Molecular community surveillance of *Plasmodium falciparum* in 6 sites of different malaria endemicity in Tanzania

INAUGURALDISSERTATION

Zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

Felista Walafried Mwingira

aus

Nyasa, Tanzania

Basel, 2014

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag

von Prof. Dr. Marcel Tanner, Dr. PD Ingrid Felger, Dr. Steffen Borrmann.

Basel, 14. Oktober 2014

Prof. Dr. Jörg Schibler

Dekan

To my Parents

Esther P. Nyotoka *and my late father* ***Walafried N. Mwingira***

and my husband

Ludovic Walter Toto Tarimo

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Summary

Malaria prevalence estimates in Tanzania have been documented to decline in the recent years. National malaria data shows prevalence rates have been reduced by half from 18% in 2008 to 9% in 2012 (THMIS 2009; 2013). This decline has been attributed to countrywide implementation of malaria interventions, including indoor residual spraying (IRS), mass distribution of insecticide treated nets (ITNs), long-lasting ITNs and the use of artemisinin combination therapy (ACT), which aim at transmission reduction. Monitoring and evaluation of malaria interventions requires accurate information on the remaining malaria burden in the community. The rapid diagnostic tests (RDTs) and light microscopy (LM) are the commonly used diagnostic tools for parasite detection and estimation of parasite prevalence rates in many resource-limited areas such as Tanzania. However, owing to the low detection limit of LM and RDTs of about 50-100 parasites/µL, their ability to capture low density infections is limited (Moody 2002; MalEra 2011). The use of molecular techniques to detect malaria parasites has been advocated to improve the accuracy of parasite prevalence estimates, especially in moderate to low endemic settings. This is because in areas of reduced endemicity, most infections occur at low densities and cannot be detected by the routine diagnostic tools. With a detection limit of about 0.034 parasites/µL of blood, molecular diagnostics are more reliable for parasite detection. In Tanzania, most of the parasites prevalence estimates have been performed by LM and RDTs, hence the most of the low density infections may remain undetected. Thus this thesis aimed to assess the usefulness of diagnostic methods for epidemiological studies by comparing the performance of routine and molecular diagnostics in parasite and gametocytes detection in community samples from Tanzania. Furthermore, the thesis investigated the occurrence of submicroscopic infections at different endemic sites in Tanzania.

For the above aims we conducted community surveys at 6 sites in Tanzania between 2011 and 2013. These sites were classified as low (Iringa), low urban (Dar-Es Salaam), moderate (coastal Tanga and Lugoba) and high (Rufiji and Morogoro) endemic sites according to district prevalence data recorded by the Tanzania HIV and Malaria indicator surveys of 2008 (THMIS 2009): A total of 2046 volunteers of all ages with signed consent forms were recruited. Finger prick blood was drawn from all individuals for parasite detection by LM,

RDT and 1*8S rRNA* qPCR. Gametocytes were detected by both LM and qRT-PCR targeting transcripts of the gametocyte specific expressed marker *pfs25*.

Generally**,** high *P. falciparum* Prevalence rates of 20% (416/2046; 95% CI 18-22%) by *18S rRNA* qPCR, 17% (349/2046; 95% CI 15.4-18.7%) by RDT and 11% (229/2046; 95% CI 9.8-12%) by LM were recorded in Tanzania. A substantial variation in molecular prevalence rates from geographically different sites was observed varying from 50% in the high endemic site, Rufiji, to 0.6% in the low endemic site, Iringa. These observed differences highlight the heterogeneity of transmission patterns in Tanzania attributed to geographical differences. Molecular parasite diagnostics unveiled that more than a half, 60% (249/416) of *P. falciparum* positive samples carried submicroscopic infections. Submicroscopic carriage was prevalent in all endemic settings. However, very few positive samples from areas of low and moderate endemicity impede a firm conclusion on the association of endemicity and submicroscopic carriage to be drawn from our samples. Molecularly determined Gametocyte prevalence was 15.3% (312/2046; 95% CI 13.6-16.8%) when data from all sites were combined. On the other hand, LM detected only 0.88% (18/2046; 95% CI 0.47-1.2%) of all samples implying only about 5% of the total gametocytes detected by molecular assay.

In conclusion molecular parasite detection revealed high parasite prevalence in Tanzania, such precise point prevalence molecular data obtained from community sampling may provide a more reliable basis of planning new tools of interventions or monitoring and evaluating the performance of existing tools in the country. Furthermore, high submicroscopic carriage of >50% in Tanzania, particularly in adults is key indicator of transmission potential of asymptomatic infections in Tanzania community and thus it is relevant for control strategies to focus on identifying submicroscopic carriers in order to successfully interrupt transmission.

Acknowledgements

I am ever grateful to the Almighty God for his constant rains of grace and blessings.

I am highly indebted to my financial sponsors the Dar Es Salaam University College of Education (DUCE) through the capacity building project-by the Science and technology and Higher Education project (STHEP) for sponsoring the first three years of my PhD, and the Swiss Tropical and Public Health Institute (SwissTPH) through which I was able to obtain stipend for the last year of my PhD studies from the Basel stipend commission and most importantly, for funding for laboratory analysis and field work during the entire period of my PhD. I remain grateful the Ifakara Health Institute (IHI) under the directorship of Dr. Salim Abdulla for providing a platform through which, I conducted my field work in Tanzania.

I recognize and appreciate the invaluable support from my supervisor Dr Ingrid Felger who has in special way instil in me her scientific passion and immense experiences, which I will cherish throughout my scientific career. I also thank her for being my support system during the entire PhD. I also thank, Dr. Steffan Borrmann from the University of Tübingen for being my Co-referee and taking his time to provide useful inputs in my study.

I am profoundly thankful to Professor Marcel Tanner for his patronage and his invaluable support in my PhD especially due to facilitating my fieldwork in Tanzania. *"ninakushukuru sana"*. I would like to thank Professor Hans-Peter Beck for his support during my PhD. Moreover, I would like to thank his family for the lovely and memorable Christmas parties which I always look forward to every time I am in Basel in December.

I wish to thank the Molecular Parasitology (MolPar) group for their support and collaboration particularly previous members such as Dania Müller, Sarah Javati for their laboratory technical assistance. I enjoyed the company of my colleagues Rahel Wampler and Natalie Hoffmann for their constant encouragement in both academics and social life even more during the write up time. I am indebted to Françoise Brand for constantly disturbing her with my orders, Sebastian Rusch for his scientific advice, Alex Oberli, Tereza Rezende, Beatrice Schibler, Musa Maganga and Pricscilla Rodrigues, Serej Ley, Christian Koepfli

Christian, Maira Bholla and Pax Masimba, Daniel Nyogea for the time we shared. I am grateful to Amanda Ross for statistical data analysis during my PhD. I must admit she was very patient and provided the best advice when needed. I also acknowledge the statistical assistance I received from my colleague Ali M. Ali. Furthermore, I would like to thank my fellow students with who I shared my Basel experience with them in the course of my studies there are too many to mention but I extend my sincere appreciation to all, Special thanks to my friend Jennie Jaribu for taking her time to proof read my work. I am very thankful to Christine Mensch and Doris Stamm their colleagues in the training office for providing assistance with academic and non-academic issues. Thanks to Margrit Slaoui, Christine Walliser, Zsuzsanna Györffy, Maya Zwygart and Dagmar Batra for assisting with administrative issues and the IT team for always being available to provide technical assistance.

I am thankful to the staff of Ifakara Health institute (IHI) - Bagamoyo whom I worked with during my PhD. Special thanks to Dr. Seif Shekalaghe and Michael Mihayo for their supervision during my fieldwork in Tanzania. I would like to thank all the laboratory technicians, drivers and fieldworkers from IHI.I also value the participation of all individuals who volunteered to be screen for in our surveys in Dar-es Salaam, Coastal Rufiji, Coastal Bagamoyo, Tanga, Morogoro and Iringa. I also thank the District Medical Officers (DMOs) of all the districts, local government leaders to hamlet leaders for taking their time to not only by allowing us to work in their respective communities, but also to mobilise people and personally be part of the cohort.

I acknowledge the encouragement from my friend and husband Ludovic Walter Toto Tarimo, to whom this thesis is dedicated. "*Nakushukuru kwa upendo wako wa dhati."* I extend my sincere gratitude to my family, my mama Esther for her love and constant encouragement my sister and confidante Catherine and brother in law Masunga Matondo with their beautiful daughter and my best friend (BFF) Ablah Nkwaya Masunga Matondo, my brother Walafried James Junior, for their prayers and constant encouragement " Nawapenda sana watu wangu wa nguvu.".

List of abbreviations

18S SSU rRNA	18S small subunit ribosomal RNA
DNA	Deoxyribonucleic Acid
LAMP	loop-mediated isothermal amplification
LM	light microscopy
LOD	limit of detection
MDA	mass drug administration
MGB	minor groove binder
mRDTs	malaria Rapid Diagnostic Tests
MSAT	mass screening and treatment
Msp	merozoite surface protein
PCR	Polymerase chain reaction
PNG	Papua New Guinea
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
QT-NASBA	Quantitative Nucleic Acid sequence Based Amplification
RNA	Ribonucleic Acid
SwissTPH	Swiss Tropical and Public Health institute
TARE-2	telomere associated repetitive element 2
TMHIS	Tanzania Malaria and HIV indicator Survey
TZ	Tanzania
*var*ATS	*var* gene acidic terminal sequence
WBC	White Blood Cells
WHO	World Health Organization
MOHSW	Ministry of health and social welfare

Introduction

Global malaria burden

Malaria is one of the major public health concerns due to the high morbidity and mortality associated with the disease. A total 104 countries globally are considered to be malaria endemic, leaving an estimated 3.4 billion people at risk of contracting the disease (Butler 2013). More than 207 million cases of malaria were recorded globally in 2012 (uncertainty range 135–287 million) and 627 000 deaths, 80% these cases and 90% deaths (occurred in Sub Saharan Africa and the highest mortality (77%) occurred among children less than 5 years old (WHO 2013).

Malaria is a protozoan infection caused by parasites of the genus *Plasmodium* of the phylum Apicomplexa. Five Plasmodium species are known to infect human namely *Plasmodium vivax, Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale* and most recently the *Plasmodium knowlesii which* was identified to infect humans (Singh et al. 2004; Antinori et al. 2013). *P*. *falciparum* and *P. vivax* are the most important species. *P. falciparum* is the most virulent due to its ability to attain high levels of parasitemia during in the life cycle. *P. falciparum* is responsible for most (91%) of the morbidity and mortality, due to its complications arising from parasite sequestrations in deep tissues.

Malaria poses a huge burden to humanity not only due to the high morbidity and mortality rates but also to huge economic burden. This ranges from poor attendance of school age children, reduction of work force and productivity by attending the sick, to cost of treatment of the disease. Globally, huge economic burden through the control and treatment of malaria has been documented (WHO 2103). For instance, up to 1.84 billion US dollars have been distributed to fight malaria in endemic countries of SSA alone in the last year (WHO 2013).

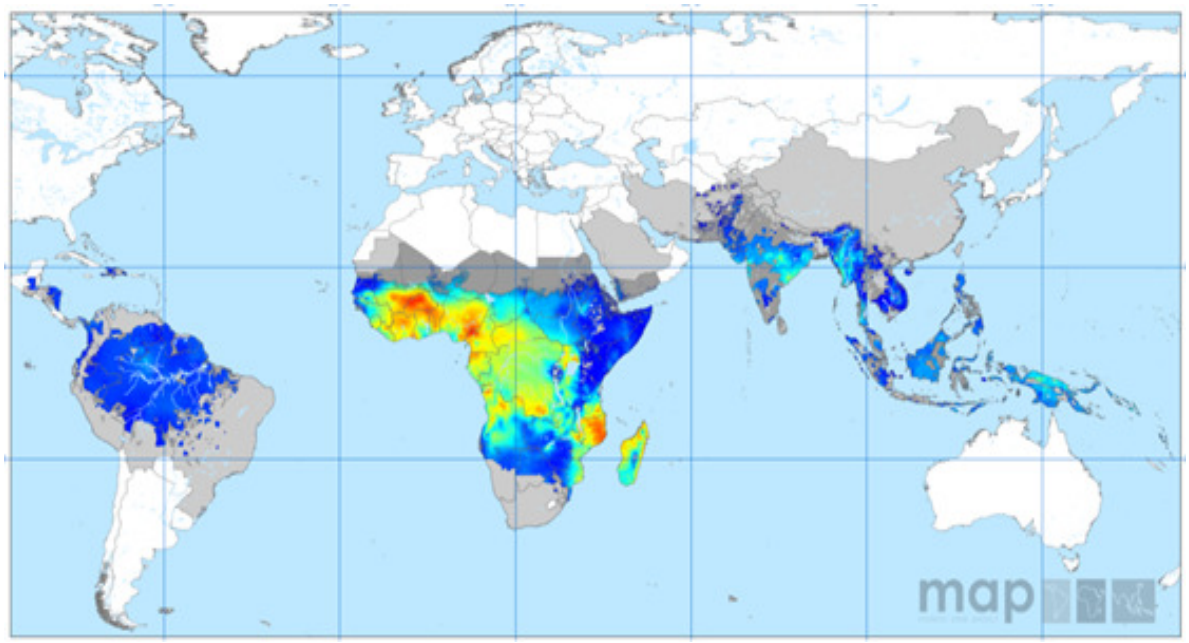

Figure 1: World map showing the spatial distribution of *P. falciparum* malaria endemicity in 2010 (blue indicates *PfPR* = 0% and red is PfPR = 70% (Gething et al. 2011). *http://www.map.ox.ac.uk/browseresources/endemicity/Pf_mean/world*

Malaria varies widely in epidemiology and clinical manifestation in different parts of the world. This variability depends on the species of malaria parasites that occur in a given area, the susceptibility to commonly used or available antimalarial drugs, the distribution and efficiency of mosquito vectors, climate and other environmental conditions, the behaviour and level of acquired immunity of the exposed human populations (Bloland 2001) and most importantly is the malaria control strategies in a given area.

Malaria Transmission-*P. falciparum* life cycle and gametocyte development

Malaria infection begins when a malaria-infected female mosquito genus Anopheles *S.L.* inoculates sporozoites into the bloodstream of a human host during a blood meal. Sporozoites migrate and infect liver cells and mature into schizonts. This cycle is known as the exo-erythrocytic schizogony cycle. Schizonts then rupture and release merozoites into the blood. The merozoites infect red blood cells where they undergo asexual multiplication resulting in the ring stage, trophozoites and finally produce schizonts which on rupture release more merozoites that continue to infect other red blood cells. This erythrocytic schizogony cycle occurs in the first 48 hours. On average, parasitemia of 50,000-500,000 per mm^3 blood and maximum parasitemia of 2.5 million per mm^3 blood can be produced; these blood stage parasites are responsible for clinical manifestations of the disease.

A small proportion about 1% of the asexual parasites differentiates into sexual stages (gametocytes) by a process called gametocytogenesis (Taylor and Read 1997). The mechanism that triggers this route is still debatable and is not limited to parasite intrinsic factors, chemical stress induced by the use of antimalarial drugs, fever and hematological disruptions (Drakeley et al. 2006; Ouédraogo et al. 2010). Commitment to the sexual pathway is believed to occur prior to the formation of the Schizont; descendants of a committed Schizont will develop into gametocytes and gametocytes produced from one sexually committed Schizont are of the same sex. *P. falciparum* gametocytes undergo five developmental stages of maturation. While stage I-IV gametocytes remain sequestered in deep tissues, only stage V is found in circulation. The mature stage V is the infectious stage (Babicker and Schneider 2008). Earlier stages I-II resembles the asexual stages, stage III-V shows distinct morphological changes and female and male gametocytes can easily be distinguished. During gametocytogenesis, molecular and biochemical levels are altered on protein level and several gametocytes specific mRNA are transcribed to enable the survival of gametocytes in once in the mosquitoes midgut. These transcribed gametocyte-specific mRNA include the *pfs16, pfg37, pfs25* and *pfs48/45* (Talman et al. 2004; Young et al. 2005; Drakeley et al. 2006 and Alano 2007).

Gametocytes are responsible for propagating transmission when ingested by an *Anopheles* mosquito during a blood meal. Male and female gametocytes develop into gametes, fuse and generate zygotes in mosquito's midgut. The motile zygotes (ookinetes) migrate to the midgut wall of the mosquito where they develop into oocysts. These grow, rupture, and release sporozoites which make their way to the mosquito's salivary glands. Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle. Gametocytes are important for perpetuating the transmission cycle. Therefore identifying and targeting gametocytes is of empirical importance for successful malaria control and elimination efforts (Babiker et al. 2008, Ouédraogo et al. 2009). Gametocytes naturally occur in lower densities under the limit of light microscopy (LM). The limit of detection of LM is roughly, 50 gametocytes / µL blood (Moody et al. 2002) and is most likely not to detect low gametocyte densities. Prevailing submicroscopic gametocytemia may contribute to transmission since they are known to also infect mosquitoes (Schneider et al. 2007). Other studies showed the submicroscopic gametocytemia showed a more than 10-fold lower infection rates in mosquito feeding experiments compared to gametocyte positive blood samples by LM and

thus able to propagate transmission (Coleman et al. 2004; Lin et al. 2014). Gametocyte identification and quantification in Tanzania in the past years was performed by light microscopy, a less sensitive tool compared to molecular detection (Drakeley 2000). Currently more sensitive assays have been developed for gametocyte detection by QT-NASBA and qRT-PCR which are able to detect less than 1 gametocyte/µL of blood (Babicker et al. 2008, Schneider et al. 2004). In recent years, a study was conducted in North Tanzania to assess gametocyte prevalence by molecular techniques (Shekalaghe et al. 2007); in this study, low gametocyte prevalence was in line with the low endemicity. Thus this thesis embarked to determine molecular –based gametocyte prevalence in crossectional survey in 6 regions of different endemic settings in Tanzania.

Malaria Burden in Tanzania

Malaria is transmitted by female mosquitoes of the genus *Anopheles*. In Africa the *Anopheles gambiae S.L* is the most important vector (WHO 2006; Hay et al. 2009). *A. Arabiensis and A. fenestus* are becoming increasingly efficient in various parts of Tanzania (Killeen at el. 2006; Lwetoijera et al. 2014). *P. falciparum* is the most prevalent species in Tanzania accounting to 98% of malaria cases in the country the rest of malaria burden is due to *P. malariae* and *P. ovale* species which are less than 0.5% of the cases (Tarimo et al. 2001; Mboera et al. 2008) and are hardly ever documented in most cases.

More than 90% of the 44 million people are at risk of contracting malaria in Tanzania. It is still the major cause of morbidity and mortality among outpatient and inpatient admissions especially children under five years and pregnant women (MOHSW 2010). Malaria transmission is reported in many parts of the country throughout the year although it is more frequent during and after the rainy season (April to June) (Figure 2).

A review of entomological inoculation rate (EIR) in Africa showed Tanzania had a mean EIR of 367 ranging (94-667) (Hay et al. 2000). Individual studies in various parts of Tanzania recorded, EIR ranges of 3-4 infectious bites per year in a low endemic area of the Usambara highlands (Oesterholt et al. 2006), a range of 29-78 in Ifakara (Drakeley et al. 2003; Russell et al. 2010). Moreover, a review of EIR recorded mean EIR of 285 in Tanzania as among the highest in sub-Saharan Africa (Kelly-Hope and Mackenzie 2009). These high EIR indicate high malaria burden in various areas of Tanzania.

Tanzania records a decline of malaria prevalence rates through national malaria indicator surveys conducted every four years. For instance, the national average malaria prevalence rate declined from 21% in 2006 to 18% by 2008. A further 50% decline was observed in the latest surveys to a national average prevalence of 9% in 2012 (MOHSW 2006; THMIS 2009; 2013). This decline in malaria burden is attributed to countrywide malaria interventions including insecticide residual spraying, mass distribution of both, insecticide treated nets (ITNs) and long lasting ITNs. In addition, the change in malaria policy to introduce the Artemisinin-based Combination Therapy (ACTs) as the first line drug in the treatment of malaria occurred in 2006. ACTs affect the asexual stage parasites and young gametocytes and hence contribute towards transmission reduction (Bousema et al. 2010; Sawa et al. 2013). The data from national malaria indicator survey show huge differences in malaria endemicity of about 80-fold differences ranging from 0.4% to 32% within regions of Tanzania (Figure 2). The different endemic settings are mainly due to geographical and environmental conditions. Regions with high altitudes and cooler temperatures (Iringa, Njombe and Mbeya), semi-arid areas such as (Singida and Dodoma) which do not favour the survival of the mosquitoes have lower malaria prevalence rates compared to warmer areas (Lindi, Mtwara and Mara) (Figure 2).

Malaria Prevalence in Tanzania is mostly measured by classical light microscopy (LM) (Drakeley et al. 2000; THMIS 2008; Mmbando et al. 2010; Ishengoma et al. 2013). Recently LM has been complemented by malaria rapid diagnostic tests (mRDTs) (THMIS 2013). RDTs are currently being scale-up for parasite confirmation in all health centres. However the supply chain of RDT to peripheral health facilities is still a challenge.

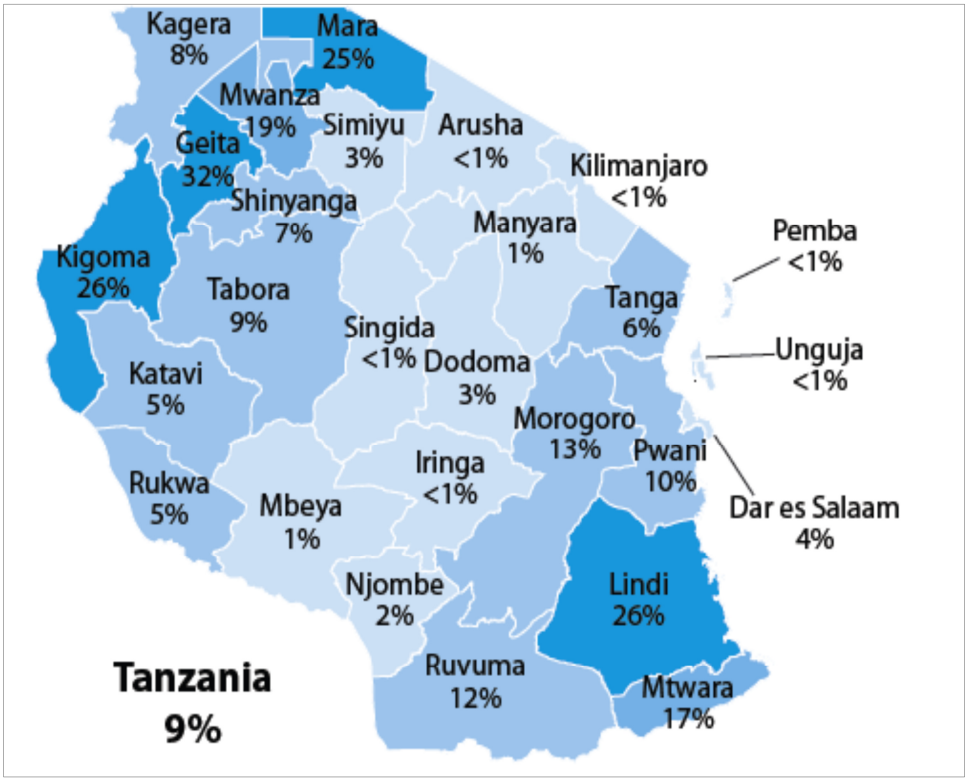

Figure 2: Malaria prevalence rates in children under five years in Tanzania national malaria indicator survey of 2011/12 (THMIS 2012)

Malaria Diagnosis and challenges

Early diagnosis and prompt treatment are essential for malaria management. LM is the standard diagnostic method of choice in many resource constrained areas in Sub-Saharan Africa. In the absence of such classical technique, diagnosis based on signs and symptoms of malaria is performed. The diagnosis based on signs and symptoms of malaria is presumptive and recommendation of parasite confirmation by laboratory diagnosis is advocated (Penhabel *et al,* 2005; MalEra 2011, WHO 2013).

LM is limited by a low detection the limit of detection (LOD) of LM is estimated at 50-100 parasite /µl blood hence low density infections are likely not detected (MalEra 2011). In fact the sensitivity of any diagnostic method is dependent on the volume of blood and parasitemia (Wampfler et al. 2013). LM uses a volume of blood of about 0.025 to 0.2 µl (Okell et al. 2009) which limits it`s sensitivity. Several other shortcomings of LM have been documented including the incorrect interpretation of blood films (Kahama - Maro et al. 2011), lack of expertise and inadequate quality control (Molyneux *et al,* 1993; WHO 1996).

In recent years, rapid diagnostic tests (RDT), which target specific *Plasmodium* antigens mainly the histidine rich proteins (HRP), *Plasmodium* lactate dehydrogenase (pLDH) and Aldolase enzyme in blood of infected humans, have been introduced. The RDTs are easier to use and their sensitivity and specificity in field settings have been established in many studies (Craig et al, 1997; Moody et al, 2002; Mueller et al, 2006). Similar to LM, RDT attains a limit of detection (LOD) of 50-100 parasites/µL blood (WHO 1996; Moody et al. 2000). The RDTs have been integrated in many health systems and used for malaria parasite detection at health care facilities for parasite confirmation prior to malaria treatment as recommended by WHO (WHO 2008, WHO 2013). Currently, RDTs are widely used in community surveys; however, due to their low LOD their performance in low endemic field settings is debatable (WHO 2000). Shortcoming of RDTs lies in its inability to quantify the parasite load, the ability of pLDH based- RDT to detect gametocytes confound treatment outcomes (Mueller et al, 2006) and generation of false positives due to residual HRP antigen even after parasite clearance (Bell et al. 2005; Batwala et al. 2010).

Low sensitivity both LM and RDTs can underestimate the malaria burden especially in areas of declining transmission. In order to precisely estimate malaria burden in Tanzania, this PhD thesis embarked on using highly sensitive molecular assays for parasite detection in order to obtain more precise estimates of malaria burden in Tanzania.

Molecular detection of *P. falciparum* parasite infections and gametocytes

Molecular assays for parasite and gametocyte detection have been introduced to several laboratories in endemic countries and are increasingly applied in interventions and epidemiological field surveys (Andrade et al. 2010; Kamau et al. 2011; Mosha et al. 2013).

These include the DNA based quantitative polymerase chain reaction (qPCR) and the RNA based quantitative reverse transcriptase PCR (qRT-PCR). RT-PCR involves the reverse transcription of RNA into cDNA and further amplifies the cDNA. The qRT-PCR uses Taqman® or SYBR® chemistry. The assay uses two primers and a minor grove binder (mgb) labeled probe. The reverse transcription makes use of the reverse primer, further a combination of both reverse and forward primers are used in the proceeding cDNA amplification. The reaction is prone to gDNA contamination hence it requires that the RNA is treated by and RNase free DNase before amplification. The qPCR and qRT-PCR are sensitive assays with ability to detect between 0.1 -0.034parasites/µL blood (Babicker et al. 1999; Abel-Wahab et al. 2002; Babicker et al. 2008; Schneider et al. 2007; Rosanas-Urgell et al. 2010; Bousema et

al. 2011; Kamau et al 2011; Wampfler et al. 2013 and Hofmann et al. 2014). The high sensitivity of molecular assays makes these assays more reliable for parasite detection (Maeno et al. 2008).

