individual drug bioavailability, as well as an often complex interpretation of PCR results [72].

1.3 MALARIA CONTROL

Between 2000 and 2012, a scale-up of malaria interventions has saved an estimated 3.3 million lives. 90%, or 3 million, of these are in the under-five age group in sub-Saharan Africa [34, 73]. Malaria control mainly focus on four areas:

- **Access to parasitologically confirmed diagnosis**

A diagnosis based on microscopy or RDT should be accessible at point of care and WHO now recommends that treatment should be based on a confirmed diagnosis [48]. This is in order to avoid over diagnosis based on clinical observations leading to overuse of anti-malarial drugs. Overuse contributes to the risk of drug resistance development and foreseeing of other causes of fever (35). The volume of RDT sales to the public and private sectors of endemic countries has increased from 88 million in 2010 to 205 million in 2012 and the proportion of suspected malaria cases receiving a diagnostic test in the public sector increased from 37% to 61% in Africa [34]. The number of patients tested by microscopic examination has also increased to >180 million in 2012, with India accounting for a majority of slide examinations.

- **Access to treatment**

Access to prompt treatment with effective antimalarial drugs in order to decrease morbidity and mortality and to interrupt transmission is a cornerstone in malaria control [34].

- **Preventive treatment**

Pregnant women are the vulnerable group most frequently targeted. They may receive, "intermittent preventive treatment" (IPTp) with antimalarial drugs given most often at antenatal consultations during the second and third trimesters of pregnancy, regardless of whether the woman is infected with malaria or not.

Intermittent preventive treatment in infants (IPTi) with SP is recommended by WHO in areas with moderate to high malaria transmission in sub-Saharan Africa that have less than 50% prevalence of *Pfdhps* mutation in the *P. falciparum* parasite [74].

- **Mosquito control**

Bed nets

Malaria vector control is intended to protect individuals from infective mosquito bites. The most effective is long lasting insecticide treated nets (LLINs), which are distributed to targeted vulnerable groups, i.e. pregnant women and children under 5 years of age, free of charge in many endemic areas. WHO recommendations are full coverage of all people at risk of malaria [75]. In 2013, an estimated 136 million impregnated bed nets (ITNs)/LLINs were delivered to endemic countries, a major increase over the 70 million bed nets that were

delivered in 2012 [34]. There are signs of mosquito resistance against insecticides used, primarily pyrethroids, and global actions are now taken against this threat by WHO and RBM.

Indoor Residual Spraying

In 2012, 135 million people (4% of the global population at risk of malaria) were protected by indoor residual spraying (IRS). IRS is the process of spraying the inside of dwellings with an insecticide where mosquitos often rest after taking a blood meal. To be effective on a population basis more than 80% of the households in an area should be sprayed. Mosquitoes are killed or repelled by the spray, preventing the transmission of the disease. Several pesticides have historically been used for IRS, the first and most well-known being DDT which has a residual efficacy of more than 6 months [76]. Recommendations are now increasingly to use non-pyrethroids for IRS because LLINs are impregnated with pyrethroids and pyrethroid resistance is increasing [34].

1.3.1 T3: Test, Treat and Track initiative

On World Malaria day 2012, WHO launched the new initiative T3 urging endemic countries, donors and the malaria community to scale up diagnostic testing, treatment and surveillance of malaria to strengthen these three fundamental pillars for control and elimination of malaria.

1.3.2 Integrated management of childhood illness

WHO and United Nations children's fund-UNICEF have developed a strategy called the Integrated Management of Childhood Illness (IMCI). IMCI is an integrated approach to child health that focuses on the well-being of the children under five years of age. IMCI aims to reduce death, illness and disability, and to promote improved growth and development among children. The IMCI guidelines was developed to improve case management and preventive interventions against leading causes of childhood mortality, i.e. pneumonia, diarrhea, malaria, measles and malnutrition [77].

Previous versions of guidelines for malaria treatment have recommended that febrile children below 5 years should be treated presumptively for malaria in high endemic areas [78]. However, there is now increasing evidence that it is safe to withhold antimalarial treatment to fever patients with a negative malaria RDT result, including also children below five years [48, 79-81]. Treatment with ACTs should therefore be restricted to children with a confirmed diagnosis. It is recommended that RDT should be used for diagnosis in areas where microscopy is not available or its quality cannot be guaranteed. The use of RDT for diagnosis has recently been implemented in local versions of IMCI guidelines, e.g. Ghana and Zanzibar [82, 83]. Adherence to the new guidelines can, however, be problematic in high endemic areas where children can develop malaria shortly after a negative test result [84].

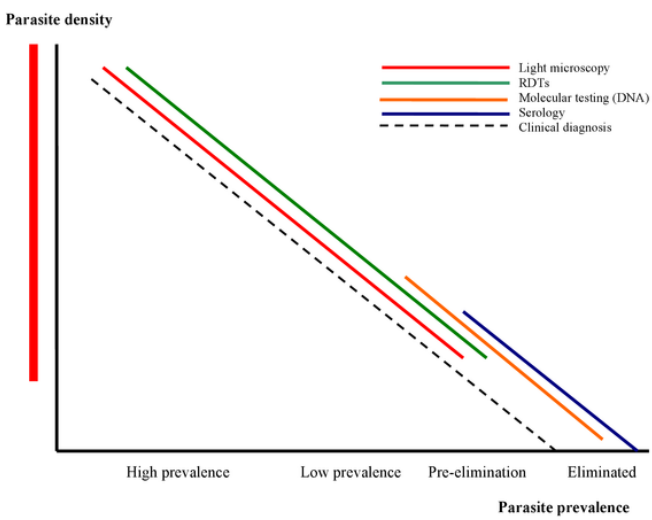
1.3.3 Malaria elimination efforts

Malaria elimination is the interruption of local mosquito-borne malaria transmission, i.e. the reduction to zero of the incidence of malaria infection in a defined geographical area. After elimination, continued surveillance is required to prevent re-establishment of transmission. At present, eight of the 97 countries with ongoing malaria transmission are classified by WHO as being in the malaria elimination phase [34].

In countries becoming low endemic and approaching a pre-elimination phase, new demands on diagnostic tests arise (Figure 3). A higher proportion of individuals in these areas will harbor parasitaemias below the detection limit of both microscopy and RDT [85]. These subpatent infections are important reservoirs for further transmission. To find and treat these carriers of low density parasitaemias is a prerequisite for an elimination strategy to be successful [86-88].

In malaria elimination settings, remaining parasite reservoirs are increasingly clustered in small geographical areas (hot spots) and parasite carriers have shifted from mainly being pregnant women and small children, to older children and men [35].

Figure 3. The usefulness of diagnostic approaches/tests in relation to parasite densities and parasite prevalence stages



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(*A research agenda for malaria eradication, PloS Medicine 8(1), 2011*)

1.3.4 Challenges in controlling malaria

Resources of health structure and commitment of health staff in endemic countries contributes to how successful malaria control activities will be [89]. The main challenges are performance and availability of diagnostics at the peripheral level and access to efficacious drugs. Provision of counterfeit and old drugs leading to substandard doses is still being provided in the private sector. Furthermore, access to and coverage of vector control are important factors. In many countries endemic for malaria, both health systems and resources for control programs have improved markedly in recent years, which have contributed to the decline in malaria incidence during the last decade.

2 MALARIA DIAGNOSIS

2.1 GENERAL BACKGROUND

A prompt and correct malaria diagnosis is a prerequisite for improved malaria control. It is important in order to restrict antimalarial treatment to those with a confirmed diagnosis, and for a rational and cost-effective use of antimalarial drugs [48, 73, 90-92]. Absence of parasite based diagnosis leaves health care workers with only clinical algorithms to diagnose malaria. The non-specific nature of symptom-based malaria diagnosis results in substantial over-diagnosis and overtreatment. Given that malaria is the most common diagnosis among African febrile children and one of the most common in adults, over- but also under-diagnosis have substantial public health implications [93, 94]. With decreasing malaria transmission as seen in many areas, the importance of a parasite based diagnosis increases. In high endemic areas a large proportion of people, including asymptomatic individuals, have parasitaemia most of the time. Thus, the detection of malaria parasites does not necessarily mean that they are responsible for the patient's illness, since they may reflect only a coincidental infection.

A correct malaria diagnosis plays an important role in the monitoring of treatment efficacy and to evaluate the impact of interventions, such as distribution of ITN/LLINs or IRS.

A comparison of diagnostic tools is presented in Table 2.

2.1.1 Clinical diagnosis

A common teaching in high endemic areas has been "fever equals malaria unless proven otherwise". In many African settings parasite based diagnostic tools are not available or not fully functional why diagnosis based on clinical observations is still the only option. Clinical malaria diagnosis leads to overdiagnosis and overtreatment with antimalarial drugs. Among reasons for overdiagnosis are that malaria traditionally is the common cause of fever, a more acceptable diagnosis and missing malaria is indefensible [94]. Clearly many lives have been saved due to access to rapid presumptive malaria treatment at community or home-based level [73], but this has also led to massive overtreatment with considerable burden on the already depleted financial resources of poor countries [95]. Symptoms of malaria often overlap with other bacterial diseases such as pneumonia [96] and clinical malaria diagnosis has therefore led to over estimation of malaria burden [97].

2.2 MALARIA MICROSCOPY

Shortly after Laveran described the malaria parasite, Romanowsky in Russia developed a method to stain the parasites, which together with improvement of microscopes made it possible to more thoroughly study them [98]. Romanowsky used a mixture of eosin and methylene blue giving the nucleus purple color and the cytoplasm blue color. The same technique remains the basis for the presently most used staining methods of malaria parasites, i.e. Giemsa and Field stain.

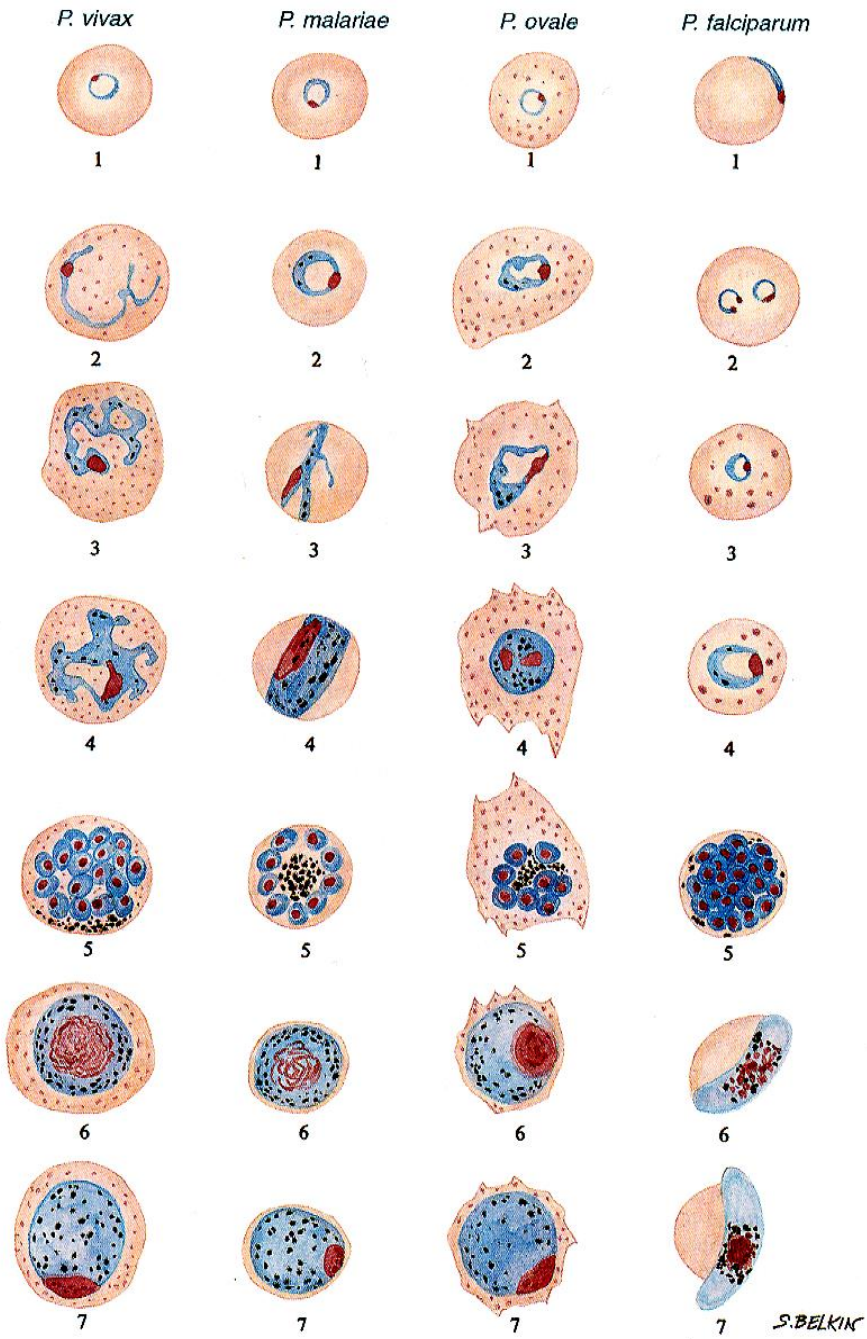
Microscopy allows for the identification and differentiation of malaria species, determination of parasite stages including gametocytes and the quantification of parasite density. Microscopy is still the method of choice for treatment follow up and investigation of malaria treatment failures. Microscopy is still considered gold standard against which other diagnostic methods are evaluated [99].

Malaria microscopy requires examination of both thin and thick smears from the same patient. Preferably capillary blood should be used for the preparation of blood films since various additives for venous puncture such as EDTA can affect the parasite morphology making it more difficult to distinguish the different species. A thick smear consists of approximately 10 microliter (µL) and a thin of 5µL blood. Optimal malaria microscopy is performed with microscopes fitted with x10 paired eyepieces and a x100 oil immersion objective (total magnification x1000) [100]. 100 or 200 microscopic fields (0.2-0.5 µL) are normally examined before a malaria infection can be excluded. In remote areas without access to electricity, microscopy can still be performed using a mirror reflecting daylight through the specimen into the eyepieces.

2.2.1 Giemsa staining method

Giemsa is a classical stain used for malaria microscopy [101]. It consists of commercially available Giemsa powder, glycerol and methyl alcohol (methanol). The stock solution is mostly purchased ready prepared and should be mixed with a phosphatase buffer solution of pH 7.2 prior to staining of the blood smears [100]. Under field conditions in endemic areas often ordinary tap water is used and that works generally well, even though a pH differing from 7.2 can affect the purple-blue contrast in the specimens. Usually a concentration of 5% Giemsa for 20-30 minutes is used for both thin and thick smears. The staining solution should be prepared within two hours prior to use. Before staining the thin smear slide should shortly be dipped in pure methanol to fix the cells. In the thick smear on the other hand, the cells should be lysed making it possible to examine a more dense layer. The sensitivity of a thick smear is 15-20 times higher than a thin film but does not allow for species determination. In the thin smear the parasites are seen within the RBC with the different characteristics of the species in terms of size, granulation and effect on the infected RBC, which generally allows for species identification if the number of parasites is not very low (Figure 4).

Figure 4. Morphological characteristics of malaria parasites



(Garcia: Diagnostic Medical Parasitology 5 ed.)

2.2.2 Field staining method

An alternative method to Giemsa is Field stain, which primarily is used for staining of thick smears. It consists of two solutions, methylene blue and eosin, and gives an excellent staining result in a few seconds if the instructions are followed carefully. Field stain also has the advantage of being very stable, allowing the same staining solutions to be used for several weeks. On the other hand, the staining can be uneven and the slide must therefore initially be scanned to find an area where both the blue and purple stains are taken up by the parasites. It is often recommended to study the colors of the leucocytes to get an idea of where to look for the parasites.

Malaria microscopy, when using Giemsa or Field stain, has an estimated cost of 0,3-1.3 USD per examination depending on number of examinations/year [102, 103].

2.2.3 Parasite quantification

There are different methods for quantification of parasites in blood smears. Until recently, this was only applicable to *P. falciparum* infection for estimation of severity of disease and treatment outcome. However, there are now reports on high parasitaemias also among severe *P. knowlesi* cases [28], which probably makes quantification important also for *P. knowlesi*.

The most common technique used in endemic areas is based on counting parasites in the thick smear against a standard number of white blood cells (WBC). The number of parasites are generally counted against 500 or 200 WBC, which with an estimated 8000 WBC per µL of blood gives a factor of 16 or 40 for calculation of parasites per µL based on the simple mathematical formula:

(parasites counted / number of WBC counted) x 8000 = parasites per µL (p/µL)

In case of very low parasite densities the numbers are often counted in 200 microscopic fields equivalent to 0.3-0.5 µL. The numbers are then given as parasites/200 microscopic fields.

Another quantification method is to estimate the percentage of infected RBCs in a thin blood smear [104, 105]. For this method the parasite density is reported as % of the RBC infected.

The thick smear method has a higher sensitivity and is the first choice in endemic areas. However, it is difficult to estimate number of parasites per WBC in high parasitaemias and the density is therefore often underestimated [106]. Then, estimation of % infected RBCs in a thin smear provides a more accurate result.

Other methods based on semiquantative estimates (+- ++++) are still used in some endemic areas [107]. All methods for estimation of parasite density are associated with potential errors due to varying WBC count and a parasite loss of up to 20% during staining of thick smears [105, 106, 108, 109]. In thin smears there is often an uneven distribution of parasites and if

not a cell counting ocular is used, the estimation of RBC per microscopic field can be arbitrary.

Parasite enumeration provides useful clinical management guidance and is a useful tool for clinical trials where serial examinations of blood smears are used to determine the parasitological response to antimalarial treatment [110].

2.2.4 Sensitivity and specificity of malaria microscopy

Microscopy remains the gold standard for assessing the outcomes of drug and vaccine trials, and for serving as a reference standard in the evaluation of new tools for malaria diagnosis [111].
The sensitivity of malaria microscopy is highly dependent on the quality of the smear, the staining and not at least the skills of the microscopist. The risk of false negative results increases with decreasing parasite densities [112]. Under optimal conditions down to 5-10 p/µL (requiring more than ten minutes of thick smear examination) can be detected by an experienced microscopist, whereas under field conditions, a detection level of about 50–100 p/µL blood is more realistic [113]. In areas with poor microscopy quality control, less skilled microscopists and poor equipment, an even higher detection limit is likely [111]. However, overestimation, i.e. interpretation of artefacts or other dots as parasites "to be on the safe side" as well as errors in species identification are also common problems [112]. Hence, wide ranges of malaria microscopy specificities have been reported [114, 115]. Moreover, evaluation of the sensitivity and specificity of malaria microscopy against PCR has shown varying results [116].

2.2.5 Advantages and disadvantages of microscopy

Advantages
Microscopy is a cheap, well established and informative method, which allows for assessment of species, stage and quantification of malaria parasites. Further, the finding of malaria pigment digested by neutrophils as a sign of a previous high parasitaemia as well as the effect on the parasite morphology by antimalarial drugs is of value ([117, 118]. In the hands of an experienced technician, microscopy can also provide additional information such as anemia, signs of bacterial infection with raised WBC and presence of other haemoparasites. Blood smears are also permanent and can be used for extraction of DNA.

Disadvantages
Microscopy is a labour-intensive, time consuming method (30min-1 hour) where the quality is highly dependent on the smear preparation, the glass slides, the fixation, the staining, the microscope and the skills of the microscopist. Field microscopy often falls short of these requirements. In the era of declining malaria incidence in many areas, it is challenging to keep up the motivation for careful microscopic examination if more than 95% of the slides

are negative. The examination is also prone to relatively high degree of subjectivity [119]. All these factors may influence compliance to test results among health care providers and despite a parasite based diagnosis, treatment decisions may still be based on clinical observations [93, 120-122].

2.2.6 Fluorescent staining techniques

Acridine orange

An alternative staining of blood smears is the use of fluorescent dyes, particularly with acridine orange. The technique uses a fluorescent dye with affinity to the nucleic acid in the parasite. A common technique is thin smears fixed with methanol, stained with 0.01% acridine orange and read in a fluorescence microscope in 400-600X magnification [123, 124]. Compared with conventional Giemsa staining, acridine orange has shown good diagnostic performance, with sensitivities of 81%-100% and specificities of 86%-100% [125]. However, the sensitivity decreases rapidly with lower parasite densities, and species differentiation is not possible [125, 126]. The most notable advantage of acridine orange over Giemsa staining is its promptness; results are readily available within 10 min. The simple design of an interference microscope has made direct acridine orange staining an accurate, rapid, simple and economically viable method for malaria diagnosis [123]. The microscopist, however, must learn to distinguish the stained cells of the parasite from other stained cells containing nucleic acids, such as WBCs or RBCs containing Howell Jolly bodies as well as cell debris and artefacts which could appear fluorescent [124].

Quantitative buffy coat

The quantitative buffy coat (QBC) method uses a combination of acridine orange staining and micro capillary tubes. After centrifugation the tubes are observed under the fluorescence microscope in the area just near the buffy coat region where parasites are concentrated. The sensitivity of the QBC method under field conditions is comparable with Giemsa staining but does not allow for parasite quantification or species identification [111, 127]. The method is also considerably more expensive which limits its usefulness for most endemic areas.

2.3 ANTIGEN BASED DETECTION OF MALARIA – RDT

2.3.1 General background to RDT

Malaria RDTs are based on immunocromatic detection of parasite antigens. The introduction of RDTs for diagnosis of malaria in the early 1990s has had a major impact on fever management in malaria endemic areas. For the first time a health worker in a remote area could rapidly and accurately distinguish between parasitaemic and non-parasitaemic febrile illness [128]. RDTs have had a major impact on the accessibility to a parasite based malaria diagnosis worldwide. The reported rate of diagnostic testing among malaria suspected cases

in the African public sector has increased to 61% in 2012, mostly attributed to increased use of RDT. Wide scale distribution of RDTs free of charge to public health facilities has become a cornerstone in malaria control program recommendations and has also been increasingly advocated in a number of malaria endemic countries [34, 129]

WHO has produced a number of guidelines evaluations and recommendations for the use of RDT [130-132], and has also set up standards for the diagnostic performance of RDT with minimum requirement of sensitivities of 95% for detection of 100 p/µL (equivalent to 0.002% parasitaemia) and specificities of minimum 90% for *P. falciparum* compared with microscopy [119, 128, 133]. WHO has also together with the Foundation for Innovative New Diagnostics (FIND), established a testing program for evaluation of the performance of commercially available RDTs. The first evaluation was published in 2008 followed by yearly reports ever since [134]. RDTs are evaluated for sensitivity and specificity in detecting *P. falciparum* and *P. vivax* at 200 and 2000 p/µL, for false positivity rate, lot variability, invalid test rate, heat stability and easy of use. The market for RDTs is enormous with more than 200 malaria RDT products currently available from more than 100 distributors worldwide [135].The volume of RDT sales to the public and private health sectors of endemic countries has increased from 88 million in 2010 to 205 million in 2012 [34]. The evaluation program together with high number of commercial products and several hundred scientific publications evaluating the use of RDTs, are factors that have encouraged the improvement and quality of RDTs. Rapid tests combining detection of malaria with that of G6PD deficiency as well as pregnancy testing are now available on the market.

2.3.2 Parasite antigens detected by RDT

Histidine Rich Protein 2

Histidine Rich Protein 2 (HRP2) is a water-soluble protein produced solely by asexual stages and young gametocytes of *P. falciparum.* HRP2 is a histidine and alanine-rich protein, which is localized in several cell compartments including the parasite cytoplasm and is expressed on the infected RBC membrane surface. Because of its abundance in *P. falciparum*, it was the first antigen used to develop a malaria RDT. The exact function of HRP2 remains incompletely understood. Studies suggest that after secretion by the parasite into the host erythrocyte cytosol, HRP2 is transported into the acidic digestive vacuole along with Hb. After Hb proteolysis, HRP2 binds the toxic haeme and mediates haemozoin (malaria pigment) formation, which is no longer toxic to the parasite [136-138].

HRP2 is being produced and secreted by the parasite during its growth and development and there are increasing concentrations of the protein during parasite maturation. HRP2 may be found in plasma, urine, cerebrospinal fluid and histological specimens. The fast secretion from the parasite makes HRP2 based RDTs suitable for detection also of parasites which are not circulating, i.e. mature stages of *P. falciparum* sequestered in the deep capillaries or placenta during infection in pregnancy [133, 139]. Plasma concentration of HRP2 has shown

to be a prognostic factor in African children with severe malaria [140]. HRP2 is also a useful tool for drug sensitivity in-vitro assays [141].

HPR2 is a very stable protein and has even been used for immunological detection of malaria among Egyptian mummies, dated about 3200 BC [142]. Despite its stability there is an extensive level of sequence diversity and deletions (up to 40% in parts of South America) of the HRP2 gene [143-146]. This has also recently been reported from the African continent [147, 148], which has implications for the performance of RDTs based on detection of HRP2.

HRP2 remains in the circulation up to several weeks after a cleared malaria infection [149-152]. A positive correlation between blood concentrations of the protein and parasite biomass has been reported [153, 154] and a strong correlation between the duration of positivity with the HRP2 based RDTs and initial parasite densities has been shown in several studies [149, 151, 155]. Conversely, a wide range of HRP2 concentrations at the same parasite densities has been found [156]. Varying concentration of HRP2 is dependent on factors like duration of infection, if the blood sample is taken soon after appearance of parasites in the blood or later during infection. Other factors such as circulating parasites may not mimic the total biomass and the anti-HRP2 immune response influences the correlation between parasite density and HRP2 concentrations [157].

***Plasmodium* lactate dehydrogenase**

LDH is a 33 kDa oxidoreductase. It is the last enzyme of the glycolytic pathway, essential for ATP generation and one of the most abundant enzymes expressed by the malaria parasite. The *Plasmodium* LDH (pLDH) isoforms can be distinguished from the human isoforms on the basis of unique epitopes within the pLDH protein as well as on its enzymatic characteristics [158]. There are no reports on antigenic variation in the pLDH gene [159].

pLDH from *P. vivax, P.malariae,* and *P.ovale* exhibit 90-92% identity with pLDH from *P. falciparum* [160] and monoclonal antibodies recognizing *P. falciparum* and *P. vivax* pLDH also recognize *P. knowlesi* in antigen capture tests [161]. However, most pLDH based RDTs have not yet been evaluated for detection of *P. knowlesi* and the available results are inconsistent [28, 162]. Detection of pLDH has been incorporated into screening methods for the identification and quantitation of parasite growth in in vitro cultures.

pLDH is produced only by viable parasites and is rapidly cleared from the blood stream following successful treatment [28, 149, 162-164]. The lack of antigen persistence after treatment could makes the pLDH test more useful compared to HRP2 based tests in predicting treatment failure. However, pLDH is produced by all asexual and sexual stages including mature gametocytes, meaning tests can persist positive due to gametocytaemia [152].

Aldolase-pan malaria antigen

Plasmodium aldolase is an enzyme of the parasite glycolytic pathway expressed by the blood stages of all five human malaria species [165, 166]. Aldolase is a highly conserved gene

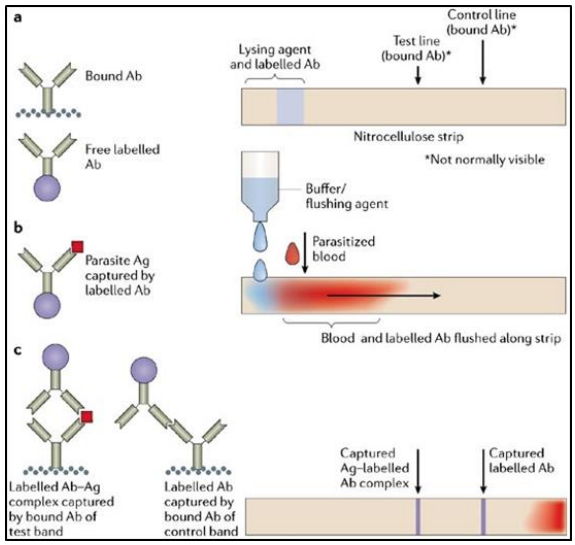
across all human malaria species and monoclonal antibodies against *Plasmodium* aldolase are pan-specific in their reaction. Aldolase is a 41 kDa protein, the presence of antibodies against p41 in the sera of human adults partially immune to malaria suggested that p41 could be involved in immune response against the parasite [167].

Low concentrations of aldolase are released from the parasites, and thus the sensitivity is dependent on the parasite density [133]. The aldolase component in RDTs has performed poorly in several studies of the aldolase-*Pf*HRP2 combo tests, with sensitivities of 30-50% for detection of *P. vivax* malaria [133, 162, 168]. and for the other species, aldolase based tests perform even worse with sensitivities below 20% [133, 169]. Due to the poor test performance of aldolase, this antigen is at the moment not commonly used in commercial RDTs and will not further be discussed in this document.