Other molecular assays include the quantitative nucleic acid sequenced based amplification (QT-NASBA) is the most sensitive assay in RNA detection and amplification compared to the qRT-PCR assay (Schneider et al. 2004). The QT-NASBA is an isothermal process at 41°C allowing a specific amplification RNA only even in the presence of DNA double strand which does not denature at 41°C. The amplification of single–strand sequences uses three enzymes namely the virus based AMV- Reverse transcriptase, RNaseH and T7 polymerase. One of the two primers carries the T7 promoter sequence. Using fluorescent probe the amplification data is collected in real time. The LOD of QT-NASBA is 0.02parasites/µL blood (Schneider et al. 2004; Mens *et al.* 2006) and its advantage over qRT-PCR is that it is affected by genomic DNA (gDNA) contamination in RNA.

Recently, the Loop-isothermal mediated DNA amplification (LAMP) molecular assay was introduced in the market (www.finddiagnostics.org). It is increasingly advocated to be used as point of care molecular malaria diagnostic tool. The performance of LAMP technique has already been tested in field surveys such as in Zanzibar-Tanzania and has shown to perform better than LM in the field (Aydin-Schmidt et al. 2014). LAMP has a detection limit of > 5parasites/µL blood. To increase sensitivity LAMP uses four primers; two outer and inner forward primers and two outer reverse primers. LAMP is isothermal operating at 65°C and has a shorter turn over time of about 15- 20 minutes. However, its inability to quantify parasites giving results by turbidity or fluorescent is a major setback of LAMP technique. Furthermore, the sensitivity of LAMP is the field in areas with low parasitemia is still debatable.

In light of the merits of molecular diagnostic tools, our study was designed to compare *P. falciparum* parasite and gametocyte carriage prevalence rates determined by LM and RDT with molecular tools in community samples in Tanzania. The higher sensitivity of PCR-based techniques can be used to assess the extent of underestimation of parasite prevalence rates.

Context of the Study

This study was conducted within the framework of two projects within the Ifakara Health Institute (IHI) (i) Artemether Lumefantrine In Vulnerable patients: Exploring health impacts (ALIVE) that have been going on for about 8 years in Kilombero and Ulanga (K-U) Districts in Morogoro and (ii) Sentinel Panel District (SPD) initiated in 2009 in 27 districts in Tanzania. This research was designed to answer very important questions that arose in the course of these on-going projects. Protocols amendments to enable blood sampling for molecular diagnosis within established in the projects were reviewed and approved by the Ifakara Health institute -ethical review board.

ALIVE project

The Ifakara Health Demographic Surveillance system (IHDSS) covers a rural population of about 84,000 people in 22,000 scattered rural households (Figure 3). Household surveys are conducted every 4 months and data on pregnancies, births, deaths, in and out migrations are recorded. The ALIVE project had over 800 households under surveillance within the IHDSS from K-U districts. Primary aim of the ALIVE project was to assess the impact on all-cause mortality in infants/children below 5 years,

Malaria prevalence in the K-U district was 25% during the first round of ALIVE study in 2005. By 2006 malaria prevalence assessed by LM had declined to 13.4%. This decline coincided with the national change on malaria policy which introduced Antimalarial combination therapy (ACTs) as first line drugs for malaria treatment. In the following years of survey RDTs were used alongside LM in the surveys. Malaria prevalence in 2007 was 11.1% by LM and 12% by RDT. A further decline to prevalence rates of 4.7% by LM and 3.9% by RDT in 2010 was observed. This decline of transmission was partly attributed to the use of ACT – Artemisin based antimalarial which has some gametocidal effects (Kabanywanyi PhD thesis 2012). However, in the entire 7 years of surveys (2004-2010) in the K-U districts molecular detection of parasitemia was not applied alongside the classical LM and RDT.

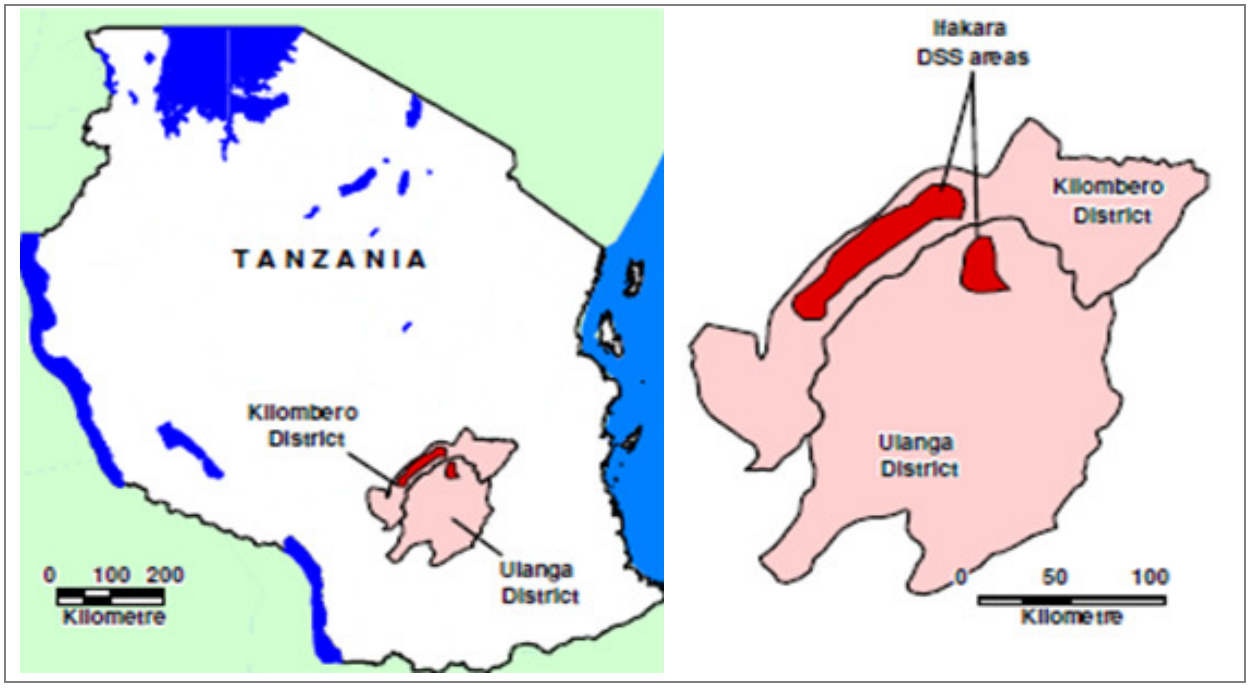

Figure 3: Map of Tanzania showing the K-U districts and the IHDSS shown in red.

Courtesy of the Ifakara Health Demographic Surveillance System www.ihi.eprints.org

Sentinel District Panel

The SPD initiative was newly established in 2009 by the IHI in collaboration with National Bureau of Statics (NBS), National Institute of Medical Research (NIMR) and Ministry Of Health and Social Welfare (MoHSW). The over-arching goal of SPD is to provide a sustainable source of reliable, national data to meet the monitoring needs of program managers, policy-makers and funding partners. In addition, it offers a national, integrated platform for impact evaluation and research. The panel comprises 23 districts (Figure 4), sampled to represent Tanzania Mainland, plus the 4 districts (Kilombero, Ulanga, Rufiji, Kigoma Urban), where IHI already operates demographic surveillance (Figure 3).

The SPD project covers a population of around 800,000 people in 167,000 households (Figure 4). The study collects birth and death registers and verbal autopsy by questionnaires in order to produce annual estimates of age- and cause-specific mortality. Field work of this PhD project was carried out in selected 6 SPD districts with different malaria endemicity (Table 1).

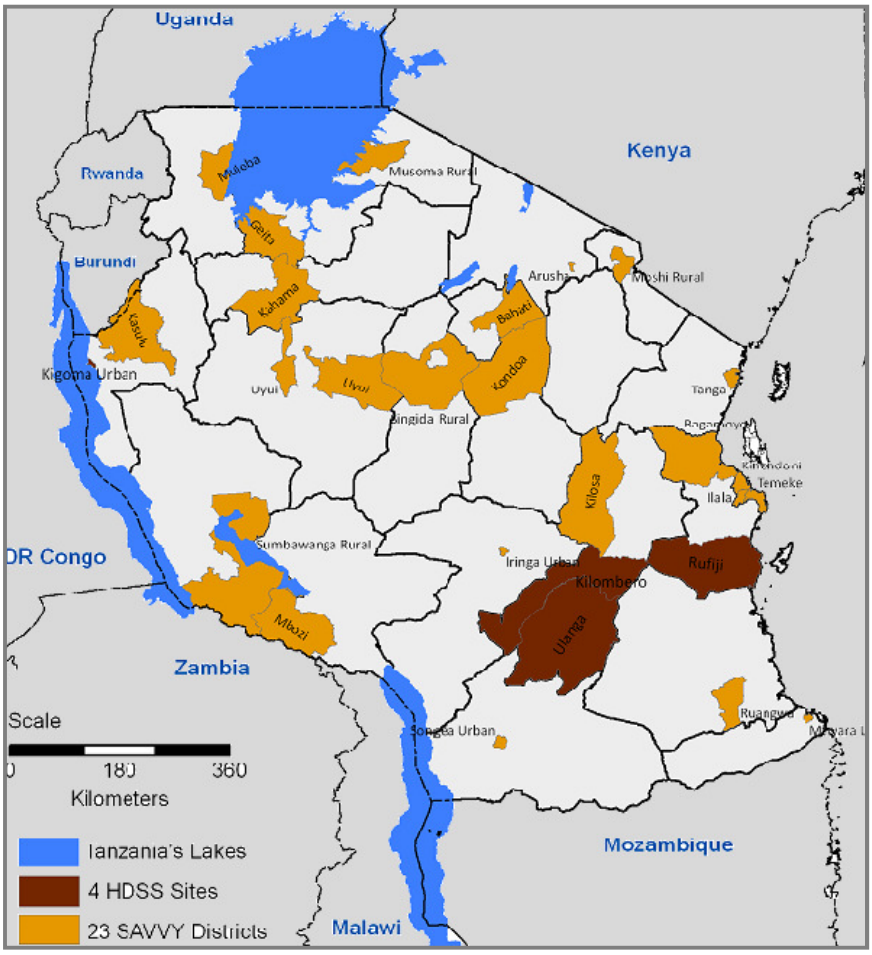

Figure 4: The Sentinel Panel Districts of– Tanzania. Courtesy of Ifakara Health Institute (IHI) spotlight issue No. 8.

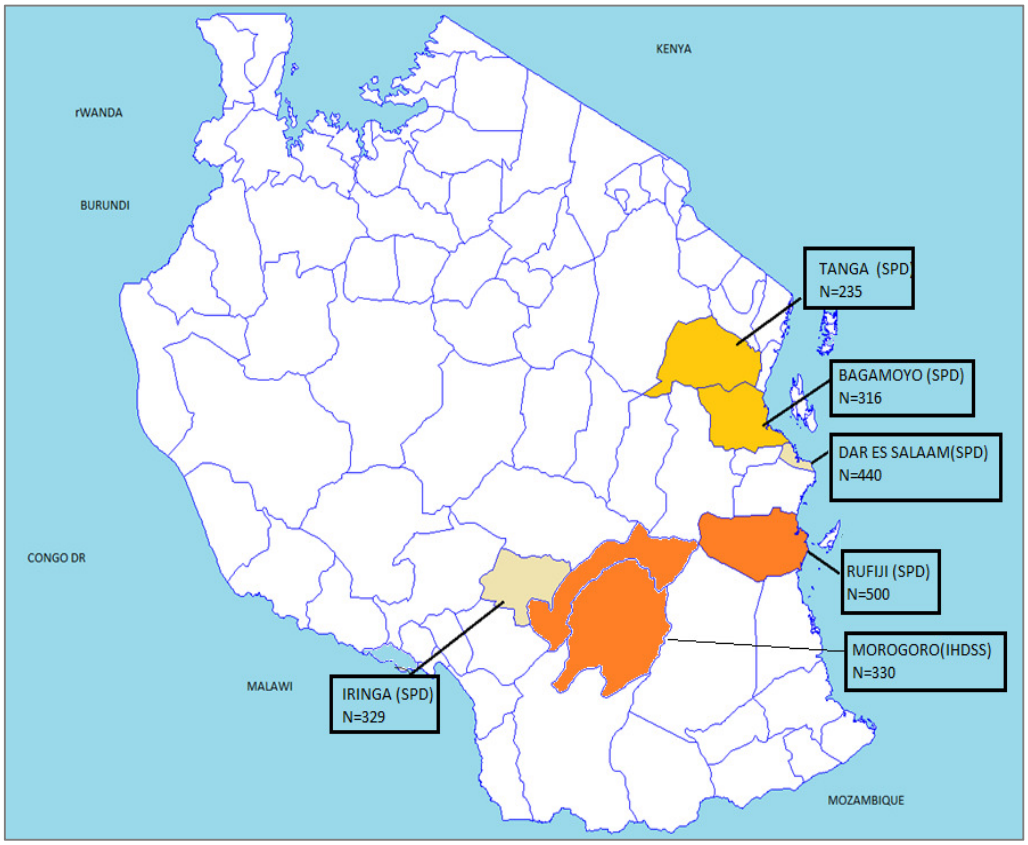

Figure 5: Map of Tanzania showing 6 selected regions within the SPD for this study with sample size from each region. Grey depicts low, Yellow -moderate and Orange depicts high endemic sites.

Table 1: The 6 selected regions from the SPD categorized into settings of different malaria endemicity using previously available prevalence data from national malaria indicator surveys (THMIS 2008).

No.	Region	THMIS-Malaria prevalence 2008	Time of sampling	sample size	malaria endemicity
1	Dar es salaam	1.2%	2013	440	
2	Iringa	3%	2013	329	Low
3	Coastal Lugoba	10%	2013	316	
4	Tanga	14%	2013	235	Medium
5	Morogoro	16%	2011	330	
6	Coastal Rufiji	21%	2013	500	High

Study Goal, Aims and Objectives

I. The main goal of this thesis was to evaluate the extent of underestimation of *P. falciparum* infection and gametocyte prevalence by the routine diagnostic tools in Tanzania (RDT and LM). For this evaluation molecular diagnostic assays were used as a gold standard.

In the course of optimizing parasite diagnostics we aimed at exploring parasite infections of very low density that are missed by most techniques, even by PCR. A novel approach to increase test sensitivity even beyond classical PCR was validated in a subset of the field samples collected. Parasite positivity results from two new highly sensitive *P. falciparum* parasite detection assays were compared to both, classical and standard molecular diagnosis.

II. Secondary objective was to identify how the diagnostic methods perform in various endemic settings in Tanzania. For this, parasites were detected in 5 selected regions: Dar es Salaam (Urban), Iringa (Low) Coastal Bagamoyo and Tanga (Moderate), Coastal Rufiji (High).

III. A further objective was to evaluate various methods of RNA sampling strategies for gametocyte detection. For this, samples from a pilot project cross sectional survey of mostly asymptomatic children (5-9) years in Papua New Guinea (PNG) were used.

The relevance and impact of this work consists in the provision of accurate prevalence estimates for evaluating and improving existing malaria interventions and to plan for new intervention strategies in the ongoing elimination attempts in Tanzania.

Research questions and rationale for specific research objectives

I. Assessment of *P. falciparum* parasite prevalence and density by classical and molecular diagnosis

Qn**:** *How useful are classical tools for malaria diagnosis?*

Most epidemiological surveys in Tanzania are performed by routine diagnostic tools LM and RDT, owing to their easy use and cheap costs. Prevalence data from RDT and LM will enable comparison with previous available data for instance, the national malaria indicator surveys and other epidemiological studies. We aimed to describe the malaria burden in Tanzania in 2012-13, a total of 2893 samples were collected in all 6 regions in Tanzania in the course of this study. Routine diagnostics was applied establish parasite prevalence rates in these regions of different malaria endemicity. LM provides asexual parasite densities which can serve to describe the age trends in parasite burden.

Qn: What are advantages offered by molecular diagnosis?

It is well established that routine diagnostic tools tend to underestimate the true prevalence rates, due to their inability to detect lower parasitemia. Lower parasitemia is characteristic in populations with acquired immunity as a result of frequent exposure to infection in endemic areas. Moreover, in areas of with interrupted transmission and declining endemicity parasitemia tends to be maintained at lower levels. Such low parasitemia remain undetected by LM and RDT. Therefore, we aimed to quantify the extent of underestimation of parasite prevalence, by determining *P. falciparum* parasite prevalence by molecular–based quantitative polymerase reaction (qPCR) targeting the *P. falciparum S- type 18S rRNA* gene.

Qn: Why is sensitive detection of gametocytes important?

Gametocytes usually persist in low densities to ensure transmission success. These low densities are likely to be missed by classical LM. Moreover studies have shown that even LM negative individuals are able to infect mosquitoes. Therefore, in order accurately estimate the gametocytemic proportions of individuals in the community all samples were further analyzed for gametocyte by LM and molecular detection targeting gametocytes - specific marker *pfs25*. Our aim was to establish and compare gametocyte prevalence rates in

Tanzania. Moreover, the improved gametocyte prevalence rates by molecular diagnosis enabled to us to identify the proportion of submicroscopic gametocytemia in different endemic settings in Tanzania. The submicroscopic gametocytemia may substantially contribute the infective reservoir.

Qn: can we further improve malaria diagnosis?

Moreover, in the course of the PhD thesis development, novel ultra-sensitive assays for *P. falciparum* parasite detection were developed targeting high-copy subtelomeric sequences of *P. falciparum* the Telomere Associated Repeat Element 2 (TARE-2), and the *var* gene Acidic Terminal Sequence (*var*ATS). These ultra-sensitive assays had a LOD 10-times higher than the 18s rRNA gene qPCR assay we routinely used in the laboratory. We compared these assays to a standard *P. falciparum* detection qPCR amplifying the S18S rRNA gene. We aimed to increase diagnostic sensitivity in field samples.

II. Comparison of malaria endemic sites in Tanzania

Qn: Does endemicity impact malaria prevalence rates?

Parasite prevalence and density are known to increase with transmission intensity. Imperfect diagnostic tools (LM) are likely not to detect lower parasitemia resulting in large proportion of submicroscopic infections in low endemic settings. Using highly sensitive molecular tools (quantitative polymerase chain reaction (qRT-PCR) & quantitative PCR (qPCR) and light microscopy (LM), we aimed to establish the proportion of submicroscopic infections (parasitemia and gametocytemia) in areas of varying endemic settings: Dar Es Salaam (Urban), Iringa (Low) Coastal Bagamoyo and Tanga (Moderate), Coastal Rufiji and Morogoro (High) in Tanzania.

III. Molecular detection of gametocytes in community samples

Qn: Do RNA sampling methods and storage conditions affect gametocyte detection?

RNA sampling, extraction and storage is difficult because RNA is unstable and easily degraded by RNases resulting into low integrity RNA. In a pilot project, we compared different blood sampling methods and storage conditions of whole blood for later gametocyte detection by qRT-PCR. Blood samples from a cross sectional survey of children

from PNG were used to define the best strategy for gametocyte detection from field samples.

Thesis Outline

This thesis consists of 6 chapters.

Chapter 2 discusses the comparison the various *P. falciparum* parasites and gametocytes detection in community samples in the Kilombero and Ulanga districts in Tanzania.

Chapter 3 focuses on the prevalence of submicroscopic infections in a community surveys in 5 regions of varying malaria endemicity in Tanzania. In light of increasing sensitivity in parasite detection

Chapter 4 discusses on the ultrasensitive *P. falciparum* parasites from field samples.

Chapter 5 introduces the various strategies for RNA sampling methods and gametocyte detection in field samples.

Chapter 6: Summarizes, discusses, and concludes the study presented in the thesis and suggests directions for future work.

The appendix shows my contributions in other projects in course of this study.

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Comparison of detection methods to estimate asexual *Plasmodium falciparum* parasite prevalence and gametocyte carriage in a community survey in Tanzania

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This chapter is under review in the Malaria Journal

ABSTRACT

Introduction

The use of molecular techniques to detect malaria parasites has been advocated to improve the accuracy of parasite prevalence estimates, especially in moderate to low endemic settings. Molecular work is time-consuming and costly, thus the effective gains of this technique need to be carefully evaluated. Light microscopy (LM) and malaria rapid diagnostic tests (mRDT) are commonly used to detect malaria infection in resource constrained areas, but their limited sensitivity results in underestimation of the proportion of people infected with *Plasmodium falciparum*. We aimed to evaluate the extent of missed infections via a community survey in Tanzania, using polymerase chain reaction (PCR) to detect *P. falciparum* parasites and gametocytes.

Methods

We enrolled 330 individuals of all ages from the Kilombero and Ulanga districts (Tanzania) in a cross-sectional survey. Finger prick blood samples were collected for parasite detection by mRDT, LM and molecular diagnosis using quantitative *18S rRNA* PCR and *msp2* nPCR. Gametocytes were detected by LM and molecularly by amplifying transcripts of the gametocyte-specific marker *pfs25*.

Results

Results from all three diagnostic methods were available for a subset of 226 individuals. Prevalence of *P. falciparum* was 38% (86/226; 95% CI 31.9 - 44.4%) by qPCR, 15.9% (36/226; 95% CI 11.1 – 20.7%) by mRDT and 5.8% (13/226; 95% CI 2.69- 8.81%) by LM. qPCR was positive for 72% (26/36) of the mRDT-positive samples. Gametocyte prevalence was 10.6% (24/226) by *pfs25*-qRT-PCR and 1.2% by LM.

Conclusions

LM showed the poorest performance, detecting only 15% of *P. falciparum* parasite carriers identified by PCR. Thus, LM is not a sufficiently accurate technique from which to inform policies and malaria control or elimination efforts. The diagnostic performance of mRDT was superior to that of LM in our survey. However, it is also insufficient when precise prevalence data are needed for monitoring intervention success or for determining point prevalence

rates in countrywide surveillance. Detection of gametocytes by PCR was 10 times more sensitive than by LM. These findings support the need for molecular techniques to accurately estimate the human infectious reservoir and hence the transmission potential in a population.

Background

Records of Tanzanian malaria indicator surveys show a general decline in malaria prevalence among children under 5 years, from 18% in 2008 to 9% in 2012 [1, 2]. This decline has been attributed to countrywide implementation of malaria interventions, including indoor residual spraying (IRS), mass distribution of insecticide treated nets (ITNs), long-lasting ITNs and the use of artemisinin combination therapy (ACT), which effectively kills both asexual blood stage parasites and immature gametocytes, thereby reducing transmission [3, 4].

Early diagnosis and prompt treatment are essential for appropriate malaria management. The World Health Organization (WHO) recommends laboratory confirmation of malaria before treatment, either by microscopy or by immuno-chromatographic malaria rapid diagnostic test (mRDT) [5]. Accurate malaria diagnosis is not only important for case management but also for estimating parasite prevalence in community surveys. Light microscopy (LM) is a standard tool for malaria diagnosis in resource constrained areas such as Tanzania. However, its performance is limited due to a lack of expertise and its low limit of detection (LOD) of about 50 parasites/µL of blood, which does not allow detection of low parasite densities [6, 7]. Although expert microscopists can attain a LOD of around 20 parasites/µL of blood [8], such high sensitivity is hardly ever achieved in field settings. mRDTs are easier to use and their sensitivity is comparable to that of LM in the field [9, 10]. Currently, mRDTs are widely used in community surveys but, owing to a low LOD, their performance in low endemic field settings is limited [11].

Recently, molecular tools for parasite detection have been introduced in many laboratories in endemic countries and are increasingly applied in monitoring interventions and epidemiological field surveys [12, 13, 14, 15]. These molecular assays have LODs between 0.34-0.002 parasites/µL of blood, which results in more sensitive and reliable parasite detection. Due to their higher sensitivity, PCR-based techniques can be used to assess the extent to which parasite prevalence has been underestimated in endemic settings such as Tanzania, where malaria prevalence is routinely measured by classical LM [1, 16, 17, 18], complemented in recent years by mRDTs [2]. So far, only a few studies in Tanzania have applied molecular techniques for blood stage parasite detection and even fewer for gametocyte detection [19, 20, 21, 22]. Therefore, we aimed to compare *P. falciparum*

parasite and sexual stage prevalence rates as determined by LM and mRDT with those obtained using molecular techniques, thereby assessing the usefulness of these different methods for epidemiological studies in Tanzania.

Methodology

Study site and design

The study was conducted in the Kilombero and Ulanga (K-U) districts in Morogoro region in southeast Tanzania. The Ifakara Demographic Surveillance System (IHDSS) covers the study area [23]. The districts are primarily rural. Transmission of malaria is perennial with two rainy periods: from October to December and from March to May. The K-U districts were among the first areas in Tanzania to implement several malaria intervention strategies. The Kilombero Net project (KINET) successfully distributed ITNs, attaining 91% coverage by late 2000 [24]. This programme led to a fourfold reduction in entomological inoculation rates (EIR) [25] to about 78 infectious bites per year [26].

Our study was conducted as an extension of the Artemether-Lumefantrine In Vulnerable Patients: Exploring Health Impacts (ALIVE) project. Its main aim was to assess the impact of introducing ACT as a first line antimalarial treatment on all-cause mortality in infants/children under 5 years of age in the K-U districts.

A cross-sectional survey was performed between May and August 2011. Randomly selected households within the IHDSS were surveyed. A subset of 330 randomly selected individuals of all ages was included in the molecular analysis. The study was granted ethical clearance by the Ifakara Health Institute (IHI) and by the National Institute for Medical Research Tanzania.

Blood collection and sample storage

Finger prick blood was used to diagnose malaria positivity by (i) mRDT SD Bioline Pan-pLDH/Pf-HRP2, (ii) blood smear and LM and (iii) PCR-based molecular diagnosis. Approximately 50µL of whole blood were collected on Whatman® grade-3 filter paper, air dried in the field and stored at ambient temperature in separate sealed plastic bags with desiccant. Two blood spots on filter paper were prepared per individual, one of which was put in 300µL TRIzol® (Invitrogen) to stabilize RNA and stored at -80°C. Samples in TRIzol® were shipped by air on refrigerant gel packs to the laboratory responsible for DNA and RNA extraction. RNA was extracted from 330 samples using the Qiagen RNeasy Plus ® protocol

with on-column DNase digestion, to ensure removal of genomic DNA (gDNA) as described elsewhere [14]. RNA was stored at -20°C for a maximum of two weeks prior to cDNA synthesis and amplification. One additional blood spot per patient was air-dried and preserved in a sealed plastic bag with desiccant at -20°C until shipped at room temperature. DNA was extracted from 226 dried blood spots using the Chelex protocol [27]. DNA was stored at -20°C for one to two weeks until used in PCR.

Microscopy blood smear reading

Thick and thin blood films were prepared in the field, air dried, Giemsa stained and read for detection and quantification of malaria parasites according to Standard Operating Procedures at the IHI laboratory. Asexual parasites were reported out of 200 leukocytes. Gametocyte detection by LM was based on a volume of blood corresponding to 500 leucocytes. Assuming 8,000 leucocytes/µL blood, parasite density (expressed/ as parasites per µL blood) was calculated by multiplying LM counts by a factor of 40 if parasites were reported out of 200 leukocytes or by 16 for 500 leukocytes. Two independent qualified technicians read all slides. In case of discrepancy between two readers, a third reader was requested. The final result was the mean of the two closest readings out of three. For cases of positive/negative discrepancy the majority decision was adopted.