2.3.3 Test principle of RDTs

RDT is a immunocromatic test based on detection of malaria specific antigens (HRP2, pLDH and Aldolase). The test device is delivered in sealed envelopes to protect it from light and humidity. The envelope should be opened just before performing the test. The nitrocellulose strip is usually in a cassette test device. Capillary (or venous blood) is applied to the test well at the proximal end of the test strip, usually 5 µL (up to 20 µL depending on brand) (Figure 5). At the sample pad, dye labelled antibodies (Ab) is mixed with the blood. A few drops of lysing buffer are added to the sample well or a special well next to sample to lyse the cells, release the antigen and facilitate antibody recognition. If antigen is present the antibody-antigen complex is flushed up with the blood-buffer mixture along the test strip until it reaches another target Ab (capture) bound to the strip in a thin line. The target Ab binds to the antigen-antibody complex which now forms a visible test line. Another Ab specific for another epitope on the labelled Ab is binding excess labelled Ab to form a control line. Note, the control band becomes positive despite no addition of blood. After passing the test band area the lysed blood is flushed into the distal end of the strip making the bands clearly visible against a bright background (Figure 5). The result should be interpreted after 15-20 minutes (depending on test brand) and not later than 30 minutes. RDTs are delivered as two band tests detecting only one malaria species, generally HRP2 based *P. falciparum* or less commonly single pLDH *P. vivax* or Pan-*Plasmodium*. Nowadays the most commonly used RDTs use a combination of HRP2 and Pan- LDH detection in a three-band format.

Figure 5.



Bell *et al. Nature Reviews* *Microbiology* **4**,2006

2.3.4 Interpretation of RDT results

To perform an RDT and to interpret the result is generally easy to learn, although there are many reports on problems and difficulties [131, 146].

The main obstacles are:

- To ensure a correct volume of blood is collected and applied into the sampling device from the patient's finger. There are a number of different blood sampling devices available on the market out of which several are not very easy to handle [170].
- To transfer the correct volume of blood into the bottom of the sample well (avoiding leaving blood on the wall of the well).
- To dispense the correct number of buffer drops into the correct well.
- To interpret the result at correct time and in good light.
- To correctly interpret weak and faint test lines. A weak band should be interpreted as positive [171, 172].
- To recognize and repeat invalid tests (no control band or the blood-buffer mixture has not migrated to the distal part of the strip, usually due to insufficient volume of buffer)

- To correctly interpret results

The two options of *P. falciparum* positivity (only HRP2-band positive or both bands including the Pan band) commonly causes confusion. The two test band positivity is often misinterpreted as mixed infection, whereas the reason usually is detection of *P. falciparum* by both HRP2 and Pan-LDH antibodies, whereas a single HRP2 band positivity is usually due to a low *P. falciparum* parasite density since the pLDH based band may be less sensitive [146, 173, 174]. However, some of the more recently developed pLDH-tests based on monoclonal antibodies have shown equal sensitivity as HRP2 based tests for detection of *P. falciparum* [163, 175].

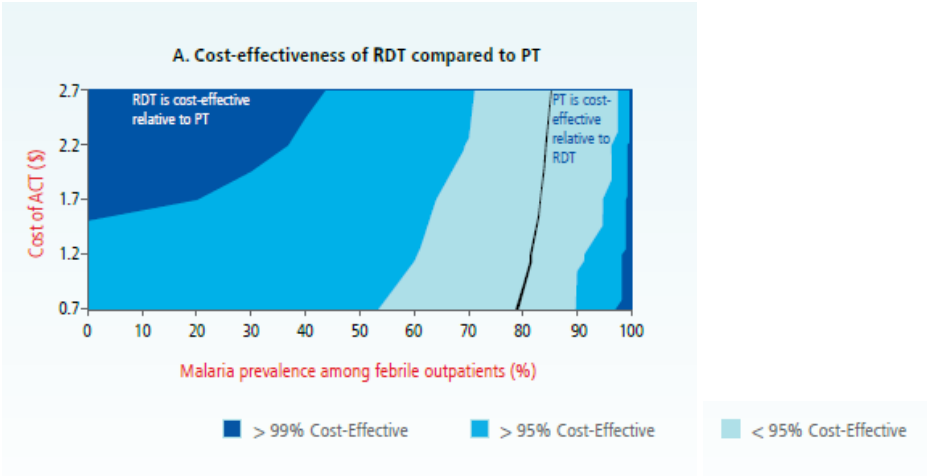
2.3.5 Cost effectiveness of RDT

The cost effectiveness of RDT use is dependent on the price per test as well as factors such as parasite prevalence, cost of prescribed treatment and adherence to test result [91, 173, 176]. The introduction of the more expensive treatment with ACT has made treatment based on parasite based diagnosis cost effective in low and moderate endemic areas where treatment based on clinical observations (fever) has led to costly over-prescription [177]. A cost effectiveness study performed by Shillcutt et al showed that this was the case also in high endemic areas if prescribers of ACTs adhered to RDT result. RDTs were cost-effective compared with presumptive treatment up to high prevalence of *P. falciparum* parasitaemia in different sub-Saharan African settings. Relative to microscopy, RDTs were more than 85% likely to be cost-effective across all parasite prevalence levels, reflecting their expected better accuracy under real-life conditions [102]. At a cost of 0.60 USD per RDT and 1.70 USD per ACT adult dose, RDTs are cost-effective with 95% confidence below a threshold of 64% malaria prevalence and 99% confidence below 20% prevalence. RDTs become more cost-effective as the cost of ACT increases (Figure 6) [178].

The cost-effectiveness of RDTs mainly reflect improved treatment and health outcomes for non-malarial febrile illness, plus savings in antimalarial drug costs [102, 177]. A study from Senegal showed a major reduction in ACT consumption with considerable cost savings after introduction of RDT at all public sector health facilities [176]. In a study from Nigeria RDT use was less costly than presumptive treatment and microscopy at 43% parasite prevalence level [103].

The excess of commercial products available on the market has also had a price-pressure effect on RDT kits.

Figure 6.



PT= presumptive treatment (WHO, Determining Cost Effectiveness of Malaria Rapid Diagnostic Test)

2.3.6 Sensitivity and specificity of RDT

In spite of over 100 published RDT trial reports, comparative assessment is difficult because (1) trials do not share common guidelines; (2) clinical and epidemiologic characteristics of the study populations, especially the parasitaemia levels vary; (3) reference standards are different; even among those using Giemsa microscopy, reading rules and microscopist skills vary; and (4) products of different lots may differ in quality or be damaged by extreme temperature or humidity during transportation and storage [111].

HRP2

RDTs based on HRP2 antigen detection are estimated to have a detection limit of 50-100 p/µL. Commercial RDTs evaluated in the WHO-FIND program show sensitivities varying from ~50-100% in detecting 200 p/µL. In real life situations the sensitivity is highly dependent on endemicity and immune status in the population tested. In low endemic/ pre elimination areas were very low parasite densities are common among asymptomatic carriers, HRP2 based RDTs are not sensitive enough for detection of asymptomatic parasite carriers (active case detection) [88, 179, 180]. False negative result with HRP2 based RDTs are seen due to *P. falciparum* genetic diversity and deletions in some areas [143, 181]. The prozone effect seen in samples with high density parasitaemias, due to blocking of binding sites for the Ab by excess antigen, can also cause false negative or very faint bands [164, 182]. The specificity of HRP2 is highly influenced by the fact that the HRP2 protein circulates in the

body up to several weeks after a cleared infection, which may cause false positive results [126, 151, 155]. This is especially of concern in high endemic areas were the population often are exposed to new infections after a recently cleared one, and health professionals are unable to distinguish the RDT positivity due to remaining antigens or a new infection. This can affect health workers adherence to and trust in RDT results. This may also frequently result in provision of antimalarial treatment to patients not infected [82, 146, 183]. RDTs based on HRP2 show higher specificity in low endemic areas where fewer individuals have recently gone through a malaria infection and unlikely carry remaining antigen [184, 185].

Another source of false positive results with HRP2 is cross-reaction with rheumatoid factor, a common problem with diagnostic tests based on IgG antibodies [186, 187]. This is, however, less of a problem with product based on IgM as either the capture or signal Ab [187]. There are also a few reports on cross-reactions with *P. vivax* and false positivity due to *Schistosoma mekongi* and dengue infection [146, 188].

pLDH

pLDH based RDTs has an overall detection limit of 100-200 p/µL for *P. falciparum* and 200-500 p/µL for *P. vivax,* and in most studies sensitivities >90% compared to microscopy [163, 164]. However, there is generally more performance variability among pLDH tests as compared to HRP2, and pLDH has a generally lower sensitivity at low parasite densities [133, 163, 177, 189]. Since pLDH is only produced by live parasites it is not circulating in the blood after a cleared malaria episode, but can produce positive result up to 2 weeks, although less frequently (5-10%) compared to HRP2 (>90%) after a cleared malaria episode [163, 164]. LDH can occasionally remain positive longer due to gametocytaemia [92, 190]. Use of LDH based RDTs in high endemic areas where high parasitaemias are frequent is therefore considered a better tool than HRP2 [163, 164].

Assessment of pLDH based tests for detection of *P. ovale* and *P. malariae* has shown poor results. With overall sensitivities ranging between 18 and 47% for *P. malariae* and between 20 and 31% for *P. ovale*, it is evident that neither test is reliable for the detection of these species [169, 191, 192]. However, pLDH antigen based RDTs are neither exposed to prozone effect [182] nor genetic diversity making the main source of false negative results low parasite densities. There are reports on cross-reactions between *P. vivax* and *P. falciparum* specific LDH [193] and pLDH can become false positive due to gametocytaemia. LDH has also been reported to be more vulnerable to high temperatures [194] even though recent evaluations have shown high stability for several pLDH based RDTs similarly as for HRP2 based tests [174].

2.3.7 The usefulness of RDT

RDT is a very efficient tool for easy-of-use, rapid, stable and accurate detection of malaria infections among fever patients with *P. vivax* or *P. falciparum* infections. A disadvantage with the use of HRP2/ Pan-LDH combo tests is that it is not possible to distinguish between the non-falciparum species, neither detects mixed infections. Other concerns are lot-to-lot variability and lack of internal control [146, 195]. However, studies on RDT in practice have shown that RDT also is a reliable tool for guidance of treatment of febrile children [80] and even effective and safe in the hands of community health care workers [196]. However, an important factor for the effectiveness of RDT use is adherence to test result among health care workers [73, 197].

2.4 MOLECULAR METHODS FOR MALARIA DETECTION

The most sensitive methods for malaria diagnosis are based on molecular detection of parasite DNA or RNA. It has been shown that when the malaria incidence in endemic areas decline in previously more high endemic areas, a large proportion of individuals carrying malaria parasites are asymptomatic with a low parasitaemia, often below the detection limit of both RDT and microscopy (Figure 3). These individuals, however, still constitute a risk for further transmission [85, 198]. Also in higher endemicity, low parasitaemias are common among adults and in chronic infections [85].To diagnose these infections, there is a need for molecular methods with high sensitivity [86, 199].

2.4.1 DNA extraction methods

Detection of malaria parasites with molecular methods is based on DNA extracted from fresh blood or blood dried on filter papers. There are several methods available for DNA extraction:

- Column based methods (e.g. Qiagen), is suitable for larger sample volumes (from fresh blood) and for long DNA fragments. The method produces high quality DNA stable for long time storage but is rather expensive and labour-intensive [200].

- Chelex-100 method, gives a high yield suitable for small sample volumes (filter paper blood spots) and for short DNA fragments. Chelex is a cheap method but is not stable for long time storage and is rather labour-intensive [201].

- Simple boil and spin methods are fast, cheap and easy but produce crude DNA sensitive to inhibition and not suitable for storage. Boil and spin methods are suitable mainly for small blood volumes [202, 203].

2.4.2 PCR

PCR is the most sensitive method for detection of malaria parasites with detection limit between 0,5-10 p/µL [28, 85, 204, 205]. In the past two decades, many PCR methods for parasite detection have been published. Snounou et al. [206] established one of the earliest nested PCR methods targeting the 18S ribosomal(r)RNA gene of the four major human *Plasmodium* species. Later, Rougemont et al. [207] and Kamau et al.[208] developed probe based real-time PCRs (qPCR) also targeting the 18S rRNA genes, and Steenkeste et al.[209] published a nested PCR method targeting the Cytochrome b (Cyt b) gene in the mitochondrial DNA (Mt-DNA). PCR methods can distinguish between all human *Plasmodium* species, identify mixed infections and also benefit antimalarial drug efficacy monitoring, vaccine studies, and screening of vulnerable populations such as pregnant woman [210]. The possibility to apply PCR methods on extracted DNA from dried blood spots preserved on filter papers has made them applicable for screening of large series of samples collected in endemic areas [202, 211]. Molecular techniques are also useful tools for screening of blood donors, thanks to their high sensitivities.

PCR methods require sophistic laboratory infrastructures including PCR machines, electrophoresis and gel analyzing equipments, well trained staff, have long turn-around time and are costly [86, 179, 212, 213]. These requirements are usually impossible to fulfill at point of care level in most malaria endemic areas. PCR based molecular methods are therefore not suitable for routine diagnosis of malaria.

Research is ongoing to find more field-friendly options for molecular diagnosis [86, 88, 214].

2.4.3 Loop Mediated Isothermal Amplification

The loop mediated isothermal amplification (LAMP) method for amplification of DNA was first published by Notomi et al in year 2000 [215]. They developed a method that could amplify a few copies of DNA up to 10^9 under isothermal conditions in less than one hour. The method for amplification of *P. falciparum* DNA was described by Poon et al, in 2006 [216].

The LAMP method uses a set of three primer pairs recognizing 4 sites of the target DNA which due to a loop formation of the amplified product acts as starting points for new primers. This autocycling strand-displacement DNA syntesis makes the amplification highly efficient and specific [215]. The amplification can occur under isothermal conditions at 65°C, with the use of the *Bacillus stearothermophilus* (BST) enzyme, robust towards inhibition and therefore also suitable for simple and field friendly DNA extraction methods such as the boil and spin method [216, 217]. The detection of amplified product can be done already after 15-40 minutes and is based on either turbidometric measurement of magnesium pyrophosfate, a white precipitate produced during DNA-amplification, or alternatively by calcein added to

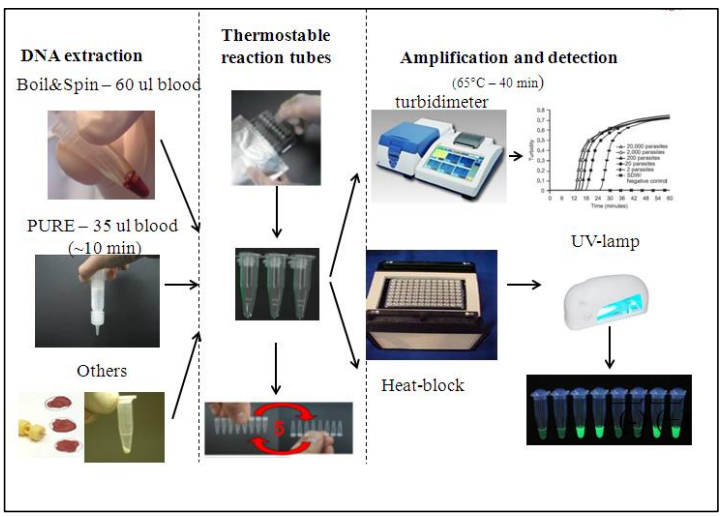
the reagent mixture in its quenched form which upon amplification is released producing fluorescence [217, 218]. Due to the very efficient amplification of DNA, there is a high risk of contamination something that users need to be aware of. To have a robust system were tubes with amplified products never allows to be opened, are of major importance [110, 218].

The LAMP method has been evaluated for detection of malaria species in a number of studies using 18s ribosomal RNA and Mt-DNA gene targets [110, 216, 217, 219, 220]. The LAMP method has been compared with microscopy and/or PCR among symptomatic patients or on cultivated parasites generally showing sensitivities and specificities of >90%. LAMP has also been suggested for detection of *Plasmodium* in mosquitos [110, 219].

A Loopamp™ MALARIA Pan/Pf detection kit was produced through a collaboration between FIND and Eiken Chemicals, Tokyo, Japan [221]. The kit consists of tubes with vacuum-dried reagent mixtures, stable at ambient temperature (<30°C), targeting the Mt-DNA sequences of all *Plasmodium* species (Pan-malaria) and *P. falciparum*, respectively. The kit has shown similar accuracy as nested PCR and sensitivities of > 95% at parasite densities of > 1/µL when it was evaluated among patients suspected for having malaria both in an endemic setting (Uganda) and in returning travelers (UK) [179, 221]. The kit can be used under field conditions with simple extraction methods, only requiring few equipment's like heating-block, centrifuge and a UV-lamp (Figure 7).

Figure 7.

Process of samples with the Loopamp MALARIA Pan/Pf detection kit



Iveth J. González FIND diagnostics, 2013

2.4.3.1 Usefulness of Loopamp MALARIA Pan/Pf detection kit

Today, the cost of a LAMP test using the commercial kit is about the same as for a PCR analysis, i.e. 3-5 USD. The cost will potentially decrease with increased use.

The use of LAMP is considered a promising tool for point of care detection of asymptomatic carriers of low parasite densities, below the detection limit for both microscopy and RDT, especially in low endemic/ pre-elimination areas [86, 88, 222]. The use of LAMP kit for screening of asymptomatic individuals under field conditions needs, however, to be further evaluated. A limitation with the LAMP kit is that it like RDTs can not distinguish between the non-falciparum species or detect mixed infections, neither quantify parasites.

2.5 MALARIA SEROLOGY

Diagnosis of malaria using serological methods is based on the detection of antibodies against asexual blood stage malaria parasites. Immunofluorescence antibody testing (IFA) or ELISA are useful tools in epidemiological surveys for assessing malaria exposure over time, especially in low endemic areas [223]. Serology is also useful for screening of potential blood donors and occasionally for providing evidence of recent infection in non-immunes. The principle of serology is that, following infection with any *Plasmodium* species, specific antibodies are produced within 2 weeks of initial infection, and persist for 3-6 months after parasite clearance [224]. Serology is therefore not a suitable method for point of care diagnosis of malaria.

2.6 FUTURE MALARIA DIAGNOSTIC OPTIONS

Mobil phone based microscopy

There are several studies ongoing to determine the feasibility of using mobile phones to capture microscopy images and transfer them to a central database for assessment [225, 226]. It could have potential to be a sensitive, robust, easy to use and cost effective system which could play a role as an alternative/complement to present diagnostic methods. However, if this is going to be a useful tool for diagnosis of malaria it has to be combined with high quality sample preparations and staining methods to prepare images suitable for computer based analysis.

Table 2. Comparison of diagnostic tools for malaria

	Microscopy		**RDT**		**Molecular methods**	
	Giemsa	**Acridine orange**	**HRP2**	**LDH**	**LAMP**	**PCR**
Sensitivity	high/low	moderate	moderate	moderate	high	high
Specificity	high/ moderate	moderat	high/low	high	high	high
Detection limit p/ µL	50	100	50	100-500*	2-5	0,5-10
Species identification	yes	no	yes for P.f	yes/no	yes/yes for P.f**	yes
Quantification	yes	no	no	no	no	yes***
Time to result minutes	40-60	10	20	20	60	> 4 hours
Lab requirements	low	moderate	no	no	moderate	high
Technical skills	high	moderate	low	low	moderate	very high
Costs	low	moderate	moderate	moderate	moderate	high/ moderate
Usefulness in clinical efficacy studies	yes	no	no	probably	probably	probably

*for *P. vivax* ** with the Loopamp Pan/Pf kit *** with qPCR P.f= *P. falciparum*

3 RATIONALE FOR THE DOCTORAL PROJECT

Malaria is a major problem from a global health perspective. However, during the last years there has been a decrease in both malaria morbidity and mortality in many African regions, e.g. Zanzibar, following wide scale deployment of combined control interventions with artemisinin-based combination therapy (ACT), long lasting insecticide treated nets (LLINs), indoor residual spraying (IRS) and intermittent preventive treatment in pregnancy (IPTp). In areas where these interventions have been successful, improved case detection and surveillance are becoming increasingly important.

Parasite-based malaria diagnosis is generally recommended by WHO to target treatment to patients with confirmed malaria infections. The introduction of RDT has been crucial to increase the diagnostic accuracy of malaria. The new rapid tests are easy to perform and interpret; they do not require access to electricity and are therefore well suited for use in rural hospitals and primary health care worldwide. The global market for these products is gigantic, where different endemic contexts may have different requirements. A continuous evaluation and development of these products is on-going. Studies on their performance and implications for case management among health care workers in different epidemical contexts are essential.

Following improved global malaria control and regional elimination efforts, there is a critical need for novel surveillance tools and strategies. *Plasmodium* DNA extracted from RDTs may become a valuable tool for molecular monitoring of malaria and drug resistance surveillance. However, comparative evaluation of different extraction methods and the applicability for use on field collected RDTs are lacking.

Currently several countries are targeting malaria elimination. To achieve this goal, molecular diagnostics that are sensitive enough to detect carriers of very low parasite densities, below the detection limit of both RDT and microscopy, are needed. LAMP is a new promising method for field use in endemic areas. The LAMP assay is faster easier and less equipment dependent compared to conventional PCR methods but evaluation of its performance and usefulness in different endemic settings is needed.

The four studies in this PhD program will provide new data on the efficiency of diagnostic tools for optimized diagnosis and surveillance of malaria in different endemic contexts

4 AIMS OF THE THESIS

4.1 OVERALL OBJECTIVE

To evaluate the use of modern diagnostic methods to improve diagnosis and surveillance of malaria in different endemic contexts.

4.2 SPECIFIC OBJECTIVES

Study 1- To assess if RDT remains an efficient tool for *P. falciparum* case detection among fever patients in an area of low malaria transmission (Zanzibar). Further, to evaluate the adherence to test results among health care workers and the performance of RDT as a part of integrated management of childhood illness (IMCI).

Study 2- To assess parasite clearance by microscopy, RDT and PCR. To compare the usefulness of two RDT devices based on detection of HRP2 and LDH antigens, respectively, for assessment of clearance and detection of recurrent *P. falciparum* infections following treatment with ACT among children in a moderately high endemic area of Tanzania.

Study 3- To compare and evaluate different methods of DNA extraction from RDTs and to test the field applicability of used RDTs versus filter papers for molecular surveillance.

Study 4- To evaluate the accuracy of a newly developed commercial LAMP kit for the detection of malaria DNA, both from symptomatic and more importantly asymptomatic low density parasite carriers, using extractions from small blood volumes spotted on filter papers.

5 MATERIALS AND METHODS

5.1 STUDY SITES

Study 1

The study was conducted in 12 primary health care facilities, 6 each including 5 primary health care units (PHCU) and 1 primary health care centre (PHCC) in North A (Unguja island) and Micheweni (Pemba island), respectively, two rural districts of Zanzibar in May-July in 2010. Zanzibar has recently undergone a rapid transition from 30% in 2003 to < 3% in 2010 of *P. falciparum* infections among fever cases. This has been achieved through wide scale implementation of combined malaria control interventions.

Zanzibar introduced *P. falciparum* specific RDT for confirmatory malaria diagnosis of fever patients in all public health care facilities in 2006. Zanzibar was also among the first regions in Africa to incorporate RDT in their local version of IMCI in 2009 [83]. ACT was introduced for first line treatment in 2003.

Study 2

The study was performed at Mlandizi PHCC in Kibaha district and Fukayosi PHCC in Bagamoyo district, both located in the Coast region of Tanzania. The study was conducted in Kibaha district in 2009-2010 and in Bagamoyo district in 2011. The region is considered a moderately high transmission area for malaria where the predominant species is *P. falciparum.* Malaria transmission occurs throughout the year with peaks following rainy seasons in March-May and October-December. ACT was introduced as first line treatment for uncomplicated malaria in 2006 in the study area. RDTs were rolled out by the National Malaria Control Programme (NMCP) for universal diagnostic testing in the whole country from 2009. During the study period (2009-2011) the monthly positivity rates among children <5 years varied between 11% and 48% in the study area.

5.2 STUDY POPULATIONS AND SAMPLING

Study 1

All patients >2 months age, seeking health care with fever or a history of fever during the preceding 24 hours, without symptoms of severe disease, were included. Upon enrolment all included individuals underwent RDT testing. All RDT positive patients and a random sample of 20% of all RDT negative patients were subjected to additional blood sampling for malaria microscopy (thick blood smear) and approximately 100 µL spotted on filter paper (Whatman 3MM) for molecular analysis. Antimalarial medicines according to national guidelines (artesunate-amodiaquine) as well as antibiotics and antipyretics were provided as required. The study included a single visit for all included individuals.

Study 2
Children below 5 years of age (6-59 months) with fever or history of fever during the preceding 24 hours and with a confirmed uncomplicated *P. falciparum* mono-infection with a parasitaemia between 2,000-250,000/ µL, willing/able to comply with a 42 day follow up was eligible to participate. All children were treated with arthemeter-lumefantrine (Coartem®, Novartis) according to national treatment guidelines. Enrolled children were requested to return for follow up on day 1, 2, 3, 7, 14, 21, 28, 35 and 42. At each visit including day 0, a finger prick blood sample was taken for thick and thin smears, two RDTs and approximately 50 µL spotted on filter paper for PCR analysis from all children.

Study 3
The RDT and filter paper samples collected in study 1 were used.

Study 4
In addition to the samples described in study 1 a subsample of asymptomatic individuals (aged 1 month-85 years) collected during a cross sectional survey performed in North A and Micheweni districts of Zanzibar in 2011 were also used in this study. All individuals were screened for malaria with RDT and approximately 50 µL blood was spotted on filter paper for molecular analysis.

5.3 LABORATORY METHODOLOGIES

5.3.1 RDTs

Study 1
The *P. falciparum* specific HRP2 based Paracheck Pf (Orchid Biomedical Systems, Goa India) was used, which at the time of the trial was the RDT device deployed by The Zanzibar malaria control programme (ZMCP).

Study 2
Two RDTs, ParaHIT ®f (Span Diagnostics Ltd, Surat, India), detecting *P. falciparum*-specific HRP2 antigen and CareStart™ Malaria (G0151), (Access Bio, Inc, NJ, USA), detecting *P. falciparum*-specific LDH antigen were used. ParaHIT was at the time of the study, approved by the Tanzanian NMCP and was the most deployed RDT. The single Pf CareStart test was chosen based on the heat stability and performance of the CareStart pan-LDH test for detection of *P. falciparum* in the WHO-FIND product testing 2009 [134]

Study 3
DNA extraction methods were compared using two RDT devices of clinical importance in Zanzibar: Paracheck-Pf and a PfHRP2/pan-LDH based RDT, SD-Bioline Malaria Ag P.f/Pan (Standard Diagnostic, Inc, USA). Zanzibar had recently changed to SD-Bioline P.f/Pan as this test also detects species other than *P. falciparum.*

The field study samples included in study 3 originated from the Paracheck-Pf RDTs performed during study 1.

Study 4
The fever patients were tested with Paracheck-Pf RDTs performed during study 1. The asymptomatic patients in the cross-sectional survey were all screened with SD-BioLine Malaria Ag P.f/Pan.

All RDTs were performed and interpreted on site according to the manufacturer's instructions. Faint bands at the test line positions were determined as positive and band intensities were not recorded.

5.3.2 Microscopy

Study 1 and 2
Giemsa stained thick blood smears (BS) for microscopy was used in both studies. Slides were stained for 20-30 minutes with 5% Giemsa solution and were examined under oil immersion (x100 magnification) by two experienced microscopists. A total of 100 (Study 1) or 200 (Study 2) microscopic fields were examined before a smear was considered negative. Asexual parasite densities were calculated by counting parasites against 200 WBC, assuming 8,000 WBC/μL of blood [100]. All blood smears with discrepant results, defined as >50% difference in parasite densities or a positive versus negative result between the readers as well as between BS and PCR results, BS and RDT results or RDT and PCR (for pLDH-RDT in Study 2) were subjected to a third decisive reading at Karolinska Institutet, Sweden.

Thin blood smears were also collected on day 0 in Study 2 and stained with Giemsa in order to confirm *P. falciparum* mono-infections.

Study 2
Thin blood smears were also examined after staining with an acridine orange solution. The smears were fixed in methanol and stained with 0.01% acridine orange and read in a fluorescence microscope at x40 magnification. Results were recorded as either negative or positive, i.e. parasite counts were not assessed.

5.3.3 Preparation of serial dilution of parasites

Study 3
For the purpose of estimating the detection limit of parasite DNA extracted from RDTs, serial dilutions of in-vitro cultured lab strain 3D7 *P. falciparum* parasites were prepared. The parasite density in the culture was estimated by microscopical examination of a Giemsa stained thin film. In contrast to ordinary counting of % infected RBCs, all parasites within the

infected RBCs were counted for a more robust assessment of parasites/μL after cell lysis. Packed RBCs from the parasite culture was then diluted 1:1 with human serum to achieve a hematocrit of approximately 50%, before it was serially diluted with uninfected male whole blood. Before preparing the dilutions, the infected and uninfected blood were freeze-thawed to lyse all cells.

Study 4.
The same method was used for evaluation of detection limit by the Pan/Pf LAMP kit.

5.4 MOLECULAR METHODOLOGIES

5.4.1 DNA extraction

Study 1 and 2
Three punches (approximately equivalent to a total blood volume of 10-15 μL) from all collected filter paper samples were extracted using the column-based ABI-6100 Nucleic Acid Prep Station protocol (Applied Biosystems, Fresno, CA, USA) [227]. DNA was eluted in 200 μL buffer and kept frozen until use.

Discordant samples in Study 1 and samples for genotyping in study 2 were extracted with Chelex-100 method using one filter paper punch (~3-5 μL) [211, 228].

Study 3
Two RDT devices seeded with 5 μL blood of parasite concentrations from 200,000 to 0.02/μL, derived from serial dilutions, were cut into 3 x 3mm pieces from four different fragments (1 cm, Proximal, Distal, Whole), all including the proximal third of the nitrocellulose strip. For comparison, filter papers (Whatman 3MM) were seeded in parallel with 5 μL (approximately equivalent to one 3-mm filter paper punch) of the serial dilutions. All cut pieces were extracted with three different methods: 1) a simple elution method [229] 2) Chelex-100 [230] and 3) a modified version (regarding regents volumes and incubation times) of the ABI-6100 protocol [227], for evaluation of extraction efficiency. The final elution volumes from the methods were 1) 50 μL, 2) ~190 μL, 3) 200 μL.

For the field study samples paired RDT and filter paper samples were extracted with the ABI-6100 Nucleic Acid Prep Station protocol (modified for the RDTs).

Study 4
The samples from the asymptomatic individuals were extracted with the Chelex-100 method using one filter paper punch [228]. For the samples from Study 1 (fever patients), DNA samples with discordant PCR versus LAMP results were subjected to DNA re-extraction with the same Chelex method.

5.4.2 PCR

Study 1

RDT positive samples were analysed with a *P. falciparum* specific nest PCR-Restriction Fragment Length Polymorphism (RFLP) method for single nucleotide polymorphism (SNPs) targeting *pfmdr*1: N86Y, Y184F, D1246Y and *pfcrt* K76T [63, 231]. The PCR products were separated in an ethidium-bromide stained 1% agarose gel by electrophoresis and visualized under UV transillumination (GelDoc 2000, BioRad, Hercules, USA). An infection was defined as mixed when both alleles were present at a particular locus. *Pfmdr1* copy number was determined by the comparative ΔΔCt method following a TaqMan probe-based real-time PCR [65].

RDT negative samples were pooled two by two and screened in duplicate for presence of *Plasmodium* DNA with an 18S ribosomal(r) DNA (targeting the rRNA genes) real-time PCR. Samples with a cycle threshold (Ct) value <42 were selected for multiplex species identification [232].

Samples with discrepant RDT and PCR results were re-analysed with a *Plasmodium* Cytochrome b (Cyt b) nested PCR [211]. PCR positivity was defined as a positive PCR that could be confirmed by another PCR method or by parasite detection by microscopy. All samples with a negative RDT but a positive microscopy and/or PCR were subjected to PCR analysis for HRP2 deletions [233].

Study 2

Samples from all time points were analysed in triplicate by 18S rDNA real-time PCR [232]. A cut-off value for positivity was set at Ct-value <40. Samples where one out of three had a Ct <40 were repeated in triplicates with the same real-time PCR. Samples with repeated single Ct-values <40 or a Ct average of >38.5 were subjected to a confirmatory/ decisive *P. falciparum*-specific nested 18S PCR [234].

Day 0 and day of recurrent infection samples from children with recurrent PCR positivity, were analysed with stepwise genotyping of *msp*1, *msp* 2 and *glurp* according to standard operating procedures to differentiate new infections from recrudescence [71].

Study 3

For the purpose of evaluating the three extraction methods from RDTs and filter papers, they were analysed for *P. falciparum* detection limits using three PCR methods: 18S rDNA nested PCR, Cyt b nested PCR and 18S rDNA real-time PCR [208, 209, 234]. The same volume of DNA was used from each extraction method (2-5 µL depending on PCR method). The *P. falciparum* detection limits were determined as the lowest consecutive positive sample in the dilution series.

For the field study samples (from Study 1) paired RDT and filter paper samples were analysed in parallel with the PCR methods described (Study 1).

Study 4

For evaluation of LAMP accuracy for detection of parasite DNA, LAMP results were compared with Cyt b nested PCR [209]. The PCR products were visualized under UV light and positive PCR products were subjected to an RFLP assay for species identification [211]. The detection limit for Cyt b nested PCR was estimated to approximately 2 p/µL.

Samples with discordant results between Pan and/or Pf-LAMP versus Cyt b nested PCR were re-amplified by a SYBR Green real-time PCR assay in triplicate, targeting the Cyt b gene of the four major human *Plasmodium* species (Xu et al, unpublished data). The PCR reaction was carried out in a final volume of 20 µL, containing 5 µL of extracted DNA, 0.25 µM of each primer, and 1 × iTaq Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA) The real-time PCR results were analysed both by melting curve and gel electrophoresis. Cyt b-real-time PCR positivity was confirmed by gel electrophoresis on a 1.5% agarose gel stained with GelRed. PCR products were digested by FspBI enzyme (Thermo Fisher, Waltham, MA) in RFLP assay for species identification. After overnight digestion in 37°C, the RFLP products were run on 2% agarose gel followed by visualization in Gel-doc system (Bio-Rad) (Xu et al, unpublished data). The detection limit for Cyt b real-time PCR was estimated to approximately 1 p/µL.

The real-time PCR corrected nested PCR result was defined as gold standard.

PCR for quantification of parasites was used for PCR positive asymptomatic individuals. DNA extracted from dilution series of 20000, 2000, 200, 20 and 2 p/µL of in-vitro cultured parasites were analysed with quantitative PCR (qPCR) targeting the *Plasmodium* 18S rRNA gene [235]. A standard curve was developed by plotting known density to quantified 18S copy numbers. The asymptomatic DNA samples were quantified by the 18S qPCR using plasmid standard [235], and the parasite densities were calculated by the acquired 18S copy numbers and the standard curve.

5.4.3 LAMP

Study 4

The extracted DNA samples were analysed with the Loopamp^{TM} MALARIA Pan/Pf detection kit (Eiken Chemical, Tokyo, Japan) according to standard operating procedures provided by FIND and the manufacturer's instructions [236]. Samples were analysed individually for Pan (all species) and Pf (*P. falciparum*) using separate reaction-tubes provided by the kit, containing dry-down reaction mixtures. 30 µL of DNA samples (diluted 1:6 in sterile water) were added to each Pan and Pf reaction tube. Each set of six samples were analysed along with a negative (nuclease free water) and a positive (purified plasmid DNA) control. After careful mixing of the DNA solution with the reaction mixture, the LAMP reaction tubes were incubated at 65°C for 40 minutes followed by a 5-minute enzyme-inactivation at 80°C in an LA-500 turbidimeter (Eiken Chemical). The real-time turbidity data were electronically recorded from the amplification curves in the control unit.

An increase in turbidity exceeding 0.1 Optical Density (OD) units per second was scored as positive [221].

5.5 ETHICAL CONSIDERATIONS

Field sample collections were conducted in accordance with the Declaration of Helsinki and Good Clinical Practice [237, 238]. The studies were approved by the Zanzibar Medical Research Ethics Committee (ZAMREC/ST/0021/09) (ZAMREC/0001/JUNE/011) (Study 1), the Directorate of Research and Publications, MUHAS (Ref.No.MU//RP/AEC/Vol.XIII/142) (Study 2). All four studies were approved by the Regional Ethics Committee, Stockholm (2009/387-31).

All study participants gave written informed consent for their participation. For children, proxy-consents from parents/legal guardians were obtained. Study 1 and 2 are registered on ClinicalTrials.gov with study identifier "NCT01002066" and "NCT01843764", respectively.

5.6 DATA ANALYSIS

Data from Study 1-4 were entered in Microsoft Excel and all statistical analyses were performed using STATA 12 (Stata Corp, Texas, USA) software. All frequencies, proportions and odds ratios (ORs) were calculated with 95% confidence intervals (CI). Statistical significance was defined as $p< 0.05$.

Study 1

Primary endpoint was adherence to RDT results and secondary endpoints included, 1) sensitivity, specificity, negative (NPV) and positive (PPV) predictive values for RDT versus PCR and blood smear (BS) microscopy and 2) performance of RDT within IMCI assessed as a subgroup analysis of the age group <5 years. Adherence to test result was assessed through drug prescription across age groups.

To account for the fact that blood sampling for BS microscopy and PCR among RDT negative patients only included a random sample of 20%, we multiplied the absolute number of observations in these groups with factors of 5.14 and 5.01 in all calculations of RDT sensitivity and specificity against PCR and microscopy, respectively. The corresponding confidence intervals (CI) were, however, based on the true sample size. Adjustment for clustering at health facility level was done using mixed effect models.

Study 2

The primary endpoints were assessment of clearance time and detection of recurrent infections by microscopy, RDT and PCR. Secondary endpoints included 1) specificity of the two RDTs, 2) identification of PCR adjusted reinfection/ recrudescence and 3) correlation between parasite density at enrolment and persistence of HRP2.

Categorical variables were compared using Fishers exact test. Pearsons linear correlation (r) was calculated in SPSS. Sensitivity and specificity of acridine orange against Giemsa-stained blood smear microscopy and both microscopic methods against PCR were calculated.

Study 3

The level of DNA extraction efficiency of the different RDT devices was determined through several PCR assays. SNPs genotyping outcomes were compared between RDT and filter paper extracted DNA by kappa analysis (κ). For determination of pfmdr1copy number variations, comparison was done by Wilcoxon rank-sum test due to many incomplete RDT/ PCR pairs.

Study 4

Sensitivity, specificity, PPV and NPV of the respective Pan and Pf-LAMP assay were calculated using real-time PCR corrected nested PCR as gold standard. When calculating the Pf-LAMP sensitivity, specificity, NPV and PPV for the asymptomatic individuals, *P. malariae* mono-infections were not included. The corresponding calculations for Pan-LAMP were based on *P. falciparum* and *P. malariae* mono-infections only. Data were also analysed after stratification by covariates that may influence the diagnostic accuracy of LAMP (e.g. parasite density and presence of non-falciparum species). Pairwise determination of non-equivalence between final outcome (including re-extraction) of Pan and Pf-LAMP as well as for Pan-LAMP and Pf-LAMP individually versus nested PCR for detection of parasite DNA was determined by the McNemar test. Kappa analysis (κ) for agreement between the methods was performed.

Table 3. Summary of the methodologies Study 1-4

	Study 1	**Study 2**	**Study 3**	**Study 4**
Study design	Observational descriptive	Exploratory descriptive	Comparative methodological	Comparative methodological
Study population age /samples	Symptomatic > 2 months	Symptomatic 5-59 months	Lab strain 3d7 RDT + FP from Study 1	Asymptomatic >1months DNA from Study 1
Study area endemicity	low	moderately high	NA	low
Sample size	3890	53	855	865 + 465
Follow up	no	day 1, 2, 3,7, 14, 21, 28, 35, 42	NA	no
Methodology	Evaluation of RDT versus microscopy and PCR , drug prescription*	Estimation of clearance and detection of recurrent infection by RDT, microscopy and PCR	Evaluation of extraction methods from RDTs, comparison of RDT and FP field samples	Evaluation of LAMP versus PCR from FP samples
Laboratory tests	microscopy, RDT and PCR	microscopy, RDT and PCR	DNA extraction and PCR	LAMP and PCR
Outcome	Usefulness of RDT, adherence	Usefulness of HRP2 and LDH-based RDT	Usefulness of RDT for molecular surveillance and field applicability	Accuracy of Pf/Pan-LAMP kit

*In relation to RDT outcome NA= not applicable FP= filter paper

6 RESULTS AND DISCUSSION STUDY 1-4

6.1 STUDY 1

Rapid diagnostic tests in the new context of low malaria transmission in Zanzibar - usefulness, adherence and performance within the IMCI algorithm

6.1.1 Results

3890 patients, aged ≥ 2 months were enrolled at the 12 health care facilities. Among these 121 (3.1%) had a positive RDT result.

The highest RDT positivity rate 6.1% (32/528) was found among children aged 5-14 years. There was an uneven distribution of RDT positivity rates between the two districts, i.e. North A 42/2225 (1.9%) and Micheweni 79/1665 (4.7%), and also between their respective health facilities with 67.1% (53) of all RDT positive patients in Micheweni district reported from only one health facility.

The three main questions we wanted to answer in this study were:

1. Does a HRP2 based RDT remain an efficient tool for *P. falciparum* detection in the new context of low malaria endemicity?

We evaluated the RDT against Giemsa stained BS microscopy but also against PCR as gold standard, considering that the importance of low parasitaemias may increase in low endemic setting (Table 4). The RDT showed a relatively low sensitivity but a high specificity when compared with both BS and PCR. Four of the six patients with negative RDT but positive blood smear had high parasite densities (3573-50190/µL). Further PCR analysis could not detect HRP2 deletions in these samples.

2. Is malaria RDT as an integrated part of IMCI a reliable tool to both identify and rule out malaria infections in children < 5 years?

The results showed no significant difference in RDT performance in children < 5 years compared to other age groups (5-14 years, >14 years).

3. Do primary health care workers continue to adhere to RDT results in this new context of low malaria transmission?

Adherence to RDT results was high, i.e. 99.9%. Only 3/3768 RDT negative patients (all >14 years) received antimalarial treatment. RDT negative patients were overall more likely to receive antibiotics compared to RDT positives [OR 3.25 (95% CI 2.15–5.01)], except for children <5 years, where prescription were equal in both groups. These children were also less likely to receive antipyretics compared to other age groups [OR 0.22 (95% CI 0.19–0.26)].

Table 4. RDT sensitivity, specificity, PPV and NPV against PCR and BS microscopy

	PCR +	PCR –	Total	BS+	BS-	Total
RDT +	117	4	121	110	11	121
RDT -	7*	726*	733	6**	733**	739
Total	124	730	854	116	744	860
Sensitivity (95% CI)	76.5% (69.0–83.9%)			78.6% (70.8–85.1%)		
Specificity (95% CI)	99.9% (99.7-100%)			99.7% (99.5-99.9%)		
Positive predictive value (95% CI)	96.7% (91.8-99.1%)			91.7% (84.3-95.4%)		
Negative predictive value (95% CI)	99.0% (98.0-99.6%)			99.2% (98.8-99.5%)		

*multiplied with a factor of 5.14 ** multiplied with a factor of 5.01

6.1.2 Discussion

Compared to a previous report from Zanzibar [197], conducted in 2005 when *P. falciparum* malaria was responsible for ~30% of fever episodes, the sensitivity of RDT against BS microscopy declined from 92% in 2005 to 79% in 2010, whereas the corresponding specificity increased from 88% to 99%. The relatively low sensitivity of Paracheck-Pf, i.e. below the reported sensitivity for *P. falciparum* detection for this RDT device in WHO/FIND evaluation in 2012 [134] is a concern. The decrease in sensitivity may reflect decreased parasite densities in the febrile patients. When the immune status in the population decreases as in this new situation of low endemicity in Zanzibar there could be more patients with fever caused by rather low density parasitaemia, high enough for detection by good quality microscopy and PCR but not enough for detection by RDT. Studies of pyrogenic threshold for *P. falciparum* in immune naive individuals have demonstrated a wide range of parasite densities at fever onset [239].

However, another and maybe a more likely explanation to the lower sensitivity in this study is suboptimal performance of individual health care workers. Four of the six RDT-/BS+ samples had high parasite densities well above the detection limit for RDT and half of all RDT-/BS+ were from the same health facility. This highlights the need for improved supervision and control of RDT use. On the other hand, the increased specificity may reflect

an overall reduced risk of repetitive malaria infections and remaining antigenemia from a previous malaria episode in the new low transmission context in Zanzibar.

Lot-to-lot variability and exposure to heat and humidity during storage are also factors that can result in differences in RDT accuracy.

The high adherence to RDT as an integrated part of IMCI indicates that the study staff members trusted the RDT results. They were encouraged to adhere to the IMCI guidelines which recommend that antimalarial treatment should be based on parasite confirmed diagnosis. However, they were free to manage according to their own clinical judgement. The high prescription of antibiotics in both RDT positive and negative children of 58% is a concern, though still lower than among children 5-14 of 66%. This shows the need for better supervision of the health care workers.

The previous study when *P. falciparum* malaria was responsible for ~30% of fever episodes showed a high adherence among health care workers [197]. It is reassuring that health workers continue to adhere to RDT results even when 97 out of 100 tests are negative and facilities for alternative diagnosis of fever are scare. The high prescription of antibiotics to study participants in all age groups shows the urgent need for studies on fever aetiology and the need for diagnosis tools to improve the management of other causes of fever.

Zanzibar is an area in the frontline of malaria control activities, which make our results probably not completely representative for other low endemic areas in sub-Saharan Africa.

6.1.3 Limitations

The high adherence among the study staff members in this study could be influenced by the fact that they had gone through a relatively extensive pre-study training and therefore not be representative for health care workers in other parts of Zanzibar. The two districts where the study was performed have been exposed to other malaria interventions during several years which also may have contributed to the high adherence.

6.2 STUDY 2

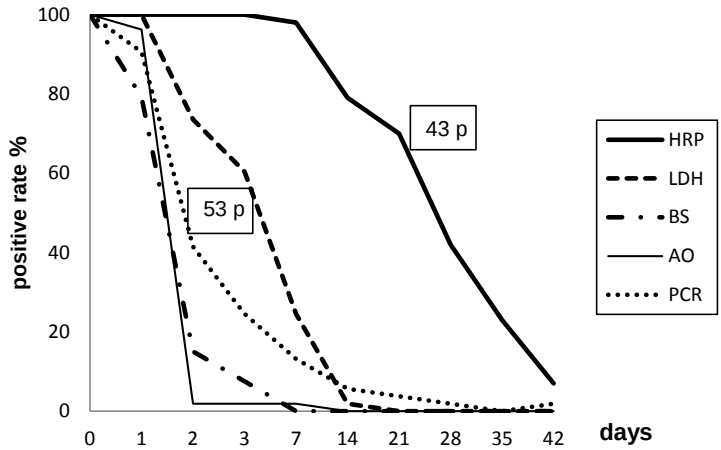
Usefulness of *Plasmodium falciparum* specific rapid diagnostic tests for assessment of parasite clearance and detection of recurrent infections after artemisinin-based combination therapy

6.2.1 Results

There were 53 children who completed the 42-day follow up. At day of inclusion the children had a fever history of 1-4 days.

Median clearance time for HRP2 was 28 (7->42) days, for LDH 7(2-14) days. PCR had a clearance time of 2 (1-28) days, BS microscopy 2 (1-7) days and AO microscopy 2 (1-14) days. Three children remained HRP2 positive up to day 42, i.e. the last follow up day.

Figure 8. Positivity rate at each sampling point by the five diagnostic tests



BS-Giemsa stained blood smear, AO-acridine orange stained blood smear, p=children

Estimation of positivity rate is based on all 53 children, except for the HRP2 based RDT where the estimation is based on the 43 children without a recurrent infection during follow up. The false positivity rates for HRP2 against PCR on days 14, 21, 28, 35 and 42 were 80% (32/40), 64% (27/42), 43% (18/42), 24% (10/42) and 7% (3/41), respectively.

The specificity against BS microscopy was for HRP2 21% on day 14 reaching 87% on day 42, whereas LDH had a specificity of $\geq$ 96% from day 14 and onwards (Table 5).

There was no significant correlation between parasite density on day 0 and duration of HRP2 positivity (r=0.13, p=0.38).

Ten children got a recurrent infection during follow up as assessed by PCR, BS and/or LDH-based RDT, one on day 14, four on day 21, two on day 28 and three on day 35. Eight of the ten children were still positive by HRP2 from the initial infection and this RDT could therefore only detect two of the new infections, whereas all other tests turned negative before parasite recurrence. LDH based RDT, identified eight at day of recurrence and another one on the next follow up visit. Out of the recurrent infections one was defined as recrudescence (treatment failure), six as reinfections (new infections) and three could not be determined by PCR. Five of the ten children had fever on day of recurrent parasitaemia and one child had only symptoms of respiratory tract infection. Four children had no symptoms.

Table 5. Specificity of HRP2 and LDH-based RDTs against BS microscopy (gold standard) during follow up

	HRP2			LDH			
Day		%	CI 95%		%	CI 95%	p-value
3		NA		20/49	41	27-56	
7		NA		40/53	76	62-86	
14	11/52	21	11-35	51/52	98	90-100	<0.0001
21	17/50	34	21-49	48/50	96	86-100	<0.0001
28	29/49	59	44-73	49/49	100	93-100	<0.0001
35	35/50	70	55-82	48/50	96	86-100	<0.005
42	45/52	87	74-94	50/52	96	87-100	>0.05

NA-not applicable

6.2.2 Discussion

We followed up the 53 enrolled children with five different diagnostic tools to thoroughly investigate the clearance time after treatment with ATC (arthemeter-lumefantrine). The mean clearance time for PCR, was 2.9 days. This rather long mean clearance time was influenced by four children with persistent positivity from the initial infection up to day 7, 14 and 21, respectively. In two of these children this may be explained by gametocytes which were found during PCR positivity. Also BS microscopy had a longer clearance than expected with a mean of 2.0 days. Four (8%) children remained positive up to day 3 and two of them were positive with very low parasite densities of 16 and 32/µL, however, with parallel negative PCR results. Both PCR and BS persistent positivity could be due to poor compliance since only the initial dose of arthemeter-lumefantrine was given under supervision.

The long clearance time observed for HRP2 (geometric mean 26.4 days), confirms the results from several other studies [151, 155]. Long clearance time has a major effect on the test specificity making the test unsuitable for assessment of treatment outcome and for diagnosis in moderately high endemic areas, especially during the peak transmission season. The long persistence of HRP2 antigen in the circulation makes it impossible to distinguish a new malaria episode from remaining positivity from a previous infection. This phenomenon can

cause incorrect interpretation as treatment failure by health workers reducing their confidence in antimalarial drug effectiveness. It may also impair health workers adherence to RDTs and have a major effect on the usefulness of these tests, especially in high endemic areas.

The pLDH-antigen showed, compared with HRP, a faster mean clearance time of 5.5 days, a high specificity (≥96% from day14) and may therefore be a better tool for detection of recurrent parasitaemia. However, despite a much faster clearance time for LDH-antigen it was still longer than for BS microscopy (2.1 days). It may therefore probably not be a useful tool for detection of prolonged parasite clearance time as a sign of emerging tolerance/resistance to antimalarial drugs.

The sensitivity for pLDH-based detection of *P. falciparum* infections among symptomatic patients, particularly in moderately-high endemic areas should also be sufficient since symptomatic patients generally have parasite densities above the detection limit of around 100 p/µL. However, in low endemic areas, especially for detection of asymptomatic malaria, probably neither of the two antigens would be sufficiently sensitive.

Importantly, pLDH is also produced by mature gametocytes, which could cause false positive result [152]. Gametocyte carriage is more seldom seen after ACT treatment compared to other antimalarial drugs and in this study there were only four children with gametocytaemia, and they were all negative by the LDH based RDT.

We saw no correlation between day 0 parasite density and persistent HRP2 positivity (r=0.13), which is in contrast to several other studies [151, 155]. Factors like duration of infection prior to blood sampling, the immune status (HRP2 antibody levels) of the patient and the total parasite biomass, including sequestered parasites, may influence the relation between HRP2 and parasite concentration.

6.2.3 Limitations

This was a small study with a limited number of recurrent infections detected during follow up. During patient inclusion there was a stock out of arthemeter-lumefantrine in the first study site, which precluded patient enrolment during the high transmission season in 2009. When the inclusion of children was continued during the following rainy season the number of parasite positive children had dropped markedly due to a massive LLIN distribution in the area. We therefore had to move to Bagamoyo district to finalize the study.

If it would have been possible to give the full treatment course under supervision, poor compliance could have been excluded as a source of prolonged clearance time by the diagnostic tools evaluated.

In clinical trials patients are usually followed daily up to day 3 followed by day 7 and thereafter weekly. For a more robust result regarding clearance time in this study, the daily sampling could have continued after day three for another one or two days. .

6.3 STUDY 3

Rapid diagnostic tests for molecular surveillance of *Plasmodium falciparum* malaria - assessment of DNA extraction methods and field applicability

6.3.1 Results

In vitro part

The *P. falciparum* DNA detection limit varied with RDT device and extraction method (Table 6). Chelex-100 extraction preformed best for both RDT devices as well as for extraction from filter paper, with a detection limit of 2 p/µL. The ABI extraction method had a 10-fold higher detection limit of 20 parasites/µL. DNA extraction was generally more efficient from SD-Bioline Malaria Ag P.f/Pan than from Paracheck-Pf. Increasing the size of the nitrocellulose strip fragment did not improve the level of detection. The PCR method used for *P. falciparum* detection influenced the detection limit by 1-2 log units. DNA extraction from RDTs was generally equal to or better than DNA extraction from an equal volume (5 µL) of blood spotted on filter paper.

Table 6. Sensitivity of DNA extraction methods from in-vitro RDT

Fragment used for DNA extraction	Simple elution	Chelex-100	ABI
Paracheck-Pf			
1 cm	§	2	200
Proximal	NA	20	200
Distal	NA	NA	20
Whole	NA	NA	20
SD-Bioline Malaria P.f/Pan			
1 cm	2	2	20
Proximal	NA	2	20
Distal	NA	NA	20
Whole	NA	NA	20
Filter paper			
5 µL blood spot	200	2	200

NA= not applicable, the size of the RDT fragment used for extraction was limited by the extraction volume

§ = Not estimated due to negative results

Field samples

There was no significant difference in PCR detection rates in DNA extracted from RDTs and filter papers. Out of 855 paired RDT and filter paper field samples, 118 (13.8%; CI 95% 11.4-16.2%) were PCR positive in both groups of samples (κ=0.94). Among the RDT negative field samples (N=734), three (0.4%; CI 95% 0.0-0.9%) and six (0.8%; CI 95% 0.1-1.5%) were PCR positive from RDT and filter paper extracted DNA, respectively (κ=0.44). Among the 121 RDT positive field samples, 115 (95.0%; CI 95% 91.1-99.0%) from RDT and 112 (92.6%; CI 95% 87.8-97.4%) from filter papers were PCR positive (κ=0.50). No observed difference was found in the ability to detect low density parasitaemias (<100 parasites/µL) even though the number was small (12).

There were no significant differences in PCR success rates for the respective SNPs in the 121 RDT positive samples (Table 7). No sample contained multiple *pfmdr1* copy number.

Table 7. PCR success rates and agreement of genotyping outcomes in field samples

	RDT PCR success rate N=121 (%; CI 95%)	Filter paper PCR success rate N=121 (%; CI 95%)	Kappa value
***Pfcrt* K76T**	114 (94.2; 90.0-98.4)	104 (86.0; 79.7-92.2)	0.72
***Pfmdr1* N86Y**	112 (92.6; 87.8-97.3)	109 (90.1; 84.7-95.5)	0.85
***Pfmdr1* Y184F**	110 (90.9; 85.7-96.1)	107 (88.4; 82.6-94.2)	0.74
***Pfmdr1* D1246Y**	113 (93.4; 88.9-97.9)	107 (88.4; 82.6-94.2)	0.77
***Pfmdr1* copy number**	84 (69.4; 61.0-77.7)	77 (63.6; 54.9-72.3)	-

6.3.2 Discussion

We performed a comprehensive comparative in-vitro evaluation of DNA extraction from RDTs and assessed the field applicability for detection of genetic markers associated with drug resistance from used RDTs collected from symptomatic patients in Zanzibar.

DNA extraction efficiency from in vitro cultured *P. falciparum* varied with RDT device and extraction method. Different designs of RDT devices affected the DNA extraction efficiency. In particular, DNA recovery from Paracheck-Pf was unsuccessful when using the simple elution method, whereas it worked well with the other two methods. It has previously been reported that plastic seals covering the nitrocellulose strip hamper DNA recovery [229]. The Chelex-100 extraction method in combination with the Cyt b or 18s real-time PCR performed best in our evaluation suggesting that Chelex is the preferable method for low density parasitaemias. However, the Chelex method is relatively labour intensive and the storage capacity for the DNA is a concern. The simple elution method on the other hand, is cheap and quick but was hampered by the small extraction volume not suitable for all devices and could also therefore not be thoroughly evaluated in our study. The high throughput ABI extraction method for RDTs was also labour-intensive and costly but provides on the other hand high quality and stable DNA. From our evaluation this extraction method seems to be more suitable for RDTs collected from symptomatic patients with higher parasite densities.