Molecular assays

A qPCR targeting the *P. falciparum* S-type 18S rRNA genes was performed on all DNA samples to determine parasite prevalence [28]. As a reference, a nested PCR (nPCR) targeting the merozoite surface protein 2 (msp2) was performed on all DNA samples [29]. Gametocytes were detected by amplifying transcripts of the gametocyte-specific expressed marker *pfs25* [14]. *pfs25* transcripts were reverse transcribed and the resulting *pfs25* cDNA was amplified by qPCR. The RNA-based quantitative reverse transcriptase PCR (qRT-PCR) assay was performed on all extracted RNA samples after complete gDNA removal had been confirmed by a qPCR assay targeting 18S rRNA genes of all *Plasmodium species* [14]. To quantify *P. falciparum* parasites and gametocytes, copy numbers of the respective template per µL blood were calculated using standard curves obtained from assay-specific plasmids routinely included on each 96-well qPCR plate.

Data analysis

All data was entered and analyzed by STATA® version 13, Texas, USA. To compare the performance of different diagnostic tests, concordance of results was recorded. Parasite density/µL blood and marker-specific template copy number/µL blood were converted to $\log_{10}$.

Results

This community survey included 330 individuals, the mean age was 18 years with an age range of 1 – 81 years. Of these, 21% were children <5 years, 44% were between 5-19 years. Individuals between 20-59 years and adults >60 years accounted for 30% and 4.5% of recruited individuals, respectively. A complete dataset including all four diagnostic methods was obtained for 226 participants and used to compare test performance.

P. falciparum prevalence and density

Prevalence of *P. falciparum* blood stages in the K-U districts was 38% (86/226; 95% CI 31.9-44.4%) by *Pf18S rRNA* qPCR. A lower parasite prevalence of 26.6% (60/226; 95% CI 19–31.2%) was observed when *msp2* nPCR was performed. Of *msp2* positive samples, 83.3% (50/60) were confirmed by *Pf18S rRNA* qPCR. Only 58% (50/86) of *Pf18S rRNA* qPCR-positive samples were positive by *msp2* nPCR (Table 1). Thus, sensitivity of qPCR was superior to that of standard nPCR.

P. falciparum prevalence was 15.9% (36/226; 95% CI 11.1 – 20.7%) by mRDT and 5.8% (13/226; 95% CI 2.69- 8.81%) by LM. mRDT was positive for 8/13 (61.5%) and qPCR for 11/13 (84.6%) of LM positive samples. Only 2/13 (15.4%) LM-positive samples were unidentified by both mRDT and qPCR, suggesting that these two LM results were false positives (Table 2). Of 36 mRDT-positive samples, 24 (66.7%) were also positive by qPCR, whereas the remaining 30% of mRDT-positive samples were negative by qPCR and LM.

LM recorded a mean of 13,483 parasites/µL (range 80 to 64,640). *Pf18S rRNA* qPCR detected a mean of 6,524 *18S rRNA* gene copies/µL (range 0.9 to 155,293). *18S rRNA* copy numbers were not converted into parasite counts because trend-line experiments using ring stage parasites were not performed for filter paper blood spots with similar storage conditions.

Moreover, original blood spots varied in size and thus whole blood content also varied. A non-linear correlation was observed between $\log_{10}$ parasite density by LM and $\log_{10}$ *18S rRNA* gene copy numbers/µL of blood for all microscopy positive samples (Figure 2).

Gametocyte prevalence

Gametocyte prevalence was determined by LM and qRT-PCR in 226 samples. Gametocyte carriage in our study population was 10.6% (24/226; 95% CI 6.6-14.7%) by qRT-PCR and 1.2% (3/226; 95% CI 0.2-2.8%) by LM. Two of the three gametocyte carriers identified by LM were confirmed by molecular gametocyte detection. A large proportion of gametocytemia (87.5%; 21/24) was submicroscopic.

The proportion of molecularly identified gametocyte carriers among *P. falciparum* positive individuals is listed in table 3 for all four independent diagnostic tests (LM, mRDT, *msp2* nPCR and *18S rRNA* qPCR). In total, 3/13(23%) LM-positive and 9/36 (25%) mRDT-positive *P. falciparum* infections harbored gametocytes detected by *pfs25* qRT-PCR. In individuals deemed positive by *msp2,* we detected 12/60 (20%) gametocyte carriers. This proportion was slightly higher than in individuals deemed positive by the more sensitive *18S rRNA* qPCR, with only 16.2% (14/86) of infections harboring gametocytes.

Discussion

Accurate estimation of malaria burden after implementation of effective malaria control programmes is of particular importance for evaluating and planning further intervention strategies. Accuracy of the diagnostic tests applied and knowledge of their limitations are essential. Therefore, we evaluated the performance of LM and mRDT, the routinely used methods for estimating *P. falciparum* prevalence in the community, and compared it with that of qPCR for determining parasite positivity. Our results highlight the poor sensitivity of LM and the high prevalence of submicroscopic infections. Malaria prevalence in the K-U districts is vastly underestimated, if detection is based on LM only.

In many parts of Tanzania, LM is still widely used as the standard parasite confirmation method because supply of mRDTs is unreliable owing to stock-outs. With the increasing success of interventions and as a consequence of reduced clinical malaria, it becomes

increasingly important to determine prevalence rates in the community to estimate the remaining malaria burden and to monitor the effect of sustained control measures. In this context, the sensitivity of the diagnostic method, which greatly influences prevalence determination, becomes increasingly important.

We observed a sevenfold difference between parasite prevalence estimated by qPCR and that estimated by LM. Other studies in Thailand, Myanmar [30, 31] and Malawi [32], as well as a systematic review [33] have also reported more than two- to fivefold difference in asexual stage parasite prevalence estimates between classical LM and molecular detection. Several limitations of LM have been documented [34], such as its dependency on the expertise of the reader, the method of slide preparation, staining and reading, and last but not least, it's LOD of about 50 parasites/µL of blood. The LOD ranges from 20-100 parasites/µL between expert and field microscopists. Thus, the high prevalence of submicroscopic infections in the K-U districts is not surprising, and even slightly higher than in studies done elsewhere. Such an abundance of submicroscopic infections is expected in areas where malaria transmission has recently been reduced successfully because parasite densities are controlled by acquired immunity of previously exposed individuals [35].

Our study revealed that two of the 13 LM-positive samples were negative by mRDT and by both molecular assays. These LM-positive samples were likely false positives that may have resulted from erroneous thick smear reads, as has been documented in other studies [36]. Massive over-diagnosis of more than twenty-fivefold difference in the prevalence rates (i.e. 53% versus 2% prevalence) has been reported in a comparative study of routine and expert LM in Tanzania [6].

Molecular methods (nPCR and qPCR) applied in the study were slightly discordant in parasite detection. This difference can be explained by a lower sensitivity of *msp2* nPCR compared to the *18S rRNA* qPCR, which is likely due to its greater amplicon size and thus less efficient amplification. Moreover, compromised integrity of parasite DNA could also lead to more efficient amplification of the shorter *18S rRNA* amplicon. However, some samples which were positive using the marker *msp2* were negative by qPCR. In samples with very low parasite densities, a chance effect in the template distribution to one reaction but not to the other could account for such discrepant results. Alternatively, PCR inhibitors could

potentially be present in a sample, which may affect the qPCR assay more than the nested PCR assay.

Our PCR data help to understand the difference between LM and mRDT results in this study. The discrepancies are likely due to the low sensitivity of LM as well as to the residual HRP antigen that remains after a cleared infection [37, 38]. The LOD of mRDTs is roughly comparable to LM in the field, although the last generation of mRDTs showed a higher sensitivity than previous ones [39]. Moreover, mRDTs performed better than LM in community surveys [12, 40]. To estimate the proportion of parasite infections undetected by LM and mRDT, we applied qPCR with a substantially higher sensitivity of up to 0.34 parasites/µL blood [15]. The use of qPCR in our study increased malaria prevalence twofold, which is similar to differences between PCR- and mRDT-detected prevalence reported elsewhere [12, 36, 40, 41]. About 30% of mRDT positive samples were negative by our most sensitive qPCR assay. Variability in the interpretation of mRDT results may have contributed to the discordance between mRDT and PCR. A direct comparison between the results of qPCR and mRDT is generally problematic because these two tests do not detect the same target molecule: while qPCR detects DNA from circulating parasites, mRDT detects circulating antigens; hence a 100% concordant result is not expected.

Another explanation for the discrepancies between mRDT, LM and PCR results could be that mRDTs are actually capturing gametocytes in the absence of asexual forms. pLDH is produced by live parasites including gametocytes [42]. In confirmed samples containing only *P. falciparum* gametocytes, mRDT was positive in 72% of samples with high gametocyte density (>500 gametocytes/µL of blood) compared to only 20.5% mRDT positives in samples with low gametocytemia(>200 gametocytes/µL of blood) , suggesting that the presence of gametocytes can compromise RDT results [43]. Similarly, among the three samples in our study that were mRDT positive but qPCR negative, all harbored gametocytes by *pfs25* qRT-PCR. This could indicate the presence of gametocytes in the absence of asexual forms. Negativity by qPCR in a gametocyte-positive sample could be explained by the presence of only three *18S rRNA* gene copies per parasite genome, whereas the numbers of *pfs25* transcripts are much higher [14]. Other molecular markers, specific for asexual parasite stages, would be needed to prove the absence of any asexual parasite.

In our study, the prevalence of gametocytes by *pfs25* qRT-PCR was ten times higher than that by LM, indicating a high proportion of submicroscopic gametocytemia in the community. Based on the low gametocyte prevalence by LM in previous years and in the same population [44], much higher gametocyte prevalence had been anticipated, but was not confirmed until now. An even greater difference in gametocyte detection between LM and molecular analysis by Quantitative Nucleic Acid Sequenced-based Amplification (QT-NASBA) has been observed in a community survey in Tanzania (0.4% and 15% positivity, respectively) [45]. In malaria epidemiology, submicroscopic gametocytemia is important. It has been shown that submicroscopic gametocyte carriage substantially contributes to the human infective reservoir for onward transmission to mosquitoes. These studies have shown that even microscopy negative individuals can infect mosquitoes [46, 47]. Therefore, the observed 10.6% gametocyte prevalence in our study population is likely to sustain malaria transmission in the presence of an efficient vector.

Over the seven-year course of malaria community surveys in the K-U districts, molecular data were generated only during the 2011 survey. Therefore, the longitudinal effect of interventions in the study area on *P. falciparum* prevalence rates can only be analyzed by classical diagnostic means. Previous LM data from the IHDSS recorded declining malaria prevalence within the K-U districts, from 25% in 2004 to 4.6% in 2009 [23, 46]. The qPCR-based prevalence rates obtained from the 2011 survey now provide a more precise picture of the malaria prevalence in the K-U districts and put the very low prevalence rate by LM into a new perspective. LM seems inadequate as a diagnostic tool for surveillance of parasite infections in Tanzania at a point when transmission intensity is shifting from high to low. The question remains whether mRDT diagnosis should be considered a suitable alternative. This test has the advantage of allowing on-site treatment for symptomatic or asymptomatic individuals with positive mRDT results. It also allows comparison of data from different areas and countries that still use conventional techniques. Lastly mRDT is quite cheap. The future might be to use both, mRDT for all individuals and PCR in a subsample, to better gauge the magnitude of underestimation of the parasite prevalence. Molecular techniques should be used especially in areas of very low endemicity, where elimination is the prime objective.

Conclusions

Light microscopy showed the poorest performance for detecting both *P. falciparum* asexual parasites and gametocytes. This implies the presence of a large proportion of submicroscopic parasitemia and gametocytemia in the K-U districts, a phenomenon that is common in areas of recently declining transmission. mRDT performed better than LM, as it detected almost half of the *P. falciparum* carriers identified by molecular tools. However, in light of our PCR results, the gain in sensitivity of mRDT over LM was still modest. However, the use of mRDT adds to our understanding of the real transmission level, in the sense that it can also detect recently cleared infections (treated or not) that are no more detectable by LM or PCR. Thus, using both tools, PCR and mRDT, which together are able to detect actual parasitemia plus recent infections, may provide the most precise information by which to assess the impact of interventions and to decide on the best control strategies. To reliably estimate the malaria reservoir in areas of high submicroscopic parasitemia, molecular tools are clearly justified.

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Acknowledgements

We would like to thank the people of the Kilombero and Ulanga districts in Tanzania for participating in the survey. We also appreciate the efforts of Jabir Namamba and the IHDSS field team for conducting the survey. We thank Dania Müller and Sarah Javati for their technical assistance at the Swiss TPH laboratory.

Funding

The study was funded by Novartis Pharma.

FM received support from Stipendienkommission für Nachwuchskräfte aus Entwicklungsländern des Kantons Basel-Stadt and the Science Technology Higher Education Project (STHEP) through Dar Es Salaam University College of Education (DUCE) in Tanzania.

Author`s contribution

Study designed by IF and BG. Field work conducted by FM and MK. Laboratory work done by FM. Manuscript written by FM, BG and IF.

All authors read and approved the manuscript.

Conflict of interest

Authors declare no competing interest.

List of tables

Table 1: Comparison of the two molecular methods *Pf18S rRNA* qPCR and *msp2* nested PCR for *P. falciparum* parasite detection

	msp2			
18S rRNA qPCR		Positive	Negative	Total
	Positive	50	36	86
	Negative	10	130	140
	Total	60	166	226
Pearson chi^2 (1) = 71.0492 **Pr** = 0.000				

Table 2: Concordance among three different diagnostic methods for detecting *P. falciparum* positivity

Patterns of test positivity by three diagnostic methods			
18S rRNA qPCR (N_{pos} = **86**)	**mRDT (N_{pos} = 36)**	**LM (N_{pos} = 13)**	**total positive samples N=98**
+	**-**	**-**	***57***
+	**+**	**-**	***18***
-	**+**	**-**	***10***
+	**+**	**+**	***8***
+	**-**	**+**	***3***
-	**-**	**+**	***2***

Table 3: Proportion of *P. falciparum* gametocyte carriers among individuals deemed positive by mRDT, LM, or molecular assays (*18S rRNA* qPCR and *msp2* nested PCR).

Malaria diagnosis	Gametocyte positive among *Pf.* positive samples **(% Gametocyte carriage among *Pf*. positives**)	Gametocyte positive by molecular *Pfs25*-qRT-PCR	Non gametocyte carriers
LM	3/13 (23%)	3	10
mRDT	9/36 (25%)	9	27
msp2 nPCR	12/60 (20%)	12	48
18S rRNA qPCR	14/86 (16%)	14	72

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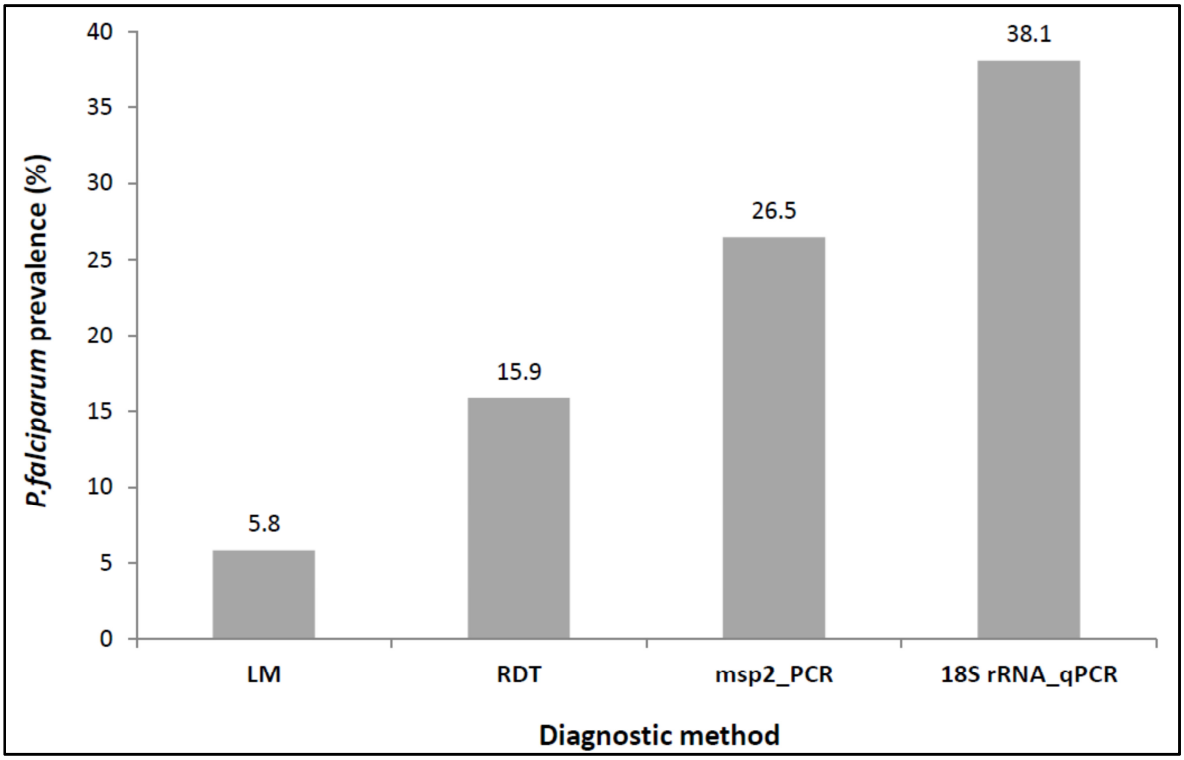

Figure 1: *P. falciparum* prevalence rates by LM, mRDT, *msp2* nested PCR and *18S rRNA* qPCR performed in N=226 samples from the Kilombero-Ulanga districts in Tanzania.

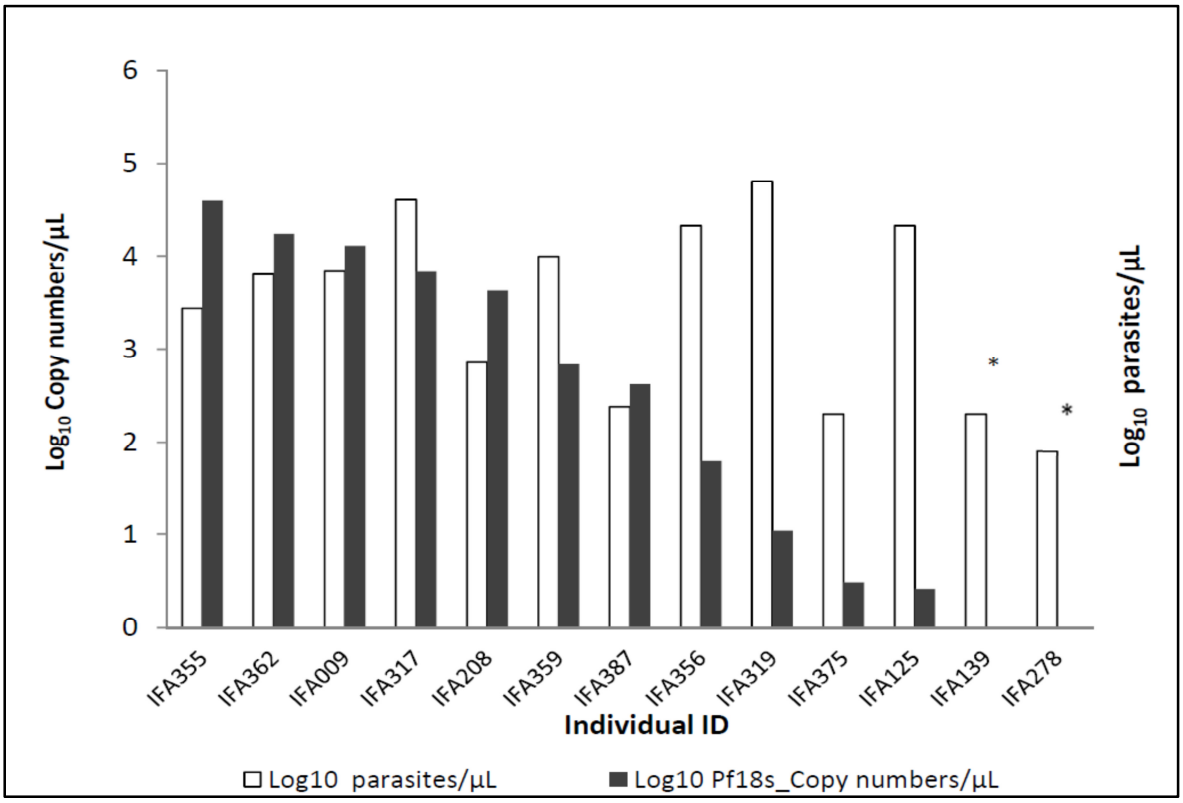

Figure 2: Comparison of $\log_{10}$ *P. falciparum 18S rRNA* gene copy numbers /µL blood by qPCR and $\log_{10}$ parasite counts /µL blood by LM. *Two LM-positive samples were negative by mRDT and molecular assays and likely represent false positive microscopy results.

Comparison of submicroscopic parasitemia and gametocytemia in five Tanzanian sites of different malaria endemicity

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This chapter will be submitted for publications after further revisions

ABSTRACT

Background

Prevalence of *Plasmodium falciparum* in community samples is one of the cornerstones for describing malaria transmission in the area. Measurements of parasite prevalence greatly depend on the diagnostic techniques applied and can lack precision in case of low parasite densities, which often remain undetected by the routinely used diagnostic tool, light microscopy (LM). Highly sensitive molecular tools are able to detect submicroscopic infections and can serve to evaluate the performance of diagnostic tools used in the field surveys. We aimed to investigate local differences in the prevalence of submicroscopic infections and gametocyte carriage at different sites of varying endemicity and to investigate age trends in submicroscopic carriage of both asexual parasites and gametocytes from Tanzanian communities.

Methods

In community surveys 1820 individuals were recruited from 5 sites of greatly varying of endemicity. Finger prick blood was collected for parasite detection by LM, RDT and *18S rRNA* qPCR. Gametocytes were detected by both LM and qRT-PCR targeting transcripts of the gametocyte-specific expressed marker *pfs25*. Submicroscopic infections were those positive by qPCR but not by LM.

Results

P. falciparum prevalence by qPCR varied from 50% at the site of high endemicity to 0.6% in low endemicity. 52.7% (174/330) of qPCR positive samples were submicroscopic. At the site of lowest endemicity only 2 samples were positive by qPCR and both were negative by LM. Submicroscopic carriage did not show a clear relationship with endemicity patterns and was 73% (11/15) in low, 34% (19/56) and 63% (5/8), in moderate and 55% (137/249) in high endemicity. Molecularly determined gametocyte prevalence at each location closely followed the parasite prevalence of this site. But the vast majority (96%; 277/288) of all gametocytemia was submicroscopic.

Conclusions

Molecular parasite detection revealed high prevalence of submicroscopic carriage in Tanzania particularly in adults. Submicroscopic infections were prevalent in all endemic settings, even in areas of high transmission intensity. Highly standardized and comparable data on submicroscopic parasitemia and gametocytemia from multiple sites within a country can help to better assess the human infective reservoir for onwards transmission and the relative contribution of asymptomatic infections. Identifying the extent of submicroscopic carriage is relevant for control strategies to successfully interrupt transmission.

Background

Measurement of parasite prevalence in communities from endemic areas is one of the most important metrics for describing levels of malaria endemicity. Prevalence rates complement entomological measures of transmission that are much more difficult to obtain, particularly in low transmission settings. Monitoring changes in prevalence rates is useful for measuring outcomes of antimalarial interventions or for informing control strategies. In particular molecular diagnosis is gaining increasing importance in the context of current efforts to scale up malaria control and to move towards malaria elimination in many countries (MalEra 2011). The merits of molecular malaria diagnostics are owing to a higher precision compared to the traditional malaria diagnostics tools, light microscopy (LM) and rapid diagnostic test (RDT), which are still widely used in all malaria endemic countries.

As a consequence of intensified malaria interventions a major decline in malaria prevalence has been observed in many areas, including highly endemic regions in sub Saharan Africa (WHO 2013) (Tanner and Hommel, 2010). Also in Tanzania the national average of malaria prevalence has halved from 18% in 2008 to 9% in 2012 (THMIS 2009, THMIS 2013). Studies conducted in various regions in Tanzania recorded intense reductions in malaria transmission (Mmbando et al. 2010; Khatib et al.2012 and Ishengoma et al. 2013).

A decline in prevalence and thus in exposure to *P. falciparum* could affect the overall parasite densities in the population and may affect the sensitivity of the commonly used diagnostic tools. In view of a worldwide malaria reduction it seems of paramount importance to investigate potential changes in the performance of LM, the main diagnostic tool. A meta-analysis using data from varying transmission intensities had indicated that in low-endemic areas submicroscopic parasite carriage was very frequent, whereas in high endemicity, 80 % of infections were detectable by LM (Okell et al. 2012). This suggests that low parasite densities could account for the low positivity by LM in community samples from low transmission settings. Such a relationship would imply that LM, the primarily used detection method, would provide insufficient sensitivity for malaria surveillance if the malaria prevalence further declines in future.

Several recent molecular-epidemiological studies in Tanzania did not conform to the results of the meta-analysis of Okell and co-workers (2012). An earlier study performed in Tanzania in an area of declining endemicity reported a large proportion of submicroscopic infections

(Shekalaghe et al. 2007). Prevalence rates in that study were 1.9% by LM and 33% by quantitative nucleic acid sequence based amplification (QT-NASBA). Submicroscopic carriage in that study did not vary by age or season, in contrast to the determinants for submicroscopic infections formulated by Okell and co-workers (2012). A further study was conducted at several Tanzanian sites of different altitude (Manjurano et al. 2011). Prevalence was found to decline with increasing altitude (and thus lesser transmission). LM detected only 50% of all infections identified by PCR and this submicroscopic carriage was similar at all altitudes. For other Tanzanian regions, in particular for settings of moderate and high malaria transmission, the prevalence of submicroscopic infections has not been investigated so far. Our comparative analysis of 5 Tanzanian sites of greatly differing malaria endemicity aimed at filling this gap.

In parallel to investigating *P. falciparum* prevalence across Tanzania, we also assessed prevalence of gametocytes, the stages solely responsible for onward transmission to mosquitoes. Again we aimed at comparing LM-based gametocyte prevalence with positivity by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Because the commitment of *P. falciparum* to sexual development is considered to be rare (about 1/100 parasites) (Bousema and Drakeley, 2011), gametocyte densities are generally much lower than those of asexual stages, and very few individuals harbour gametocytemia detectable by LM (Taylor et al 1997). Importantly, also gametocytemia at submicroscopic levels contributes to transmission of the parasite (Schneider et al. 2007). Mosquito membrane feeding experiments revealed that blood samples with submicroscopic gametocytemia could infect mosquitoes, but the mosquito infection rate was 10-fold lower compared to the rate observed for blood samples that were gametocyte-positive by LM (Coleman et al. 2004; Lin et al. 2014). Quantification of submicroscopic gametocytes by qRT-PCR or NASBA and their contribution to transmission has been investigated in several studies (Schneider et al 2006; Shekalaghe et al 2007; Ouédraogo et al 2009; Harris et al.2010; Manjurano et al 2011; Mosha et al 2013; Lin et al 2014). In an attempt to define the relationship between gametocyte density and mosquito infection rate, Churcher and co-workers (2013) showed that this relationship was complex and non-linear. These results indicated that molecularly determined gametocyte densities cannot be used as a surrogate for a simple prediction of the impact of transmission reducing interventions, other factors such as host age, asexual parasite densities and gametocyte maturity also seem to play a role (Churcher et al. 2013).

However, gametocyte quantification is useful for describing the human infectious reservoir to mosquitoes.