The field analysis showed that collected RDTs are adequate alternatives to filter papers for DNA storage under field conditions. In areas of low endemicity where clinical trials are

logistically challenging to conduct, analysis of used RDTs, routinely taken for diagnosis would be an alternative for molecular surveillance. It also reduces the need for additional blood samplings of RDT positive individuals if only one finger prick is required for both malaria case detection and for preservation of biological material. DNA extracted from RDTs collected in the field could also be used for more sensitive parasite detection with PCR and for quality control of RDTs.

6.3.3 Limitations

To get a more robust comparison between the three extraction methods it would have been an advantage to use the same volume of elution buffer in all three. However, the blood volumes used in this study was in accordance with the published protocols for the three methods, meaning that the ABI method used a four times higher volume compared with simple elution, i.e. DNA was four times more diluted. A DNA quantification of the extracts with a qPCR method comparing the Ct-values would have added information in this study.

In the article we claim that the higher detection limit by the ABI method compared to Chelex did not influence the field sample results when we compared RDT and filter paper extraction. However, we do not know the outcome if these samples would have been extracted with another (Chelex) method.

6.4 STUDY 4

Loop Mediated Isothermal Amplification (LAMP) accurately detects malaria DNA from filter paper blood samples of low density parasitaemias

6.4.1 Results

Fever patients

Overall 115/865 (13.3%) patients were positive by nested PCR and another two were positive with real-time PCR. The geometric mean parasite density was 7491p/µL (range 6-782,400) as assessed by microscopy. All PCR positive samples were *P. falciparum* mono-infections, of which 105 (91%) were detected by Pan-LAMP and 101 (88%) by Pf-LAMP. There were 21 samples with discordant nested PCR versus Pan and/or Pf-LAMP results. All 21 samples were positive when analysed with real-time PCR. After repeat PCR and LAMP analysis with Chelex re-extracted DNA from the 21 samples (and 21 control samples) 115 (98,3%) of the

117 real-time PCR positive samples were positive by both Pan-LAMP and Pf-LAMP (Table 8)

Asymptomatic individuals:

Out of the 465 samples from the cross-sectional screening, 50 (11%) were positive by nested PCR and another four with real-time PCR, 33 *P. falciparum*, 13 *P. malariae* and 8 mixed *P. falciparum/P. malariae* infections. The geometric mean parasite density determined by qPCR was 10 p/µL (range 0-4972). Pf-LAMP had a sensitivity of 92.7% (95%CI 80.1-98.5) for detection of the 41 *P. falciparum* infections. Pan-LAMP had sensitivities of 97% (95%CI 84.2-99.9) and 76.9% (95%CI 46.2-95) for detection of *P. falciparum* and *P. malariae* mono-infections, respectively. There was no significant difference in parasite densities between the three Pan-LAMP negative (range 1-2p/µL) versus the ten Pan-LAMP positive (range 0-3p/µL) *P. malariae* samples ($p \geq 0.05$). Pan and Pf-LAMP both detected seven, whereas nested PCR detected five of the eight samples with mixed *P. falciparum*/*P. malariae* infections (Table 8).

The detection limit for *P. falciparum* was ≤ 2 parasites/µL for both Pan and Pf-LAMP as assessed by serial dilutions of lab strain parasites.

Table 8. Sensitivities and kappa analysis for detection of malaria DNA from fever patients and asymptomatic individuals with Pan and Pf-LAMP versus gold standard (real- time PCR corrected Cytochrome b nested PCR)

Fever patients ABI-extracted DNA (n=865)		
	Sensitivity % (95% CI)	**Kappa value**
Pan-LAMP	91.5 (84.8-95.8)	0.95
Pf-LAMP	86.3 (78.7-92.0)	0.92
Fever patients: including Chelex re-extracted DNA (n=823+42)		
	Sensitivity % (95% CI)	**Kappa value**
Pan-LAMP	98.3 (94.0-99.8)	0.99
Pf-LAMP	98.3 (94.0-99.8)	0.99
Asymptomatic individuals Chelex extracted DNA (n=465)		
	Sensitivity % (95% CI)	**Kappa value**
Pan-LAMP all species	90.7 (79.7-96.9)	0.95
Pan-LAMP *P.f.**	97 (84.2-99.9)	0.97
Pan-LAMP P.m.**	76.9 (46.2-95)	0.89
Pf-LAMP *P.f.****	92.7 (80.1-98.5)	0.96

*P.f.**= *P. falciparum* mono infections (n=33), P.m.**= *P. malariae* mono infections (n=13), P.f.*** *P. falciparum* mono and mixed infections (n=41), CI= confidence interval

The specificities for both Pan and Pf LAMP were 100% in both study groups.

6.4.2 Discussion

In this study we have shown that LAMP had a high diagnostic accuracy for detection of low density parasitaemias from minute blood volumes preserved on filter papers, a relatively low-cost and practical solution for population screening purposes. This supports the potential role of LAMP for improved passive and active malaria case detection in pre-elimination settings.

LAMP performed very well on samples recently extracted with the Chelex method but less well for samples with the column-based ABI-extraction method. Long storage and several freeze-thawing episodes may have influenced the quality of DNA suitable for LAMP more than for PCR, since several samples with microscopically detectable parasitaemias were negative with LAMP but positive with both PCRs. This shows that the quality of DNA extract could be of importance despite the use of *Bst-polymerase*, an enzyme considered suitable for simple and ruff extraction methods [216].

The LAMP kit evaluated in this study was easy to handle and interpret and could be a useful tool for screen and treat activities in areas of low endemicity/pre elimination. Considering that the relative importance of *non-falciparum* infections increases in such areas, screening with Pan-LAMP only, which had a slightly lower detection limit compared to Pf- LAMP [236] followed by Pf-LAMP analysis of Pan-LAMP positive samples may represent a cost effective strategy. However, at very low *P. falciparum* parasite densities the Pf-LAMP could then give a false negative result, leading to overestimation of the *non-falciparum* prevalence.

6.4.3 Limitations

The two sets of samples used in this trial were extracted with two different methods. The fever patient samples were extracted with the column based ABI high throughput method already in 2010. This method is considered suitable for long time storage and to produce a high quality DNA. However, these samples have since then been freeze-thawed several times. We could not re-extract with the same method neither use it for the asymptomatic individuals, since the method was then replaced by the Chelex-method in our laboratory. The samples with concordant PCR and LAMP results were not re-extracted, meaning we do not know the outcome if we had re-analysed these samples; however they were previously investigated with several PCR methods.

6.5 OVERALL DISCUSSION STUDY 1-4

The usefulness of new diagnostic tools like RDT and LAMP are highly dependent on the malaria transmission intensity in the area where they are going to be used. The choice of diagnostic tool is also dependent on the target population, i.e. whether it is asymptomatic individuals or fever patients that are going to be tested. In study 1 and 2 we have evaluated the usefulness of *P. falciparum* specific RDT as diagnostic tool among fever patients in both a low and a moderately high endemic areas, however, from different perspectives. In study 1

our data suggest that probably there is need for a continuous quality control of RDT use, despite a relatively extensive pre-study training and in an area where the health infrastructure and surveillance system is relatively well functioning.

In study 2 we thoroughly investigated parasite clearance after treatment with ACT both with microscopy, RDT and PCR. Parasite clearance time provides useful information in drug trials, where prolonged clearance could be an early sign of increased tolerance to the given antimalarial treatment. The evaluation of the two antigens HRP2 and pLDH for use in RDTs in an area where the risk of reinfection was relatively high, could also provide useful information for choosing an appropriate RDT device by malaria control programs in such areas.

In study 3 we evaluated RDT from another perspective, namely as a source of parasite DNA. We could show how RDTs can be used for retrospective analyses with more sensitive methods such as PCR for parasite detection. We could also show that RDT is a good alternative to filter papers for storage of DNA, already packed in individual casettes with minimal risk of contamination between samples. Collection of used RDTs in low endemic/pre-elimination areas can be applied for more sensitive molecular methods both for improved case detection and surveillance.

In study 4 we evaluated a new molecular method- LAMP, for detection of malaria parasites using a commercial kit. The evaluation of LAMP showed that it is a promising method, with equal high sensitivity compared to real-time PCR also when small blood volumes preserved on filter papers are used. However, the need for highly sensitive methods is primarily for screening of asymptomatic populations, whereas RDT and microscopy generally are sufficient tools for detection of symptomatic parasite carriers. The LAMP kit evaluated in this project needs, however, to be further evaluated under field conditions, especially for detection of low parasite densities in low transmission areas. User-friendliness and turn-around time are important aspects if LAMP is going to be useful in the future.

7 CONCLUSIONS

7.1 OVERALL CONCLUSION

The four studies in my doctoral project have provided new information regarding the usefulness of new diagnostic tools in different endemic contexts. To develop and determine the optimal methods for malaria diagnosis is a challenge. However, research and development for new and improved diagnostic methods together with better knowledge among users are promising opportunities in the fight against malaria.

7.2 SPECIFIC CONCLUSIONS

Study 1

- The sensitivity of RDT in the hands of primary health care workers compared with both PCR and microscopy for *P. falciparum* case detection was relatively low in the new situation of low malaria transmission in Zanzibar. This highlights the need for improved quality control of RDT use in primary health care facilities as well as need for more sensitive point-of-care malaria diagnostic tools in Zanzibar.

- Adherence to test results with antimalarial treatment in the study area of Zanzibar was generally excellent and the results also provide evidence that RDT can be reliably integrated in IMCI as a tool for improved childhood fever management.

Study 2

- Parasite clearance time after ACT treatment as estimated by PCR and microscopy was two days for the majority of children.

- The LDH-based RDT was superior to HRP2-based for monitoring of treatment outcome and detection of recurrent *P. falciparum* infections after ACT treatment in a moderately high endemic area of Tanzania. These results may have implications for the choice of RDT devices in similar transmission settings for improved malaria case management.

- There was no correlation between parasite density at enrolment and persistence of HRP2-based RDT positivity.

Study 3

- RDT is a valuable source of parasite DNA which can be a useful tool for improved malaria case detection, molecular drug resistance surveillance, and RDT quality control.

- The purpose of DNA extraction from RDTs should be considered when choosing which extraction method best suits the type of samples to be analyzed.

Study 4

- The Loopamp™ MALARIA kit revealed high diagnostic accuracy for parasite detection among both fever patients and asymptomatic individuals with low parasite densities.

- Using DNA extracted from minute blood volumes for the LAMP assay may be of value, particularly when blood sampling on filter paper from capillary finger pricks would be advantageous for practical and logistical reasons.

- The LAMP method has a potential role for the implementation of active case detection activities in malaria pre-elimination settings.

8 PERSONAL REFLECTIONS AND FUTURE PERSPECTIVES

I have a background as a biomedical technologist with a long experience of malaria microscopy. I have had the opportunity to participate in many clinical trials for malaria, both on site in Africa but also with quality control of blood smears sent to Sweden from the field. This experience has been invaluable for my understanding of all the problems and difficulties with achieving good quality of malaria microscopy under field conditions, and also for my doctoral project focusing on diagnosis.

The diagnosis of malaria has during the last decade become one of the most important tools for surveillance and control. From being something the locals dealt with "down in Africa" it has now become a hot topic in the research community. The perception of how important a proper diagnosis is for correct management of malaria in combination with increased awareness that malaria microscopy, which was the only available tool, demanded good techniques and skilled personnel, something that was lacking in most areas suffering from the disease, led to the development of rapid diagnostic tests some twenty years ago. Many commercial companies saw the potential for this product on the global market endemic for malaria as well as from increased global travelling leading to more demands for good diagnostic tools also in non-endemic countries. The high and growing number of available RDT products and brands made WHO-FIND, to initiate the yearly RDT product testing program. This initiative has been very important for guiding and information regarding RDT use. The evaluation in combination with the competition between commercial producers and hundreds of research articles evaluating RDTs from different aspects, has resulted in the development of new and better products. Access to a diagnostic tool that is easy to use, prompt, relatively cheap and sensitive as well as specific has had a major impact on the possibilities for a parasite based diagnosis.

However, there are still a number of issues with the present RDTs available, which need to be considered if they are going to be useful tools in the future. The need for increased sensitivity is probably the most important; a detection limit of more than 50 p/µL for *P. falciparum* with significantly higher limits for the other species is not enough in the era of declining malaria transmission. Specificity of HRP2 for detection of ongoing infection is also an issue, especially in high endemic areas. LDH-based *P. falciparum* detection would be a better option in such areas but until now it seems that most guidelines for malaria diagnosis recommend HRP2 based RDTs due to their slightly higher sensitivity. There are suggestions that a combo test with HRP2 and pLDH may be useful for treatment follow up [189, 240],

meaning when pLDH becomes negative the HRP2 positivity should be interpreted as remaining antigenemia. However, I think it would be challenging to ensure correct interpretations of such tests in most endemic areas, since health workers are aware of the lower HRP2 detection limit. The combo test detecting *P. falciparum* in either only the HRP2 band or also in the pan-LDH band is not a good solution and still a common cause of confusion. There are a few recent developed RDTs detecting the non- *falciparum* species solely, excluding *P. falciparum* from the Pan-LDH that could be an option. However, a test based on pLDH (or another antigen) with different combinations of species-specific detection of all five human malaria species with high sensitivity and specificity in an RDT format would be an optimal diagnostic tool in the future. This would also diminish the problems with other shortcomings of HRP2 based RDTs, such as gene deletions and prozone effect.

In the era of decreased malaria transmission and with the possibility of achieving elimination in areas like Zanzibar there are urgent demands for field friendly and affordable tools for detection of the remaining parasite carriers. Molecular detection of parasite DNA with LAMP has recently shown to be promising also for use in mass screen and treat activities in Zanzibar (unpublished data). I think the LAMP-kit evaluated in this project has the potential to become very useful in the future. However, like for RDT the kit can only distinguish *P. falciparum* from Pan, detecting all four (five) human species. It is also a concern that Pan is slightly more sensitive than Pf-LAMP for *P. falciparum* at densities close to the detection limit of 2 p/µL which could lead to incorrect estimations of prevalence of the *non-falciparum* species. LAMP can also not determine parasite densities, due to the very efficient DNA amplification giving rise to equally high fluorescence or turbidity from very low densities as from very high. However, the use of a real-time turbidimeter could maybe be a future tool since time to start of increase in turbidity in the samples is dependent on the DNA concentration. The efficient amplification means a high risk of contamination; something that really has to be considered when the method is set up in laboratories as well as in field settings. Development of a robust high throughput system for extraction and analysis of large sample volumes is a priority if LAMP is going to be an even better tool for mass screen and treat activities. There is also a need for specific detection of other malaria species, primarily *P. vivax*, which is a common cause of malaria infections in many areas, with the need for other control and elimination strategies.

Finally, it is problematic to use a method as reference (gold) standard (blood smear microscopy), which often is less sensitive than the methods being evaluated.

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The Usefulness of Rapid Diagnostic Tests in the New Context of Low Malaria Transmission in Zanzibar

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Abstract

Background: We assessed if histidine-rich-protein-2 (HRP2) based rapid diagnostic test (RDT) remains an efficient tool for Plasmodium falciparum case detection among fever patients in Zanzibar and if primary health care workers continue to adhere to RDT results in the new epidemiological context of low malaria transmission. Further, we evaluated the performance of RDT within the newly adopted integrated management of childhood illness (IMCI) algorithm in Zanzibar.

Methods and Findings: We enrolled 3890 patients aged ≥2 months with uncomplicated febrile illness in this health facility based observational study conducted in 12 primary health care facilities in Zanzibar, between May-July 2010. One patient had an inconclusive RDT result. Overall 121/3889 (3.1%) patients were RDT positive. The highest RDT positivity rate, 32/528 (6.1%), was found in children aged 5–14 years. RDT sensitivity and specificity against PCR was 76.5% (95% CI 69.0–83.9%) and 99.9% (95% CI 99.7–100%), and against blood smear microscopy 78.6% (95% CI 70.8–85.1%) and 99.7% (95% CI 99.6–99.9%), respectively. All RDT positive, but only 3/3768 RDT negative patients received anti-malarial treatment. Adherence to RDT results was thus 3887/3889 (99.9%). RDT performed well in the IMCI algorithm with equally high adherence among children <5 years as compared with other age groups.

Conclusions: The sensitivity of HRP-2 based RDT in the hands of health care workers compared with both PCR and microscopy for P. falciparum case detection was relatively low, whereas adherence to test results with anti-malarial treatment was excellent. Moreover, the results provide evidence that RDT can be reliably integrated in IMCI as a tool for improved childhood fever management. However, the relatively low RDT sensitivity highlights the need for improved quality control of RDT use in primary health care facilities, but also for more sensitive point-of-care malaria diagnostic tools in the new epidemiological context of low malaria transmission in Zanzibar.

Trial registration: ClinicalTrials.gov NCT01002066

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Introduction

Zanzibar has recently undergone a rapid transition from high to low transmission of *Plasmodium falciparum* malaria following wide scale, high coverage implementation of combined malaria control interventions [1]. In this new epidemiological context, efficient malaria case detection and targeted malaria treatment with artemisinin-based combination therapy (ACT) to fever patients with parasitologically confirmed malaria infection is critical. Therefore, Zanzibar introduced *P. falciparum* specific rapid diagnostic testing (RDT), based on antigen detection of histidine-rich protein 2 (HRP2), for confirmatory malaria diagnosis of fever patients in all public health care facilities previously not equipped with microscopy service [2]. Importantly, malaria RDT has also been recently incorporated in the local version of the integrated management of childhood illness (IMCI) guidelines of 2009 [3,4]. These guidelines are used for management of illness in children aged 2–59 months in low and middle-income countries. The performance of RDT as an integrated tool in IMCI for improved childhood fever management in Africa and Zanzibar in particular has previously not been evaluated.

There are concerns regarding the usefulness of RDT, both with regards to case detection with differences in test performances in various epidemiological settings and to adherence to negative test results among health care workers [5,6,7,8,9]. However, we have previously shown that HRP2 based RDT aided diagnosis of fever patients in primary health care facilities in Zanzibar was efficient for *P. falciparum* detection when compared with blood smear (BS) microscopy with a sensitivity of 92% and a specificity of 88%; moreover, RDTs resulted in improved adequate treatment and health outcomes [10]. Importantly, the previous study was conducted when *P. falciparum* malaria was responsible for approximately 30% of febrile illness in primary health care facilities in Zanzibar [10,11]. This is in marked contrast to the present situation, when overall <3% of fever patients have confirmed *P. falciparum* infection [12].

Based on the above, three critical scientific questions can be identified: Firstly, does HRP2 based RDT remain an efficient tool for *P. falciparum* detection in the new epidemiological context in Zanzibar? This should be evaluated not only against conventional BS microscopy but also against highly sensitive PCR as gold standard, considering that the relative importance of parasitemias below the detection limit of RDT/microscopy may increase in low transmission settings [13,14,15]. Secondly, is malaria RDT, as an integrated part of the recently adopted local version of the IMCI algorithm, a reliable tool to both identify and rule out malaria infection in children below five years of age? Thirdly, and importantly, do primary health care workers continue to adhere to RDT results when over 95% of the tests are negative and alternative diagnostic tools for fever management are scarce?

The aim of this study was therefore to evaluate the usefulness of HRP2 based RDT in the hands of primary health care workers, including its performance within the local version of the IMCI algorithm, for *P. falciparum* case detection, and adherence to test results among fever patients in the new epidemiological context of low malaria transmission in Zanzibar.

Methods

Study Design, Area and Sites

This work was a health facility based observational study conducted in twelve primary health care facilities, six each in North A (Unguja Island) and Micheweni (Pemba Island) districts of Zanzibar, between May and July 2010**.** None of these health facilities were part of our previous RDT study from Zanzibar [10]. The two study districts are mainly rural with approximately 100,000 inhabitants each. *P. falciparum* is the predominant malaria species and *Anopheles gambiae* complex the main vector. Malaria transmission has historically been stable with peaks related to the seasonal rainfalls in March to May and October to December. Following a successful malaria control programme the BS/RDT positivity rate among fever patients has markedly declined during the last decade in Zanzibar to reach 0.6% and 2.2%, respectively, in North A and Micheweni districts in 2009 [12].

Public health care in the study area is delivered through twelve primary health care units (PHCU) and one primary health care centre (PHCC) per district. The PHCUs provide basic out-patient care including malaria RDT for fever management, whereas the PHCCs in addition to basic out-patient care also have facilities for in-patient care and laboratory services, e.g. routine use of microscopy for confirmatory malaria diagnosis.

The twelve study sites were purposely selected, i.e. one PHCC and five PHCU from each district. This was done to ensure an adequate manpower capacity, with at least two health workers available per study site during the conduct of the trial, and to provide a balanced geographical distribution of the study sites in the respective districts.

Study Staff and Pre-study Training

A total of 33 nurses and clinical officers with formal prescription rights from Zanzibar Ministry of Health were responsible for the outpatient care in the twelve study sites. All study staff members were previously trained in malaria case management by the Zanzibar Malaria Control Programme (ZMCP). Based on previous IMCI training, the health workers received either a six-day refresher course or an eleven-day full course on the recently adopted IMCI guidelines in Zanzibar in which malaria RDT has been integrated in the algorithm [3]. In addition to the IMCI training and prior to the study start, all study staff members also received a one-week study specific training including good clinical practice, ethical considerations and provision of informed consent, data recording in case record forms (CRF), blood sampling for BS and filter papers as well as performance and interpretation of RDT (Paracheck Pf®) according to the manufacturer's instructions. During the pre-study training the study staff members were encouraged to adhere to the IMCI guidelines for management of children below five years of age and the national malaria guidelines, which recommend anti-malarial medicines to be prescribed based on laboratory confirmation of malaria [2]. However, they were free to manage patients according to their own clinical judgement during the conduct of the trial. Prior to the first enrolment a pilot study with "dummy runs" was conducted in all participating study sites.

All health workers received a fixed monthly salary as top ups for their study participation according to standard practice in Zanzibar, whereas study participants did not receive any incentives.

Patient Enrolment and Management

Patients were eligible if aged ≥2 months and presenting at the study sites with fever, i.e. measured axillary temperature of ≥37.5°C or a history of fever during the preceding 24 hours, and willing to provide written informed consent (proxy consent from parents/guardians for children) to participate in the study. Patients were excluded and referred in case of any symptoms of severe disease or danger signs. Patients were recruited Monday to Friday between eight am to four pm by the study health worker on duty in the respective study sites. Children aged 2–59 months, hereafter referred to as "<5 years", were managed according to the local IMCI guidelines. The study included a single visit for each participant.

Upon enrolment a sealed envelope was opened containing a study reference number and information on whether the individual had been randomly selected for additional blood sampling (see below). This was followed by a capillary finger prick for performance of RDT. All RDT positive patients and a pre-defined computer generated random sample (www.randomization.com) of 20% of all study participants were subjected to additional capillary blood sampling for malaria microscopy (thick BS) and PCR (approximately100 µL spotted on a Whatmann 3 MM filter paper).

Basic demographical data, axillary temperature (measured with a digital thermometer), history of fever in the preceding 24 hours, other symptoms, previous anti-malarial treatment (in case of pregnancy use of intermittent presumptive treatment (IPTp)), whether the patient slept under an insecticide treated nets/long lasting insecticidal nets (ITN/LLIN) the night before study enrolment and travel history (defined as having spent at least one night outside the home Shehia, i.e. the smallest administrative

unit in Zanzibar, in the last 28 days) were recorded in a CRF together with information on RDT-result and clinical management.

The RDT results were provided directly to the patients by the study staff. Anti-malarial medicines according to national guidelines, i.e. artesunate-amodiaquine (first-line) or oral quinine (second-line), antibiotics (cotrimoxazole, ampicillin, amoxicillin or erythromycin) and antipyretics (e.g. paracetamol) were available free of charge to all study participants. Patients and guardians were encouraged to return if the clinical condition deteriorated or if the fever persisted.

In case malaria parasitemia was first detected by first BS microscopy reading and missed by RDT diagnostic (see below), the result was reported to the health facility and the patient was traced and offered treatment if he/she did not receive anti-malarial medicines at the time of the study visit.

All CRFs were collected by trained health staff at the end of each day and stored centrally in Zanzibar. During the conduct of the study, no results were disclosed to the health workers. However, after study completion, preliminary results were disseminated to all health workers in both districts.

Laboratory Methods

RDT. We used Paracheck Pf (Orchid Biomedical Systems, Goa, India) detecting *P. falciparum* specific HRP2 antigen, which by the time of trial was the RDT device deployed by ZMCP. The tests were performed and interpreted according to the manufacturer's instructions. Very faint bands at the test line position were to be assessed as a positive test result. Band intensity was not recorded.

Blood smear microscopy. BS were prepared on site, stored in slide boxes and transported daily to the district central level, where all slides were stained with 5% Giemsa for 30 minutes and examined under oil immersion (×100 magnification) by two independent and experienced microscopists blinded to both the RDT and each other's microscopy result [16]. Asexual parasite densities were calculated against 200 white blood cells (WBC), assuming 8,000 WBC per microliter of blood. BS were defined negative if no parasites were found after examining 100 high power microscopy fields. BS with discordant results between the two readers, i.e. positive versus negative, difference in species diagnosis or a difference of >50% in parasite density, were re-examined by a third expert microscopist at Karolinska Institutet, Stockholm, Sweden. The mean of the two most concordant parasite counts were used for calculating the final parasite density [17]. Moreover, all BS from patients with discrepancies between RDT and PCR results, RDT and BS results or PCR and BS results were subjected to a third decisive microscopy reading at Karolinska Institutet.

Filter paper. The blood samples (approximately 100 µL) collected on filter papers (Whatmann 3 MM) were dried thoroughly, put in individual zipped plastic bags containing desiccant and stored in room temperature (<25°C) in Zanzibar until completion of the study and then transported to Karolinska Institutet for molecular analyses.

DNA extraction. DNA was extracted from three filter paper punches (Ø 3 mm) using a modified version of the ABI 6100 Nucleic Acid Prep Station protocol (Applied Biosystems, Fresno, CA) [18]. In samples with discrepancies between RDT and PCR results, DNA was re-extracted from the filter papers using Chelex-100 [19].

DNA extraction quality control. The presence of human DNA was analysed by real-time PCR in case of PCR-negative results in RDT positive samples [20].

Parasite detection by PCR. For the purpose of an ongoing genotyping project all RDT positive samples were analysed with three previously described *P. falciparum* specific nested PCR methods targeting the four antimalarial drug resistance markers *P. falciparum* multidrug resistance gene 1 (*pfmdr1*) N86Y, Y184F, D1246Y and *P. falciparum* chloroquine resistance transporter gene (*pfcrt*) K76T [21,22]. The RDT negative samples were screened in duplicate for human plasmodial infection with an 18s real-time PCR [23] Samples with a cycle threshold (Ct) value <42 were selected for species identification. Samples with discrepant RDT and PCR results were re-extracted and subjected to a confirmatory nested PCR analysis targeting *Plasmodium* cytochrome b [19]. PCR positivity was defined as a positive PCR result that could be validated by at least one other PCR method or by parasite detection by microscopy.

Analysis of P. falciparum HRP2 deletion. All samples with a negative RDT but a positive microscopy and/or PCR result were subjected to PCR analysis of HRP2 deletion according to a previously described protocol [24].

Adherence to RDT Results

Assessment of adherence to RDT results was done based on a comparison between the reported RDT outcome and prescription of anti-malarial drugs. Adherence was defined as prescription and absence of prescription of anti-malarial drug (first or second line) in RDT positive and negative patients, respectively.

Sample Size Calculation, Study Endpoints, Data Management and Statistical Analysis

The sample size calculation was based on the primary endpoint, i.e. adherence to malaria RDT results, with the assumption that approximately 10% of the RDT negative patients are prescribed ACT in Zanzibar [10]. The minimal sample size for independent observations would be 864 patients when requiring a maximum length of +/−2% for a 95% confidence interval (CI). Considering a potential clustering effect on health facility level we calculated that a sample of minimum 3054 patients would allow for an intra-cluster coefficient of slightly over 0.01 and still fulfil the given level of precision.

Secondary endpoints included sensitivity, specificity, positive and negative predictive values of HRP2 based RDT for *P. falciparum* detection compared with both PCR and BS microscopy; performance of malaria RDT as an integrated part of the local IMCI algorithm in Zanzibar (assessed as a sub-group analysis of sensitivity, specificity, positive and negative predictive values in children aged <5 years compared with other age groups); and antibiotic and antipyretic treatment across age-groups, i.e. <5, 5–14 and >14 years, among RDT positive and negative patients.