The aim of our multi-site study was to identify local differences in the proportion of submicroscopic parasitemia and to investigate how well LM performs in malaria surveillance over the full range of local transmission intensities. This also provides a useful and precise description of malaria prevalence in the country. By determining the extent of submicroscopic parasitemia particularly in areas of very low transmission and urban areas in Tanzania we aimed to test the hypothesis that such settings most *P. falciparum* infections are detectable only by molecular methods. Secondary objective was to document the molecular gametocyte prevalence in the different transmission settings and to confirm their presence also in samples of very low or urban transmission, despite their apparent absence by LM.

Methods and study design

Study sites

The study was conducted at 5 sites in Tanzania, which were classified in urban, low, medium and high endemic settings according to district prevalence data recorded by the Tanzania HIV and Malaria indicator surveys of 2008 (THMIS 2009): Dar Es Salaam (urban), coastal Rufiji (high), coastal Bagamoyo and Tanga (Moderate) and Iringa (very low). The prevalence rates reported in the THMIS surveys and our classification of endemicity levels for the 5 study sites are listed in **supplementary Table S1**.

Study Design

Cross-sectional surveys were conducted at all study sites between May and August 2013. 1820 individuals of all ages (>6 months to 90 years) with signed consent forms were enrolled. Finger prick blood was collected and malaria screening was performed by Pan PLDH/Pf (HRP2) SD Bioline® rapid diagnostic tests (RDT). All individuals positive by RDT were treated at the time of blood sampling with a recommended full dose of ACT (Coartem®). Thick blood smears were made for parasite diagnosis by LM. In the first survey conducted in Dar Es Salaam approximately 50µL blood were spotted onto Whatman® grade 3 filter paper, air dried and transferred into 300µL of TRIzol® RNA stabilate within 1-7 days as described elsewhere (Wampfler et al. 2013). At 4 of our study sites about 100-150µL whole blood from finger prick were collected in EDTA tubes and kept at ambient temperature until later that

same day 100µL EDTA blood were transferred to microtubes containing 500µL RNA Protect® reagent and mixed. Both types of samples for RNA extraction were stored at -20°C until shipped on ice packs to the molecular laboratory. RNA and DNA were co-extracted in a 96-well plate format, RNA was extracted using the Qiagen RNeasy® plus extraction kit. DNA was obtained by washing gDNA eliminator columns from the RNA extraction using buffers from the modified protocols in the QiAmp® DNA mini kit. The obtained nucleic acids were stored temporarily at 20°C storage prior to analysis by PCR. Details of nucleic acid (RNA and DNA) extraction methods have been described previously in (Wampfler et al. 2013, Mwingira et al. under review).

Ethical clearance

Approval for the TZ cross-sectional study was obtained from the Institutional Review Board of the Ifakara Health Institute (IHI), Dar Es Salaam (no. 13-2013). This study was carried out within the framework of the Sentinel Panel District Project of IHI (IRB and NIMRI clearance). Approval was also sought from the District Medical Officers and from the local governing bodies of respective administrative wards, streets and hamlet level. Before blood sampling an informed written consent was obtained from all participants or parents/guardians of all children.

Light microscopy

Thick films were made from finger prick blood, air dried in the field and stained with 10% Giemsa in the IHI laboratory. Gametocytes were read separately from asexual blood stage forms. Parasite densities were quantified by counting the number of asexual and gametocytes per 200 and 500 leucocytes respectively. The results were converted to parasites/µL assuming a total of 8000 WBC/µL blood.

P. falciparum detection

P. falciparum asexual stages were analyzed by a DNA-based *P. falciparum* 18S rRNA qPCR assay as described elsewhere (Wampfler et al. 2013).

Gametocyte detection

P. falciparum gametocyte detection was performed on RNA from all samples irrespective of their DNA-based positivity but only after confirming the presence of parasite RNA in the sample. Transcripts of the *pfs25* mRNA gene, a gametocyte-specifically expressed gene, were reverse transcribed and the resulting *pfs25* cDNA was amplified by qPCR in a single reaction. Details of this assay have been described previously (Wampfler et al. 2013). Standard curves of assay-specific plasmids in triplicates were analysed on each plate together with the test samples to enable conversion of Ct values into template copy numbers/µL blood. No further conversion of the copy numbers/µL blood was done.

Data analysis

Data were entered and analysed by STATA® version 13, Texas USA. Prevalence rates by LM, RDT and qPCR were determined and compared between 4 endemic settings. Marker-specific transcript copy numbers/µL blood were converted to $\log_{10}$. Age of participants was categorized in 8 groups as follows: <1, 1-2, 3-4, 5-9, 10-19, 20-39,40-59, 60+ years. Samples with missing age data were excluded from the analysis.

Results

P. falciparum prevalence and density at 5 study sites

Our study included 1820 participants from 5 sites in Tanzania, numbers of participants by study site and the endemicity classification of sites are shown in **Supplementary Table S1.**

The *P. falciparum* parasite prevalence rates determined by the 3 diagnostic tests for all sites combined were 18.1% (330/1820; 95% CI 16.3-19.9%) by *18S rRNA* qPCR, 17.2% (313/1820; 95% CI 15.5-18.9%) by RDT and 11.8% (216/1820; 95% CI 10.3-13.3%) by LM. The molecular prevalence rates varied substantially between geographic sites and ranged roughly 50% in Rufiji, the site of highest endemicity, to 0.6% in Iringa, the site of lowest endemicity (**Supplementary Table S2**). These figures highlight the variation of malariometric indices in Tanzania. As expected, the regions in the moderate and high endemic sites according to the THMIS classification showed higher molecular prevalence rates than sites in low and urban endemicity (Figure 1 and **Supplementary Table S2**). LM and RDT mirrored the molecular

findings (Figure 1). The discrepancy between molecular and LM prevalence rates was large at the highly endemic site, whereby about 50% of total parasite infections remained undetected by LM. An exception to the poor performance of LM was observed at the urban site Dar Es Salaam, where LM recorded a prevalence of 6.6% (29/440), which was 4-fold higher than the prevalence by RDT (7/440) and 2-fold higher than that by qPCR (15/440). Yet, these differences in parasite detection by the three methods LM, RDT and qPCR at the urban endemic site were not significant. The three diagnostic methods differed only marginally in prevalence rates recorded in Iringa (low endemicity), Tanga and Bagamoyo (both low and moderate endemicity). This implies that submicroscopic infections prevail at high endemic sites but are rare in low and moderate transmission sites, where LM performed more or less similar to RDT and molecular assays.

To investigate whether higher parasite densities in areas of low and moderate transmission could account for the good LM diagnosis observed, we analysed parasite counts by LM (Figure 2). The geometric mean parasite density by LM was 4407 parasites/µL blood 95% CI: 3131-6203 parasites/µL blood in Rufiji, the highly endemic site, while the urban low endemic site, Dar recorded a mean parasite density of 317 parasites/µL blood 95% CI: 269-374 parasites/µL blood (Figure 2). The difference in mean density by LM between the sites of highest and lowest endemicity was 14-fold. However, we obtained very low numbers of *P. falciparum* positive samples in the low and moderate endemic settings, thus the estimates of mean parasite load at these sites may not be very robust.

Submicroscopic infections in different endemic sites

Submicroscopic parasitemia was defined as samples that were parasite positive only by molecular detection but not by LM. Overall, 52.7% (174/330) of samples positive for *P. falciparum* were submicroscopic according to the above definition. At our site of lowest endemicity (Iringa), LM did not detect any positive sample, whereas 2 *P. falciparum* positive samples were found by qPCR and confirmed by RDT. The very low numbers of positive samples precludes any firm conclusion on submicroscopic prevalence in the two low endemicity sites. In contrast, high numbers of submicroscopic carriers (55%; 137/249) were observed in Rufiji. No clear trend in the relationship between submicroscopic carriage and endemicity was observed (Table 1).

Prevalence and submicroscopic carriage of gametocytes

Gametocytes were diagnosed in all samples by LM and qRT-PCR. The molecular gametocyte prevalence in all community samples from the 5 sites was 15.8% (288/1820; 95% CI 14.1-17.5%). When only *P. falciparum* positive samples were considered, 87.2% (288/330) carried gametocytes by qRT-PCR. By LM the overall gametocyte prevalence was 0.82% (15/1820; 95% CI 0.47-1.2%). Thus, LM identified only 5.2% (15/288) of all gametocyte carriers identified by qRT-PCR. When only slide reading results were considered, 4.5% (15/330) of the *P. falciparum* positive individuals would be gametocyte carriers.

The lowest molecular gametocyte prevalence of <1% was observed in Iringa and Dar Es Salaam (**Supplementary Table S2**). This sharply contrasts with findings from the high endemic site Rufiji, where 43.6% of the population carried gametocytes. In high endemicity LM-based gametocyte detection was 15-fold lower compared to molecular diagnosis. In most areas of low and moderate endemicity (Iringa, Tanga and Bagamoyo) LM did not detect any gametocytes, while qRT-PCR had detected gametocytes at all sites. When all data was combined, 96% (277/288) of all *P. falciparum* gametocyte positive samples were submicroscopic. Endemicity did not seem to affect the prevalence submicroscopic gametocytemia in a major way (Table 2).

Comparison of parasite and gametocyte load

The molecular gametocyte load in the high endemic site, Rufiji, had geometric mean of 86.8 *pfs25* transcripts/µL blood (95% CI 63.2-119.1 *pfs25* transcripts/µL blood). Only one of the two parasite positive samples in the lowest endemic site, Iringa, carried gametocytes with 16 *pfs25* transcripts/µL blood. By LM the overall mean gametocyte density was 52 gametocytes/µL blood, ranging from 30-90 gametocytes/µL blood. Rufiji, the high endemic site displayed a geometric mean of 54 gametocytes/ µL blood, while only one positive samples in Dar, urban low endemic had a geometric mean of 32 gametocytes/µL blood. We observed a positive linear correlation (r^2=0.47) between *P. falciparum* parasite load depicted by the log transformed 18S rRNA copies/µL blood and the gametocyte load in the same sample (Figure 3).

Age trends in prevalence and submicroscopic carriage of P. falciparum parasites and gametocytes

To investigate age trends in parasite load, prevalence and submicroscopic carriage we combined the molecular data from all sites. In age groups 1-2, 3-4, and 5-9 years both high prevalence rates and high densities were observed (Figure 4 and Supplementary Figure S1). The highest prevalence rate by qPCR peaked at 5-9 yrs. Similarly observation was shown by LM. Parasite density by LM and PCR was highest at 3-4 yrs followed closely by age groups 1-2 yrs and 5-9 yrs). A decline of both prevalence rates and parasite load by LM and qPCR was observed in children older than 10 yrs and adults. The prevalence of submicroscopic infections increased steadily over the younger age groups and peaked at 20-39 yrs followed by a decline in the oldest age groups (Supplementary Figure S2). Thus, the age trend in submicroscopic infections was non-monotonous with a clear increase over the younger age groups.

Discussion

Our comparison of 5 sites in Tanzania revealed huge variations in P*. falciparum* parasite and gametocyte prevalence rates across the varying endemic sites. The strength of this study consisted in a good comparability of the data generated at each site. Parasite detection by LM, RDT and qPCR was performed for the entire data set by the same field and laboratory team using a generalized protocol. This approach minimized the technical variability and has major advantages over a comparison of data from unrelated studies performed with different techniques. The observed differences between the study sites should thus be attributed to geographical factors, particularly those that favour the development and survival of the mosquito vectors. Highland areas, such as rural Iringa with low temperatures of about 15°C and an altitude >1600m, are known to have low or no malaria transmission (Mboera et al. 2008; Talundzic et al. 2014). Urban areas of Dar Es Salaam have much less malaria (Strøm et al. 2013[a]) compared to the rural areas of Tanga (Mmbando et al. 2010), Rufiji (Khatib et al. 2012) that are well documented and known to be highly endemic (THMIS 2013).

In our low and moderate endemic areas the prevalence rates obtained by the 3 independent diagnostic tools did not differ substantially. This implies that most infections were captured even by LM and RDTs. In contrast, major discrepancies between LM and qPCR were observed at the high endemicity site Rufiji. Because the highly sensitive detection of 0.34 parasites/µL by our 18S rRNA qPCR (Hofmann et al. 2014) massively exceeds the limit of detection of LM of around 50-100 parasites/µL (Wongsrichanalai et al. 2007), we expected and confirmed that molecular detection yielded the highest prevalence rates at all sites except Dar Es Salaam, where LM outperformed both RDT and PCR. We assume that the 14/440 samples that were positive only by LM could represent technical problems at this site or due to erroneous reading of thick smears. Under the condition of low endemicity LM sometimes creates false positives. High levels of false positivity by LM have been reported from other areas of low endemicity (Wongsrichanalai et al. 2007; Kahama-Maro et al. 2011; Fançony et al. 2013; Strøm et al. 2013 [b]).

This study confirms the predominance of submicroscopic *P. falciparum* infections in Tanzania with 52.7% of all molecularly detected infections being submicroscopic. This is consistent

with data from the Usambara and Kilimanjaro areas in Tanzania where, 50% and 33% of the total infections were submicroscopic (Manjurano et al. 2011; Shekalaghe et al. 2007). Our results also agree with an earlier meta-analysis that had indicated that LM detects on average only 50% of total infections determined by PCR (Okell et al. 2009). Submicroscopic infections in our study were more prevalent in older children and adults with peak prevalence at 20-39 yrs. A similar observation was reported from north-east Tanzania, where older children were 3-times more likely to carry submicroscopic infections compared to younger children (Manjurano et al. 2011). Similarly, a meta-analysis by Okell and co-workers (2012) had shown that submicroscopic infections were common in adults, owing to the ability of acquired immunity to control parasite densities to levels under the detection limit of LM. In contrast to this meta-analysis we did not observe an increase of submicroscopic carriage with decreasing endemicity. Our data of submicroscopic carriage did not show substantial differences between the sites of different endemicity.

The detection limit of LM very often does not permit to diagnose low density infections, and thus prevalence estimates generated by LM will depend greatly on the average parasite density of the population studied. In our study we did not detect significant differences in parasite densities nor in the prevalence of submicroscopic carriage. This indicates that at our sites parasite densities in high as well as low endemic settings might be well controlled by acquired immunity as a consequence of previous exposure to malaria parasites (Alves et al. 2002; Proietti et al. 2011; Okell et al. 2012; Harris et al. 2010; Sturrock et al. 2013 and Lindblade et al. 2013). It has been argued that a recent decline in transmission in formerly highly endemic areas keeps parasite densities low despite reduced transmission (Gatton et al. 2010; Golassa et al 2013). Similarly, a study in Tanzania indicated an inverse relation between parasite densities and endemicity, suggesting that submicroscopic infections are likely to occur in high endemic areas where exposure to infection is more frequent and densities are low (Mosha et al. 2013). That study and our own data are inconsistent with the meta-analysis by Okell et al. (2012), which had indicated that in low endemic settings with a *P. falciparum* prevalence of <10% the great majority of infections (88%) should be submicroscopic, while in high endemic settings with a prevalence >75% the submicroscopic carriage was estimated to be as low as 25%. A major problem for this kind of comparison is that the positive cases in low endemicity settings are very few (e.g. we observed only 2

infections by qPCR at our lowest endemicity site), thus, not sufficient data is generated to draw firm conclusions on submicroscopic carriage. These limitations became clear in view of the very few qPCR-positive samples even in low (positivity: 2/329 and 15/440) and moderate (positivity: 8/235 and 56/316) transmission settings. We observed high *P. falciparum* prevalence rates by both LM and qPCR in young children with a peak at age 5-9 yrs. This was expected as high incidence rates for clinical malaria and infections of high parasite densities mainly occur in children. Similar studies in Ghana and Tanzania have also recorded a peak in malaria prevalence at 5-9 yrs (Owusu-Agyei et al. 2002; Drakeley et al. 2006; Mosha, et al. 2013).

An additional objective of this study was to investigate whether malaria endemicity affects the prevalence of submicroscopic gametocyte carriers. As expected, applying molecular tools for gametocyte detection was far better than LM. The molecular assay detected 15.8% gametocyte carriers compared to <1% by LM and 96% of the gametocyte positive samples were submicroscopic. The occurrence of a high proportion of submicroscopic gametocytemia (95-100% at all sites) irrespective of endemicity is alarming, since several studies involving mosquito feeding experiments had indicated a substantial contribution of submicroscopic gametocyte carriage to transmission (Coleman et al. 2004; Schneider et al. 2006; Churcher et al. 2013; Lin et al.2014).

Despite great benefits of molecular tools for parasite detection, PCR will unlikely replace the two standard diagnostic tools in the field, LM and RDT, which will continue to be heavily used in resource limited settings (Mwingira et al. manuscript under review). However, PCR can play an essential role in assessing the quality of LM. By routinely conducting PCR-based studies in parallel to LM diagnosis in some malariological surveys, valuable estimates of this otherwise undetected parasite burden can be generated. With a country-wide cross sectional surveys combined with PCR diagnosis we were able to provide an assessment of the LM quality for the widely differing transmission intensities in Tanzania.

Conclusions

Our study confirms a high prevalence of submicroscopic parasitemia and gametocytemia in Tanzania. Submicroscopic infections were observed in all endemic settings and did not show a specified trend with decreasing endemicity. Our results can inform control strategies by drawing attention to asymptomatic individuals, mostly adults with primarily submicroscopic

infections, whose contribution to onwards transmission should not be ignored. Our molecular parasite detection assays unveiled 52% of infections and 96% of gametocyte carriers that would have been missed if the survey had relied on LM alone. Imperfect diagnosis will likely jeopardize malaria control efforts. Thus our qPCR-based point prevalence rates for areas of different endemicity contribute to a solid basis for planning and monitoring elimination efforts in Tanzania.

Acknowledgments

We thank the study participants, Michael Mihayo and the field team from Ifakara Health Institute for participating in field surveys in Tanzania. We thank Honorati Masanja for providing logistics within the sentinel Panel of Districts (SPD). We also thank Amanda Ross from SwissTPH for her support in data analysis.

Competing interests

The authors declare no conflict of interest.

Funding

This work was funded by the SwissTPH. FM received funding from the Science and Technology Higher Education Project (STHEP) through the Dar Es Salaam University College of Education (DUCE) and the Stipendienkommission Basel Stadt.

Authors' contribution

FM: Execution of Field work and laboratory analyses; manuscript writing

SS: Field work supervision and review of the manuscript

SA: Study concept and design; review of manuscript

MT: Study design; review of manuscript

IF: Study concept, molecular epidemiological study design, supervision of molecular work, manuscript writing

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List of tables

Table 1: Proportion of submicroscopic parasitemia in 5 areas of different endemicity in Tanzania

Region	qPCR positives % (n/N)	LM positives in qPCR positives % (n/N)	Proportion submicroscopic carriers (n/N)	Prevalence of submicroscopic carriers (n/N)	Submicroscopic carriers %
Iringa	0.6 (2/329)	0	2/2	2/329	n.a.[1)]
Dar	3.4 (15/440)	26.7 (4/15)	11/15	11/440	73
Tanga	3.4 (8/235)	37.5 (3/8)	5/8	5/235	63
Bagamoyo	17.7 (56/316)	66.1 (37/56)	19/56	19/316	34
Rufiji	49.8 (249/500)	45 (112/249)	137/249	137/500	55

[1)] Number too low to support any firm conclusion

Table 2: Proportion of submicroscopic gametocytemia in 5 areas of different endemicity in Tanzania (N denotes samples per site)

Region	*Pfs25* qRT-PCR % (n/N)	LM positive in *pfs25* positive % (n/N)	Proportion submicroscopic gametocyte carriers (n/N)	Submicroscopic gametocytemia %
Iringa	0.3 (1/329)	0	1/1	n.a.[1)]
Dar	0.68 (3/440)	0	3/3	n.a.[1)]
Tanga	6.4 (15/235)	0	15/15	100
Bagamoyo	16.1 (51/316)	0	51/51	100
Rufiji	43.6 (218/500)	5 (11/218)	207/218	95

[1)] Number too low to support any firm conclusion

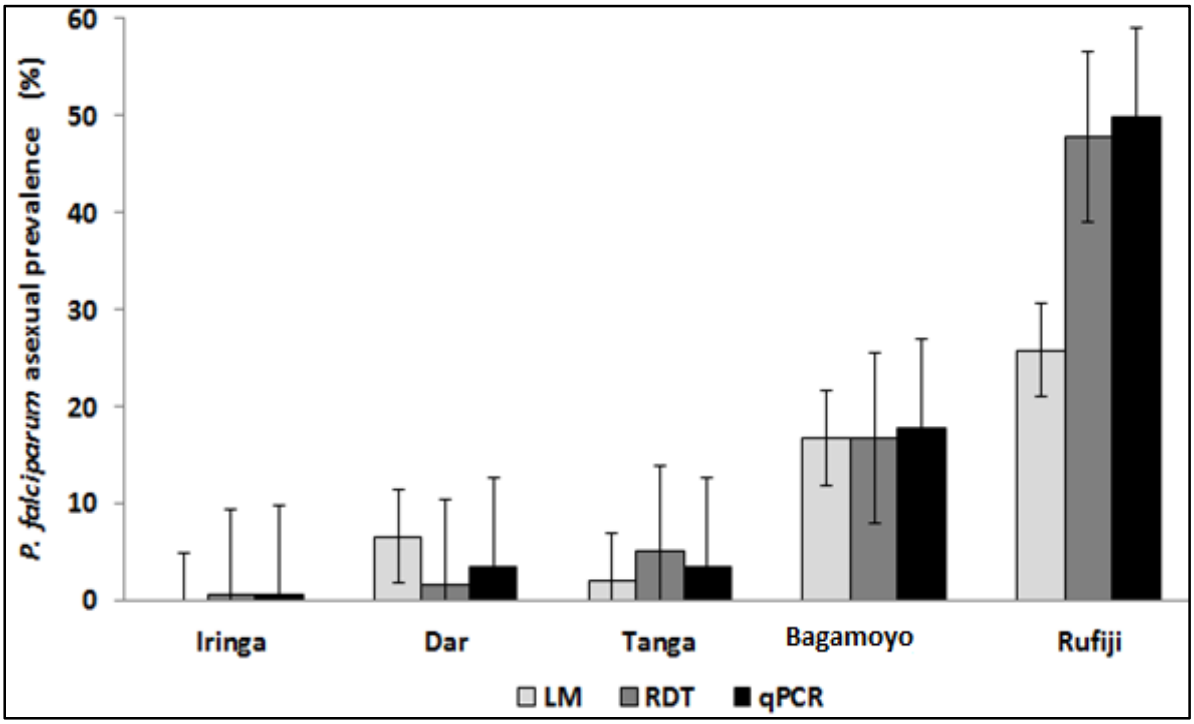

Figure 1: *P. falciparum* prevalence by three independent diagnostic methods in 5 Tanzanian regions with greatly varying malaria endemicity.

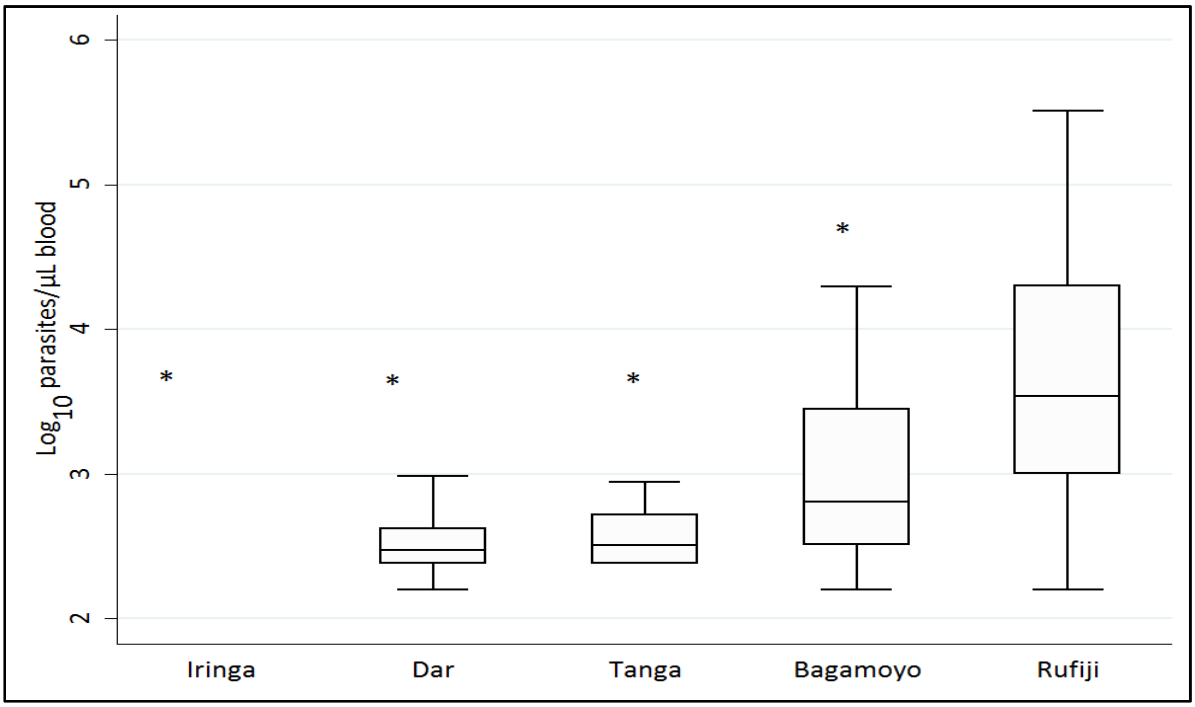

Figure 2: parasite load quantified by LM (log $_{10}$ parasites/µL blood) observed at 5 study sites. The median is shown as solid line with 25 and 75% quartiles.

* Very few or no samples at all were detected at sites of low and moderate endemicity.

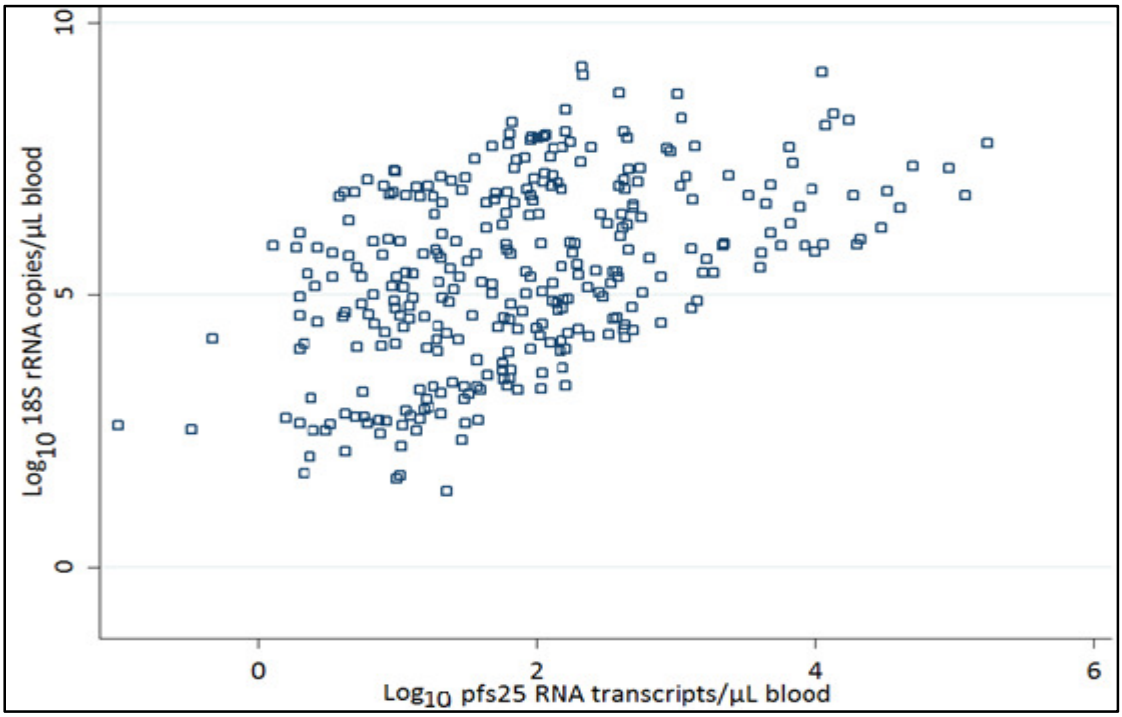

Figure 3: Correlation of *P. falciparum* parasite load (expressed as Log_{10} 18S rRNA copy numbers/µL blood) and gametocyte load by (expressed as Log_{10} *pfs25* copy numbers/µL blood) in samples positive for both molecular assays. $r^2 = 0.47$. Samples from all cross sectional surveys were combined.

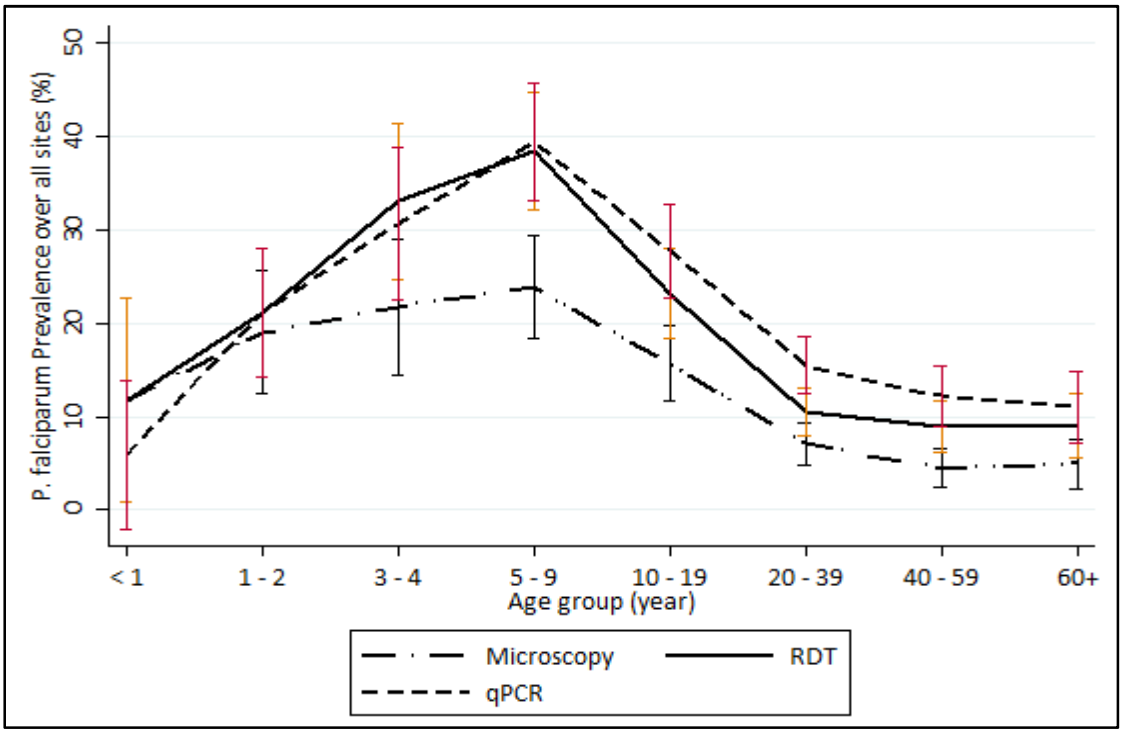

Figure 4: Age trends in *P. falciparum* prevalence diagnosed by LM, RDT and qPCR for the combined data set from all study sites.

Supplementary Tables

Supplementary Table S1: Study sites classified into different endemic settings according to prevalence results from the national malaria indicator survey of 2008 (THMIS 2009);N denotes the number of samples per study site..

Region	**THMIS-Malaria prevalence of 2008**	**Time of sampling (N)**
Dar Es Salaam	1%	2013 (440)
Iringa	3%	2013(329)
Coastal Bagamoyo	10%	2013(316)
Tanga	14%	2013(235)
Coastal Rufiji	21%	2013(500)

Supplementary Table S2: *P. falciparum* parasite and gametocyte prevalence detected by LM, RDT and molecular assays per study site.

	Parasite prevalence % (p/N)			**Gametocyte prevalence % (p/N)**	
Region	**LM**	**RDT**	**18S qPCR**	**LM**	**Pfs25 RT-PCR**
Iringa	**0** (0/329)	**0.6** (2/329)	**0.6** (2/329)	**0** (0/329)	**0.3** (1/329)
Dar	**6.6** (29/440)	**1.6** (7/440)	**3.4** (15/440)	**0.2** (1/440)	**0.7** (3/440)
Tanga	**2.1** (5/235)	**5.1** (12/325)	**3.4**(8/325)	**0** (0/235)	**6.4** (15/235)
Bagamoyo	**16.8** (53/316)	**16.8** (53/316)	**17.7** (56/316)	**0** (0/316)	**16.1** (51/316)
Rufiji	**25.8** (129/500)	**47.8** (239/500)	**49.8** (249/500)	**2.8** (14/500)	**43.6** (218/500)

Supplementary figures

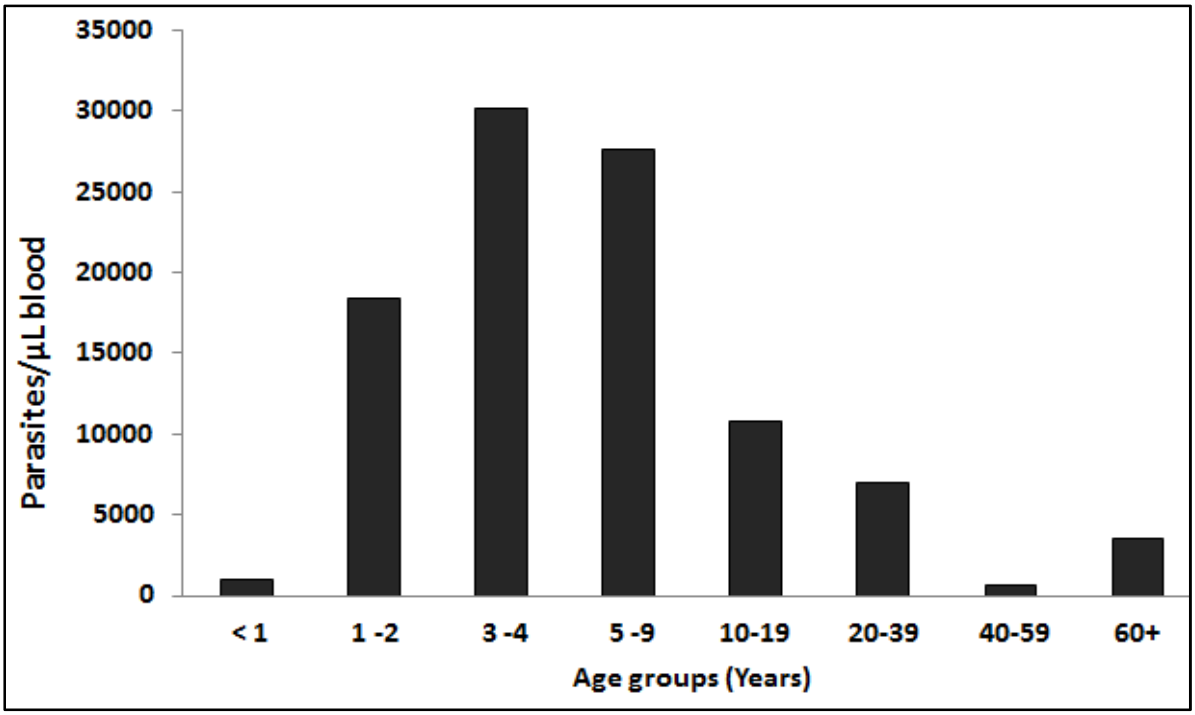

Figure S1: Mean parasite density (parasites/µL blood) by age established by LM.

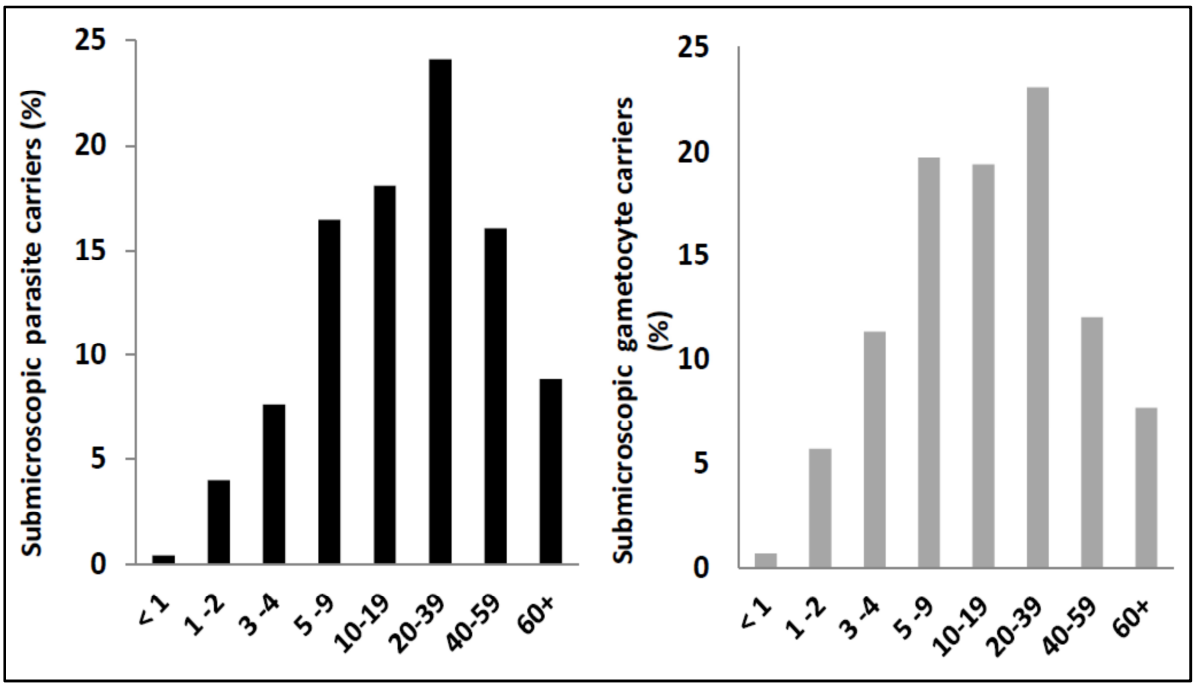

Figure S2: Percentage of submicroscopic parasitemia by age in black bars. Percentage of submicroscopic gametocytemia by age is shown in grey bars.

Ultra-sensitive detection of *Plasmodium falciparum* by amplification of multi-copy subtelomeric targets reveals underestimation of parasite prevalence

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KEYWORDS

Malaria, diagnosis, elimination, quantitative, highly sensitive PCR, multi-copy target, Tanzania, Papua New Guinea

Submitted to PLOS Medicine August 2014

ABSTRACT

Background

Planning and evaluating malaria control strategies relies on accurate definition of parasite prevalence in the population. A large proportion of asymptomatic parasite carriers are identified by surveillance with molecular methods only, yet these infections also contribute to onward transmission to mosquitoes. Sensitivity of molecular detection by PCR is limited by the abundance of the target sequence in a DNA sample, thus detection becomes imperfect at low densities. We aimed to increase PCR diagnostic sensitivity by targeting multi-copy genomic sequences for a reliable detection of low-density infections and investigated the impact of these tools on community prevalence data.

Methods and findings

Two qPCR assays were developed for ultra-sensitive detection of *P. falciparum*, targeting the high-copy telomere-associated repeat element 2 (TARE-2, ~250 copies/genome) and the *var* gene acidic terminal sequence (*var*ATS, 59 copies/genome). Using parasite culture, our assays reached a limit of detection of 0.034 parasites/µl blood and are thus 10x more sensitive than standard 18S rRNA qPCR. In a cross-sectional study in Tanzania, 297/500 samples tested positive with one or both of the ultra-sensitive assays, whereas 18S rRNA qPCR failed to identify 50 (17%) of these. Light microscopy missed 170 (57%) infections. To judge their suitability for high-throughput screens, TARE-2 and *var*ATS assay performance was tested on sample pools. Both ultra-sensitive assays correctly detected all pools containing one low-density *P. falciparum* positive sample among nine negatives, which went undetected by 18S rRNA qPCR.

Conclusions

Malaria prevalence in communities is largely determined by the sensitivity of the diagnostic tool used. Prevalence in our study population was underestimated by 10% even when applying molecular diagnostics. Our findings highlight the need for highly sensitive tools such as TARE-2 and *var*ATS qPCRs in community surveillance and for monitoring interventions to better describe malaria epidemiology and inform malaria elimination efforts.

Introduction

Accurate and sensitive detection of malaria parasites is a key factor in planning, targeting and evaluating malaria control efforts and requires different strategies at different elimination stages [1–3]. One major challenge is the identification of remaining reservoirs of human-to-mosquito transmission in asymptomatic individuals carrying low-density infections. The true extent of this predominantly submicroscopic reservoir became better defined with the wider application of molecular detection techniques in epidemiological studies [4,5] and its relevance to sustained malaria control has been brought into focus [1–3]. It was recently estimated that submicroscopic but PCR-detectable infections make up 20% of all malaria infections in high-transmission areas and as much as 70% in low-endemic areas, where they contribute 40% of all transmission to mosquitoes [5]. Mass drug administration (MDA) interventions include treatment of these undetected carriers and can thereby reduce parasite prevalence for several months in low-to-moderate prevalence settings, with even longer effects predicted at low-transmission levels [6,7]. According to modelling predictions, mass screening and treatment (MSAT) strategies have a lower impact than MDA-based interventions [7], as MSAT is limited by the sensitivity of the diagnostic tool used. A recent study in Burkina Faso found no sustained effect of anti-malarial treatment on incidence of clinical episodes nine months after MSAT using conventional rapid diagnostic test (RDT)-based diagnosis [8]. This finding is likely attributable to the large proportion of undetected low-density infections. The true parasite burden could be better defined using nucleic-acid based diagnostics, but even then, very low-density infections might be missed. Such low-density infections might be particularly prevalent in areas with a recent and drastic decline in *P. falciparum* force of infection, where high parasite densities and disease are controlled by residual immunity. As more countries successfully reduce malaria prevalence [9], the proportion of low-density infections can be expected to rise and more sensitive diagnostics that surpass even conventional PCR are urgently needed to detect potential hidden reservoirs.

Of the current molecular detection methods available for malaria diagnosis (summarized in Table 1), RNA-based techniques such as quantitative Reverse Transcription-PCR (qRT-PCR) [10–12], nucleic acid-sequence based amplification (NASBA) [13–15] or ELISA-like hybridization assays [16] reach highest sensitivities by targeting the highly abundant 18S

small subunit ribosomal RNA (18S rRNA). However, due to the unstable nature of RNA, these assays require dedicated and controlled sample collection and storage and thus only have a limited application in field settings. DNA-based techniques are generally easier field-adaptable and include nested PCR [17–22], quantitative PCR (qPCR) [23–31], loop-mediated isothermal amplification (LAMP) [32–35], isothermal recombinase polymerase amplification (RPA) [36], and alternative PCR-based detection methods [19,37–41]. Of the DNA-based assays, only qPCR allows to robustly quantify copy numbers of the template DNA in the reaction as a measure of parasite load in the sample.

Due to the lower number of target molecules in the sample, DNA-based techniques have a reduced sensitivity compared to their RNA-based counterparts, but sampling for DNA-based diagnosis is more robust. The most prominent molecular marker is the 18S rRNA gene, present at 5-8 copies per genome, depending on the parasite strain [42]. Lately, several attempts have been made to increase DNA-based PCR sensitivity by choosing mitochondrial [19,27,32,43] or other nuclear multi-copy targets [40,44]. Already in 1997, Cheng *et al*. designed a nested PCR detecting the conserved region of the subtelomeric *stevor* gene group with many copies per genome [45], which had improved sensitivity over single-copy PCRs [46].

We have taken this approach further and chose high-copy subtelomeric sequences to develop novel qPCR assays for highly sensitive detection and quantification of *P. falciparum* in low-density infections. The telomere associated repetitive element 2 (TARE-2) is a 1.6-kb long block consisting of ten to twelve 135-bp repeat units with slightly degenerate sequences, interspersed by two 21-bp sequences [47,48]. The TARE-2 repeat is present at 24 of 28 subtelomeres in *3D7* [48], which amounts to ca. 250-280 copies per genome, and is specific to *P. falciparum* strains [47].

The *var* gene family is located primarily in the subtelomere and was chosen to develop a second qPCR with multi-copy target. The genome of the *3D7* culture strain harbours 59 *var* genes [48], and an estimated 50-150 copies are present in other parasite lines [49,50]. *Var* genes encode the *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) and possess a transmembrane domain and one intron, with exon 1 and 2 encoding for the extra- and intracellular parts of *Pf*EMP1. In contrast to the highly variable extracellular domain, the

intracellular acidic terminal sequence (*var*ATS) comprises some well-conserved stretches and can thus be targeted by qPCR [49,50].

With the aim to increase test sensitivity at least tenfold and to improve the robustness of parasite detection at low densities, we developed two novel qPCR assays using the multi-copy TARE-2 and *var*ATS sequences as targets. We then investigated the potential of both assays to detect ultra-low density infections that are beyond the detection limit even of 18S rRNA qPCR. We further hypothesized that the abundance of the PCR target in the parasite genome would counterbalance the diluting effect of sample pooling, and thus tested the suitability of our assays for application to sample pools.

Methods

Primer design and qPCR conditions

For *var*ATS primer design, all 59 *var*ATS sequences per *P. falciparum* genome (strain *3D7*; PlasmoDB) were aligned using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Within the size polymorphic *var*ATS domain (size range 1-1.5 kb), the most conserved domain was selected for primer and MGB-probe design. One wobble was each inserted into the forward primer and probe to improve annealing, whereas the reverse *var*ATS primer matched very well with all *3D7 var*ATS sequences. We expect that only about 40% of *3D7* sequences match sufficiently well with the selected oligonucleotides to yield an amplification product. Attempts to further increase assay sensitivity by using additional wobbles and combinations of primers were not successful. Primer and probe sequences as well as qPCR reaction and cycling conditions are listed in Supplementary Table S1.

The TARE-2 repeat region was identified in the genome of *P. falciparum* strains *3D7* (NCBI, http://www.ncbi.nlm.nih.gov/) and *IT* (PlasmoDB, http://plasmodb.org/plasmo/) using the Tandem Repeats Finder tool (http://tandem.bu.edu). TARE-2 sequences of other *P. falciparum* strains were retrieved by BLAST search using *3D7* and *IT* repeat units (http://blast.ncbi.nlm.nih.gov). All repeat units were aligned using Clustal Omega (http://www.ebi.ac.uk) and primers were designed on the most conserved stretches so that 8 nucleotides prior to the 3' end matched with the majority of repeat sequences. One wobble was inserted into each primer for better annealing. Owing to repeat degeneration and therefore difficult probe design, probe-free SYBR® Green-based real-time quantification

of amplicons was chosen. Primer sequences and qPCR reaction and cycling conditions are specified in Supplementary Table S1. Melt curves of amplicons were inspected in each experiment to detect false positivity. True positive samples differed clearly from primer dimer and unspecific PCR products based on the amplicon's melting temperature (Supplementary Figure S2).

Samples were quantified using a standard curve of plasmid (*var*ATS) or parasite genomic DNA (gDNA, TARE-2). As *var*ATS standard, the *var*ATS amplicon was amplified from *3D7* genomic DNA and inserted into the TOPO®-TA vector (Invitrogen). The purified plasmid was diluted to 10^6, 10^4 and 10^2 plasmids/µl in TE buffer. As TARE-2 standard, gDNA of a 10-fold dilution of ring-stage *3D7* parasite culture was used ($6.8x10^3$ to $6.8x10^{-2}$ parasites/µl, described in Supplementary Protocol S3).
The reference 18S rRNA qPCR was performed as described previously [23], using a MBG-probe (6FAM-5'-ACGGGTAGTCATGATTGAGTT-3'-NFQ-MGB) in a total volume of 12 µl. DNA volume matched that of *var*ATS and TARE-2 qPCRs. The amount of target DNA in each sample was calculated from the C_t value using a plasmid standard curve as described above (18S rRNA amplicon inserted in TOPO®-TA vector, Invitrogen).

Analytical specificity and sensitivity

The analytical specificity of TARE-2 and *var*ATS qPCRs was assessed both *in silico* using BLAST search and experimentally using *human* genomic DNA from a healthy malaria-free volunteer and *P. malariae* and *P. ovale* genomic DNA from archived anonymized diagnostic blood samples. No amplification from non-*falciparum Plasmodium* or *human* DNA was observed using the *var*ATS and TARE-2 qPCRs.

For assessment of *P. vivax* cross-reactivity, 14 samples with low to medium number of genomic *P. vivax* 18S rRNA copies (22 - 393 *Pv*18S rRNA copies/µl, light microscopy (LM): 0 - 219 parasites/µl) were selected from a previously analysed sample pool [11]. All 14 selected *P. vivax* DNA samples had been diagnosed *P. falciparum*-negative by 18S rRNA qPCR. All 14 samples were *var*ATS and TARE-2-negative.

Analytical sensitivity and qPCR efficiency were validated on a *P. falciparum* dilution row of *in vitro* cultured ring stages (*3D7* strain). Details on generation of the dilution row are

presented in Supplementary Protocol S3. TARE-2 and *var*ATS qPCR efficiencies were comparable to that of 18S rRNA qPCR, however, all qPCR efficiencies were slightly outside the desirable efficiency range of 90-105% (Table 2). The limit of detection (LOD) was defined as the last dilution at which more than 50% of replicates were positive. The TARE-2 and *var*ATS assays were 10x more sensitive than 18S rRNA qPCR and reached a LOD of 0.034 parasites/µl blood (Table 3). This corresponds to 7 parasites in 200 µl whole blood, which is the typical volume normally processed for DNA extraction from finger prick blood samples.

Field samples and DNA extraction

Field samples used for these analyses were derived from a cohort study conducted in Maprik district, PNG [11] and a cross-sectional survey conducted in Rufiji, TZ, in 2013. Scientific approval and ethical clearance for the PNG cohort study was obtained from the Medical Research and Advisory Committee of the Ministry of Health in PNG (MRAC no. 09.24) and the Ethics Commission of Basel Land and Basel Stadt (no. 237/11). Approval for the TZ cross-sectional study was obtained from the Institutional Review Board of the Ifakara Health Institute, Dar es Salaam, TZ (no. 13-2013). Informed consent was obtained from all study participants in PNG and TZ, for children from parents or legal guardians prior to sampling.

The 60 DNA samples from PNG were selected from a larger pool of previously analysed samples based on their positivity in 18S rRNA qPCR (33 positives, 27 negatives) and using 18S rRNA copy numbers in theses sample to select a wide range of parasite densities [11]. DNA of PNG samples was extracted using the FavorPrep^{TM} 96-well genomic DNA extraction kit (Favorgen) from 50-150 µl blood cell fraction, eluted in 200 µl elution buffer and stored at -20°C.

The 500 TZ samples were age-stratified randomly selected from the larger cross-sectional sample set. DNA was co-extracted during RNA extraction from 50 µl whole blood in 250 µl RNAprotect Cell reagent (Qiagen) using the RNeasy Plus 96 kit (Qiagen). Briefly, DNA was recovered from the gDNA eliminator column after two washing steps according to the QIAamp 96 DNA blood kit protocol (500 µl AW1 buffer, 500 µl AW2 buffer) and eluted in 100 µl AE elution buffer. After the first round of performing all three qPCRs, TZ samples positive in only one assay were repeated in duplicate for all assays. TZ samples positive in two out of three assays were repeated in duplicate for the negative assay only. Samples were defined as positive if two out of three replicates were positive.

Generation of pooled samples

Low-density *P. falciparum* positive samples (< 2 parasites/µl by TARE-2 qPCR, LM negative) were selected from the TZ collection and mixed with 4 or 9 *P. falciparum* negative blood samples to create pools of 5 or 10 samples. Negative samples were prepared by mixing 50µl blood from a malaria-negative blood donor with 250µl RNAprotect Cell reagent (Qiagen) to permit simultaneous DNA and RNA isolation. Per sample 100 µl of whole blood in RNAprotect Cell reagent were added to the pool, resulting in a total sample volume of 500 µl or 1 ml (for 5- and 10-sample pools, respectively). DNA was extracted from the entire volume of these pools using the RNeasy Plus 96 kit (Qiagen) as described above and DNA was eluted in 100 µl (5-sample pools) or 200 µl (10-sample pools). In total we generated 20 pools of 5 samples, 5 of which contained a *P. falciparum* positive sample, and 10 pools of 10 samples, 2 of which contained a positive sample.

Results

Detection of ultra-low density infections in Maprik area, PNG

We compared the ability of the three qPCRs to detect low-density *P. falciparum* infections in 60 DNA samples from PNG. All 33 samples that were positive in 18S rRNA qPCR were also positive using both ultra-sensitive assays. Out of the 27 samples negative by 18S rRNA qPCR, four were positive in *var*ATS qPCR. The same four samples plus five additional samples were positive by TARE-2 qPCR. Since samples were not randomly selected but chosen deliberately to include a wide parasite density range, this result does not reflect the true *P. falciparum* prevalence in Maprik area, PNG. Nevertheless the number of additional samples positive for *P. falciparum* demonstrates that a considerable proportion of infections may persist at ultra-low densities and remain undetected by standard qPCR.

Prevalence of ultra-low density infections in Rufiji, TZ

P. falciparum prevalence in Rufiji, TZ, was assessed on 500 samples randomly selected from a larger cross-sectional study conducted in 2013. *P. falciparum* prevalence was significantly higher using ultra-sensitive detection methods as compared to 18S rRNA qPCR ($p < 0.05$). Prevalence values were 58.4% (95% CI: 54.0 – 62.6) by TARE-2 qPCR, 58.0% (95% CI: 53.6 – 62.2) by *var*ATS qPCR and 49.4% (95% CI: 45.0 – 53.7) by 18S rRNA qPCR (Figure 1A). The gain in *P. falciparum* prevalence thus was 9% and 8.6% by TARE-2 and *var*ATS qPCR,

respectively. This corresponds to roughly a fifth of *P. falciparum* carriers that were missed using standard 18S rRNA qPCR.

The gain in prevalence by use of ultra-sensitive versus standard qPCR was similar across all age groups. We observed the highest gain in diagnosed infections in infants <1 year when using TARE-2 qPCR (16.7%%, p = 0.21, Figure 1B). For *var*ATS qPCR-based prevalence the gain in infants was small with 5.6% (p = 0.63). Parasite densities or target copy numbers in the blood sample were low in infants <1 year, peaked in 2-3 year-old children and thereafter decreased continuously with age for all assays (Figure 1C). Parasite loads observed in infants were lower, equal or higher than those observed in >60 year-olds using TARE-2, *var*ATS and 18S rRNA qPCRs respectively. This suggests that infections carrying the lowest parasite densities are found in infants and were missed using *var*ATS qPCR and even more so using 18S rRNA qPCRs.