Data were double entered in CSPro, validated using Microsoft Excel and subsequently exported to STATA 12 software where all statistical analyses were performed. All frequencies, proportions and odds ratios (ORs) were calculated with 95% CIs and corresponding p-values, as appropriate. Adjustments for clustering on health facility level were done using mixed effect models.

To account for the fact that blood sampling for BS microscopy and PCR among RDT negative patients only included a random sample of 20%, we multiplied the absolute number of observations in these groups with a factors of 5.14 and 5.01 in all calculations of RDT sensitivity and specificity against PCR and microscopy, respectively. The corresponding confidence intervals were, however, based on the true sample size. Statistical significances were stated at the 5% level.

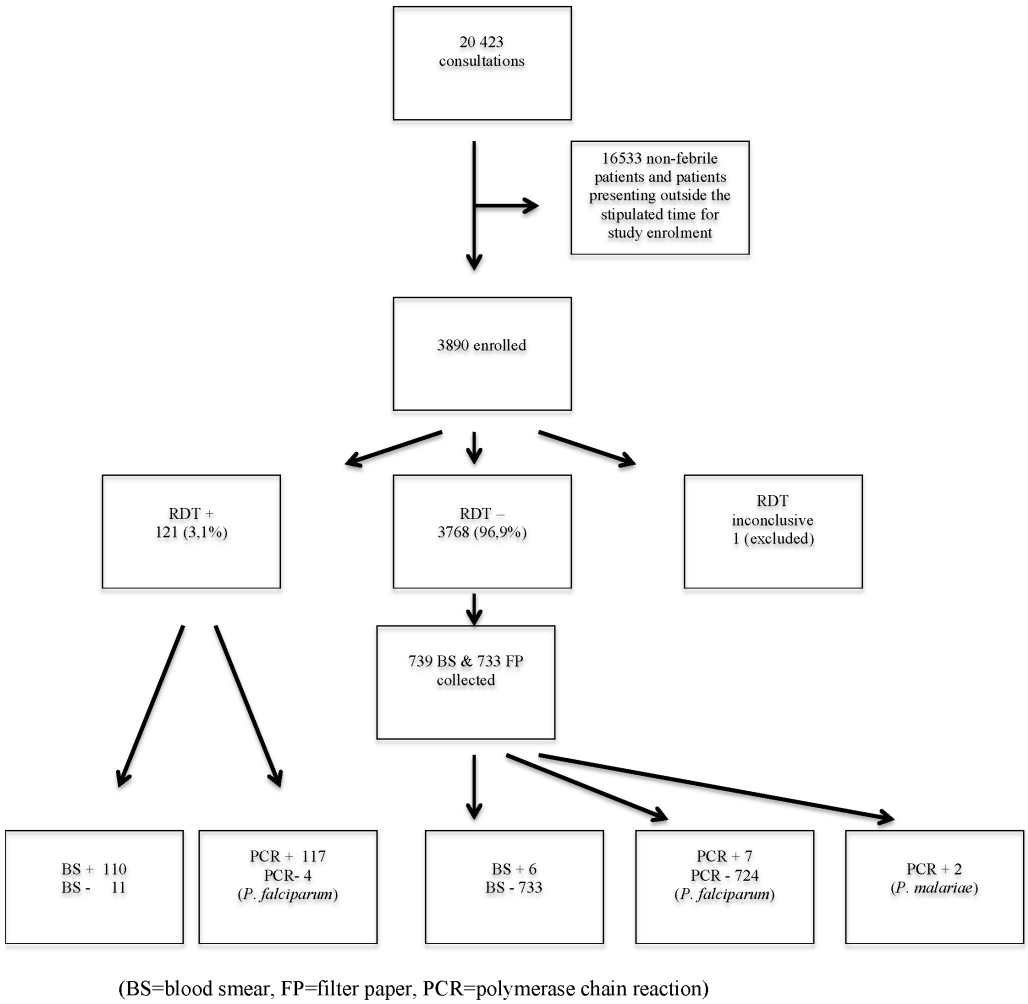

Figure 1. Study flow chart. (BS = blood smear, FP = filter paper, PCR = polymerase chain reaction.).
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Ethical Considerations

The study was conducted in accordance with the principles stated in the latest version of the Declaration of Helsinki and Good Clinical Practice [25]. It was approved by the Zanzibar Medical Research Ethics Committee (ZAMREC) and the Regional Ethics Committee, Stockholm, Sweden. The study is registered on ClinicalTrials.gov with study identifier "NCT01002066".

Results

The flow of patients through the trial is outlined in Figure 1 and baseline characteristics of the RDT positive and negative groups are presented in Table 1. Among the 3890 patients enrolled one was excluded due to an inconclusive RDT result. Overall 121/3889 (3.1%) patients were RDT positive. The highest RDT positivity rate, i.e. 32/528 (6.1%), was found in children aged 5–14 years. There was an uneven distribution of RDT positivity rates between the two districts, i.e. North A 42/2225 (1.9%) and Micheweni 79/1665 (4.7%), and also between their respective study sites with 53 (67.1%) of all RDT positive patients in Micheweni district reported from Tumbe PHCU only. RDT positivity was statistically significantly associated with absence of ITN/LLIN use [OR 4.78 (95% CI 3.24–7.02)] and history of travel within the last 28 days [OR 3.27 (95% CI 2.05–5.07)] (Table 1).

The distribution of positive RDT, PCR and BS results as well as microscopy determined parasite densities across age groups are shown in Table 2.

Table 1. Baseline characteristics.

		RDT +	RDT −	Total (N)
District	North A	42 (34.7%)	2182 (57.9%)	2224
	Micheweni	79 (65.3%)	1586 (42.1%)	1665
Age	5 y	36 (29.8%)	1786 (47.4%)	1822
	5–14 y	32 (26.4%)	496 (13.2%)	528
	>14 y	53 (43.8%)	1472 (39.1%)	1525
	Data missing	0	14 (0.3%)	14
Sex	Male	65 (53.7%)	1611 (42.8%)	1676
	Female	56 (46.3%)	2156 (57.2%)	2212
	Data missing	0	2 (100%)	2
Pregnancy		3 (2.5%)	87 (2.3%)	90
		RDT +	**RDT −**	**P-value**
Mean axillary temp◦C		38.3	37.4	<0.001
Proportion withaxillary temp. ≥37.5◦C		74 (66.7%)	1465 (41.5%)	<0.001
Data missing		10 (8.3%)	240 (6.4%)	0.350
ITN/LLIN use		65 (53.7%)	3193 (84.7%)	<0.001
Travel history	≤28 days	30 (24.8%)	356 (9.5%)	<0.001
	Domestic	21 (17.4%)	325 (8.6%)	0.003
	Abroad	9 (7.4%)	31 (0.8%)	<0.001

Domestic = History of travel within Zanzibar.
Abroad = History of travel outside Zanzibar.
ITN = Insecticide-treated nets.
LLIN = long-lasting insecticidal nets.
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The RDT sensitivity, specificity, positive and negative predictive values against PCR and BS microscopy are presented in Table 3. There was no significant difference in RDT performance in children aged <5 years, i.e. tested and managed within the IMCI algorithm, and other age groups (data not shown).

There were four and eleven RDT positive patients with negative PCR and BS results, respectively. Importantly seven patients had both positive RDT and PCR verified *P. falciparum* infection, but BS microscopy could not detect parasitemia. Some, 6/739 (0.8%) RDT negative patients had detectable parasitemia by BS microscopy; all but one aged ≥5 years, of whom three were from Tumbe PHCU (Micheweni). Four of these six patients had high parasite densities, i.e. 3573, 14420, 25779, 50190 parasites/μL, respectively, whereas the remaining two had low parasite counts of ten and 50 parasites/μL each. All six were also positive by PCR, five for *P. falciparum* and one for *P. malariae*. The *P. malariae* PCR positive sample had a low parasite density of ten parasites/μL, as determined by BS microscopy, i.e. insufficient to allow accurate species identification. Further PCR analysis could not detect *P. falciparum* HRP2 deletions in any of these samples. Four of the RDT negative patients had a positive first BS microscopy reading. They were all successfully traced at home and received treatment with artesunate-amodiaquine. None had developed symptoms/signs of severe malaria between day of study visit and time of active follow-up.

Prescription of anti-malarial medicines, antibiotics and antipyretics by RDT result and age group are shown in Table 4. All 121 RDT positive, but only 3/3768 (0.1%) of RDT negative patients, all aged >14 years, were prescribed anti-malarial medicines. Overall adherence to RDT results was thus 3886/3889 (99.9%).

Table 2. Distribution of *P. falciparum* positive rapid diagnostic test (RDT), polymerase chain reaction (PCR) and blood smear (BS) microscopy results as well as microscopy determined parasite density across age groups.

	Age groups			**Total**
	<5 y n = 1822	**5–14 y n = 528***	**>14 y n = 1525***	
RDT +	36 (2.0%)	32 (6.1%)	53 (3.5%)	121
PCR +	34 (1.9%)	34 (6.4%)	56 (3.7%)	124
BS +	31 (1.7%)	33 (6.3%)	52 (3.4%)	116
Geometric mean parasite density/μL (range)	13815 (50–597,500)	14172 (12–782,400)	3739 (6–184,500)	

*Data on specific age categories (5–14 y and >14 y) were missing from14 patients.
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Table 3. Rapid diagnostic test (RDT) sensitivity, specificity, positive and negative predictive value against polymerase chain reaction (PCR) and blood smear (BS) microscopy.

	PCR +	PCR –	Total	BS+	BS−	Total
RDT +	117	4	121	110	11	121
RDT –	7*	726*	733	6**	733**	739
Total	124	730	854	116	744	860
Sensitivity	76.5% (95% CI 69.0– 83.9%)			78.6% (95% CI 70.8–85.1)		
Specificity	99.9% (95% CI 99.7– 100%)			99.7% (95% CI 99.5–99.9%)		
Positive predictive value	96.7% (95% CI 91.8– 99.1%)			91.7% (95% CI 84.3–95.4%)		
Negative predictive value	99.0% (95% CI 98.0– 99.6%)			99.2% (95% CI 98.8–99.5%)		

*For calculations of sensitivity and specificity the absolute numbers in the RDT negative group were multiplied with a factor of 5.14 to account for that only a sub-sample, i.e. 733 of 3768, in this group were subjected to blood sampling on filter paper for PCR.
**For calculations of sensitivity and specificity the absolute numbers in the RDT negative group were multiplied with a factor of 5.01 to account for that only a sub-sample, i.e. 739 of 3768, in this group were subjected to blood sampling for BS microscopy.
doi:10.1371/journal.pone.0072912.t003

Among the 124 patients that were prescribed anti-malarial medicines, 119 (96.0%) received artesunate-amodiaquine, four (3.2%) quinine and the remaining one (0.8%) a combination of artesunate-amodiaquine and quinine.

Antibiotics were prescribed to a total of 2218/3889 (57.0%) patients, of whom 24 (1.1%) received prescription of more than one antibiotic. Some 1338 (60.3%) received cotrimoxazole, 573 (25.8%) ampicillin/amoxicillin, 288 (13.0%) penicillin, 33 (1.5%) erythromycin, 29 (1.3%) metronidazole and 18 (0.8%) ciprofloxacin, respectively. RDT negative patients were overall statistically significantly more likely to receive antibiotics than RDT positive [OR 3.25 (95% CI 2.15–5.01)]. However, among children <5 years of age the rate of antibiotic prescription was similar between the groups, i.e. 57% and 58% in the RDT negative and RDT positive group, respectively. Among RDT positive patients, no statistically significant difference between children<5 years and patients aged ≥5 years was observed [OR 1.36 (95% CI 0.77–2.30)]."

Antipyretics were prescribed to 1689/3889 (43.4%) patients of whom 1558 (92.2%) received paracetamol and the remaining 122 (7.2%) non-steroidal anti-inflammatory drugs including acetylsalicylic acid. There was a balanced distribution of antipyretic prescription between the RDT positive and RDT negative group. However, children <5 years were overall statistically significantly less likely to receive antipyretics than patients aged ≥5 years [OR 0.22 (95% CI 0.19–0.26)].

Table 4. Prescription of anti-malarial medicines, antibiotics and antipyretics by rapid diagnostic test (RDT) result and age group.

Prescription	Age	RDT +	RDT −	P-value
Anti-malarial medicines	<5 y	36/36 (100%)	0/1786 (0%)	<0.001
	5–14 y	32/32 (100%)	0/496* (0%)	<0.001
	>14 y	53/53 (100%)	3/1472* (0.2%)	<0.001
Antibiotics	<5 y	20/36 (55.6%)	1032/1786 (57.8%)	>0.999
	5–14 y	8/32 (25.0%)	341/496* (68.8%)	<0.001
	>14 y	6/53 (11.3%)	804/1472* (54.6%)	<0.001
Antipyretics	<5 y	14/36 (38.9%)	437/1786 (24.5%)	0.053
	5–14 y	19/32 (59.4%)	278/496* (56.0%)	0.855
	>14 y	34/53 (64.2%)	894/1472* (60.7%)	0.669

*Data on specific age categories (5–14 y and >14 y) were missing from14 patients.
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Discussion

The rapid transition from high to low malaria transmission recently observed in various areas of sub-Saharan Africa, including Zanzibar, constitutes a major challenge to ensure sustained high parasite-based *P. falciparum* diagnostic accuracy with malaria RDT among fever patients and high adherence to RDT results among health care workers. This is critical for safe and rational fever case management, including targeted ACT treatment to patients with confirmed malaria infection. The present study was therefore undertaken to assess if HRP2 based RDT remains an efficient tool for *P. falciparum* case detection among fever patients in Zanzibar, not only against BS microscopy but also against highly sensitive PCR, and if primary health care workers continue to adhere to RDT results in the new epidemiological context of low malaria transmission. Further, we scientifically evaluated, for the first time in Africa, the performance of RDT as an integrated part of the newly adopted IMCI algorithm in Zanzibar.

The observed diagnostic accuracy of HRP2 based RDT (Paracheck-Pf) for *P. falciparum* detection in this study indicates a relatively low sensitivity but a high specificity when compared with both BS microscopy and PCR. Since our previous report from Zanzibar [10], conducted when *P. falciparum* malaria was responsible for approximately 30% of fever episodes, the sensitivity of RDT against BS microscopy declined from 92% to 79%, whereas the corresponding specificity increased from 88% to 99%. The latter may reflect an overall reduced risk of repetitive malaria infections and remaining antigenemia from a previous malaria episode in the new low transmission context in Zanzibar. However, the relatively low sensitivity of Paracheck-Pf, i.e. below the reported sensitivity for *P. falciparum* detection for this RDT device in the most recent WHO/FIND evaluation [26], is a concern especially since the fact that similar observations have been documented in other low transmission areas [14]. Even lower RDT sensitivities than we observed have been reported from other studies i.e. 65% against BS microscopy in Rufiji district of Tanzania [27] and 69% against PCR in two Tanzanian hospitals [8].

The relatively low sensitivity may be at least partly due to sub-optimal RDT performance of individual health care workers, considering that approximately half of all RDT negative but BS positive cases were reported from the same health facility. This calls for improved systems for RDT supervision and quality control in primary health care facilities, but also for more sensitive point-of-care malaria diagnostic tools. Other factors, unrelated to health care workers performance, such as presence of *P. falciparum* HRP2 deletions and sequence variation as well as the so called "prozone effect", need to be considered as a potential cause of the relatively low RDT sensitivity, particularly since the HRP2 based RDT failed to detect four cases of relatively high *P. falciparum* density, i.e. 3573, 14420, 25779 and 50190 parasites/μL, respectively [24,28,29]. Importantly, we did not detect any case of *P. falciparum* HRP2 deletions in these samples, which is in contrast to observation from Mali in West Africa [30]. However, HRP2 sequence variation was not analysed, since previous studies have not been able to link RDT sensitivity with sequence variation in clinical malaria infections with parasite densities ≥200 parasites/μL. Whether a prozone effect, defined as a false-negative result in an immunological reaction due to an excess of either antigens or antibodies, occurred in any of our samples could not be assessed retrospectively since blood was not available for serial dilution. Of note is though that the prozone phenomenon has previously been described primarily in patients with *P. falciparum* hyperparasitemia (≥250,000 parasites/μL) [31].

This study confirms our previous observations from Zanzibar of an excellent adherence to RDT results among primary health workers [10]. It is indeed reassuring that health care workers in Zanzibar continue to rely on RDT results in a context where overall 97 out of 100 tests are negative and scarce tests facilities for diagnosing alternative causes of fever causes are available. With our results in mind and considering the contradictory reports of adherence to RDT results from various levels of health care and epidemiological settings in sub-Saharan Africa [5,6,8,32], we have conducted a qualitative pilot study to explore context specific determinants of RDT perception among health care workers in Zanzibar in order to improve the understanding of factors influencing adherence to RDTs [33]. However, it should be acknowledged that the health care workers enrolled in our study all received a relatively extensive pre-study training. Moreover, they are all working in an area where other malaria research activities have been conducted in recent years. These factors may have contributed to the high adherence to RDT results, which may not necessarily be representative for the general primary health care worker in Zanzibar.

The incorporation of malaria RDT in IMCI provides an opportunity for improved childhood fever case management in Africa. This study is, to our knowledge, the first scientific evidence from sub-Saharan Africa that RDT can be reliably integrated in IMCI. There was neither any difference in RDT performance nor in adherence to RDT test results in children <5 years of age compared with other age groups. The ability of HRP2 based RDT to reliably rule out malaria infection in a low transmission setting is critical. However, to ensure a sustainable adherence to particularly RDT negative results, health care workers need to be trained and equipped with tools for improved management of non-malarial fevers. A prerequisite for such improved non-malarial fever management is to study the etiology of acute uncomplicated fevers in rural settings of Zanzibar. Such studies, which are ongoing, will in addition provide data on antibiotic requiring bacterial infections among fever patients in Zanzibar. This is critical, since the observed antibiotic prescription rate of 55–65% among RDT negative patients in all age groups as well as RDT positive children <5 years of age (in the form of dual treatment with ACT) probably represents a substantial overuse, which may fuel development and spread of antibiotic resistance.

Our observation of low *P. falciparum* parasite prevalence among fever patients in Zanzibar is coherent with official programmatic data [12]. Interestingly, the highest RDT positivity rate was found in children aged 5–14 years, not in children <5 years. Furthermore, there was an apparent uneven spatial distribution of RDT positivity rate both within and between the study districts. These changing malaria epidemiological patterns, together with the association between the risk of RDT positivity and absence of ITN/LLIN use as well as travel history, need to be considered in future malaria control activities in Zanzibar.

In summary, the sensitivity of HRP-2 based RDT in the hands of primary health care workers compared with both PCR and microscopy for *P. falciparum* case detection was relatively low, whereas adherence to test results with anti-malarial treatment was excellent. Moreover, the results provide evidence that RDT can be reliably integrated in IMCI as a tool for improved childhood fever management. However, the overall relatively low RDT sensitivity highlights the need for improved quality control of RDT use in primary health care facilities, but also for more sensitive point-of-care malaria diagnostic tools in the new epidemiological context of low malaria transmission in Zanzibar.

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Author Contributions

Conceived and designed the experiments: DS KE MIM BG KAB ASA AB AM. Analyzed the data: DS KE BAS UM MP AB AM. Wrote the paper: DS KE BAS UM MP AB AM. ICMJE criteria for authorship read and met: DS KE BAS MIM UM RO XW MP BG KAB ASA AB AM. Agreed with the manuscript's results and conclusions: DS KE BAS MIM UM RO XW MP BG KAB ASA AB AM. Collected data/did experiments for the study: DS KE RO MIM BAS UM WX. Supervised the study: DS KE AM. Wrote the first draft of the paper: DS KE BAS AM. Contributed to the writing of the paper: DS KE BAS UM MP AB AM.

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Usefulness of *Plasmodium falciparum*-specific rapid diagnostic tests for assessment of parasite clearance and detection of recurrent infections after artemisinin-based combination therapy

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Abstract

Background: Rapid diagnostic test (RDT) is an important tool for parasite-based malaria diagnosis. High specificity of RDTs to distinguish an active *Plasmodium falciparum* infection from residual antigens from a previous infection is crucial in endemic areas where residents are repeatedly exposed to malaria. The efficiency of two RDTs based on histidine-rich protein 2 (HRP2) and lactate dehydrogenase (LDH) antigens were studied and compared with two microscopy techniques (Giemsa and acridine orange-stained blood smears) and real-time polymerase chain reaction (PCR) for assessment of initial clearance and detection of recurrent *P. falciparum* infections after artemisinin-based combination therapy (ACT) in a moderately high endemic area of rural Tanzania.

Methods: In this exploratory study 53 children < five years with uncomplicated *P. falciparum* malaria infection were followed up on nine occasions, i.e., day 1, 2, 3, 7, 14, 21, 28, 35 and 42, after initiation of artemether-lumefantrine treatment. At each visit capillary blood samples was collected for the HRP2 and LDH-based RDTs, Giemsa and acridine orange-stained blood smears for microscopy and real-time PCR. Assessment of clearance times and detection of recurrent *P. falciparum* infections were done for all diagnostic methods.

Results: The median clearance times were 28 (range seven to >42) and seven (two to 14) days for HRP2 and LDH-based RDTs, two (one to seven) and two (one to 14) days for Giemsa and acridine orange-stained blood smear and two (one to 28) days for real-time PCR. RDT specificity against Giemsa-stained blood smear microscopy was 21% for HRP2 on day 14, reaching 87% on day 42, and ≥96% from day 14 to 42 for LDH. There was no significant correlation between parasite density at enrolment and duration of HRP2 positivity ($r = 0.13$, $p = 0.34$). Recurrent malaria infections occurred in ten (19%) children. The HRP2 and LDH-based RDTs did not detect eight and two of the recurrent infections, respectively.

Conclusion: The LDH-based RDT was superior to HRP2-based for monitoring of treatment outcome and detection of recurrent infections after ACT in this moderately high transmission setting. The results may have implications for the choice of RDT devices in similar transmission settings for improved malaria case management.

Trial registration: Clinicaltrials.gov, NCT01843764

Keywords: Malaria, RDT, LDH, HRP2, Clearance, Recurrent parasitaemia, Treatment follow-up

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Background

Parasite-based *Plasmodium falciparum* diagnosis is generally recommended by the World Health Organization (WHO) to target artemisinin-based combination therapy (ACT) to patients with confirmed malaria infections [1]. This is important in order to prevent overuse of ACT, reduce costs, minimize development and spread of anti-malarial drug resistance and to improve management of other causes of fever [2,3]. The availability of malaria rapid diagnostic tests (RDTs) constitutes an opportunity for parasite-based malaria diagnosis in rural African settings beyond the reach of microscopy services [4].

Malaria RDTs are based on detection of parasite antigens. The main antigen targeted is histidine-rich protein 2 (HRP2), which has proven to be a highly sensitive and stable marker for identification of *P. falciparum* infection. However, a concern with HRP2-based RDTs is the presence of residual antigenaemia resulting in persistent positive test results during several weeks after a successful treatment [5,6]. This is of particular concern in moderate/high transmission areas where false positive RDTs may frequently result in provision of anti-malarial treatment to patients who are not malaria infected. This phenomenon may also impair health workers trust in and adherence to RDT results [7,8].

Another antigen used in RDTs is parasite-specific lactate dehydrogenase (LDH), either species specific, i.e., *P. falciparum* LDH (*Pf*LDH) and *Plasmodium vivax* (*Pv*LDH), or pan-*Plasmodium* (pLDH), detecting all five human malaria species [9]. LDH-based RDTs have generally been shown to be less heat stable and sensitive than HRP2-based RDTs for detection of *P. falciparum*, but they are more specific since LDH is rapidly cleared from the blood following a successful anti-malarial treatment. Consequently, LDH-based RDTs do not remain positive after parasite clearance [2,10]. Interestingly, some recently available *Pf*LDH-based RDTs have shown sensitivities and heat stabilities similar to HRP2-based RDTs [11-14].

Previous RDT studies in this particular field have mainly focused on post-treatment clearance and have primarily followed patients until the tests have become negative [12,13]. Thus, insufficient data are available on the efficiency of RDTs to identify recurrent infections after recently cleared infections in moderate/high transmission areas. This may be of particular importance in an era of increasing drug resistant malaria [15]. The aim of this study was therefore to investigate clearance and detection of recurrent *P. falciparum* infections of HRP2 and LDH-based RDTs during 42 days after initiation of artemether-lumefantrine treatment in children with uncomplicated malaria in a moderately high endemic area of rural Tanzania. Furthermore, since no previous study has included clearance by polymerase chain reaction (PCR) as a comparator, this was done to allow a more comprehensive evaluation of RDT for monitoring of anti-malarial treatment outcome. The entire assessment herein reported thus included two RDTs (HRP2 and LDH-based) for antigen detection, compared with two microscopy techniques (Giemsa-stained thick blood smears and acridine orange-stained thin blood smears) for whole parasite detection and real-time PCR for detection of parasite DNA.

Methods

Study site and population

This health facility-based study was conducted during the peak seasons for malaria transmission, in June to September 2009 and July to October 2010 at Mlandizi health centre, Kibaha district and during March to May 2011 in Fukayosi dispensary, Bagamoyo district, both located in Coast Region, Tanzania. Artemether-lumefantrine was introduced as first-line treatment for uncomplicated malaria in 2006 in the study area, whereas RDT had not yet been implemented for parasite-based malaria diagnosis. At the time of the trial malaria transmission was considered to be moderately high in both study sites.

The Mlandizi health centre provides basic in- and outpatient care for a population of approximately 33,000. Laboratory services, including malaria microscopy, are available during office hours. The monthly blood smear positivity rate among febrile children < five years of age was 33% (range 20-48%) and 15% (range 11-21%) during the sampling period in 2009 and 2010, respectively. There was a period of artemether-lumefantrine stock-out in Mlandizi in 2009. During this period patient recruitment was stopped. There was an on-going insecticide-treated bed net campaign in the area markedly reducing the incidence of malaria among children < five years between 2009 and 2010.

Fukayosi dispensary provides basic outpatient care for a population of about 7,000. Malaria microscopy service is available seven days a week. Malaria blood smear positivity rate was 19% (74/384) among febrile children < five years of age during the conduct of the trial.

Participating laboratory staff and study nurses at the two study sites received one day's training in performance and interpretation of both RDTs before the start of the study.

Study design and sample collection

Children between six and 59 months presenting at the study sites with fever, i.e., measured axillary temperature of ≥37.5°C or a history of fever during the preceding 24 hours, and a positive screening blood slide for *P. falciparum* mono-infection with a parasite density of 2,000-250,000/μL, and willing/able to comply with the

42 days follow-up were eligible to participate in the study. Children with a history of anti-malarial drug intake within two weeks or symptoms/signs of severe disease were excluded. Written informed consent was obtained from a parent/guardian of all enrolled children.

At enrolment, i.e., day 0, a complementary finger-prick capillary blood sample was taken for two thick and thin smears, and two RDTs. In addition, approximately 50 μL of blood was spotted on a filter paper. All enrolled children were treated with artemether-lumefantrine (Coartem®) in standard doses based on body weight, according to national treatment guidelines [16]. Only the initial drug dose was given under supervision. Enrolled children were requested to return for clinical review and blood sampling on days 1, 2, 3, 7, 14, 21, 28, 35 and 42, or anytime if condition deteriorated or fever re-occurred. At each follow-up visit all day 0 blood tests were repeated, except for the Giemsa-stained thin smear, which was used for confirmation of *P. falciparum* mono-infection solely on day 0. A case record form was completed at enrolment by a clinical officer, with clinical and demographic information, including age, sex and information on use of insecticide-treated bed nets. Body temperature, symptoms and prescription of drugs were recorded in the case record forms on all visits.

Whenever fever and/or any other symptoms/signs of disease re-occurred during follow-up, a Giemsa-stained blood slide was to be read directly at the health centre and if positive for *P. falciparum* the child was retreated with artemether-lumefantrine.

The only incentive given to the study participants was bus fares to cover travel costs during follow up visits.

Laboratory procedures

Rapid diagnostic tests

Two RDTs, ParaHIT *f* (Span Diagnostics Ltd, Surat, India), detecting *P. falciparum*-specific HRP2 antigen (hereafter referred to as HRP2) and CareStart™ Malaria (G0151), (Access Bio, Inc, NJ, USA), detecting *P. falciparum*-specific LDH antigen (hereafter referred to as LDH), were performed and interpreted on site according to the manufacturer's instructions. ParaHit was, at the time of the study, approved by the Tanzanian National Malaria Control Programme and was the most deployed RDT. The single *Pf* CareStart test was chosen based on the heat stability and performance of the CareStart pan-LDH test in the WHO product testing 2009, where it was among the best performing LDH-based tests for *P. falciparum* detection [17]. Both RDTs are two band tests, i.e., one test band specific for *P. falciparum* and one control band.

A laboratory technician or study nurse, blinded to any Giemsa-stained blood smear result, performed, interpreted and recorded the RDT results. Very faint bands at the test line position were to be defined as positive. Band intensity was not recorded. In case the control line did not appear, the result was considered invalid and the test was repeated. The RDT kits were stored at <30°C prior to use, the temperature being recorded daily.

Giemsa-stained blood smear microscopy

One of the two thin blood smears collected on day 0 and the thick blood smears from all sampling points were stained with 5% Giemsa for 20 minutes at the health facilities, after which they were transported once weekly to Muhimbili University of Health and Allied Sciences (MUHAS) in Dar es Salaam. The day 0 Giemsa-stained thin smear was examined for confirmation of *P. falciparum* mono-infection. One of the two thick smears was examined by two independent, experienced microscopists, who were unaware of the RDT results, at MUHAS. A total of 200 microscopic fields (×100 magnification) were examined before a smear was considered negative. Asexual parasite densities were calculated by counting parasites against 200 white blood cells (WBC), assuming 8,000 WBC/μL of blood. If less than 10 parasites were detected per 200 WBC, estimates were made against 500 WBC [18]. Gametocytaemia was assessed by reading 200 microscopic fields. All blood slides with discrepant results, defined as >50% difference in parasite density or a positive *versus* negative result between the readers, were subjected to a third blinded reading at Karolinska Institutet (KI), Sweden. In addition, all blood slides from children showing a negative thick blood smear and a positive PCR at the same time point, as well as a random sample of 10% of all blood slides, plus the blood slides from the time point of each participant with the last CareStart positive, and the first CareStart negative results, were subjected to microscopy reading at KI for quality control. The mean of the two most concordant counts were used to calculate the final parasite density [18]. In case of discrepancy regarding positive *versus* negative results between the first two readers and the third, the third reading at KI was defined as decisive.

Acridine orange blood slide microscopy

The thin blood smears from all sampling points were subjected to acridine orange staining and reading at Muhimbili University Hospital. The thin smears were fixed in methanol. A solution of 0.01% acridine orange in phosphate buffer (pH 7.2) with 5% glycerine was then applied to the smears, which were read in a fluorescence microscope at ×40 magnification [19]. The results were recorded as either positive or negative, i.e., parasite counts were not assessed. The microscopists were unaware of all previous RDT and microscopy results.

DNA detection by PCR

Approximately 50 μL of blood seeded on a filter paper (Whatman 3MM®) from all sampling points were collected for molecular analysis. The filter papers were dried and put in individual plastic bags and transported to KI. The filter papers were stored at <30°C until processed. Three 3-mm punches (approximately 10–15 μL) from each filter paper sample were extracted with a modified version of the ABI 6100 Nucleic Acid Prep Station protocol (Applied Biosystems, USA) as previously described [20]. DNA was eluted in 200 μL of buffer. All samples were analysed for presence of *Plasmodium* DNA by an 18S rDNA probe based real-time PCR assay [21]. A cut-off value for positivity was set at a cycle threshold (Ct) of <40. All samples were run in triplicates. Samples where one out of three had a Ct-value <40 were repeated in triplicates with the same real-time PCR. Samples with repeated single Ct-values <40 or a Ct average of >38.5 were subjected to a confirmatory/decisive *P. falciparum*-specific nested PCR [22]. Two positive *P. falciparum* controls (5, 10 or 50 parasites/μL) as well as negative controls were included in each 96-well PCR plate.

PCR genotyping to distinguish re-infections from recrudescence

Filter-paper blood spots from patients with recurrent PCR positivity during follow-up were re-extracted using the Chelex-100 method [23]. Stepwise genotyping with three highly polymorphic genetic markers, i.e., the *merozoite surface protein* (*msp*) 1, *msp* 2 and *glutamate rich protein* (*glurp*) was performed according to standard protocols to differentiate re-infection (new infection) from recrudescence (treatment failure). For each marker, recrudescence was defined as the presence of at least one matching allelic band and re-infections were defined as the absence of any matching allelic band in samples at enrolment (day 0) and at day of recurrent infection [24,25].

Definition of clearance time and recurrent infections

Clearance time was defined as the first sampling day after initiation of treatment when a test result was negative. There were two exceptions to this. First, when a RDT result turned negative for one sampling day, followed by a positive RDT result again the following sampling day, the negative result was ignored if the PCR and/or BS results did not indicate presence of a recurrent infection. Second, when one negative PCR result was followed by a positive PCR result the following sampling day up to day 7, the negative result was ignored. From day 14 and onwards the clearance time was calculated from the first day with negative result.

Recurrent infection was defined as detection of *P. falciparum* DNA (PCR) confirmed by a positive Giemsa-stained blood smear microscopy and/or LDH during follow-up after the initial infection had been cleared.

Study outcomes and statistical analysis

The primary outcomes were clearance time and detection of recurrent infection with the five diagnostic tests. Secondary outcomes included specificity of the two RDTs against Giemsa-stained blood smear microscopy (gold standard), identification of PCR-adjusted re-infection/recrudescence among the recurrent infections and correlation between parasite density at enrolment and persistence of HRP2. In the calculation of correlation between day 0 parasite densities and duration of HRP2 positivity, seven children were included who had cleared HRP2 positivity before they were lost to follow-up. These seven children were not included in any other analysis.

The study was considered exploratory, which precludes a power calculation. A sample of ≥50 children was predefined.

Data were entered in Microsoft Excel® and analysed using STATA 12® software. Categorical variables were compared using Fisher's exact test. Pearson linear correlations were calculated in SPSS. Sensitivity and specificity of acridine orange against Giemsa-stained blood smear microscopy (gold standard) and both microscopic methods against PCR (gold standard) was calculated. Statistical significance was stated at the 5% level and 95% confidence intervals (CI) are presented.

Ethical considerations

The study was conducted in accordance with the Declaration of Helsinki and Good Clinical and Laboratory Practices. It was approved by the Directorate of Research and Publications, MUHAS (Ref.No.MU//RP/AEC/Vol.XIII/142) and the Regional Ethics Committee, Stockholm, Sweden.

The study is registered at www.clinicaltrials.gov with study identifier NCT01843764.

Results

Study subjects

The flow of patients through the trial is outlined in Figure 1. Two children were excluded from the analysis due to high parasite densities at enrolment, i.e., 992,000 and 494,400/μL. Another 16 children were not able to fulfil the stipulated follow-up, the most common reason being leaving the study area or long distance to the health centre.

All children were positive with both RDTs at day of inclusion. Baseline characteristics of the 53 children included in the analysis are presented in Table 1.

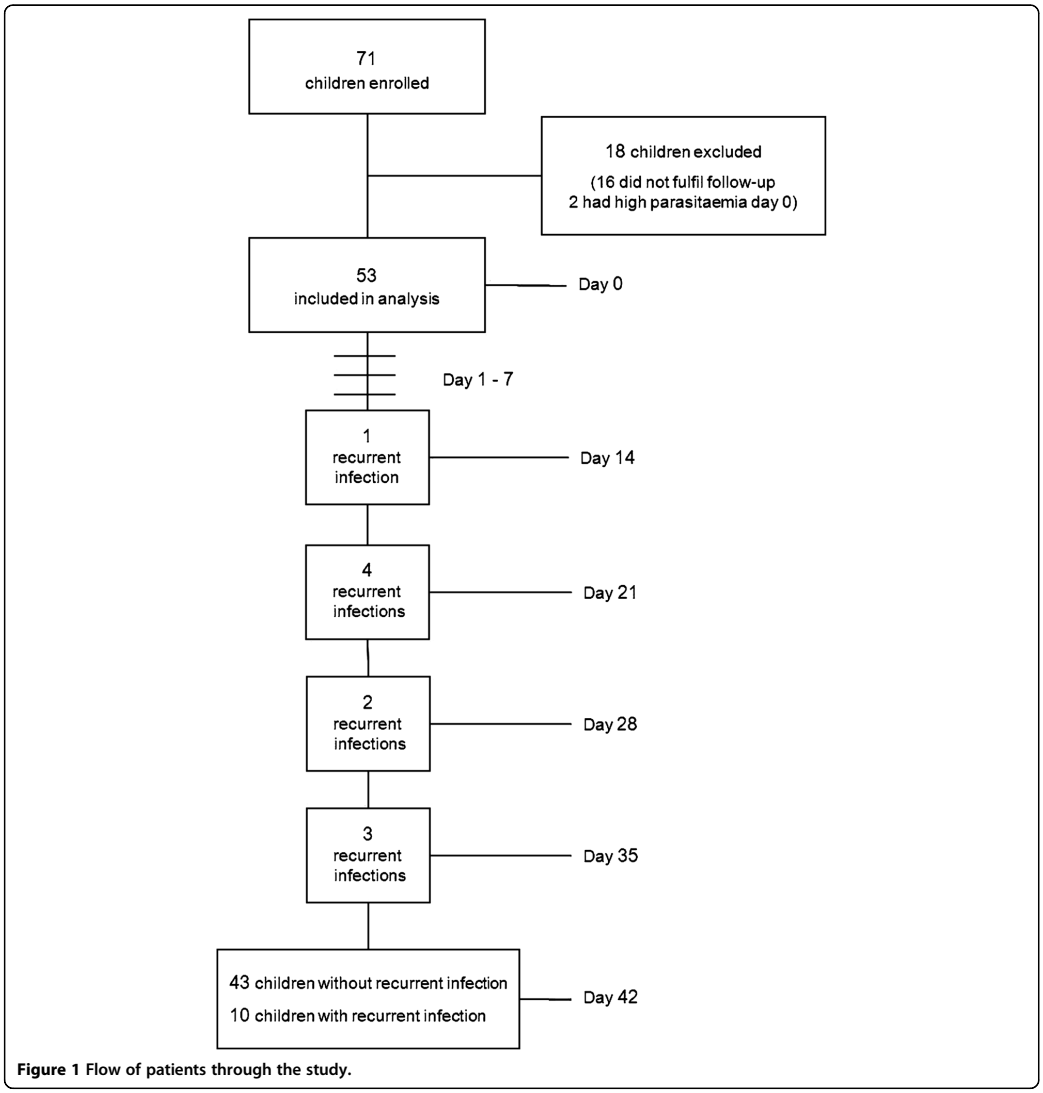

Figure 1 Flow of patients through the study.

Clearance time

The calculations for HRP2 clearance are based on the 43 children without recurrent infection during follow-up, since eight of the ten children with parasite recurrence remained HRP2-positive from enrolment up to the time of recurrent infection. All other tests, i.e., LDH, Giemsa and acridine orange-stained blood smears as well as PCR cleared before parasite recurrence. Consequently, clearance calculations for these tests are based on data from all 53 children. The median clearance times for the five diagnostic tests are presented in Figure 2 and their respective positivity rates at each sampling point are shown in Figure 3.

The geometric mean clearance time for HRP2 was 26.4 (95% CI 23.0-30.3) days. One patient cleared HRP2 by day 7 and three remained positive up to day 42 (last day of follow-up). The false positivity rates for HRP2 against PCR on days 14, 21, 28, 35 and 42 were 80% (32/40), 64% (27/42), 43% (18/42), 24% (10/42) and 7% (3/41), respectively. All PCR positive results were also

Table 1 Baseline characteristics of the 53 children included in the analysis

Age in months, mean	42 (range 10–59)
Sex male/female	35/18
Axillary temperature °C, mean	37.7 (range 37.0-39.0)
Axillary temperature ≥37.5°C	83% (44/53)
Duration of fever, mean days	2.5 (range 1–4)
Other complaints*	64% (34/53)
Geometric mean parasite density /μL	37,640 (range 2,000-250,000)
Sleeping under insecticide-treated bed net	85% (45/53)

*vomiting n = 18.
abdominal pain n = 9.
cough n = 11.
diarrhoea n = 3.

HRP2-positive throughout the study. There was no significant correlation between parasite density at enrolment and duration of HRP2 positivity (r = 0.13, p = 0.38) (Figure 4). For LDH the mean clearance time was 5.5 days (95% CI 4.5-6.7). No significant correlation between parasite density at enrolment and duration of LDH positivity was observed (r = 0.21, p = 0.11).

The mean clearance time for Giemsa-stained blood smears was 2.0 days (95% CI 1.8-2.3). Four children (8%) remained positive by microscopy until day 3. Two children were microscopy positive day 2 and 3, respectively, with parasite densities of 16 and 32/μL each. However, PCR was negative for both samples at these time points. There was no significant association between clearance times of Giemsa-stained blood smears and HRP2 (p = 0.50).

Acridine orange-stained blood smears had a mean clearance time of 2.1 days (95% CI 1.9-2.3). The mean clearance time for PCR was 2.9 days (95% CI 2.3-3.6). Persistent PCR positivity up to day 7, 14 and 21 was observed in one, one and two children, respectively. In two of these four children, solely gametocytes were detected by Giemsa-stained blood smear microscopy at two and three sampling points, respectively, during the time of persistent PCR positivity.

Recurrent infections

Ten children had recurrent *P. falciparum* infection during the 42 day follow-up as assessed by PCR, Giemsa-stained blood smear and/or LDH. One recurrent infection was defined as recrudescence, six as re-infections, whereas three were undetermined by PCR genotyping (Table 2). Eight of these ten children had remaining HRP2 positivity from the initial infection. Thus, only two recurrent infections, both occurred on day 35, were detected by HRP2. Conversely, LDH detected eight (80%) recurrent infections at the day of parasite recurrence. Furthermore, one additional child had a positive LDH result on the following visit. Giemsa-stained blood smear microscopy identified eight of the ten recurrent infections, whereas acridine orange detected one. PCR identified all ten infections at the time of parasite recurrence. However, six more patients experienced a single occasion of PCR positivity during follow-up between days 21 and 42. All these six children were asymptomatic by the time of the transient PCR positivity. Except for two patients who were still HRP2-positive, all other tests were negative at the time of PCR positivity. Thus, none of the six children fulfilled the definition of recurrent infection. Cumulative positivity by

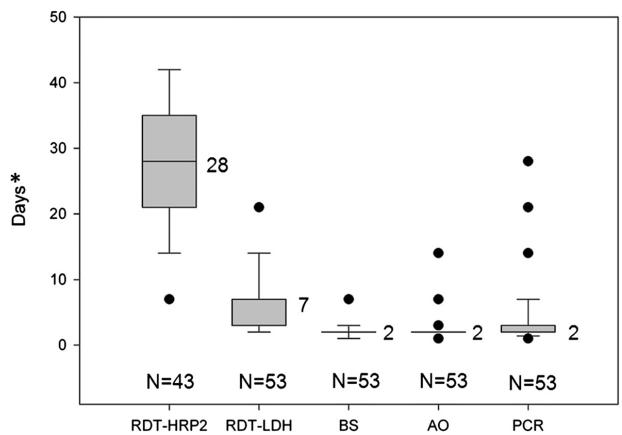

Figure 2 Median clearance times for the five diagnostic tests. N = number of patients included in the analysis. BS = Giemsa-stained blood smear. AO = acridine orange-stained blood smear. * Last day of follow-up = day 42.

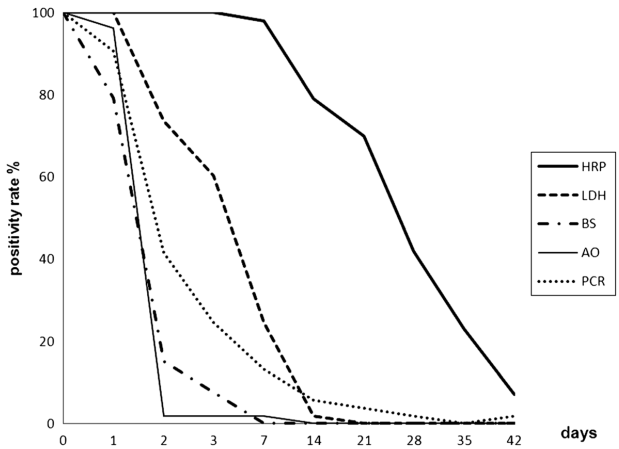

Figure 3 Positivity rates at each sampling point by the five diagnostic tests. HRP2 data represent 43 patients and the remaining diagnostic tests 53 patients. HRP = HRP2 based RDT. LDH = LDH based RDT. BS = Giemsa-stained blood smear. AO = acridine orange-stained blood smear. PCR = real-time PCR.

the respective diagnostic tests for the ten recurrent infections detected during follow-up is shown in Figure 5.

Five children had fever at the time of detection of recurrent infection (Table 2). Four of them had their Giemsa-stained blood smear read on site and were immediately diagnosed and retreated with artemether-lumefantrine. The fifth child did not have the blood smear read on site due to symptoms of respiratory tract infection. This child received antibiotics and improved clinically.

Sensitivity and specificity

The specificities of HRP2 and LDH against Giemsa-stained blood smear microscopy between days 3 and 42 are shown in Table 3. Compared with PCR the overall (days 0–42) sensitivities and specificities were 68% (95% CI 61–75) and 99% (CI % 98–100) for Giemsa, and 61% (95% CI 53–68) and 98% (95% CI 96–99) for acridine orange-stained blood smear microscopy, respectively. The sensitivity and specificity of acridine orange against

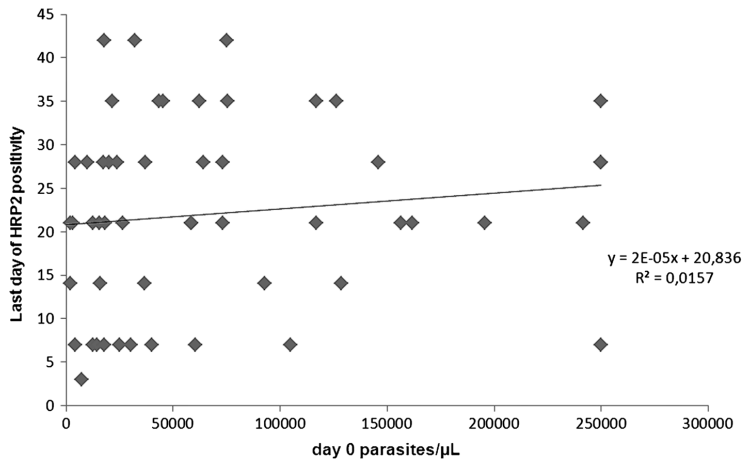

Figure 4 Correlation between parasite density at enrolment and duration of HRP2 positivity. N = 52, including 43 children without re-infection, two with cleared positivity before re-infection and seven with cleared positivity before being lost to follow-up. Correlation coefficient (r) = 0.13 (p = 0.38).

Table 2 Characterization of the ten recurrent *Plasmodium falciparum* infections detected during follow-up

	Diagnosis day	Symptom	Diagnostic tool			Parasites /μL	New infection/ Recrudescence[2]
			PCR	Giemsa	LDH-RDT		
1	14	A	+	+		80	New
2	21	A	+	+	+	1,600	New
3	21	R	+		+		undertermined
4	21	[1]	+	+	[1]	600	recrudescence
5	21	F	+		+		undertermined
6	28	A	+	+	+	316,000	new
7	28	F	+	+	+	229,600	new
8	35	F	+	+	+	61,240	undertermined
9	35	F	+	+	+	9,800	new
10	35	F	+	+	+	33,920	new

A = asymptomatic F = fever R = respiratory tract infection.
[1]turned positive with fever following visit.
[2]PCR corrected outcome.

Giemsa-stained blood smear microscopy were 81% (95 CI 73–88) and 97% (95% CI 95–98).

Discussion

The efficiency of two *P. falciparum*-specific RDTs, i.e., HRP2 and LDH were studied for assessment of clearance and detection of recurrent infections during 42 days after initiation of artemether-lumefantrine treatment. This was done through a comparison with two microscopy techniques and real-time PCR. HRP2 had a significantly longer median clearance time (28 days) compared with LDH (seven days). Due to persistent HRP2 positivity from the initial infection, only two out of the ten children with recurrent *P. falciparum* infections during follow-up were identified by HRP2, whereas LDH was able to recognize eight at the time of parasite recurrence. Acridine orange blood smear microscopy did not provide any additional information compared with the other tests used in this study.

Clearance

Long clearance times for HRP2-based RDTs after ACT treatment have previously been shown [6,26,27], with remaining HRP2 positivity by day 35 in up to 73% of

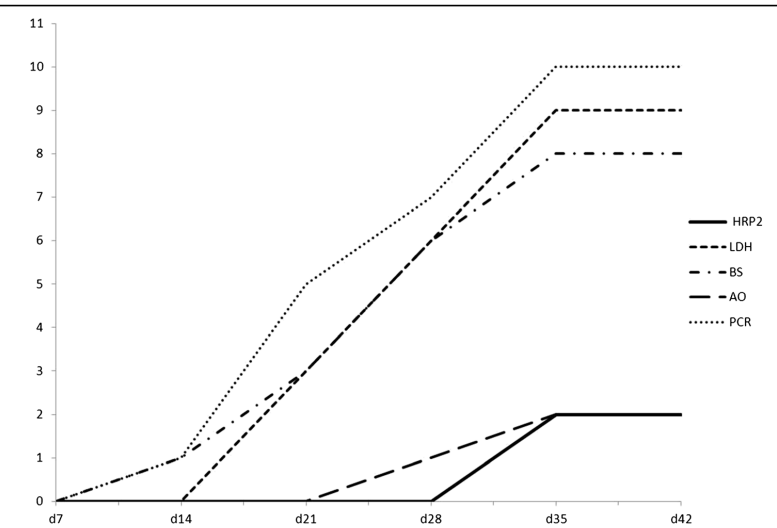

Figure 5 Cumulative positivity by the diagnostic tests for the ten recurrent *Plasmodium falciparum* infections detected during follow-up. BS = Giemsa-stained blood smear. AO = acridine orange-stained blood smear.

Table 3 Specificities of the *Plasmodium falciparum* HRP2 and LDH-based rapid diagnostic tests against Giemsa-stained blood slide microscopy (gold standard) between days 3 and 42

	HRP2			LDH			
Day		%	CI 95%		%	CI 95%	p-value
3		NA		20/49	41	27-56	
7		NA		40/53	76	62-86	
14	11/52	21	11-35	51/52	98	90-100	<0.0001
21	17/50	34	21-49	48/50	96	86-100	<0.0001
28	29/49	59	44-73	49/49	100	93-100	<0.0001
35	35/50	70	55-82	48/50	96	86-100	<0.005
42	45/52	87	74-94	50/52	96	87-100	>0.05

NA = not applicable.

patients despite efficacious treatment. Initial parasite density has been suggested to influence the duration of HRP2 positivity, but a patient's immune status may also be a contributing factor [28].

Similarly with the present findings, a relatively rapid clearance of LDH has previously been documented [10,12]. It has been claimed that especially HRP2 but also LDH are released by immature gametocytes, which may result in persistent positive test results [5,29]. However, this opinion has been challenged by others who argue that the number of gametocytes after ACT treatment are too few to cause persistent positivity [30]. Importantly, in our this study, only four solely gametocyte carriers were detected by Giemsa-stained blood smear microscopy, of whom three and none were HRP2 and LDH positive, respectively, during gametocyte carriage.

Expert blood smear microscopy remains gold standard for estimation of parasite clearance in clinical trials of anti-malarial drugs. Blood smear positivity by day 3, i.e., 72 hours after initiation of ACT treatment, was observed in 4/53 (8%) of the patients. This is a relatively uncommon finding among African children treated with ACT for uncomplicated malaria [27,31]. The prolonged Giemsa-stained blood smear positivity may be explained by poor compliance since only the initial artemether-lumefantrine dose, i.e., one out of six doses, was given under supervision. Similarly, the relatively long mean PCR clearance time observed may possibly be explained by poor compliance. The presence of gametocyte carriage in patients with prolonged PCR positivity during follow-up may also have influenced the PCR clearance time.

Parasite density day 0 and persistent HRP2

No correlation was observed between parasite density at enrolment and duration of HRP2 positivity. This is in contrast to several previous publications that report a strong positive correlation [5,6,26,27]. However, these studies have generally included a wider range of parasite densities down to <1,000/μL, where clustering has shown a shorter HRP2 clearance time in parasitaemias <10,000/μL. Conversely, Choidini *et al.* identified a wide range of HRP2 concentrations at equal parasite densities both in panels of cultures and of field isolates [32]. Varying HRP2 concentrations may be dependent on factors such as the duration of infection, the total parasite biomass including the sequestered parasites [33] and anti-HRP2 immune response.

Detection of recurrent infections

In the moderately high transmission area where the study was conducted, ten (19%) children had recurrent infection detected between days 14 and 35 during follow-up. All recurrent infections were detected by PCR, whereas both the LDH-based RDT and Giemsa-stained blood smear detected eight, HRP2 identified two and acridine orange smear microscopy identified only one of these infections at the time of parasite recurrence. Few other studies have looked at the efficiency of RDTs for detection of recurrent infections during follow-up after anti-malarial treatment. Maxay *et al.* found in a study performed in Thailand that 40/92 (43%) patients experienced recurrent infection during a 28-day follow-up [5]. All these patients had persistent HRP2 positivity from enrolment up to day of recurrent infection.

RDTs for assessment of treatment outcome

The usefulness of RDTs for monitoring of anti-malarial treatment, i.e., clearance time as well as identification of treatment failure/re-infection, is highly dependent on test specificity. HRP2-based RDTs appear not to be a sufficient tool for this. LDH, with a limited but still longer clearance time as compared with blood smear microscopy, may be useful but for detection of prolonged parasite clearance as a sign of emerging artemisinin tolerance/resistance, it is probably not sufficient.

RDTs for case detection

Sensitivity and specificity of the HRP2 and LDH antigens for case detection are highly dependent on malaria endemicity [27,28]. In high endemic areas HRP2 generally shows a low specificity, especially among febrile children, because a large proportion of residents have remaining antigenaemia from previous infections. The generally higher parasite densities observed in high endemic areas may also cause longer HRP2 clearance times affecting the specificity. However, Abeku *et al.* showed that in an area of high transmission the specificity increased towards the end of the rainy seasons, and in older age groups probably due to increased HRP2 antibody levels in the population. In low endemic areas the specificity of HRP2 among febrile patients generally

increases due to the low malaria incidence and thus the low risk of detecting remaining antigenaemia after cleared infections. LDH, on the other hand, which has shown a comparatively lower sensitivity for detection of *P. falciparum* infections with densities <200/μL, may in areas of low transmission where parasite densities generally are lower especially among asymptomatic cases [34], be a less efficient diagnostic tool than HRP2.

Other limitations with HRP2-based RDTs are the genetic diversity, including the deletions recently described among African *P. falciparum* isolates [35,36], as well as false HRP2 negative results due to the prozone effect [37]. To date, no genetic LDH diversity/deletion has been described and LDH-based RDTs are not susceptible to any prozone effect [38,39]. All these factors should be considered for the choice of RDT in a specific epidemiological setting.

What does the present study add to the knowledge base of RDTs?

Previous RDT studies in this particular field have mainly followed up the study participants until the RDTs have become negative [12,13]. Conversely, all five diagnostic tests were assessed up to day 42 after treatment initiation in this report. This study design provides a more comprehensive assessment both of clearance times and of recurrent malaria infections for all diagnostic methods, and importantly assesses the efficiency of the two RDTs to detect recurrent malaria infections, which has previously not been reported. Eight of the ten recurrent infections in this study were recognized by LDH at the day of infection and another one on the following sampling day, whereas HRP2 detected only two of the recurrent infections. These results, in combination with the significantly shorter median clearance time of LDH compared with HRP2, provide evidence for the former to be a better tool for monitoring of anti-malarial treatment outcome among children with symptomatic infection in this moderately high endemic area. In addition, this study is the first to incorporate PCR as a comparator for assessment of clearance.

Limitations

A general limitation when assessing performance of parasite based diagnostic tests that indeed detect different things such as antigens (RDT), DNA (PCR) or whole parasites (microscopy) is that the results are not fully comparable. Furthermore, this is a small study with a limited number of patients with recurrent infections detected during follow-up.

Conclusion

The LDH-based RDT was superior to HRP2-based for monitoring of treatment outcome and detection of recurrent infections after ACT in this moderately high transmission setting. The results may have implications for the choice of RDT devices in similar transmission settings for improved malaria case management.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

BAS, MM, AM, AB, and ZP conceived and designed the study. MM, BAS, ZP, and BN carried out and supervised the field work. BAS and UM performed the laboratory analyses. BAS, UM, MM, AB, and AM analysed the data and drafted the manuscript. MP gave important intellectual input in the statistical analysis. All authors read and approved the final manuscript.

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Rapid diagnostic tests for molecular surveillance of *Plasmodium falciparum* malaria -assessment of DNA extraction methods and field applicability

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Abstract

Background: The need for new malaria surveillance tools and strategies is critical, given improved global malaria control and regional elimination efforts. High quality *Plasmodium falciparum* DNA can reliably be extracted from malaria rapid diagnostic tests (RDTs). Together with highly sensitive molecular assays, wide scale collection of used RDTs may serve as a modern tool for improved malaria case detection and drug resistance surveillance. However, comparative studies of DNA extraction efficiency from RDTs and the field applicability are lacking. The aim of this study was to compare and evaluate different methods of DNA extraction from RDTs and to test the field applicability for the purpose of molecular epidemiological investigations.