Inter-assay agreement and correlation of *P. falciparum* quantification in Rufiji, TZ

The level of agreement between assays was almost perfect with K = 0.86 for all assays (Fleiss' Kappa) as well as in pairwise comparisons (Cohen's Kappa K = 0.80-0.95) in the TZ sample set. 297/500 samples (59.4%) were *P. falciparum* positive by any assay. 50/297 samples were not detected by 18S rRNA qPCR but were positive in either *var*ATS or TARE-2 qPCR (Figure 2A). 80% (40/50) of these samples were detected by both ultra-sensitive assays. All samples detected by 18S rRNA qPCR were also detected by *var*ATS qPCR and all but two by TARE-2 qPCR.

Good correlation of *P. falciparum* parasite quantification by 18S rRNA qPCR and microscopy was demonstrated previously [23]. Similarly, quantification by 18S rRNA qPCR also correlated very well with *var*ATS and TARE-2 quantification in field samples from Rufiji (R^2 = 0.98 and R^2 = 0.95, respectively, p < 0.001, Figure 2B). Correlation of *var*ATS and TARE-2 quantification was also very high with R^2 = 0.97 (p < 0.001). Parasite loads by TARE-2 qPCR in samples negative by 18S rRNA qPCR were, except for few outliers, within the lowest quartile of all parasite loads by TARE-2 quantification. The same was observed for *var*ATS copy numbers of 18S rRNA negative samples.

Performance on sample pools

To investigate the potential of our assays for a wider application in malaria surveillance or epidemiological field studies, we tested the power of all three qPCR assays to identify *P. falciparum* positive samples in pools of 5 or 10 samples, each containing one low-density *P. falciparum* infection.18S rRNA qPCR failed to identify the two positive 10-sample pools and only identified 1 of 5 positive 5-sample pools. In contrast, *var*ATS and TARE-2 qPCR correctly detected all positive 5- and 10-sample pools. No amplification was observed from negative control-pools. Our ultra-sensitive assays thus proved suitable for detection of low-grade infections after dilution in nine negative samples. These infections would be missed by 18S rRNA qPCR after pooling. In a setting with 2% *P. falciparum* prevalence as simulated here, the cost of sample processing and detection can therefore be reduced by at least 70% without loss in sensitivity if ultra-sensitive assays are applied on pools of 10 samples.

Discussion

Accurate data on parasite prevalence in the community are imperative for targeting antimalarial interventions and for monitoring their outcome. In this study, we provide first evidence of very low-grade infections in individuals who had previously been considered parasite-free, even after molecular diagnosis. In Rufiji, a high-endemic area in TZ, microscopic and submicroscopic infections each made up roughly 40% of all *P. falciparum* infections. The remaining approx. 20% were of ultra-low density and not detected by regular 18S rRNA qPCR. Also in Maprik area in PNG, 18S rRNA qPCR failed to identify ultra-low density infections, which resulted in underestimation of the parasite burden. Improved measures of prevalence have consequences for monitoring and evaluating malaria control activities as well as for assessing the potential for onward transmission from human hosts to mosquitoes. In malaria-endemic areas with formerly high infection rates and recent drop in transmission due to successful control programmes, in particular, most *P. falciparum* infections are asymptomatic and often submicroscopic. In such areas, detection of infection rather than assessment of malaria-associated illness could serve as a better measure of the malaria burden and a better parameter for surveillance and evaluation [1].

A meta-analysis of infection prevalence across the endemicity spectrum has indicated that submicroscopic infections are generally more prevalent in low transmission settings than in

high transmission areas [5], probably as a result of a recently reduced force of infection and the long duration of asymptomatic untreated infections [51–53]. This suggests that prevalence of ultra-low-density infections and, thus, underestimation of the infection burden may also be greatest in low-endemic areas. Few studies have investigated the transmission potential of submicroscopic infections. Microscopically patent infections with gametocyte densities below the microscopical threshold can infect mosquitoes albeit at lower rates than microscopically gametocyte-positive samples (13.2 vs. 2.3% infected mosquitoes) [54]. Similarly, data from the mid-20th century and from two recent studies showed that even blood from infections without any microscopically detectable parasite resulted in 0.2-3.2% infected mosquitoes [5,55–57]. Despite low infectivity of each individual infection, the relative contribution of low-density infections to forward transmission to mosquitoes becomes substantial in areas where these account for a large proportion of infections [5,54]. Accurately defining the full extent of the submicroscopic infection burden, including the lowest-grade infections only detectable with ultra-sensitive assays, is therefore a prerequisite for evaluating its relevance to maintaining malaria transmission.

Our results highlight the fact that prevalence data are strongly dependent on the sensitivity of the diagnostic technique applied. Even if parasite prevalence is measured using standard qPCR protocols, many low-key infections remain undetected. Standard PCR is widely considered to be the gold standard of malaria diagnosis, yet our results suggest that this notion requires revision. It becomes increasingly clear that the volume of blood analysed and the use of multi-copy markers to increase representation of a PCR template in the diagnostic assay have great influence on the prevalence outcome. Our findings shed new light on MSAT strategies for interruption of transmission in elimination settings, particularly those that rely on RDT-based diagnosis only, as it becomes clear that the ignored proportion of submicroscopic infections is even larger than anticipated. Following a recent MSAT campaign in TZ, RDT-undetected infections were given as a plausible explanation for the short-lived effect on malaria episode incidence [58]. In this study, more than 45% of PCR-detectable infections were missed, which, given our results, is very likely a substantial underestimation. A major task now consists in adapting molecular methods with enhanced sensitivity to meet the requirements of a robust, field-compatible diagnostic assay. Such tools become increasingly important to determine the infection burden irrespective of endemicity level.

We have presented here two ultra-sensitive qPCR assays for improved detection of low-grade P. *falciparum* infections and application to sample pools. The *var*ATS qPCR is very robust, highly specific and allows fast and easy data analysis through the use of a sequence-specific probe. The TARE-2 assay is more susceptibility to changes in chemical composition of the DNA solution and requires melt curve analysis of amplicons, which can be a potential draw-back, particularly when performed by less-trained personnel. Surprisingly, the TARE-2 qPCR did not outperform the *var*ATS assay despite substantially higher target numbers in the genome. This might be explained by the degenerate sequence of the TARE-2 repeat units or by the clustered distribution of the repeats at chromosome ends. In the *3D7* genome about ten TARE-2 tandem repeats are present at 24 chromosome ends and, in this arrangement, are unlikely to be separated during DNA extraction. The 59 *var*ATS targets of strain *3D7* also localize to chromosome ends and a few intracellular loci. We assume an equal probability for both targets to be represented in a PCR reaction, but certainly both assays surpass that of 18S rRNA assays with three copies on different chromosomes.

Because of the need for advanced laboratory infrastructure and staff training, use of our TARE-2 and *var*ATS qPCRs in their current setup is not feasible in remote field settings. However, they are ideally suited for use in reference laboratories, for example for quality assurance or for centralized processing of large sample numbers in sample pools. Several strategies for pooling samples for malaria surveys have been described, comprising one or several pooling steps before [59–61]or after [62,63] DNA extraction. Pooling is severely limited by its inherent diluting effect and is therefore not recommended in the malERA strategy [1]. In low-endemic settings, in particular, where pooling would be most cost- and labour-effective, submicroscopic infections are highly prevalent [5] but are most likely missed in pools due to their low densities. Our *var*ATS and TARE-2 proved to be useful for sample pooling as they counterbalance the diluting effect through multiple marker copies per parasite. In our hands even the lowest-density infections diluted with nine negative samples were still detectable. This may be further enhanced by increasing the volume of blood samples and concentrating material before qPCR [64]. Availability of ultra-sensitive assays such as our TARE-2 and *var*ATS qPCRs makes sample pooling without loss in sensitivity feasible and allows achieving higher throughput in the context of limited resources in large-scale field studies.

In conclusion, we encourage employing assays with enhanced sensitivity, such as the TARE-2 or *var*ATS qPCRs, in any malaria survey aiming to obtain accurate prevalence data and for monitoring intervention success, and recommend them particularly for screening of community samples in areas of low endemicity. The fact that parasites are more prevalent than currently thought has consequences for malaria control efforts, some of which are based on identifying all infected individuals, and must be acknowledged by all users of prevalence data such as health officials, strategy planners or mathematical modellers. Until the infectiousness to mosquitoes of low-density infections has been clarified, applying the most sensitive tools is essential for better defining the true infection burden and informing elimination strategies.

Acknowledgments

We thank the study participants and their parents or guardians, and the field teams in PNG and Tanzania.

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Author contributions

IF conceived the study, NH and FM conducted lab and field work. IM, LR and SS supported field work in PNG and Tanzania. NH and IF wrote the manuscript.

Funding

This work was supported by Swiss National Science Foundation [grant number 310030_134889], International Centers of Excellence in Malaria Research [grant number U19 AI089686) and Bill and Melinda Gates Foundation [grant number OPP1034577]. FM received funding from the Science and Technology Higher Education Project (STHEP) through the Dar-Es-Salaam University College of Education (DUCE) and the Stipendienkommission Basel Stadt. IM is supported by an NHMRC Senior Research Fellowship (GNT1043345).

Competing interests

The authors declare no conflict of interest.

Ethics statement

Scientific approval and ethical clearance for the PNG cohort study was obtained from the Medical Research and Advisory Committee of the Ministry of Health in PNG (MRAC no. 09.24) and the Ethics Commission of Basel Land and Basel Stadt (no. 237/11). Approval for the TZ cross-sectional study was obtained from the Institutional Review Board of the Ifakara Health Institute, Dar es Salaam, TZ (no. 13-2013). Informed consent was obtained from all study participants in PNG and TZ, for children from parents or legal guardians prior to sampling.

Abbreviations

18S rRNA, 18S small subunit ribosomal RNA

ELISA, enzyme-linked immunosorbent assay

LAMP, loop-mediated isothermal amplification

LM, light microscopy

LOD, limit of detection

MDA, mass drug administration

MGB, minor groove binder

MSAT, mass screening and treatment

NASBA, nucleic acid-sequence based amplification

PfEMP1, *P. falciparum* erythrocyte membrane protein 1

PNG, Papua New Guinea

qPCR, quantitative PCR

qRT-PCR, quantitative Reverse-Transcription PCR

RDT, rapid diagnostic test

RPA, isothermal recombinase polymerase amplification

TARE-2, telomere associated repetitive element 2

TZ, Tanzania

*var*ATS, *var* gene acidic terminal sequence

Tables

Table 1. Assay characteristics and limit of detection (LOD) of published *P. falciparum* detection assays.

Method	Template molecule	Target gene	Quantification	LOD (parasites/ µl blood)	Reference
nPCR	DNA	18S rRNA, dhfr-ts, 28S rRNA, Stevor	No	0.1 - 10	[17-22,45]
PCR	DNA	mtDNA	No	0.5	[43]
qPCR	DNA	18S rRNA, Cox1, Cytb	Yes	0.02 - 3	[23-31]
PCR-based	DNA	18S rRNA, Cox1	Yes/No	0.5 - 1	[19,37-41]
LAMP [$]	DNA	18S rRNA, mtDNA	No	1 - 10	[32-35]
RPA[$]	DNA	18S rRNA	No	4	[36]
qRT-PCR	RNA	18S rRNA	Yes	0.002-0.02	[10-12]
(QT-)NASBA[$]	RNA	18S rRNA	Yes/No	0.02	[13-15]

[$] Isothermal amplification process

Table 2: qPCR details and efficiencies of the 18S rRNA, *var*ATS and TARE-2 assays

Assay	Slope	Efficiency (%)	Intercept [a]	R^2	Platform	Amplicon length[b]	Amplified copy numbers in genome
18S rRNA	-3.63	88.5	41.09	1.0	Taqman	221 bp	3
*var*ATS	-3.63	88.6	34.50	1.0	Taqman	65 bp	<59[c]
TARE-2	-3.75	84.7	32.08	0.97	Sybr Green	93 bp	<250-280[c]

[a] Intercept equals the C_t value of the DNA equivalent of 5 parasites added to the qPCR reaction.

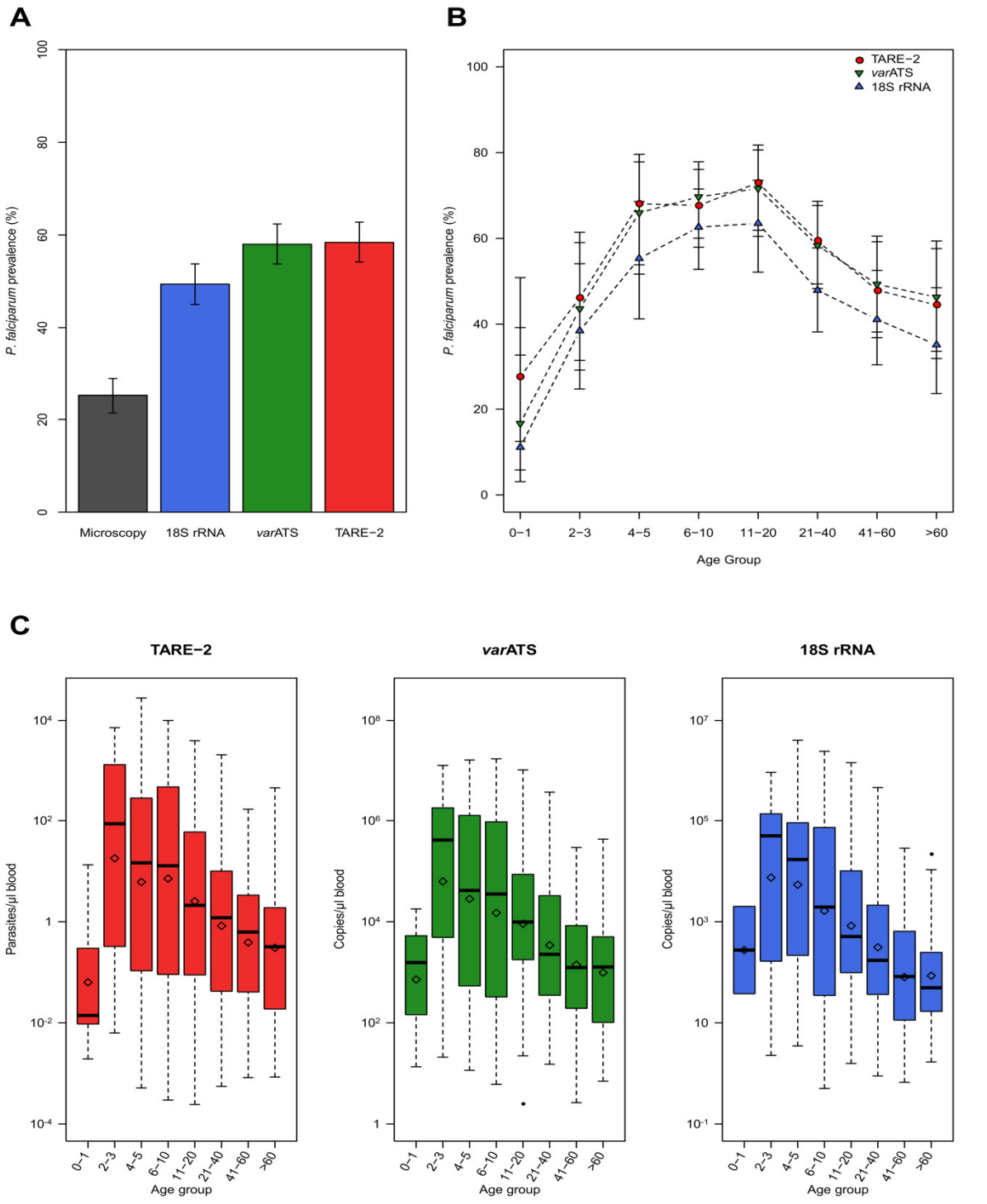
[b] Length of consensus sequence.

[c] Polymorphism in primer binding sites likely does not permit efficient amplification of all genomic copies. Number of target sequences present in parasite genomes from field samples cannot be determined in absence of the respective genome data. Therefore this assay is not suitable for absolute parasite quantification.

Table 3: LOD of TARE-2, *var*ATS assays and 18S rRNA qPCR determined by serial dilution of ring-stage parasite culture (*3D7* strain)

	18S rRNA		***var*ATS**		**TARE-2**	
Parasites/µl blood	Positivity	Mean C_t (±StDev)	Positivity	Mean C_t (±StDev)	Positivity	Mean C_t (±StDev)
6800	3/3	26.3 ± 0.5	3/3	19.9 ± 0.3	3/3	16.9 ± 1.0
680	3/3	30.4 ± 0.5	3/3	23.8 ± 0.4	3/3	21.1 ± 1.4
68	3/3	34.1 ± 0.5	3/3	27.5 ± 0.2	3/3	24.7 ± 1.7
6.8	3/3	37.6 ± 0.7	3/3	30.8 ± 0.1	3/3	28.6 ± 1.6
3.4	3/3	38.7 ± 0.3	6/6	32.0 ± 0.2	6/6	29.7 ± 1.4
0.68	3/3	40.8 ± 1.2	6/6	34.4 ± 0.3	6/6	31.9 ± 1.3
0.34	3/3	42.7 ± 0.8	6/6	35.5 ± 0.2	6/6	32.6 ± 1.7
0.068	1/3	-	6/6	37.3 ± 0.3	6/6	38.5 ± 2.0
0.034	0/3	-	6/6	37.7 ± 0.5	5/6	42.0 ± 1.5
0.0068	0/3	-	1/6	-	0/6	-
0.0034	2/3	-	2/6	-	2/6	-
0.00068	0/3	-	1/6	-	1/6	-

Figures



(A) Overall *P. falciparum* prevalence by different diagnostic methods. Error bars represent 95% CIs. (B) *P. falciparum* prevalence based on TARE-2, *var*ATS and 18S rRNA qPCRs by age. Error bars represent 95% CIs. (C) *P. falciparum* densities based on TARE-2, *var*ATS and 18S rRNA qPCRs by age. The geometric mean in each age group is marked by a diamond; the median is denoted by a black line.

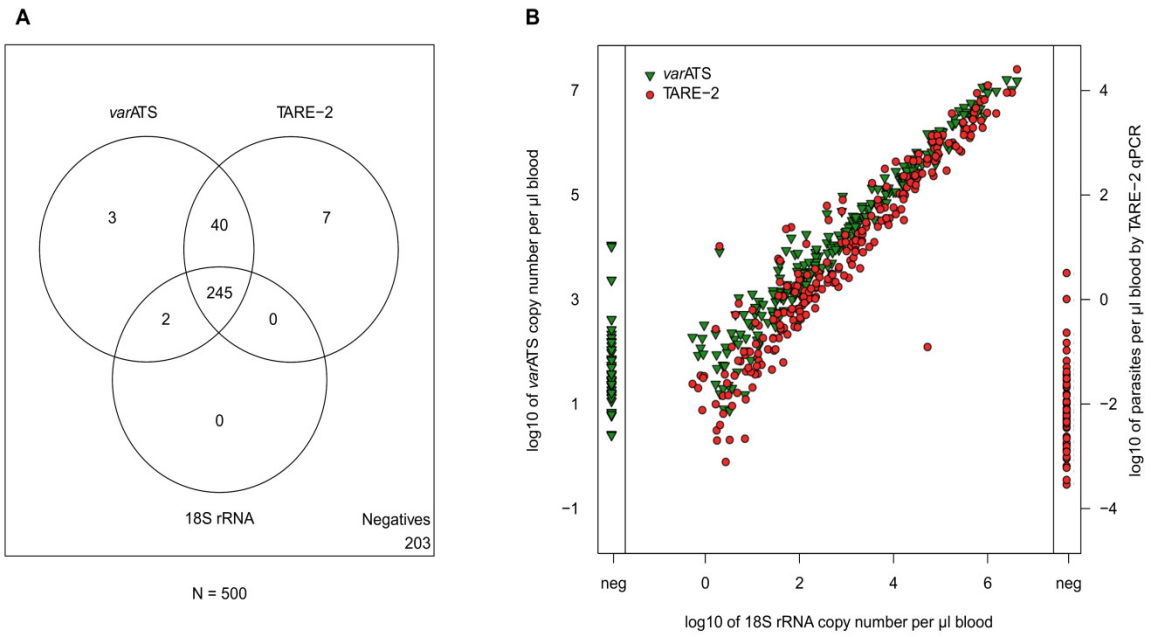


Figure 2: Correlation of sample positivity and quantification using *var*ATS, TARE-2 and 18S rRNA qPCRs.

(A) Venn diagram of positivity by *var*ATS, TARE-2 and 18S rRNA qPCR in samples from Rufiji, TZ. (B) Parasite quantities determined by ultra-sensitive assays and their correlation with 18S rRNA quantification. Quantification was done relative to copy numbers of plasmid standards (18S rRNA, *var*ATS) or a parasite dilution row (TARE-2). Quantities of samples negative in 18S rRNA qPCR but positive in ultra-sensitive assays are shown in the left (*var*ATS) and right panel (TARE-2).

Supporting information captions

Table S1: Oligonucleotide sequences and qPCR conditions for *var*ATS and TARE-2 assays. Primers were purchased from Eurofins. The *var*ATS-probe and all qPCR reagents were purchased from Applied Biosystems/Life technologies.

	varATS	**TARE-2**
Oligonucleotide sequences		
Primer-fw (5'-3')	cccatacacaaccaaytgga	ctatgttgcacttacatgcayaat
Primer-rev (5'-3')	ttcgcacatatctctatgtctatct	tgacctaagaagtavaataatgatga
Probe (5'-3')	6-FAM-trttccataaatggt-NFQ-MGB	-
qPCR reaction conditions (final concentration in qPCR mix)		
Total volume	12 (25)[$]	25 (25)[$]
DNA volume	4 (5)[$]	4 (5)[$]
TaqMan® Gene Expression Mastermix	1x	-
Power SYBR® Green mix	-	1x
Primer (each fw & rev)	800 nM	200 nM
Probe	400 nM	-
qPCR cycling conditions		
Pre-incubation	2 min – 50°C	2 min – 50°C
Initial denaturation	10 min – 95°C	10 min – 95°C
Denaturation	15 sec – 95 °C	15 sec – 95 °C
Annealing & Elongation	1 min – 55°C	1 min – 57°C
Number of cycles	45	45
Melt Curve	-	57-95°C, 0.3°C increment
Positivity threshold	0.07	0.07
Standard material for quantification	Plasmid	gDNA of parasite dilution row
Platform	StepOne Plus® Real-Time PCR System (Applied Biosystems)	StepOne Plus® Real-Time PCR System (Applied Biosystems)

[$] Brackets: volumes used for sensitivity and specificity tests on parasite culture and for PNG samples

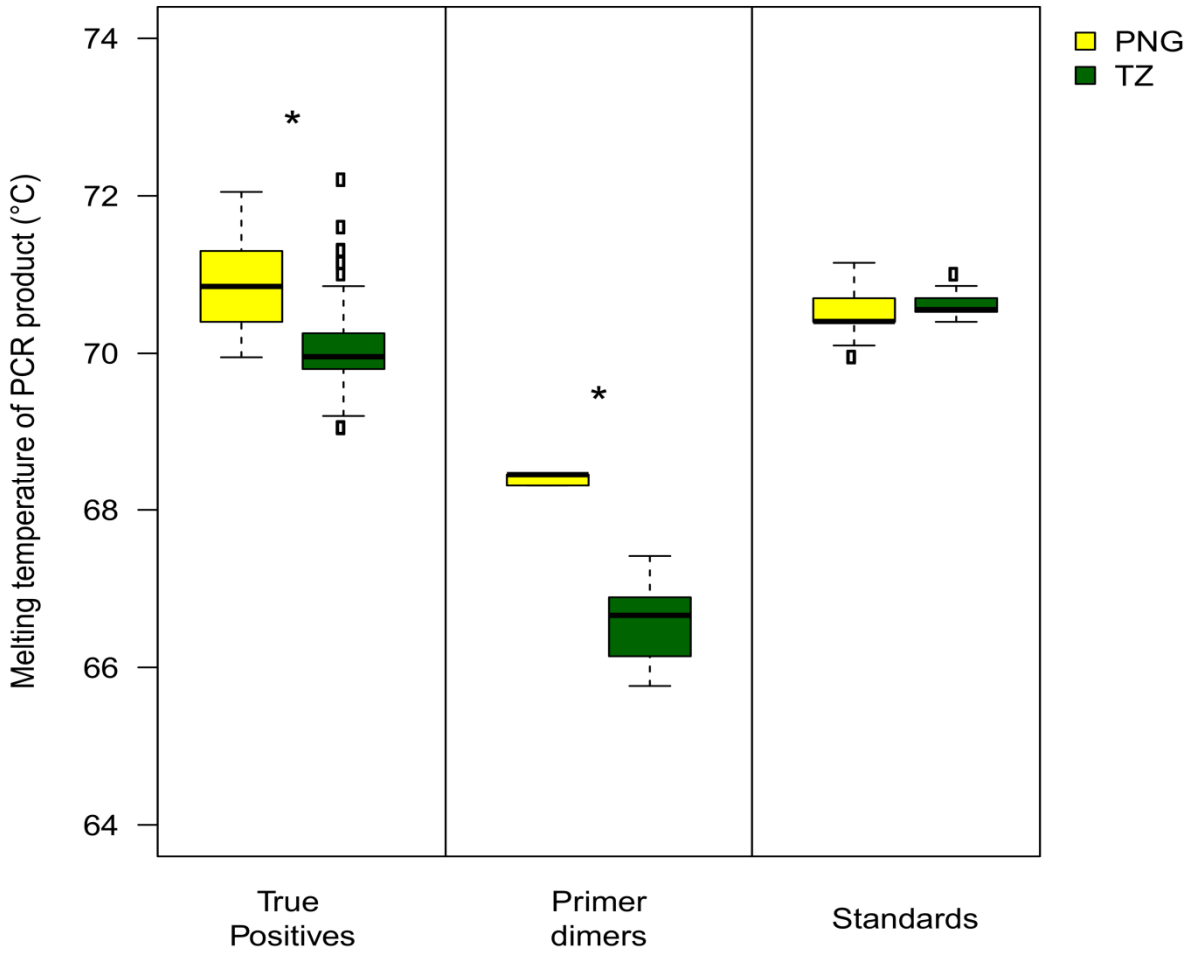


Figure S2: Melting temperature of TARE-2 amplicons using DNA samples from 2 different sources (surveys in TZ and PNG).

Melting temperature (T_m) of true positives (TP, as in positive control/standards) differ significantly from false positive signals (primer dimer, $p < 0.001$). Owing to the degenerate character of the TARE-2 repeat unit, PCR products vary in sequence composition, which is reflected in slight variations in the T_m of TP (TZ 68.6 – 72.2 °C; PNG 70.0 – 72.1 °C). Different DNA extraction kits and dilution buffers used in the PNG and TZ surveys cause shifts in T_m for both specific amplicons and primer dimer. The mean T_m of TP and primer dimer was significantly different between PNG and TZ samples ($p < 0.001$), while qPCR amplicons amplified from 3D7 DNA standard included on both the TZ and PNG qPCR plates showed no significant differences in their mean T_m. The T_m of specific amplicon and primer dimer was hence established separately for each of our two sets of field samples.

Protocol S3: Generation of a *P. falciparum* dilution row from cultured ring stages (*3D7* strain)

After 2 rounds of synchronization (5% sorbitol), ring-stage parasites were quantified by microscopic examination of 15 fields of a Giemsa-stained thin film by counting parasites versus red blood cells (RBC). In total >2000 RBC were screened. Parasite concentration in the blood pellet was calculated based on RBC counts of two representative blood pellets in a Neubauer-counting chamber. A serial dilution of parasite culture was produced in PBS and diluted 1:10 in whole blood from a malaria-negative blood donor. For each dilution, DNA was isolated in triplicate from 100 µl blood using the QIAamp DNA Blood Kit (Qiagen), eluted in 100 µl TE-buffer and stored at -20°C. For determination of assay sensitivity, each assay was performed on the extracted triplicates of all dilutions, once for high-density dilutions ($6.8x10^4$ to 6.8 parasites/µl) and twice for low-density dilutions (3.4 to 0.00068 parasites/µl).

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Strategies for Detection of *Plasmodium species* Gametocytes

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Abstract

Carriage and density of gametocytes, the transmission stages of malaria parasites, are determined for predicting the infectiousness of humans to mosquitoes. This measure is used for evaluating interventions that aim at reducing malaria transmission. Gametocytes need to be detected by amplification of stage-specific transcripts, which requires RNA-preserving blood sampling. For simultaneous, highly sensitive quantification of both, blood stages and gametocytes, we have compared and optimized different strategies for field and laboratory procedures in a cross sectional survey in 315 5-9 yr old children from Papua New Guinea. qRT-PCR was performed for gametocyte markers pfs25 and pvs25, *Plasmodium* species prevalence was determined by targeting both, 18S rRNA genes and transcripts. RNA-based parasite detection resulted in a *P. falciparum* positivity of 24.1%; of these 40.8% carried gametocytes. *P. vivax* positivity was 38.4%, with 38.0% of these carrying gametocytes. Sensitivity of DNA-based parasite detection was substantially lower with 14.1% for *P. falciparum* and 19.6% for *P. vivax*. Using the lower DNA-based prevalence of asexual stages as a denominator increased the percentage of gametocyte-positive infections to 59.1% for *P. falciparum* and 52.4% for *P. vivax*. For studies requiring highly sensitive and simultaneous quantification of sexual and asexual parasite stages, 18S rRNA transcript-based detection saves efforts and costs. RNA-based positivity is considerably higher than other methods. On the other hand, DNA-based parasite quantification is robust and permits comparison with other globally generated molecular prevalence data. Molecular monitoring of low density asexual and sexual parasitaemia will support the evaluation of effects of up-scaled antimalarial intervention programs and can also inform about small scale spatial variability in transmission intensity.

Citation: Wampfler R, Mwingira F, Javati S, Robinson L, Betuela I, et al. (2013) Strategies for Detection of *Plasmodium species* Gametocytes. PLoS ONE 8(9): e76316. doi:10.1371/journal.pone.0076316

Editor: Rick Edward Paul, Institut Pasteur, France

Received April 24, 2013; **Accepted** August 23, 2013; **Published** September 27, 2013

Funding: This work was supported by the Swiss National Science Foundation (grant no. 310030_134889), the International Centers of Excellence in Malaria Research (grant no. U19 AI089686-03) and the Brazilian Swiss Joint Research Programme (grant no. BJRP 0112-07). L. Robinson was supported by an Australian National Health and Medical Research Council Early Career Fellowship #1016443. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

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Introduction

The importance of molecular monitoring of gametocytes of *Plasmodium* parasites is increasingly acknowledged, because it provides fast and sensitive quantification of the parasite stage required for transmission from humans to mosquito vectors. Molecular techniques detect particularly very low gametocyte densities that escape detection by light microscopy (LM). Gametocyte densities are an important measure for evaluating effects of interventions that aim at reducing transmission, such as specific drugs, vaccines or bednets [1–3]. Monitoring gametocytes in population studies can inform about the human infective reservoir and provides relevant data for transmission models.

Mature stage V gametocytes circulate in the peripheral blood of infected humans for a mean period of 6.4 days or a maximum of 3 weeks, but often at sub-microscopic levels [2,4]. The proportion of gametocytes among total parasites per host ranged from 0.2% in young children to 5.7% in adults [5]. By molecular techniques gametocytes are differentiated from concurrent asexual forms by targeting RNA transcripts of gametocyte-specifically expressed genes. In the past, detection of submicroscopic gametocytaemia of *P. falciparum* and *P. vivax* was achieved by two non-quantitative methods, reverse transcription-PCR (RT-PCR) and nucleic acid sequence based amplification techniques (NASBA) [4,6–9]. Currently quantitative NASBA techniques are applied increasingly for gametocyte detection of both, *P. falciparum* [10] and *P. vivax*

[11]. For this work we have developed and validated quantitative qRT-PCR TaqMan probe-based assays for *P. falciparum* and *P. vivax* gametocytes.

In view of large field studies planned to monitor effects of antimalarial interventions, robust sampling strategies and laboratory assays are needed. For the detection of gametocytes of malaria parasites, we have evaluated and compared several approaches adapted specifically to those meso- to highly endemic settings where several *Plasmodium* species occur together. Diagnosis of multiple species incurs substantial costs for cross sectional surveys or surveillance, when mostly uninfected individuals or asymptomatic parasite carriers require testing. This issue was addressed by introducing a generic screening assay to determine initially in a single experiment all those samples positive for any *Plasmodium* species. Only samples positive in the generic test were carried forward to species-specific and gametocyte assays. Our aim was to devise a parsimonious but sensitive diagnostic approach, generating robust information on asexual stage and gametocyte densities. These strategies should be useful for investigating transmission dynamics in longitudinal studies.

Prompted by previous reports of successful usage of finger prick blood collected on various filter paper brands in the field [6,7], we have compared the efficiency of sampling and storage on filter paper versus in solution. Three different sampling strategies were applied in the field: (i) whole blood stored in RNAprotect® cell reagent, (ii) whole blood spotted onto Whatman® 3MM filter paper, air dried and stored in TRIzol® reagent thereafter, and (iii) Whatman FTA classic cards. The focus of this study was on the practical field work in the endemic settings with realistic time periods and limited access to freezers. This adds to some recent comparisons of laboratory cultured gametocytes under a variety of controlled conditions and stored for a maximum period of 3 months until processing of samples [8,12]. Both these studies provided a good overview on various brands of filter papers. However, under the specific conditions of malaria surveillance or intervention programs, time intervals from sample collection to processing in the molecular laboratory will likely extend beyond 3 months. Therefore we have investigated the stability of RNA in stored blood samples and that of extracted RNA over a two year interval.

High throughput of samples requiring RNA extraction for gametocyte detection becomes a technical challenge in the context of intensified malaria surveillance. RNA extraction from filter papers is more tedious and contamination prone than handling liquid samples in 96-well format. We have therefore investigated the field applicability of RNAprotect solution (Qiagen) which stabilizes RNA for short term storage and transport at ambient temperature and permits RNA extraction in 96 well plates. We made use of the availability of DNA and RNA of each sample for evaluating the diagnostic sensitivity of DNA-based versus RNA-based parasite detection in field samples. Due to the high abundance of transcripts of our molecular marker 18S rRNA we expected detection of very low density infections, even below the detection limit of standard PCR.

In Papua New Guinea (PNG) all four major *Plasmodium* spp. infecting humans co-occur, whereby *P. falciparum* and *P. vivax* are the predominant *Plasmodium* species with similar frequency [13]. Malaria endemicity is geographically variable throughout PNG with variations not only along broad environmental gradients, but also between villages only a few kilometers apart [14] and even between different clusters of houses within the same villages [15,16]. In this work we have compared several sampling methods and molecular diagnostic approaches, the summary of which lead us to propose a parsimonious strategy for high throughput molecular monitoring in endemic areas with several sympatric *Plasmodium* species.

Materials and Methods

Study population and ethics

Samples were collected from February to March 2010 from 315 mostly asymptomatic children aged 5 to 9 years. This cross-sectional survey formed part of a major cohort study conducted in the Albinama region of Maprik District, East Sepik Province, a malaria endemic area in PNG. Written informed consent was obtained from parents or guardians of each child. Ethical clearance for all molecular analyses was obtained from the Medical Research Advisory Committee of the PNG Ministry of Health (MRAC no. 1206) and from the Ethics Committee of Basel (no. 237/11).

Blood collection and sample storage and transport

From each study participant approximately 250µl of blood was collected in the village by finger prick into a BD microtainer™ containing EDTA. Samples were stored by three different methods: (i) 50 µl whole blood spotted on Whatman® 3MM filter paper directly after bleeding, air dried and stored for 2-4 weeks at +4 °C. Then each blood spot was cut into multiple pieces, transferred into a microfuge tube containing 300 µl TRIzol® reagent (Life Technologies, Zug, Switzerland) and stored at -80°C until shipment on wet ice packs to the molecular laboratory; upon arrival filter papers again were stored at -80°C until RNA extraction. When handling filter papers, RNase was eliminated by RNase away™ Reagent (Ambion) (ii). 50 µl whole blood spotted on FTA classic cards (Whatman, cat. number: WB120205) directly after bleeding, air-dried completely, stored at +4°C and shipped with desiccant at ambient temperature, then again stored at -20°C until RNA extraction (iii). After transport to the field laboratory and within a maximum of 4 hours following blood collection, 50 µl whole blood was transferred from the microtainer into 250 µl RNAprotect® cell reagent (Qiagen). The mixture was stored at -20°C until transported with wet ice pack cooling to the molecular laboratory. RNAprotect and FTA card samples were stored for 5-8 months before nucleic acids were extracted. Filter papers in TRIzol were stored for up to 1 year.

The sampling methods described should not be considered optimized procedures, but rather reflect the best possible option under the specific field conditions. Clearly, the shorter the storage at ambient temperature and the time span to RNA extraction, the better. But stricter protocols are often only

realizable under trial conditions or when working with in vitro cultured parasites or blood from travel clinics.

Extraction of nucleic acids

RNA extraction. All three blood samples collected from 315 study participants were subject to different RNA extraction protocols that had been optimized with *P. falciparum* 3D7 in vitro culture.

(i) Whatman 3MM filter paper: RNA was extracted from whole blood spotted on Whatman 3MM filter paper (corresponding to 25µl whole blood) and stored in TRIzol. Filter papers were transferred from TRIzol into 600 µl RLT lysis buffer containing β-mercaptoethanol (Qiagen RNeasy® plus mini kit) and incubated for 15 min at 30°C on a shaker at 1000 rpm. After centrifugation for 30 sec at 13000g the aqueous phase was transferred to a gDNA eliminator column, a kit component. From this step onwards, the instructions of the kit supplier (Qiagen) were followed closely. The now following procedure included an on-column DNase digest performed after the first washing step with buffer RW1. 10 µl RNase-free DNase (Qiagen) was mixed with 70 µl RDD buffer and added to the membrane. DNA digestion was allowed to proceed for 15 min at room temperature and then terminated by a wash step with buffer RW1 and the supplier's protocol was continued. Finally RNA was eluted in 50 µl RNase-free dH2O and stored for short term at -20°C, for long term at -80°C.

(ii) Whatman® FTA classic cards: RNA was extracted from FTA cards using the Qiagen RNeasy® plus mini kit protocol to 5 filter discs punched out from FTA cards impregnated with whole blood using a Harris Micro-Punch (tip diameter 3.0 mm). Discs were vortexed in buffer RLT Plus for 1 minute and the incubated for 30 min at room temperature (24-27°C) followed by a gDNA eliminator column and an on-column DNase digestion (all Qiagen) according to the manufacturer's protocols. Yields at room temperature were higher than those at incubation temperature of 50°C. We also tested an alternative strategy for RNA extraction from FTA cards following the Whatman FTA Protocol BR01 (http://www.whatman.com/UserFiles/File/Protocols/Bioscience/BR01). Substantial costs for the recommended RNA processing buffer and our RNA yields severely compromised by the necessity for DNase digestion of extracted RNA prompted us to discontinue this approach.

(iii) RNAprotect® cell reagent: RNA was extracted from 300 µl total volume (50µl whole blood plus 250µl RNAprotect reagent) using the RNeasy® plus mini kit protocol for spin column followed by on-column DNase digestion (all from Qiagen). All procedures followed those described in (i) with exception of the first step. This protocol starts with centrifugation for 15 min at 14'000 g. If a pellet was visible, all supernatant was removed and stored at -80°C for subsequent RNA extractions. If no pellet was visible, the centrifugation was repeated. If still no pellet was visible, 250 µl supernatant were removed and to the remaining 50 µl left in the tube RLT lysis buffer was added and RNA was extracted as described above in procedure (i).

The outcome in common of all our attempts to optimize RNA extraction points towards the use of on-column DNase digestion for minimizing loss of RNA.

DNA extraction. Parasite genomic DNA was extracted from all 311 blood samples, from 4 study participants these samples were missing. After removal of plasma and storage of blood pellets at -20°C for a maximum of two years, 50-150 µl blood pellet was used for DNA extraction (individual volumes were recorded) using FavorPrep™ 96-well genomic DNA extraction kit (Favorgen, Taiwan). DNA was eluted in 200 µl elution buffer and stored at -20°C.

Molecular detection of *Plasmodium* parasites

The workflow in Figure **1** depicts the series of consecutive assays performed with both RNA and DNA samples. The infecting Plasmodium species was determined by quantitative PCR (qPCR) using a gDNA template extracted from blood pellets and in parallel also by quantitative reverse-transcription PCR (qRT-PCR) using RNA obtained from the 3 different sampling methods.

RNA-based *Plasmodium species* diagnosis. After extraction all RNA samples were tested by qPCR targeting genes encoding 18S rRNA to confirm complete digestion of gDNA in a StepOne Plus® Real-Time PCR system (Applied Biosystems). This was followed by a generic qRT-PCR using the same generic primers and probe but on RNA in a one-tube reaction combining the reverse transcription and amplification reaction. Primers and probes are listed in Table **S1**, reaction mixes and PCR profiles are listed in Table **S2**. The RNA-based *P. falciparum* assay targeted A-type 18S rRNA transcripts expressed in asexual stages [17], whereas the generic assay targeting conserved regions would amplify all 5 copies of 18S rRNA genes in the genomes of *P. falciparum* and *P. vivax* [18]. All samples positive by the generic *Plasmodium* sp. assay were further analyzed by species–specific qRT-PCR reactions in a simplex reaction for *P. falciparum* and *P. ovale*, and as a duplex reaction for *P. vivax* and *P. malariae*. Primers and probes are listed in Table **S1**. Due to the generally higher parasitaemia in *P. falciparum* than in *P. vivax* infections, 18S rRNA transcripts in *P. falciparum* samples were highly abundant compared to 18S rRNA of for *P. vivax*. During extensive test evaluation we have observed a low level of aerosol-derived contamination when introducing negative controls, i.e. extraction of water. This low level of air-bourne contamination found in some, but not all negative samples, was corrected for by introducing a cut-off of 10 copies/µl extracted RNA for *P. falciparum* 18S rRNA qRT-PCR; for *P. vivax* no cut off was required. The cut off was identified in a plot of all measured transcript copy numbers by the point from which copy numbers rose above a steady baseline. By introducing a cut-off for *P. falciparum*, 51 previously *Pf* qRT-PCR positive samples were considered false positive. All except one sample had been *P. falciparum* negative by qPCR. This finding gave support to the choice of 10 copies/µl extracted RNA as our cut off.

Each plate carried a dilution series of assay-specific control plasmids with the respective template inserted at concentrations of 10^6,10^4 and 10^2 copies of template/reaction in

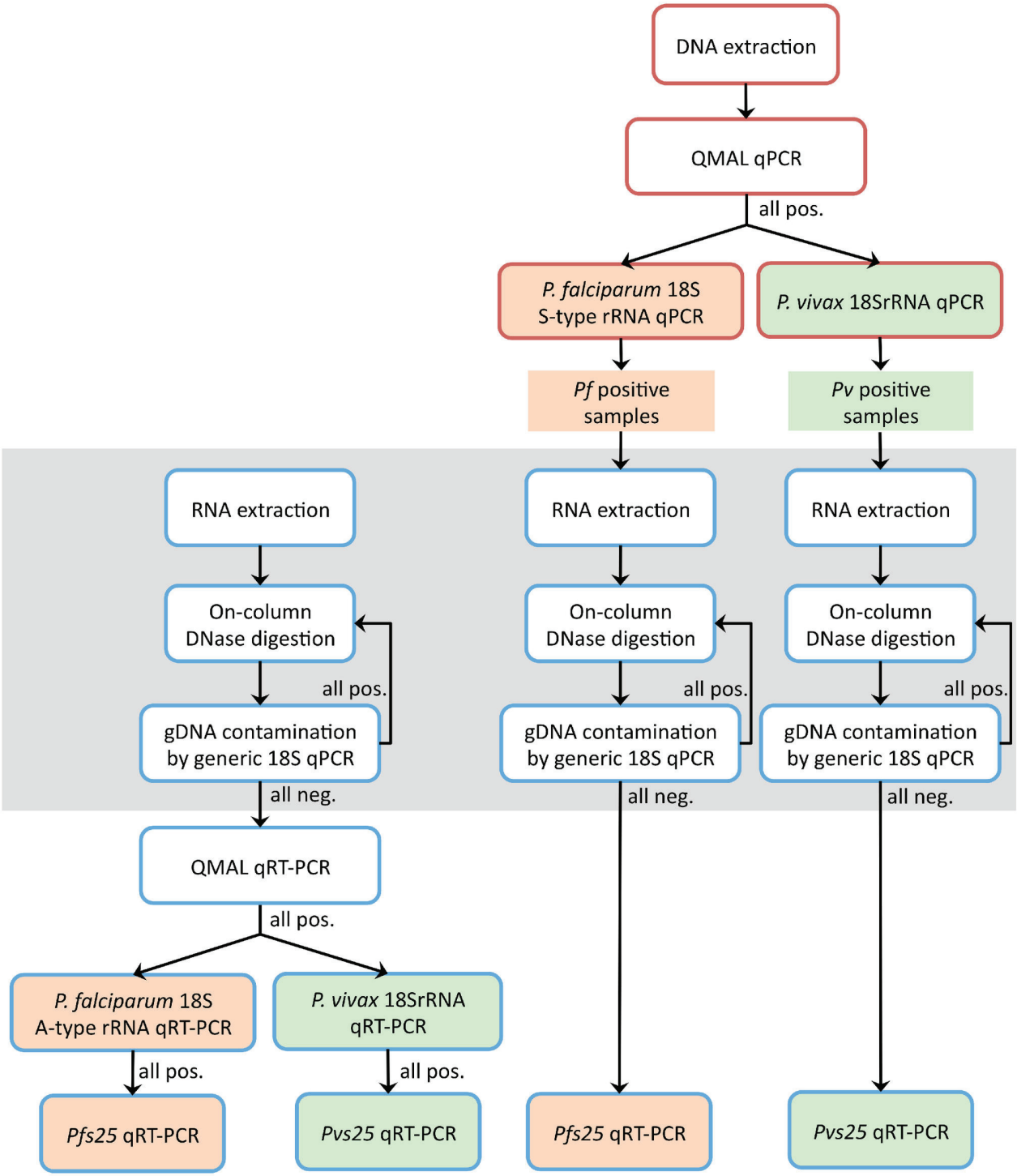

Figure 1. Flow diagram of molecular analyses performed for detection of asexual and sexual parasite stages of *P. vivax* and *P. falciparum* in field samples from PNG. Red and blue frames indicate assays done on DNA and on RNA, respectively. Orange and green boxes are *P. falciparum* and *P. vivax*-specific assays, respectively. *P. malariae* and *P. ovale* assays are not included in the diagram.

doi: 10.1371/journal.pone.0076316.g001

duplicates. For each plate standard curves were generated from these values for quantification of copy numbers in test samples.

DNA-based species diagnosis. As depicted in Figure **1**, all DNA samples were first tested for positivity for any *Plasmodium* parasite species by a generic assay. All samples positive by generic assay were quantified by simplex qPCRs for *P. falciparum* and *P. vivax*. *P. malariae* and *P. ovale* assays were not performed on DNA level, only on RNA level.

Gametocyte-specific assays

For detection and quantification of *P. falciparum* and *P. vivax* gametocytes qRT-PCRs targeting the two orthologues *pfs25* and *pvs25* transcripts (GenBank accession no: AF193769.1 and GU256271.1, respectively) were developed and validated. These genes are the most frequently used markers for gametocyte detection in NASBA. *Pfs25* is highly expressed in mature gametocytes [4]. Sequences of oligonucleotides are given in Table **S1**, the composition of reaction mixes and thermo profiles are shown in Table **S2**. *Pfs25 and pvs25* primers as well as the *pfs25* HEX-BHQ1-labeled and *pvs25* FAM-BHQ1-labeled probes were selected within non-polymorphic positions identified by alignment of all publicly available nucleotide sequences. Of 138 *P. vivax pvs25* sequences, none showed polymorphism at sequences targeted by our assay, with the exception of a SNP observed in a single isolate, however, this DNA was no more available from the authors for confirmation of sequencing [19]. The region of *pfs25* targeted by our qRT-PCR assay is similar to that of the *pfs25* qNASBA assay of Schneider and coworkers [10], whose molecular beacon overlaps with the forward primer of our qRT-PCR assay. *Pvs25* primers and probe target the gene region also chosen by Beurskens and coworkers [11], whose molecular beacon is identical with our probe.

Gametocyte trend line used for conversion of *pfs25* transcript copy numbers into gametocyte counts

A synchronized (5% sorbitol) 3D7 ring culture of 8% parasitaemia was induced to undergo gametocytogenesis at day -2 by reducing the hematocrit and doubling the Albumax concentration in the medium. At day -1, induced trophozoites were diluted to 2% and a second stress medium was added. At day 0, regular medium was added to the gametocytes (modified after [20]). At day 1 and until day 9, 50mM N-acetylglucosamine was added to the gametocytes to reduce surviving of asexual stages [21]. At day 12, gametocytes were purified by a percoll gradient [22] and counted in a Neubauer Cell Count Chamber at 3 different concentrations. The purified gametocytes were diluted in full medium to concentrations of 10'000, 1'000, 100, 50, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, 0.005 gametocytes/µl in triplicates. 50 µl of gametocyte dilution was added to 250 µl RNAprotect cell reagent (Qiagen) and frozen at -20°C. RNA was extracted using the RNeasy plus 96well Kit (Qiagen) with an additional on column DNase digestion step and amplified on *pfs25* qRT-PCR and QMAL qPCR. A linear regression was applied on the $\log_{10}$ transformed copy numbers of the gametocyte trend line by R version 2.14.0 [23]. The regression coefficient (r^2) was 0.95 ($p<0.0001$). The conversion from transcript copy numbers to gametocytes was as follows: gametocyte counts/µl whole blood = $10^{-1.6225}$ * (copy number pfs25 transcripts/µl whole blood) $^{0.8518}$.

Light microscopy (LM)

Blood slides with thick and thin smears were collected in duplicate for each patient and examined microscopically for *Plasmodium* parasite density (asexual stages and gametocytes) and species identification. Three reads were done and densities counted over 200 white blood cells, which were then converted into parasite s/µl by assuming 8000 white blood cells/µl whole blood.

Statistical analysis

Parasite counts by LM were multiplied by 40 (200WBC ◊ 8000WBC/µl blood) and $\log_{10}$ transformed. Template copy number/µl whole blood obtained from qPCR and qRT-PCR were $\log_{10}$ transformed. Only samples with positive cell counts in both tests were considered. For comparing molecular and microscopic parasite quantification methods, linear regression was applied to the log-transformed densities and DNA or transcript copy numbers. Correlation coefficients were calculated with R [23]. F-statistic was applied to test whether regressions (e.g., LM versus molecular detection) were significant.

Results

Validation of assays and Limit of Detection (LOD) for amplification of gDNA and cDNA

For each assay the respective PCR fragment was inserted into a plasmid as described previously [24]. Serial dilutions of these control plasmids were made in quintuplicates. LOD was defined as the lowest concentration of control plasmid (in copy number/µl) yielding positive results in >50% of parallel samples tested. LOD and amplification efficiencies (calculated as Efficiency = $10^{(-1/\text{Slope})}$ -1) of each assay is listed in Table **S3**.

Prevalence of *Plasmodium sp.* in study population by RNA-based versus DNA-based detection

Detection of any Plasmodium species (generic assay) as well as specific detection of *P. falciparum* or *P. vivax* was performed in parallel on DNA and RNA for all samples. DNA-based detection followed our previously described protocol [24]. For RNA-based detection by qRT-PCR the same primers and probes were utilized, except for the *P. falciparum* assay, which on RNA level targets the A-type 18S rRNA instead of the S-type gene as in qPCR [17]. The generic assays on RNA and DNA level were carried out on all samples, whereas the species-specific assays were only performed in samples previously positive by the generic assay according to Figure **1**. Due to the low local prevalence of *P. malariae* and *P. ovale*, both these species were only detected by RNA-based assays, DNA-based assays were omitted. To reduce complexity in Figure **1**, the performed *P. malariae* and *P. ovale* assays were not included.

Table 1. Prevalence of asexual and sexual stages of *P. falciparum* and *P. vivax* detected by microscopy, qPCR or qRT-PCR in samples from 315 children from PNG.

	Detection method of asexual *Plasmodium* stages		
	Light microscopy	DNA-based approach	RNA-based approach
Sample size (N)	301	311	315
P. falciparum prevalence	20/301 (6.6%)	44/311 (14.1%)	76/315 (24.1%)
Pf gametocyte carriers in *Pf* pos.	7/20 (35.0%)	26/44 (59.1%)	31/76 (40.8%)
P. vivax prevalence	40/301 (13.3%)	61/311 (19.6%)	121/315 (38.4%)
Pv gametocyte carriers in *Pv* pos.	23/40 (57.5%)	32/61 (52.4%)	46/121 (38.0%)

doi: 10.1371/journal.pone.0076316.t001

The initial analysis, carried out to screen for the presence of any malaria parasite, detected 112/311 parasite positive DNA samples, whereas RNA-based 169/315 samples were positive. Such discrepancy in positivity mirrors the high sensitivity of detection when targeting highly abundant 18S rRNA transcripts (probably >10^6 per cell), as opposed to only 5 copies of the 18S rRNA gene per genome [18].

All DNA or RNA samples positive by the generic assays were further analyzed by species specific qPCR or qRT-PCR assays, which also targeted 18S rRNA sequences, yet not the conserved part, but stretches instead that differed between *Plasmodium* species. We have compared parasite positivity obtained by both qPCR and qRT-PCR and by light microscopy in our samples (Table **1**). *P. falciparum* prevalence was 14.1% in DNA samples, but 24.1% in RNA samples. The discrepancy was even larger for *P. vivax*, with 19.6% DNA-based and twice as high RNA-based positivity (38.4%). As expected light microscopy provided the lowest prevalence rate (6.6% for *P. falciparum* and 13.3% for *P. vivax*). Because most parasite carriers were asymptomatic, many of these infections likely harbored low parasite densities around the detection limit of microscopy.

Quantification of parasites

For most research questions quantitative parasitological data is desirable. When introducing molecular measures for parasite quantification, their performance with respect to the classical techniques needs to be evaluated. We therefore compared parasite counts by the different methods. Quantification of *P. vivax* was expected to be particularly difficult for two reasons: firstly, in our study area *P. vivax* densities are about 10 fold lower than *P. falciparum* densities [25], thus detection by PCR is more likely affected by the so called “Monte Carlo effect”, i.e., the random presence or absence of template in a tested DNA aliquot deriving from a blood sample of very low parasitaemia. The additional detection of a large number of scanty parasitaemias by the molecular assay of high sensitivity will lower the median parasite density compared to microscopy. Secondly, due to the presence of *P. vivax* schizont stages in the peripheral blood, a single parasite is not equivalent to one genome, but could account for a per parasite >20 fold higher copy number of the target gene.