Methods: DNA was extracted from two RDT devices (Paracheck-Pf® and SD Bioline Malaria Pf/Pan®), seeded *in vitro* with 10-fold dilutions of cultured 3D7 *P. falciparum* parasites diluted in malaria negative whole blood. The level of *P. falciparum* detection was determined for each extraction method and RDT device with multiple nested-PCR and real-time PCR assays. The field applicability was tested on 855 paired RDT (Paracheck-Pf) and filter paper (Whatman® 3MM) blood samples (734 RDT negative and 121 RDT positive samples) collected from febrile patients in Zanzibar 2010. RDT positive samples were genotyped at four key single nucleotide polymorphisms (SNPs) in *pfmdr1* and *pfcrt* as well as for *pfmdr1* copy number, all associated with anti-malarial drug resistance.

Results: The *P. falciparum* DNA detection limit varied with RDT device and extraction method. Chelex-100 extraction performed best for all extraction matrixes. There was no statistically significant difference in PCR detection rates in DNA extracted from RDTs and filter paper field samples. Similarly there were no significant differences in the PCR success rates and genotyping outcomes for the respective SNPs in the 121 RDT positive samples.

Conclusions: The results support RDTs as a valuable source of parasite DNA and provide evidence for RDT-DNA extraction for improved malaria case detection, molecular drug resistance surveillance, and RDT quality control.

Keywords: *Plasmodium falciparum*, Malaria, Rapid diagnostic test, DNA extraction, Molecular surveillance

Background

The World Health Organization has recommended the use of malaria rapid diagnostic tests (RDTs) for prompt and accurate parasitological confirmation of *Plasmodium falciparum* malaria in settings where microscopy services are not available. However, the ability to detect individuals with asymptomatic low density parasitaemia, i.e., below detection limit of both RDTs and microscopy (~100 parasites/μL blood), in low endemic settings has been increasingly acknowledged as a challenge to achieve malaria elimination [1]. In this context there is a need for novel sensitive molecular tools and strategies for improved malaria case detection. Furthermore, molecular tools for monitoring the selection of genotypes associated with anti-malarial drug resistance are critical since they may provide an early warning system of development and spread of tolerance/resistance to artemisinin-based combination therapy (ACT) before clinical treatment failures are apparent.

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The possibility of recovering parasite DNA from RDTs was first shown by Veron *et al.* [2]. Thereafter, two additional methods of DNA extraction from RDTs have been published [3,4] suggesting that RDTs are a reliable source for parasite DNA preservation. This provides an opportunity for improved molecular surveillance and RDT quality control. However, and importantly, comparative studies on the efficiency of the published DNA extraction methods from RDTs are lacking. This is critical since there is evidence that the efficiency of DNA extraction may be highly influenced by choice of extraction matrix and method [3-6]. Furthermore, comprehensive studies are needed to investigate whether wide-scale collection of RDTs can provide the basis for modern molecular surveillance of malaria, including both improved malaria case detection and anti-malarial drug resistance genotyping.

The aims of this study were, therefore, to evaluate two published DNA extraction methods, in comparison with a previously unpublished, high-throughput method (Ferreira P E, unpublished data), and to assess the field applicability of RDT-DNA extraction for molecular surveillance, in comparison to DNA extraction from filter paper.

Methods

RDT-DNA extraction methods

Three DNA extraction methods from RDTs were evaluated: 1) A simple elution method [3]; 2) Chelex-100 extraction [4]; and, 3) a previously unpublished method following a modified version of the protocol "isolation of DNA from fresh or frozen whole blood" employing an ABI PRISM 6100 Nucleic Acid PrepStation™ and NucPrep reagents (Applied Biosystems, USA) (Ferreira P E, unpublished data). In brief, for the third method, the biological samples were lysed in three-fold volume of NucPrep reagents. The lysate mixture was incubated for 1h (instead of 10 min) at 58°C, and the lysed samples were incubated at 4°C overnight before preforming the extraction (as previously reported) [7]. On day two, the solid material was separated from the lysate by passing the content through a 5 mL syringe. The full lysate was flowed through one column per sample in a DNA purification tray II (Applied Biosystems, USA) by three consecutive loadings. DNA washing was performed as recommended by the manufacturer. Incubation with DNA elution solution 1 was increased from three to five minutes. The final DNA containing elution volume was 200 μL for ABI extraction, 50 μL in the simple elution method and ~190 μL in the Chelex-100 extraction (after deduction of 5% Chelex-100 from a total volume of 200 μL).

RDT devices

DNA extraction was compared from two RDT devices of clinical importance in Zanzibar: Paracheck-Pf® (Orchid Biomedical Systems, Goa, India) and SD-Bioline Malaria Ag P.f/Pan® (Standard Diagnostic, Inc, USA). Paracheck-Pf has been widely used in sub-Saharan Africa, and was the first RDT to be implemented in Zanzibar in 2006. Zanzibar has recently changed to SD-Bioline P.f/Pan as this test also detects species other than *P. falciparum*.

Plasmodium falciparum in vitro samples and analysis

Both RDT devices were seeded according to the manufacturers' instructions with 5 μL of 10-fold serial dilutions of laboratory cultured 3D7 *P. falciparum* (200,000-0.02 parasites/μL) as well as with malaria negative whole blood (negative control). For comparison, Whatman® 3MM filter paper was seeded in parallel with 5 μL (approximately equivalent to one 3-mm punch) of the serial dilution. Parasite cultures and malaria negative whole blood were lysed by freeze-thawing prior to serial dilution.

The RDTs and filter papers were allowed to air dry for a minimum of 16 hours at room temperature (20°C), whereafter RDT cassettes were opened using a thin metal spatula. The nitrocellulose strip was held at the buffer pad with forceps and cut into 3 × 3 mm pieces using scissors. In between each sample, forceps and scissors were washed in 70% ethanol and dried on clean tissue paper, to minimise cross-contamination during sample preparation. RDT preparation was in accordance with the worldwide antimalarial resistance network (WWARN) guidelines, with minor modifications [8]. DNA extraction was compared from four different RDT fragments (Figure 1), all including the proximal third of the nitrocellulose strip that has been reported to generate best results upon DNA extraction [3]. DNA was extracted using the three methods described above.

The *P. falciparum* DNA detection limits were determined using three PCR techniques: 18S ribosomal DNA (rDNA) nested PCR [9], cytochrome b nested PCR [10], and 18S rDNA probe-based real-time PCR [11]. The same volume of DNA was used from each extraction method (2-5 μL depending on PCR). The *P. falciparum* detection limits were determined as the lowest consecutive positive sample in the dilution series.

Field study sampling

The field samples were collected during an RDT effectiveness study conducted in 12 public health facilities, six each in North A and Micheweni districts, Zanzibar, May-July 2010 (Shakely *et al.*, submitted). Febrile patients were tested for *P. falciparum* malaria with Paracheck-Pf. In parallel approximately 100 μL of blood was spotted onto Whatman 3MM filter paper. Paired RDT and filter paper samples were available from 121 RDT positive and 734 RDT negative patients. Informed consent was obtained from enrolled patients or parent/guardians of children. The study was conducted in accordance with the Declaration of Helsinki [12] and Good Clinical Practice [13]. The study is registered as NCT01002066. Ethical approvals

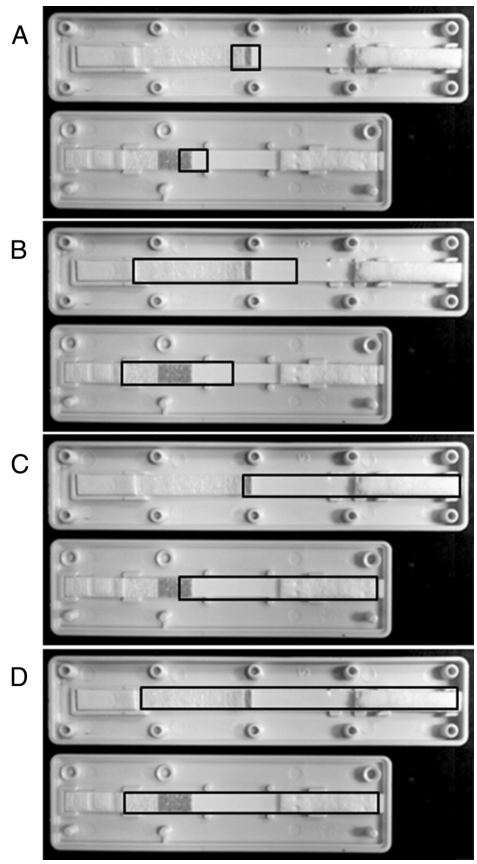

Figure 1 RDT fragments used for DNA extraction. Each panel shows Paracheck-Pf (top) and SD-Bioline Malaria Ag P.f/Pan (bottom). The RDTs are orientated with the buffer pad and sample site to the left (proximal end), and the absorption pad to the right (distal end). The fragments used for extraction are outlined by the black boxes **A**) 1cm **B**) Proximal **C**) Distal **D**) Whole.

were obtained from the ethical committee in Zanzibar (ZAMREC/ST/0021/09) and the Regional Ethics Committee in Stockholm, Sweden (2009/387-31). All samples were stored at room temperature with desiccants until the time of DNA extraction. Samples were transported to Karolinska Institutet, Sweden, August 2010, where DNA extraction from RDT and filter paper samples was conducted in parallel within six months after sample collection.

Field sample analysis

DNA was extracted from the paired RDT and filter paper field samples (N=855) using the ABI PRISM 6100 Nucleic Acid PrepStation™ method. DNA was extracted from the distal two thirds (Figure 1C) of the RDT strip, containing ~5 μL blood, as described above, and from three 3-mm punches of filter paper, containing ~15 μL blood, as described previously [7].

RDT negative samples (N=734) were screened using an 18S rDNA real-time PCR assay, that detects all five species of *Plasmodium* [14]. Samples were pooled two by two in 384 well plates. Each PCR was performed twice at different time points. Pools with a single Cycle threshold (Ct) value <40, or a Ct average <42 were selected for multiplex species identification for *P. falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae*. Samples were considered PCR positive if positive in the species identification analysis.

Single nucleotide polymorphisms (SNPs) in *pfcrt* K76T, *pfmdr1* N86Y, Y184F and D1246Y were analysed with previously described PCR-RFLP methods in all RDT positive samples (N=121) [15-17]. An infection was defined as mixed when both alleles were present at a particular locus. *Pfmdr1* copy number was determined by the comparative ΔΔCt method following a TaqMan probe-based real-time PCR [18]. Samples were considered PCR positive if positive in at least one PCR.

All PCRs were run in parallel on RDT and filter paper extracted DNA.

Statistical analysis

SNP genotyping outcomes were compared between RDT and filter paper extracted DNA by kappa analysis (κ). The SNP and haplotype prevalences were analysed and published elsewhere [17]. The spread of Ct values below

Table 1 Sensitivity of RDT-DNA extraction methods in *in vitro* cultured parasites

RDT Fragment	Simple elution	Chelex-100	ABI
Paracheck-Pf			
1 cm	§	2	200
Proximal	NA	20	200
Distal	NA	NA	20
Whole	NA	NA	20
SD-Bioline Malaria P.f/Pan			
1 cm	2	2	20
Proximal	NA	2	20
Distal	NA	NA	20
Whole	NA	NA	20
Filter paper			
5 μL blood spot	200	2	200

Lowest achieved parasite detection levels (parasites/μL) for DNA extraction from Paracheck-Pf, SD-Bioline Malaria P.f/Pan and from 5 μL blood spotted on filter paper.
§ = Not estimated due to negative results.
NA = Not applicable, the size of the RDT fragment used for extraction was limited by the extraction volume.

Table 2 PCR success rates and agreement of genotyping outcomes in field samples

	RDT PCR success rates N = 121 (%; CI 95%)	Filter paper PCR success rates N = 121 (%; CI 95%)	Kappa value
Pfcrt K76T	114 (94.2; 89.9-98.5)	104 (86.0; 79.6-92.3)	0.72
Pfmdr1 N86Y	112 (92.6; 87.8-97.4)	109 (90.1; 84.6-95.5)	0.85
Pfmdr1 Y184F	110 (90.9; 85.7-96.2)	107 (88.4; 82.6-94.3)	0.74
Pfmdr1 D1246Y	113 (93.4; 88.8-97.9)	107 (88.4; 82.6-94.3)	0.77
Pfmdr1 copy number	84 (69.4; 61.0-77.8)	77 (63.6; 54.9-72.4)	-

Analyses of single nucleotide polymorphisms and *pfmdr1* gene copy numbers associated with anti-malarial drug resistance, from RDT and filter paper extracted DNA collected from 121 RDT positive field samples.

the cut-off of Ct 35, in the TaqMan probe-based real-time PCR for determining *pfmdr1* copy number, were compared by Wilcoxon rank-sum test, as there were many incomplete pairs. All calculations were done with Stata/SE 12.0, StataCorp LP USA. Statistical significance was defined as $p<0.05$.

Results

In vitro cultured parasites - sensitivity of RDT-DNA extraction methods

The *P. falciparum* DNA detection limit varied with RDT device and extraction method (Table 1). When following the respective protocols, Chelex-100 extraction preformed best for both RDT devices as well as for extraction from filter paper, with a detection limit of two parasites/μL. The ABI extraction method had a 10-fold higher detection limit of 20 parasites/μL. DNA extraction was generally more efficient from SD-Bioline Malaria Ag P.f/Pan than from Paracheck-Pf. The simple elution method was unsuccessful for DNA extraction from Paracheck-Pf. Increasing the size of the nitrocellulose strip fragment (as seen in Figure 1) did not improve the level of detection. The method of *P. falciparum* detection influenced the detection limit by one to two log units (see Additional file 1). DNA extraction from RDTs was generally equal to or better than DNA extraction from an equal volume (5 μL) of blood spotted on filter paper.

Field samples - parasite detection and drug resistance genotyping

There was no significant difference in PCR detection rates in DNA extracted from RDTs and filter paper. Out of 855 paired RDT and filter paper field samples, 118 (13.8%; CI 95% 11.4-16.2%) were PCR positive in both groups of samples (κ=0.94). Among the RDT negative field samples (N=734), three (0.4%; CI 95% 0.0-0.9%) and six (0.8%; CI 95% 0.1-1.5%) were PCR positive from RDT and filter paper extracted DNA, respectively (κ=0.44). Among the 121 RDT positive field samples, 115 (95.0%; CI 95% 91.1-99.0%) and 112 (92.6%; CI 95% 87.8-97.4%) were PCR positive (κ=0.50). No observed difference was found in the ability to detect low density

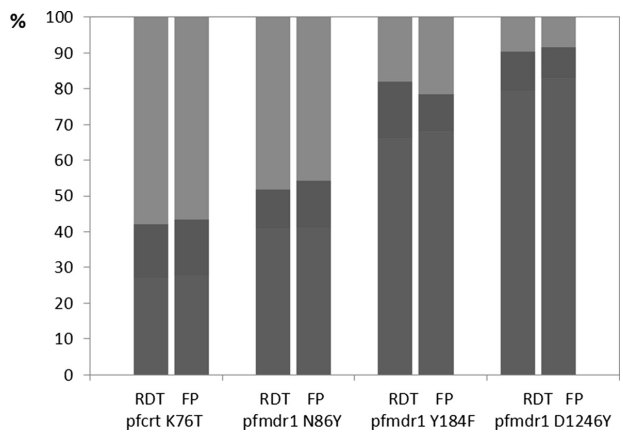

Figure 2 Genotyping outcomes for RDT and filter paper extracted DNA at four key drug-resistance associated SNPs. Stacked bar graph comparing the genotyping outcomes for RDT and filter paper extracted DNA for *pfcrt* K76T, *pfmdr1* N86Y, *pfmdr1* Y184F and *pfmdr1* D1246Y. Blue colour represents *pfcrt* K76, *pfmdr1* N86, Y184 and D1246, whereas red represents mixed infections, and green represents *pfcrt* 76T, *pfmdr1* 86Y, 184F and 1246Y, respectively.

parasitaemia (<100 parasites/μL), although the numbers were too small to allow for statistical analysis (12 PCR positives in both RDT and filter paper extracted DNA).

There were no significant differences in PCR success rates and genotyping outcomes for the respective SNPs in the 121 RDT positive samples (Table 2). Furthermore, there was no difference between RDT and filter paper extracted DNA in the overall ability to detect mixed infections (Figure 2). Similarly, no statistically significant differences in the distribution of real-time PCR Ct values below Ct 35 from RDT and filter paper extracted DNA were observed (P_{FAM}=0.10; P_{VIC}=0.24). No sample contained multiple *pfmdr1* copy number.

Discussion

This is to date the most comprehensive comparative study of DNA extraction efficiency from malaria RDTs and assessment of the field applicability of RDT-DNA extraction for molecular surveillance, including detection of infections and key genetic markers associated with anti-malarial drug resistance.

DNA extraction efficiency from *in vitro* cultured *P. falciparum* varied with RDT device and extraction method. The same level of parasite detection as seen in previous publications was not achieved in this study [3,4]. Different designs of RDT devices affected the DNA extraction efficiency. In particular, DNA recovery from Paracheck-Pf was unsuccessful when employing the simple elution method, supporting as previously reported that plastic seals covering the nitrocellulose strip hamper DNA recovery [3].

The cost, time, final template volume and the purpose for DNA extraction should be considered when choosing extraction method. Although simple elution is the cheapest and fastest alternative, it is a crude method of DNA extraction and its use may be limited by RDT design and choice of PCR [3]. Chelex-100 is relatively inexpensive. The higher sensitivity observed with Chelex-100 extraction indicates that this method is particularly suitable for low density parasitaemia in low endemic settings. However, the Chelex-100 method is moderately labour-intensive and the DNA may be of lower quality than DNA extracted with commercially available column-based extraction kits [6]. Another concern is the storage capacity of Chelex-100 extracted DNA, which is thought to be more susceptible to DNA degradation during sample freeze-thawing [19]. Conversely, ABI extraction is a high throughput method providing high quality DNA, but at a substantial cost and requiring specialised equipment. This method had a higher *P. falciparum* detection level when compared with Chelex-100, perhaps explained by loss of DNA on the column. However, this did not appear to have influenced the field sample results (see below). Thus, ABI extraction could be suitable for analyses of RDT positive, symptomatic malaria patients enrolled in clinical trials. The final DNA containing volume is also important to take into consideration, as the concentration of the DNA will affect the parasite detection limits.

In the field analysis, RDTs provided DNA of equal quality as filter papers, suggesting that RDTs are a valuable alternative to filter paper for DNA storage in the field. High PCR success rates were obtained from DNA extracted from RDTs, for key loci in *pfcrt* and *pfmdr1* associated with anti-malarial drug resistance. SNP and haplotype prevalences were analysed and discussed elsewhere [17]. Wide scale collection of used RDTs is currently being implemented as an integral part of molecular surveillance of malaria in Zanzibar.

Increased deployment of RDTs in health care facilities and cross-sectional surveys facilitates passive and active collection of biological material for molecular surveillance. The advantages of using RDTs for DNA storage include reducing invasive procedures in the field. RDTs require just one finger prick for both malaria case detection and preservation of biological material. DNA storage on filter papers, on the other hand, requires an initial finger prick for malaria case detection by RDT or microscopy, followed by a second finger prick, for individuals with a positive diagnosis for collection of blood on filter paper. Multiple blood sampling can especially be problematic in small children and may increase the risk of mixing/miss-labelling of samples during collection. RDTs are also easily stored and have either a plastic or cardboard case that protects against cross-contamination. A disadvantage of RDT-DNA extraction is the limited amount of biological material (5–15 μL blood). This makes RDT-DNA extraction a "one shot operation" with no possibilities for re-extraction, unlike filter paper sampling where a larger amount of blood is usually collected (50–100 μL).

Conclusions

The results support RDTs as a valuable source of parasite DNA and provide evidence for RDT-DNA extraction for improved malaria case detection, molecular drug resistance surveillance, and RDT quality control. However, the purpose of DNA extraction should be considered when choosing which extraction method best suits the type of samples to be analysed.

Additional file

Additional file 1: Sensitivity of RDT-DNA extraction methods in *in vitro* cultured parasites. Description: Raw data showing detection levels for the three PCR methods used in the *in vitro* part of the study.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions

PF, UM, BA, AM, JPG and AB conceived and designed the study. DS, ASA and MIM carried out the field work. UM, BA and LJ carried out the molecular analyses. UM, BA, LJ, PF, AM and AB analysed the data. MP gave important intellectual input in the statistical analysis. UM drafted the manuscript. All authors read and approved the final manuscript.

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IV

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Loop Mediated Isothermal Amplification (LAMP) Accurately Detects Malaria DNA from Filter Paper Blood Samples of Low Density Parasitaemias

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Abstract

Background: Loop mediated isothermal amplification (LAMP) provides an opportunity for improved, field-friendly detection of malaria infections in endemic areas. However data on the diagnostic accuracy of LAMP for active case detection, particularly low-density parasitaemias, are lacking. We therefore evaluated the performance of a new LAMP kit compared with PCR using DNA from filter paper blood spots.

Methods and Findings: Samples from 865 fever patients and 465 asymptomatic individuals collected in Zanzibar were analysed for Pan (all species) and Pf (*P. falciparum*) DNA with the Loopamp MALARIA Pan/Pf kit. Samples were amplified at 65°C for 40 minutes in a real-time turbidimeter and results were compared with nested PCR. Samples with discordant results between LAMP and nested PCR were analysed with real-time PCR. The real-time PCR corrected nested PCR result was defined as gold standard. Among the 117 (13.5%) PCR detected *P. falciparum* infections from fever patients (mean parasite density 7491/μL, range 6–782,400) 115, 115 and 111 were positive by Pan-LAMP, Pf-LAMP and nested PCR, respectively. The sensitivities were 98.3% (95%CI 94–99.8) for both Pan and Pf-LAMP. Among the 54 (11.6%) PCR positive samples from asymptomatic individuals (mean parasite density 10/μL, range 0–4972) Pf-LAMP had a sensitivity of 92.7% (95%CI 80.1–98.5) for detection of the 41 *P. falciparum* infections. Pan-LAMP had sensitivities of 97% (95%CI 84.2–99.9) and 76.9% (95%CI 46.2–95) for detection of *P. falciparum* and *P. malariae*, respectively. The specificities for both Pan and Pf-LAMP were 100% (95%CI 99.1–100) in both study groups.

Conclusion: Both components of the Loopamp MALARIA Pan/Pf detection kit revealed high diagnostic accuracy for parasite detection among fever patients and importantly also among asymptomatic individuals of low parasite densities from minute blood volumes preserved on filter paper. These data support LAMPs potential role for improved detection of low-density malaria infections in pre-elimination settings.

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Background

In areas of sub-Saharan Africa where malaria elimination is targeted, new tools and strategies are needed to achieve this ambitious goal. A critical component to pursue malaria elimination is to identify and treat all malaria parasite carriers, both symptomatic patients and asymptomatic individuals through passive and active case detection, respectively [1]. Importantly, during the transition from malaria control to pre-elimination and eventually elimination, the relative importance of low-density parasitaemias as a reservoir of on-going transmission is expected to gradually increase [2,3,4]. Conventional point-of-care malaria diagnostic tools, i.e. microscopical investigation of stained blood smears and rapid diagnostic tests (RDTs), are not sensitive enough for reliable detection of low-density parasitaemias, considering their limit of detection of approximately 100 parasites/μL of blood in field use [5,6,7]. Conversely, highly sensitive diagnostic tools like

PCR are hampered by their complexity, need for specialized laboratory infrastructure and know-how, relatively long time-to-result and thus lack of field friendliness [8].

Newly described molecular based diagnostic methods, such as loop-mediated isothermal amplification of DNA (LAMP), provide an opportunity to raise the target of both efficient passive and active malaria case detection to include also low-density parasitaemias in field settings of endemic areas [2,5,9]. The new Loopamp MALARIA Pan/Pf detection kit ((LMC 562, Eiken Chemical Co., Ltd. Tokyo, Japan) consists of two separate reagents tubes containing vacuum dried reaction mixtures with primers targeting the mitochondrial DNA sequences of all human *Plasmodium* species (Pan-malaria) and *Plasmodium falciparum* (Pf), respectively. The kit is designed to address the need for a molecular test that achieves higher sensitivity and specificity than microscopy and RDT and at the same time it is more field-friendly than PCR since it employs temperature stable vacuum dried reagents requiring no cold transport chain, is easily reconstituted on site and neither requires a thermocycler for amplification nor a gel imaging system for result reading [5,10]. The *Bacillus stearothermophilus* (*Bst*) polymerase used in the LAMP method is also considered more robust than *Taq* polymerase with regards to inhibition of the reaction, making it suitable for simple and rapid DNA extraction methods [11]. A feasibility study has previously demonstrated that the Loopamp MALARIA Pan/Pf kit can amplify *Plasmodium* DNA in less than 40 minutes from samples containing as low as 2 parasites per microliter (p/μL) of blood, with a sensitivity and specificity of 93.3% and 100%, respectively, using nested PCR as reference standard [12]. A recent field study conducted in a high transmission area of Uganda using venous blood samples from fever patients has also shown that technicians without previous molecular training could reliably perform LAMP in a simple laboratory space without specialized equipment after a short training period [5]. Under these conditions LAMP had sensitivity and specificity equivalent to nested PCR performed upon paired samples in a reference level laboratory, but with a significantly faster time-to-result.

Both these studies employed liquid blood from venipuncture [12]. However, convincing evidence that the LAMP method can reliably detect parasite DNA extracted from finger prick blood samples spotted and dried onto filter paper from asymptomatic carriers of low parasite densities (necessary for an effective field screening programme) is lacking. Moreover, the performance of LAMP for overall improved malaria case detection in low-endemic/malaria pre-elimination settings remains to be shown. The aim of this study was therefore to evaluate the accuracy of the Loopamp MALARIA Pan/Pf kit as a comparator to PCR for detection of malaria parasite carriers in Zanzibar, a malaria pre-elimination setting of sub-Saharan Africa. Samples were derived from archived dried blood spots collected both from fever patients attending primary health care facilities and asymptomatic individuals participating in a cross-sectional survey.

Methods

Study design, blood samples and their origin

This comparative study on the diagnostic accuracy of the Loopamp MALARIA Pan/Pf detection kit for detection of pan-*Plasmodium* and *P. falciparum* DNA, respectively, versus PCR as reference standard, included a total of 1330 archived blood samples collected on filter papers, of which 865 originated from fever patients and 465 from asymptomatic individuals in Zanzibar.

The study was conducted according to the STARD (Standards for reporting of Diagnostic Accuracy Studies) guidelines [13].

Fever patients. The 865 blood samples were collected from fever patients presenting at primary health care facilities in two districts, i.e. North A (Unguja Island) and Micheweni (Pemba Island), in Zanzibar, 2010. All patients were enrolled in a previously published malaria RDT study [14]. Briefly, 3890 fever patients (defined as either documented axillary temperature $\geq$ 37.5°C at enrolment or history of fever during the preceding 24 hours) aged $\geq$2 months were tested for *P. falciparum* malaria with a histidine rich protein 2 (HRP2) detecting RDT (Paracheck-Pf,Orchid Biomedical Systems, India). Overall, 121 (3.1%) patients were RDT positive. Blood smears for microscopy and a blood spot on filter paper (Whatman^{TM}3MM, GE Health care, UK) for molecular analysis were collected from capillary finger pricks, from all RDT positive patients and 744 (~20%) randomly selected RDT negative patients. All RDT positive samples were previously analysed with three standard *P. falciparum* specific nested PCR methods [14], whereas the RDT negative samples were screened in duplicate for human plasmodial infection with an 18 s real-time PCR [15]. In total 122 of the 865 (121 RDT positive+744 RDT negative) patients were PCR positive for *P. falciparum*. No other species was found. Four of the 121 (3.3%) RDT positive patients were negative by PCR, whereas five of the 744 (0.7%) RDT negative patients were positive by PCR [14]. All samples were analysed with Cyt b nested PCR in association with the LAMP assays.

Asymptomatic individuals. The 465 blood samples were selected from asymptomatic individuals participating in a community based cross sectional survey conducted in North A and Micheweni districts in Zanzibar, 2011. A total of 2977 individuals were screened with a *Pf*HRP2/pan-*Plasmodium* lactate dehydrogenase (pLDH) based RDT (SD-BioLine Malaria Ag P.f/Pan,Standard Diagnostic, Inc, Republic of Korea). Blood was also collected on filter paper (Whatman 3MM) and subsequently analysed with a nested [16] and a real-time PCR, both targeting the Cytochrome b (Cyt b) gene. The RDT positivity rate was 13/2977 (0.4%), of which 6 (46%) samples were confirmed positive by PCR, all *P. falciparum* mono- infections. The corresponding overall PCR positivity rate was 65/2977 (2.2%), 61 (93.8%) were detected by nested PCR and additionally four by real-time PCR. All 65 PCR positive samples and 400 randomly selected PCR negative samples were included in the present analysis. Prior to the present study, DNA was re-extracted with the Chelex method from all 65 original PCR positive samples due to insufficient amount of remaining DNA.

Molecular analyses

DNA extraction from dried blood spots on filter paper. *Fever patients*: DNA was extracted from three Ø 3 mm filter paper punches, equivalent to approximately 10–15 μL blood, using a modified version of the column-based ABI 6100 Nucleic Acid Prep Station protocol (Applied Biosystems, Fresno, CA) [17]. Each DNA sample was eluted in 200 μL of buffer and stored in −20°C until use.

Asymptomatic individuals: DNA was extracted from one Ø 3 mm filter paper punch, equivalent to approximately 3–5 μL blood. Each filter paper was suspended in 0.5% saponin PBS buffer (Sigma, St Louis, MO), washed in 1× PBS buffer (Gibco, Paisley, UK), boiled in 100 μL of 10% Chelex (Bio-Rad, Hercules, CA) H_2O solution at 95°C for 10 min and centrifuged [18]. The supernatant containing DNA was obtained and transferred to a new plate, and stored at −20°C before further molecular analysis.

PCR methods. *Nested PCR:* Cyt b nested PCR as described by Steenkeste et al, 2009 [16] was performed on all samples prior to the LAMP assay. Briefly, primer pairs of 5′-TAATGCCTA-

GACGTA TTCCTGATTATCCAG-3′/5′-TGTTTGCTTGGGAGC TGTAATCATAATGTG-3′ and 5′-GAGAATTATGGAGT GGATGGTG-3′/5′-TGGTAATTGA CATCCAATCC-3′ were used in the first and nested PCR, respectively. PCR master mix was prepared [16] using 5 μL of extracted DNA and PCRs was run on the ABI Thermal Cycler 2700 using the same cycling conditions for the first and nested PCR runs except for the extension step. The conditions were 95°C for 3 min; 40 cycles of 95°C for 15 sec, 60°C for 1 min and 72°C for 1.5 min (first PCR) or 1 min (nested PCR); and final extension at 72°C for 5 min. The nested PCR products were visualized under UV light after gel electrophoresis, and positive PCR products were subjected to a Restriction Fragment Length Polymorphism (RFLP) assay for species identification [19]. The detection limit for Cyt b nested PCR was estimated to approximately 2 p/μL as assessed by *P. falciparum* 3D7 laboratory culture dilution series seeded on filter papers.

Real-time PCR: A recently developed Cyt b real-time PCR was performed on the Pan and/or Pf-LAMP/nested PCR discordant samples, using the primer pairs of 5′-TGGTAGCA-CAAATCCTTTAGGG-3′ and 5′-TGGTAATTGACATC-CAATCC-3′ targeting the Cyt b gene of the five human *Plasmodia* species (unpublished data). The real-time PCR master mix was prepared containing 5 μL of extracted DNA, 1× iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA), and 0.25 μM of each primer in 20 μL volume, and run on the ABI Prism 7000 system. The real-time PCR conditions were 95°C for 4 min; 40 cycles of 95°C for 15 sec, 60°C for 1.5 min with fluorescence detection; 72°C for 5 min; and melting curve acquisition. The real-time PCR results were analysed by both the melting curve and gel electrophoresis. Positive real-time PCR products were digested by FspBI enzyme (Thermo Fisher, Waltham, MA) in RFLP assay for species identification.The RFLP reaction was carried out in a total reaction volume of 20 μL including 5 μL of real-time PCR products and 5 units of FspBI enzyme in 1× reaction buffer in accordance with manufacture instructions. After overnight digestion in 37°C, the RFLP products were run on 2% agarose gel followed by visualization in Gel-doc system (Bio-Rad). The detection limit for Cyt b real-time PCR was estimated to approximately 1 p/μL as assessed by *P. falciparum* 3D7 laboratory culture dilution series seeded on filter papers.

Quantitative PCR determined parasite densities. All nested PCR positive samples from asymptomatic individuals were subjected to parasite density determination by *P. falciparum* 3D7 dilutions and quantitative PCR (qPCR) targeting the *Plasmodium* 18S rRNA gene [20]. Firstly, filter papers (Whatman 3MM) were prepared by diluting laboratory cultured *P. falciparum* 3D7 to densities of 20000, 2000, 200, 20 and 2 p/μL, spotting 30 μL from each density in the dilution series on to a separate filter papers and air dried. Secondly, filter papers were subjected to Chelex extraction and qPCR quantification targeting 18S rRNA gene using plasmid standards. Thirdly, a standard curve was developed by plotting known density to quantified 18S copy numbers. Fourthly, asymptomatic DNA samples were quantified by the 18S qPCR using plasmid standard [20], and the parasite densities were calculated by the acquired 18S copy numbers and the standard curve.

LAMP procedures

All DNA samples were analysed with the Loopamp MALARIA Pan/Pf detection kit according to standard operating procedures provided by FIND and the manufacturer's instructions (available at: http://www.finddiagnostics.org/export/sites/default/programs/malaria-afs/docs/SOPs_LAMP_Malaria_AUG12.pdf) [21]. Samples were analysed individually for Pan and Pf using separate reaction-tubes containing specific primers. In brief, 30 μL of DNA samples (diluted 1:6) were added to each Pan and Pf reaction tube. Each set of six samples were analysed along with a negative and a positive control included in the kit. After mixing the DNA solution with the dried reagents, the LAMP reaction tubes were incubated at 65°C for 40 minutes followed by a 5-minute enzyme-inactivation at 80°C in an LA-500 turbidimeter (Eiken Chemical). Results were electronically recorded from the amplification curves in the control unit. An increase in turbidity exceeding 0.1 Optical Density (OD) units per second was scored as positive [22]. In case a sample showed invalid result (questionable curve) or the controls did not show expected results the whole strip of eight tubes were re-analysed.

Limit of detection

Parasite densities of *P. falciparum* (3D7) laboratory strain cultures were assessed by microscopy and diluted to concentrations of 2000-0.2 p/μL and seeded on filter papers. Detection limits for the Loopamp MALARIA Pan/Pf kit were determined after DNA extractions with Chelex and found to be ≤2 parasites/μL for both Pan and Pf-LAMP.

Lot testing

Incoming quality check (IQC) for lot release was performed for the two lots of Loopamp MALARIA Pan/Pf detection kit used during the trial with two *P. falciparum* samples of 5 and 50 p/μL, one *P. malariae* sample and three negative samples.

Re-testing of blood samples with discordant LAMP and PCR results

For the 865 fever patients, DNA samples with discordant Cyt b nested PCR versus Pan and/or Pf-LAMP results were subjected to DNA re-extraction with the Chelex method and re-testing with Cyt b nested PCR and Pan and Pf-LAMP. Any samples which continued to give discordant results were then re-amplified by Cyt b real-time PCR in triplicate. DNA samples from asymptomatic individuals with discordant results between nested PCR versus Pan and/or Pf-LAMP were also re-amplified by Cyt b real-time PCR in triplicate.

Reference PCR methods

The method used for comparison of PCR versus Pan and Pf-LAMP was Cyt b nested PCR, herein referred to as reference standard. Cyt b real-time PCR corrected nested PCR result was defined as gold standard for evaluation of LAMP in this study.

Blinding of samples

The person performing the LAMP assays and re-testing of discordant PCR and LAMP samples was blinded to all clinical data and previously obtained results by all other diagnostic methods (microscopy, RDT and PCRs). All samples were anonymized before testing and re-testing.

Ethical considerations

Sample collections were conducted in accordance with the Declaration of Helsinki and Good Clinical Practice [23,24] and approved by the Zanzibar Medical Research Ethics Committee (ZAMREC/ST/0021/09) (ZAMREC/0001/JUNE/011) and the Regional Ethics Committee, Stockholm (2009/387-31). All participants in both studies gave written informed consent for their participation. For children, proxy-consents from parents/legal guardians were obtained. The RDT study on fever patients

[14] is registered on ClinicalTrials.gov with study identifier "NCT01002066".

Data management, sample size calculation and statistical analysis

Data were entered and statistical analyses performed using STATA v.12 (Stata Corp, Texas, USA). The primary endpoint was to assess the accuracy with which the Loopamp MALARIA Pan/Pf detection kit detected *Plasmodium* DNA in fever patients and asymptomatic individuals using dried blood spots collected on filter paper in a malaria pre-elimination setting. The power calculation was based on the assumption that LAMP (both Pan and Pf reactions) should have an average sensitivity of 90% and specificity of 95% when compared with PCR. The proposed sample size, including 121 RDT positive fever patients and 61 asymptomatic PCR positives, would then provide confidence intervals (CIs) within 10% of the sensitivity point estimate (83.6–94.1% and 80.0–95.3%, respectively). Specificity was estimated much more narrowly given that the 748 fever patient RDT negatives and 400 asymptomatic PCR negatives would provide CIs of 93.2–96.3% and 92.4–96.7%, respectively. Sensitivity, specificity, negative (NPV) and positive (PPV) predictive values of the respective Pan and Pf-LAMP assay with corresponding 95% CIs were calculated using real-time PCR corrected nested PCR as gold standard. When calculating the Pf-LAMP sensitivity, specificity, NPV and PPV for the asymptomatic individuals, *P. malariae* mono-infections were not included. The corresponding calculations for Pan-LAMP were based on *P. falciparum* and *P. malariae* mono-infections only, i.e. the eight mixed infections were not included in the calculations. Data were also analysed after stratification by covariates that may influence the diagnostic accuracy of LAMP (e.g. parasite density and presence of non-falciparum species).The immediate commands of cii was used to calculate the 95% CI (STATA v.12). Pairwise determination of non-equivalence between final outcome (including re-extraction) of Pan and Pf-LAMP as well as for Pan-LAMP and Pf-LAMP individually versus nested PCR for detection of parasite DNA was determined by the McNemar test. Statistical significance was defined as $p<0.05$. Kappa statistics for agreement between the methods were also performed.

Results

Baseline characteristics of the included subjects are presented in Table 1 and the study flow is outlined in Figure 1.

Fever patients

Overall 115/865 (13.3%) fever patients were positive by Cyt b nested PCR. The geometric mean parasite density (p/μL) determined by blood smear microscopy was 7491 (range 6–782400). All 115 nested PCR positives were *P. falciparum* mono-infections, of which 105 (91%) were detected by Pan-LAMP and 101 (88%) by Pf-LAMP. Two nested PCR negative samples were positive by Pan-LAMP (Figure 1).

There were thus 21 samples with discordant nested PCR versus Pan and/or Pf-LAMP results. Among these, five had negative blood smears and the remaining 16 microscopy positive samples had parasite densities assessed by microscopy ranging from 9 to 5331 p/μL (Table 2). All 21 samples were positive when analysed with real-time PCR in triplicate.

After repeat PCR and LAMP analysis with Chelex re-extracted DNA from the 21 discordant and 21 randomly selected concordant negative samples as controls, four discordant samples remained, i.e. Pan and Pf-LAMP positive/nested PCR negative. Among these, two samples had parasite densities of 3930 and 679 p/μL, respectively, as assessed by microscopy whereas the other two were negative. All these four samples were again determined positive by Cyt b real-time PCR, see table 2 for detailed results. Two patients negative by Pan and Pf-LAMP were after Chelex-extraction also negative by Cyt b nested PCR. Both samples were negative by microscopy (Table 2). Nested PCR detected 15/21 (71%) of the real-time PCR positive re-extracted samples. All 21 previously concordant negative samples remained negative when re-analyzed.

Asymptomatic individuals

Overall 50/465 (11%) asymptomatic individuals were positive by Cyt b nested PCR, 29 samples were determined positive for *P. falciparum*, 16 for *P. malariae* and five for *P. falciparum*/*P. malariae* mixed infections. The geometric mean parasite density (p/μL) determined by qPCR was 10 (range 0–4972). Pan-LAMP was positive for 49 (10.5%) and Pf-LAMP for 38 (8.2%) subjects. There were 12 samples with discordant nested PCR and Pan and/or Pf-LAMP results. Their detailed results including qPCR determined parasite densities are presented in Table 3.

The 12 discordant samples along with the same number of randomly selected concordant negative samples as controls were re-analysed with Cyt b real-time PCR in triplicate. This resulted in another 4 PCR positives among the discordant samples. Among these 54 (50+4) PCR positive samples, the RFLP assay with FspBI digestion showed 33 *P. falciparum*, 13 *P. malariae* and 8 mixed *P. falciparum*/*P. malariae* infections. Out of the 41 (33+8) *P. falciparum* real-time PCR positive samples, 38 (93%) and 34 (83%) were positive by Pf-LAMP and nested PCR, respectively. Pan-LAMP was positive in 49/54 (91%) of all PCR positive samples and in 32/33 (97%) and 10/13 (77%) of the PCR determined *P. falciparum and P. malariae* mono-infections, respectively. Nested PCR was positive for all 13 *P. malariae* samples. There was no significant difference in parasite densities between the three Pan-LAMP negative (range 1–2 p/μL) versus the ten Pan-LAMP positive (range 0–3 p/μL) *P. malariae* samples ($p\geq0.05$). Pan and Pf-LAMP both detected seven, whereas nested PCR detected five of the eight samples with mixed *P. falciparum*/*P. malariae* infections. All 12 previously concordant negative samples remained negative when re-analysed.

Sensitivities and Specificities for LAMP

Fever patients. The sensitivity, specificity and predictive values for parasite detection in ABI-extracted samples for Pan and Pf-LAMP versus real-time PCR corrected nested PCR (gold standard) are presented in Table 4. Final outcome for Pan and Pf-LAMP among fever patients including the results from the analysis using Chelex re-extracted DNA samples (823+42) and the respective kappa-values are also presented in Table 4. Statistical equivalence between the performance of Pan versus Pf-LAMP and between Pan-LAMP and Pf-LAMP individually versus nested PCR for detection of *P. falciparum* DNA revealed p-values of 1.00, 0.13 and 0.13, respectively.

Asymptomatic individuals. The sensitivity, specificity and predictive values for parasite DNA detection for the respective Pan and Pf-LAMP compared with the defined PCR gold standard are presented in Table 4. Statistical equivalence between the performances of Pan versus Pf-LAMP and between Pf-LAMP versus nested PCR for detection of *P. falciparum* among asymptomatic individuals revealed no significant difference, with p-values of 1.00 and 0.29, respectively. Similarly, the performance of Pan-LAMP versus nested PCR for detection of all malaria positives also showed equivalence ($p = 1.00$).

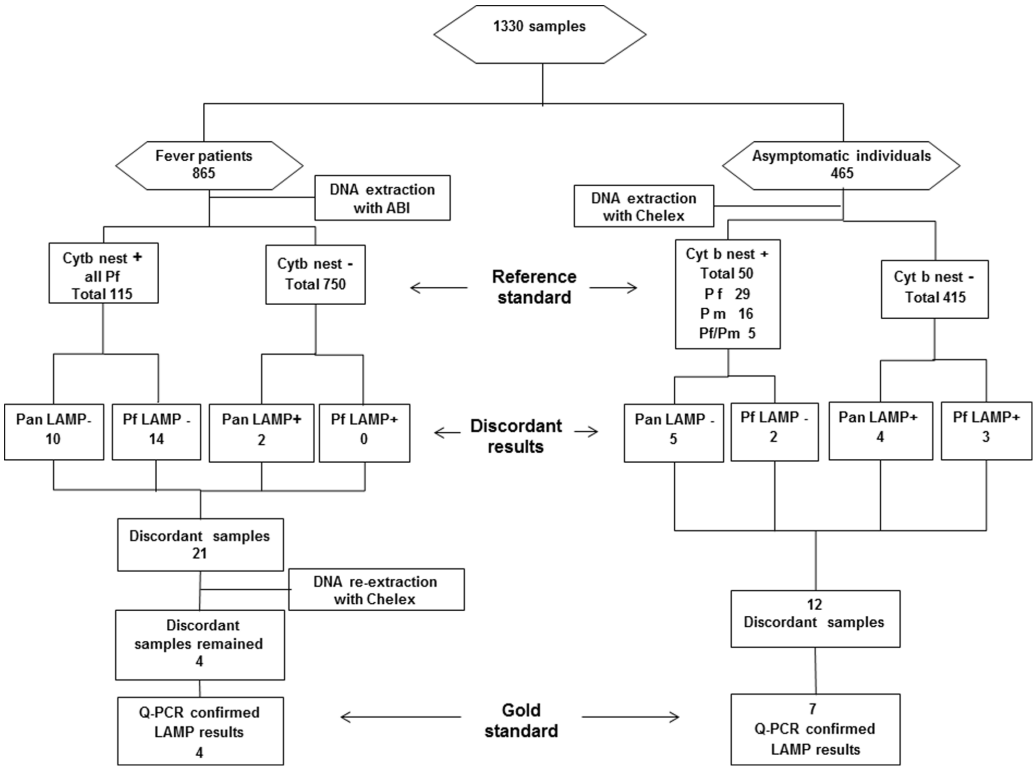

Figure 1. Flow chart of study. Reference standard = Cytochrome B nested PCR. **Gold standard** = Cytochrome B real-time PCR corrected nested PCR.
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Discussion

The Loopamp MALARIA Pan/Pf detection kit evaluated in this study revealed high diagnostic accuracy both with Pan and Pf-LAMP for parasite DNA detection among fever patients and asymptomatic individuals from filter paper blood samples collected in Zanzibar. This is, to our knowledge, the first evidence of high diagnostic accuracy of LAMP using parasite DNA extracted from minute blood volumes spotted on filter paper from fever patients

Table 1. Baseline characteristics of fever patients and asymptomatic individuals.

	Fever patients (n = 865)	Asymptomatic individuals (n = 465)
Median age	19 (2 months-92 years)	14 (1month-85 years)
Sex male %/female %	42/58	40/60
RDT positive *(P. falciparum)*	121	13
RDT positive (non *P. falciparum*)	ND	0
Microscopy positive (all species)	116	ND
Geometric mean parasite density* p/μL	7491 (6–782,400)	ND
PCR positive (all species)	122	65
Geometric mean parasite density** p/μL	ND	10 (0–4972)

*determined by microscopy,
**determined by quantitative PCR,
ND = not done, p/μL = parasites/microliter, values in ( ) = range.
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Table 2. Fever patient samples with discordant results.

			ABI extracted DNA				Chelex re-extracted DNA			
ID	Parasites/μL*	RDT	Pan LAMP	Pf LAMP	Nested PCR	Real-time PCR	Pan LAMP	Pf LAMP	Nested PCR	Real-time PCR
1	360	+	−	+	+	+	+	+	+	
2	0	+	−	−	+	+	+	+	−	+
3	0	−	−	−	+	+	−	−	−	
4	0	−	+	−	+	+	−	−	−	
5	2691	+	+	−	+	+	+	+	+	
6	0	+	+	−	+	+	+	+	+	
7	1307	+	−	+	+	+	+	+	+	
8	2760	+	+	−	+	+	+	+	+	
9	0	+	+	−	−	+	+	+	−	+
10	3513	+	−	+	+	+	+	+	+	
11	3930	+	+	−	−	+	+	+	−	+
12	340	+	−	−	+	+	+	+	+	
13	9	+	+	−	+	+	+	+	+	
14	319	+	+	−	+	+	+	+	+	
15	233	+	−	−	+	+	+	+	+	
16	0	+	−	−	+	+	+	+	+	
17	1637	+	+	−	+	+	+	+	+	
18	5331	+	−	+	+	+	+	+	+	
19	679	+	−	+	+	+	+	+	−	+
20	2820	+	+	−	+	+	+	+	+	
21	679	+	+	−	+	+	+	+	+	

*Determined by blood smear microscopy, RDT = rapid diagnostic test + positive, − negative.

Outcome summery of fever patient samples with discordant results between Pan and/or Pf-LAMP and nested PCR using ABI extracted DNA and after DNA re-extraction with Chelex.

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Table 3. Asymptomatic individuals with discordant results.

ID	Parasites/μL*	Pan LAMP	Pf LAMP	Nested PCR	Real-time PCR
1	3	+	+	-	Pf
2	3	+	+	-	Pf
3	10	+	+	Pm	Pf/Pm
4	<1	+	+	-	Pf
5	5	−	−	Pf/Pm	Pf/Pm
6	5	+	+	Pm	Pf/Pm
7	2	−	−	Pm	Pm
8	3	+	+	Pm	Pf/Pm
9	1	+	−	-	Pf
10	2	−	−	Pf	Pf
11	1	−	−	Pm	Pm
12	2	−	−	Pm	Pm

*determined by quantitative PCR, Pf = *Plasmodium falciparum* Pm = *Plasmodium malariae*,
P/μL = parasites/microliter, + positive, − negative.
Outcome summary of asymptomatic individuals with discordant results between.
Pan and/or Pf-LAMP and nested PCR using Chelex extracted DNA.
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and importantly asymptomatic low-density parasitaemias. These data support LAMPs potential role for improved passive and active malaria case detection in pre-elimination settings.

The LAMP method has previously been evaluated against microscopy and PCR among symptomatic patients suspected of having malaria infection, already diagnosed malaria cases and from samples obtained from malaria cultures using larger blood volumes [10,11,12,22,25] showing sensitivities and specificities > 90%. Importantly, the present study shows similar high diagnostic accuracy from asymptomatic individuals using minute blood volumes preserved on filter paper. This is in agreement with a recently published study using a RealAmp assay with high diagnostic accuracy with DNA extracted from dried blood spots from asymptomatic individuals in Thailand, although on a very small number of positive samples [26].

The Loopamp MALARIA Pan/Pf detection kit has previously been shown to be stable, user-friendly and robust [22]. The kit is also considered safe with minimal risk of contamination [12,27]. The high amplification capacity provides highly sensitive parasite detection with either a turbidimeter or under UV-light after 40 minutes incubation. Moreover, the high amplification capacity of LAMP makes the obtained results easy to interpret; with a few

Table 4. Sensitivities, specificities, positive and negative predictive values and kappa analysis.

Fever patients ABI-extracted DNA (n = 865)					
	Sensitivity % (95% CI)	**Specificity % (95% CI)**	**PPV % (95% CI)**	**NPV % (95% CI)**	**Kappa value**
Pan-LAMP	91.5 (84.8–95.8)	100 (99.5–100)	100 (96.6–100)	98.7 (97.6–99.4)	0.95
Pf-LAMP	86.3 (78.7–92.0)	100 (99.5–100)	100 (96.4–100)	97.9 (96.6–98.8)	0.92
Fever patients: including Chelex re-extracted DNA (n = 823+42)					
	Sensitivity % (95% CI)	**Specificity % (95% CI)**	**PPV % (95% CI)**	**NPV % (95% CI)**	**Kappa value**
Pan-LAMP	98.3 (94.0–99.8)	100 (99.5–100)	100 (96.8–100)	99.7 (99.0–100)	0.99
Pf-LAMP	98.3 (94.0–99.8)	100 (99.5–100)	100 (96.8–100)	99.7 (99.0–100)	0.99
Asymptomatic individuals Chelex extracted DNA (n = 465)					
	Sensitivity % (95% CI)	**Specificity % (95% CI)**	**PPV % (95% CI)**	**NPV % (95% CI)**	**Kappa value**
Pan-LAMP all species	90.7 (79.7–96.9)	100 (99.1–100)	100 (92.7–100)	98.8 (97.2–99.6)	0.95
Pan-LAMP *P.f.**	97 (84.2–99.9)	100 (99.1–100)	100 (89.1–100)	99.8 (98.7–100)	0.97
Pan-LAMP P.m.**	76.9 (46.2–95)	100 (99.1–100)	100 (69.2–100)	99.3 (97.9–99.9)	0.89
Pf-LAMP *P.f.****	92.7 (80.1–98.5)	100 (99.1–100)	100 (90.7–100)	99.3 (98.0–99.9)	0.96

*P.f.** = *P. falciparum* mono infections (n = 33), P.m.** = *P.malariae* mono infections (n = 13), P.f.*** *P. falciparum* mono and mixed.
infections (n = 41), CI = confidence interval, PPV = positive predictive value, NPV = negative predictive value.
Sensitivities, specificities, positive and negative predictive values and kappa analysis for detection of malaria DNA from fever patients and asymptomatic individuals with Pan and Pf-LAMP versus gold standard (real- time PCR corrected Cytochrome b nested PCR).
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parasites per microliter of blood generating equally high turbidity or fluorescence as high-density parasitaemias [12].

In the present study, LAMP performed less well on DNA samples extracted with an automated ABI platform. However, LAMP analysis of the same blood samples, but DNA extracted with Chelex resulted in an excellent agreement between PCR and LAMP and importantly, we did not find any false positive LAMP results.

Among asymptomatic individuals, both Pan and Pf-LAMP had high sensitivities in identifying infections with low parasite densities. LAMP appeared also to be a better tool to identify mixed *P. falciparum*/*P. malariae* infections compared to nested PCR. Moreover, Pan-LAMP appeared to have slightly lower sensitivity in detecting low-density *P. malariae* infections compared to nested PCR, on the other hand LAMP was superior for *P. falciparum* detection. Our results are in accordance with a previous evaluation of Pan and Pf-LAMP for detection of the various *Plasmodium* species showing a detection limit of 2–5 p/μL for all human malaria species [12]. Similarly with the results retrieved from fever patient samples, no false positive LAMP result (specificity and PPV 100%) was detected among the asymptomatic individuals.

Our results show that the Loopamp MALARIA Pan/Pf detection kit may represent a promising opportunity for improved malaria case detection in screen and treat activities within malaria pre-elimination settings. Considering that the relative importance of *non-falciparum* infections appear to increase in such areas [2,7] screening with Pan-LAMP only, which also has a slightly lower detection limit compared to Pf-LAMP (5 versus 7.5 DNA copies/test) [21], may represent a cost effective strategy followed by Pf-LAMP analysis of Pan-LAMP positive samples. However, there is a need for vivax-specific LAMP assays especially in areas outside Africa where *P.vivax* infections predominate [26]. Previous studies have demonstrated high diagnostic accuracy of LAMP with fresh or frozen blood [10,11,12,22,25]. The present study provides evidence that the LAMP kit performs equally well with blood samples from finger pricks collected and stored on filter paper, a low-cost and practical solution for population screening purposes. However, simpler and faster sample preparation for LAMP has been evaluated in the field [5]. Such simplification of sample processing and thus at low cost and improved throughput of high number of samples represents a priority if LAMP is going to be a useful tool in areas aiming at malaria elimination. Importantly, and based on our data, future field evaluations of the LAMP assays for detection of asymptomatic low-density parasite carriers need to be conducted. Further studies on the impact on overall transmission of low-density parasite detection by LAMP followed by adequate treatment need to be conducted.

Limitations

In this study two sets of DNA samples previously extracted with two different methods and stored at −20°C were used. Samples from fever patients were extracted with a column-based ABI method, a high throughput set-up used in our laboratory at the time of the trial [14,17]. This method has been replaced by the Chelex-100 extraction method in our laboratory. The discordant samples between nested PCR and LAMP were therefore re-extracted using the latter method. The concordant ABI extracted PCR positive and negative samples which were not re-extracted, were earlier investigated with several nested and real-time PCR methods [14].

Long storage of frozen DNA extracted with the ABI method, in combination with several freeze-thawing episodes may have influenced the DNA quality suitable for the LAMP reaction more than for ordinary PCRs since several samples with microscopically detectable parasitaemias were negative for Pan and/or Pf-LAMP, but positive for Cyt b nested and real-time PCR. When recently extracted DNA with the Chelex method was used both Pan and Pf-LAMP showed even higher sensitivity than nested PCR.

Conclusion

Both components, Pan- and Pf-detection, of the Loopamp MALARIA kit evaluated in this study revealed high diagnostic accuracy for parasite detection among both fever patients and asymptomatic individuals. This study provides, to our knowledge, the first published evidence of high diagnostic accuracy of LAMP for parasite detection from minute blood volumes spotted on filter paper from asymptomatic individuals in a population where elimination strategies such as focal screening and treatment may be of value, particularly when blood sampling on filter paper from capillary finger pricks would be advantageous for practical and logistical reasons. These data support LAMPs potential role for the implementation of active case detection activities in malaria pre-elimination settings.

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Author Contributions

Conceived and designed the experiments: BAS WX IG SP AB AM. Performed the experiments: BAS WX. Analyzed the data: BAS WX AB AM. Contributed reagents/materials/analysis tools: BAS IG WX DS MM. Contributed to the writing of the manuscript: BAS WX IG SP DB DS MM AB AM. Collected data: BAS WX DS MM. Supervised the study: IG SP DB AB AM.

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