To permit parasite quantification based on target gene or transcript copy numbers detected by our 18S rRNA assays, we have plotted densities by light microscopy (LM) versus DNA- or RNA-based quantification (Figure **2**). Only samples positive by both compared tests were considered. For *P. falciparum* 17 LM/DNA, 20 LM/RNA and 42 DNA/RNA positive pairs were available, for *P. vivax* these were 29 LM/DNA, 37 LM/RNA and 58 DNA/RNA pairs. For *P. falciparum* (upper panel Figure **2**), parasite quantification by LM and qPCR correlated well (r^2=0.81), when assuming presence of mainly ring/early trophozoite stage parasites in peripheral blood samples (equal to1 genome/parasite).

For *P. vivax* (lower panel Figure **2**) LM parasite counts and DNA copy numbers correlated less well (r^2=0.35). Confidence intervals wider than those for *P. falciparum* denote a less robust quantification for *P. vivax*, likely due to the presence of schizonts or the overall lower densities. For both parasite species the correlation between the measurements for RNA transcripts versus gene copies was around 50% (Figure **2**, panels on the right). In Figure **2** all regressions on the log-transformed data were significant to a p-value less than 0.001.

Due to the lower sensitivity of LM compared to both molecular methods, only a very limited number of samples was available with data from all quantification methods, which does not represent a robust basis for conversion of molecular data. Nevertheless, we have explored this possibility for conversion and calculated the median parasite densities quantified by qPCR or qRT-PCR (Table **S4**) by using the algorithm determined in the regression analyses (shown in Figure **2**). As expected, mean parasite densities by DNA based quantification were lower than by LM, reflecting the contribution of the additional samples sub-patent by LM and with presumably lower parasite densities. RNA-based quantification was not consistent between *P. falciparum* and *P vivax*.

Our efforts to generate molecularly determined parasite counts provided preliminary evidence for a good predictive relationship between DNA copy numbers and microscopic parasite density, especially for *P. falciparum*. Yet, this approach needs further validation by a larger sample set to provide a solid mathematical function for this relationship.

Co-infections with multiple *Plasmodium species*

To determine the overall prevalence of any *Plasmodium species* or of each specific species, we took into account all positive test results from DNA- and RNA-based assays (Table **S5**). Overall malaria parasite prevalence in the 315 children was 54.3%. Of these, almost a quarter had P. falciparum/P. vivax mixed infections (21.1%). Triple infections of *P. falciparum, P. vivax* and either *P. malariae* or *P. ovale* were seen in very few cases (0.6% and 3.5%). P. malariae single infections were less than 1%; no *P. ovale* single infection was observed.

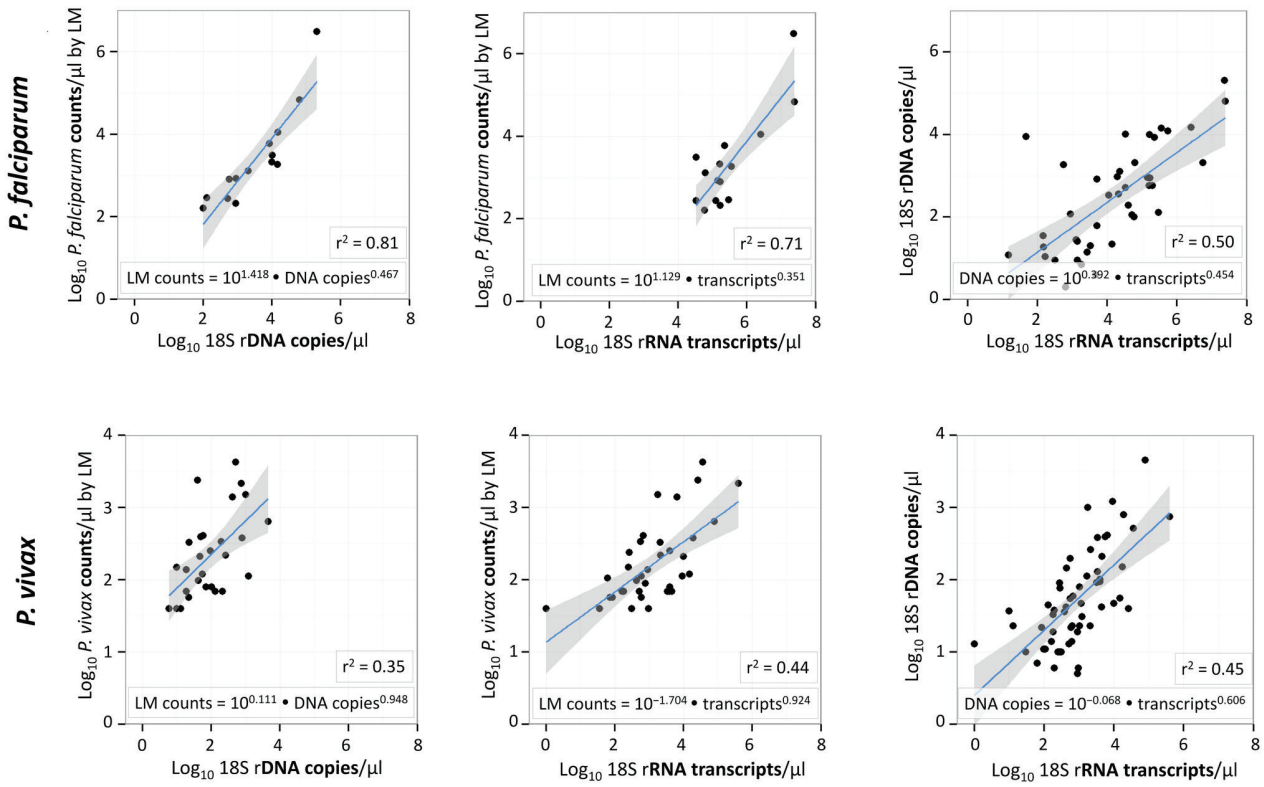

Figure 2. DNA- versus RNA-based quantification of *Plasmodium* parasites by qPCR and qRT-PCR of 18S rRNA genes or transcripts in comparison to light microscopy (LM). *P. falciparum* (upper panel) and *P. vivax* (lower panel). Boxed values indicate the correlation coefficient (r^2) and the conversion functions extracted from these data. All correlation coefficients (r^2) were significant (p-value < 0.001).

doi: 10.1371/journal.pone.0076316.g002

Gametocyte prevalence rates for *P. falciparum* and *P. vivax*

In our hands RNA extraction from FTA classic cards did not yield satisfactory results, despite efforts in optimizing the extraction protocol with the Qiagen RNeasy Plus mini kit. In contrast, gDNA could be extracted from these cards, but positivity in the *Plasmodium* species assays was reduced compared to results from the two alternative sampling methods (data not shown). Storage time >3 months or another, by us unnoticed problem during sampling, shipment or storage, all could have compromised RNA integrity on FTA cards. Our failure to detect gametocyte-specific RNA is in line with a similar work on field samples from Brazil [26].

RNA was extracted successfully from all blood samples collected by both strategies, RNAprotect® and filterpaper/TRIzol®. Of 315 children tested by any sampling method, 32 and 46 carried *P. falciparum* and *P. vivax* gametocytes, respectively (Table **1**). To evaluate the differential performance of the two sampling approaches, we have compared the gametocyte positivity and transcripts numbers by either method for *P. falciparum* and *P. vivax* (Figure **3**). RNAprotect® sampling yielded more *P. falciparum* positive samples than filterpaper/TRIzol®. In samples positive by both methods, transcript copy numbers were higher for RNAprotect® sampling as shown in a comparison of paired results (Figure **3**, bar chart). For *P. vivax*, each of the methods missed about one third of gametocyte positive samples as compared to the summary result. The great fluctuation in *P. vivax* positivity and quantification is likely due to the overall lower density of *P. vivax* asexual stages and gametocytes. By LM no *P. falciparum* or *P. vivax* gametocytes were observed, thus confirming the well established superiority of molecular gametocytes detection. We have assessed how far gametocyte prevalence is associated with asexual densities (Figure **S1**). A positive association of high 18S rRNA transcripts and gametocyte prevalence was observed for *P. vivax* and *P. falciparum*. This association was also seen for 18S rDNA copy numbers of *P. vivax*, but not *P. falciparum*. The data available was rather limited; a more robust investigation of these relationships would require a larger sample set.

Evaluation of *P. falciparum* and *P. vivax* gametocyte quantification assays

For quantification of gametocytes in a blood sample, the number of detected transcripts per sample is not meaningful without a standard curve that permits transforming copy

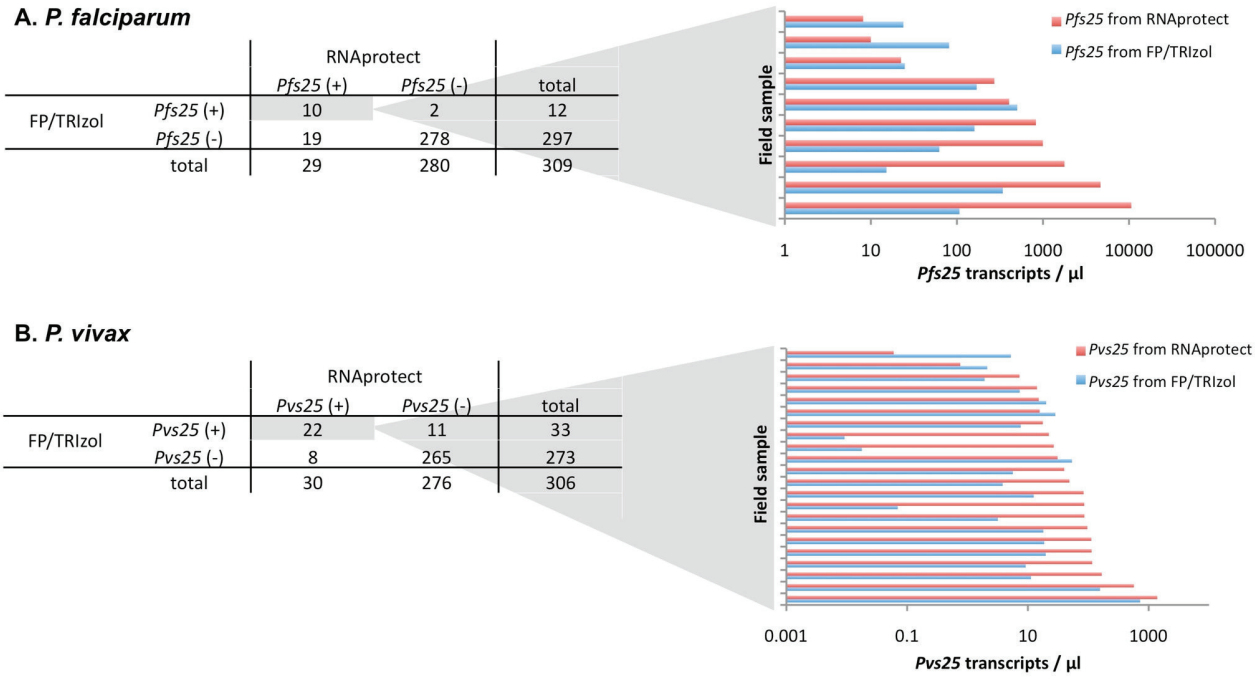

Figure 3. Comparison of two blood sampling strategies for measuring gametocyte prevalence rates. (A) *P. falciparum*, (B) *P. vivax*. Gametocyte positivity (left panel) and transcript copy numbers (right panel) are shown for RNAprotect solution versus filter paper soaked in TRIzol. Only samples were compared for which both measurements were available.

doi: 10.1371/journal.pone.0076316.g003

numbers into gametocyte counts. Therefore a gametocyte trend line was generated from 3D7 in vitro culture by counting gametocytes prior to harvesting RNA. Figure **4** presents the standard curve used for estimating gametocyte loads in our samples and for establishing the detection limit of our qRT-PCR assays. According to the conversion factor obtained from regression analysis (see method section), one *P. falciparum* gametocyte corresponds to 87.05 *pfs25* transcript copies (95% CI: 65.55-115.60). This translates into a limit of detection (LOD) of 0.02-0.05 gametocyte / µl blood when 50 µl blood or parasite culture was subject to RNA extraction. If volumes larger than 50 µl of blood would be sampled, our *pfs25* qRT-PCR would even permit an almost 2-fold greater sensitivity (1 gametocyte / 100 µl blood). This example indicates that LOD based on transcript copy numbers rather than gametocytes only describes detection potential, whereas presence or absence of gametocytes determines the effective sensitivity. Our LOD compares to that published for qNASBA-based gametocyte detection [27]. Due to the lack of *P. vivax in vitro* culture, we had to use the *P. falciparum* based conversion factor for calculating *P. vivax* gametocyte loads. Median gametocyte numbers per µl blood were 0.99 (1st quartile, 3rd quartile: 0.27, 4.95) for *P. falciparum* and 0.34 (1st quartile, 3rd quartile: 0.11, 0.68) for *P. vivax*. So far we failed to establish gametocyte assays for *P. malariae* and *P. ovale* due to yet little success to find *pfs25* orthologues in these species.

Effect of storage duration on stability of *pfs25* transcripts

Two years after extraction of RNA from both types of samples, RNAprotect® and filter paper/TRIzol®, we have repeated *pfs25* qRT-PCR of a subset of all samples representing the full range of transcript copy numbers. Sample pairs plotted side by side did not indicate compromised RNA stability after 2 years of storage at -80°C (Figure **5**). Other protein coding transcripts were not tested and the stability of *pfs25* RNA may represent an exception rather than the rule.

Discussion

The difference in DNA- versus RNA-based *Plasmodium* species determination derives from the dramatic difference in the number of templates per parasite. Each *Plasmodium* parasite harbors only 5 copies of the *18S rRNA* gene, 3 S-type (detected by our qPCR assay) and 2 A-type genes [18], whereas many thousands or even some million copies of *18S rRNA* transcripts can be expected per cell. The use of these extremely abundant transcripts for parasite detection warrants great care during RNA extraction, a large number of negative controls and precise definition of a cut-off to avoid false positives through potential aerosols (low level of signal caused by airborne templates). Depending on the research question, outermost sensitivity may be desired, e.g. when searching for very rare infections in a close to elimination setting. When

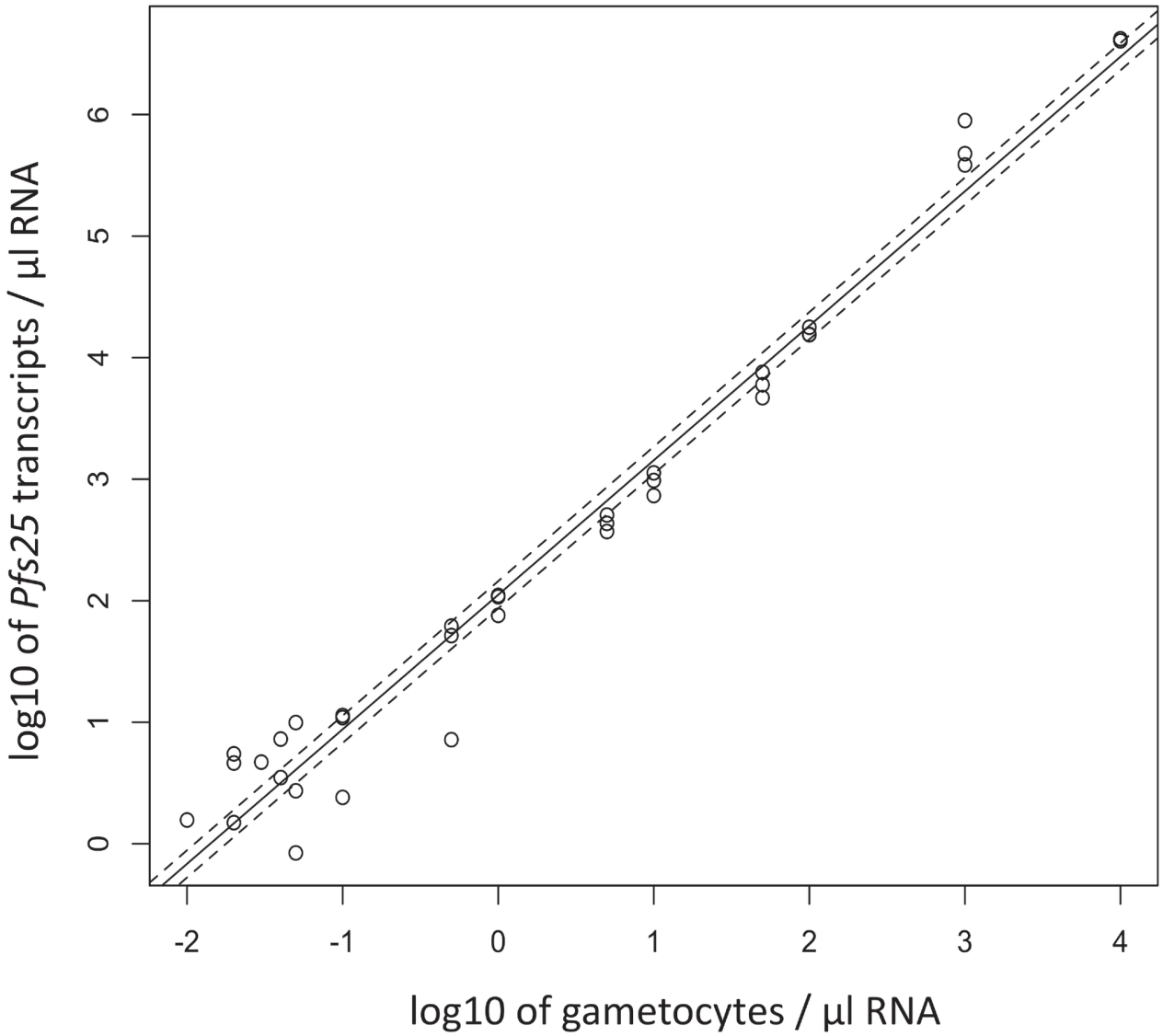

Figure 4. Gametocyte trend line generated with 3D7 *P. falciparum* in vitro culture for converting *pfs25* transcript copy numbers into gametocyte counts. Dashed lines indicate 95% confidence interval of intercept.

doi: 10.1371/journal.pone.0076316.g004

pooling samples for massive parasitological screening, such high sensitivity is a prerequisite. These issues might attract further attention in the eliminations context when sensitive diagnosis is required for detection of asymptomatic parasite carriers with low density, as well as for tracking both asexual and sexual stages in parallel [26].

Sensitivity of detection of blood-borne parasites is defined in a major way by the volume of blood analyzed, which for very low parasitaemias may or may not contain a parasite by chance. In asymptomatic infections the probability of detection of malaria parasites is hampered by generally low densities. Accordingly, the chances of gametocyte detection are even more limited. Our gametocyte assay was found to be sufficiently sensitive to reproducibly detect a single gametocyte in 50 µl whole blood. Because 1 gametocyte corresponded on average to 87 *pfs25* transcript copies, it seems likely that extracting RNA from 100 µl whole blood could have improved detection of scarce gametocytaemia. Generally the blood volume collected in population studies is limited, as up to 250 µl of whole blood can be obtained by finger prick, the usual sampling method for large-scale field surveys and cohort studies. After setting aside aliquots for blood films, serology and DNA extraction, starting material for gametocyte detection is restricted, but should be maximized according to the above results.

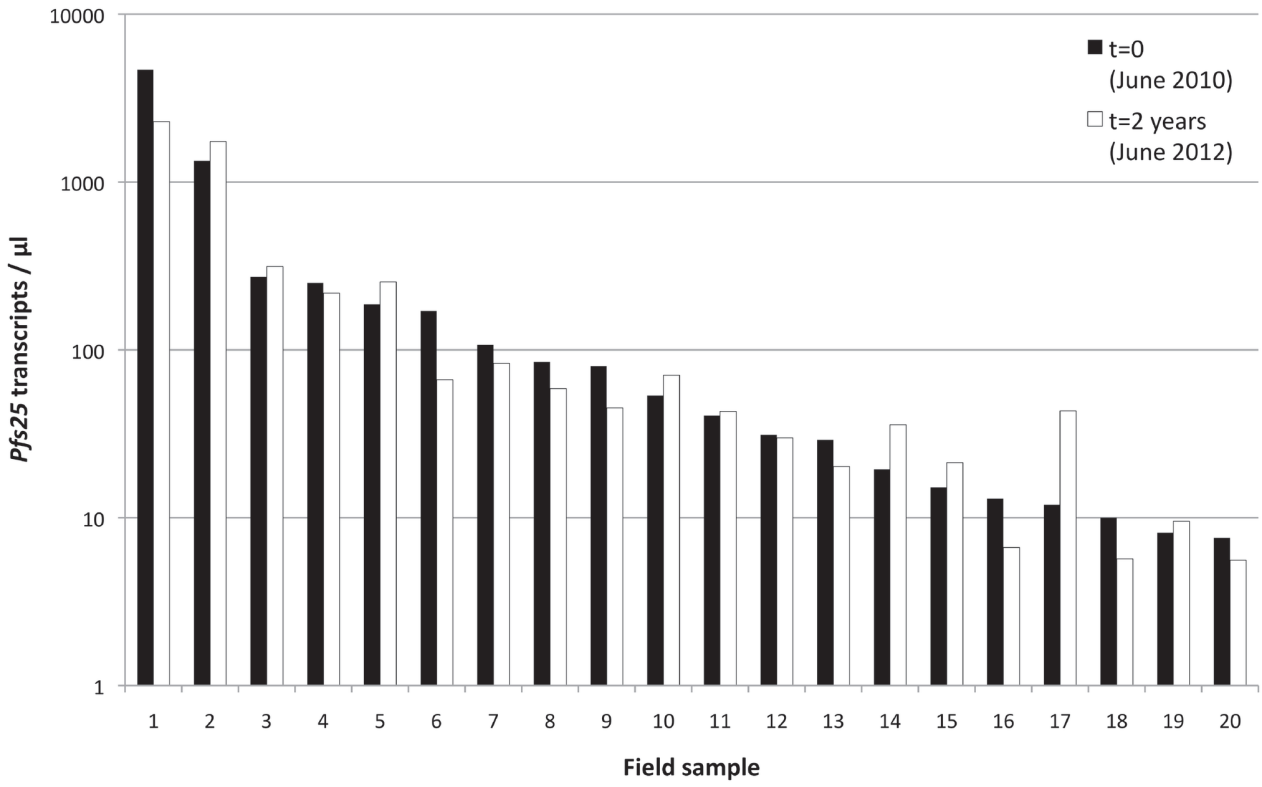

Figure 5. Effect of extended storage time on *pfs25* transcripts. 20 samples were chosen to represent a wide range of transcript copy numbers at start of the 2 yr storage period. The initial copy numbers (black bars) are shown next to copy number detected 2 yrs later in the same RNA sample (white bars).

doi: 10.1371/journal.pone.0076316.g005

When describing DNA- or RNA-based diagnostic assays, the sensitivity of parasite detection is generally presented on the basis of experiments with *in vitro* cultured parasites under optimal laboratory conditions [28]. Yet, for *P. vixax in vitro* culture is not available. The approach used in our study takes into account the conditions under which field samples were collected and shipped, mirroring the setting of routine malariological surveillance. To permit direct comparison of *P. falciparum* and *P. vivax* densities, parasites of both species ideally should be quantified in the same way by extracting an algorithm for converting template copy numbers detected in each sample into parasite s/µl whole blood. We have determined this relationship (Figure **2**) and quantified the median parasite densities for *P. falciparum* and *P. vivax* based on the correlations observed (Table **S4**). For both species DNA based quantification produced mean parasite densities lower that those by LM, because the increased molecular detection contributed primarily samples with parasite densities below the detection limit of LM. RNA-based parasite densities revealed a much greater variance than DNA-based quantification and thus provided less precise estimates of parasite density. This likely reflects small variations in sampling or processing, or could derive from longer delays in reaching the molecular laboratory for some of the samples. The generally adverse and variable field conditions could differentially impair the RNA quality in certain samples. Our data suggests that quantification based on RNA needs further validation.

Our study aimed at improving blood-sampling techniques in the field at remote sites. For this we have optimized gametocyte detection and quantification and compared RNA stability achieved by three sampling methods. We were only able to extract RNA reliably from samples stored in TRIzol and RNAprotect. Samples stored on FTA cards did not give satisfactory results. In contrast, Pritsch and co-workers [12] reported successful RNA extraction from Whatman FTA classic cards. The comparability of these sampling procedures with our results is limited, because our starting material consisted of low density asymptomatic field samples that had been stored for 6 months, whereas storage time was not specified in the earlier publication [12]. In a comprehensive comparison of several filter papers for collecting low density gametocytes, Jones and coworkers [8] reported a much lower amplification success of pfs25 transcripts from FTA classic cards compared to Whatman 3MM untreated filter paper. This is in line with our observations. But also in that study filter papers were stored only for up to 3 months. In our experience, samples from major field surveys very often are stored for periods longer than 3 months and longer than originally anticipated. Storage periods

of 4 weeks of 3 months, which is normally evaluated in comparative analysis of sampling materials, is very brief given the average duration of large scale field studies. Particularly cohort studies may well last over a year, e.g. to capture seasonal changes. We therefore reported the sensitivity of asexual stage and gametocyte detection after extended storage periods of up to 1 year and confirmed the stability of *pfs25* transcripts even after long term storage of 2 years.

Whatman 3MM non-impregnated filter paper has been used successfully in several recent studies [7,12,29], all reporting good RNA yields from blood spotted on filter paper without addition of any RNA-stabilizing reagent such as RNAprotect or TRIzol, but storage duration in these studies was up to 3 months only. We have shown that even long term storage of the blood impregnated 3MM filter paper in TRIzol is possible.

RNAprotect sampling showed best results, despite a delay of several hours until whole blood samples were transferred from EDTA microtainer into RNAprotect reagent. This contrasts with conclusions of a previous study that suggested compromised RNA stability after a 6 hrs delay prior to addition of RNAprotect [7]. The optimal sampling of whole blood in a RNA stabilizing agent would involve blood collection directly into tubes containing RNAprotect. But skin contact to RNAprotect, an irritant substance, should be avoided; thus finger prick blood is first collected in microtainers prior to transferring a 50 µl or larger aliquot into a tube pre-filled with 5 volumes RNAprotect solution. In this field survey we attempted to limit the delay until transfer in stabilizing agent to a maximum of 4 hours. A recent major field study conducted in Burkina Faso has demonstrated the feasibility of mixing whole blood with RNAprotect reagent directly at the site of blood collection without any delay [30]. In conclusion, for field settings far away from laboratory facilities, the latter approach of transferring whole blood directly into RNAprotect immediately after blood collection represents the optimal strategy, which, however, requires thorough training of field staff, e.g. on contamination-free pipetting of an aliquot whole blood from a microtainer into the prepared RNAprotect tube.

By detecting transcripts from late stage V gametocytes, we targeted specifically the parasite population in the human host most relevant for transmission. This depicts the impact of transmission-reducing interventions more closely than markers of earlier gametocyte stages, as not all committed rings might successfully develop into mature gametocytes. The presence of gametocytes is no evidence for subsequent transmission success of gametocytes to the vector. The relationship of gametocyte densities and successful infection of mosquitoes is of great relevance for molecular monitoring of interventions, but only recently first results were published on the prediction of mosquito infection from gametocyte densities [3]. It remains to be shown how molecular gametocyte counts in the host compare to the classical measure of transmission, the entomological inoculation rate [31]. We have evaluated procedures for gametocyte detection and quantification. Sampling strategy and molecular assays can be considered robust tools for molecular epidemiological studies and might prove valuable for estimating the impact of transmission-reducing interventions, such as drugs, vaccines, or vector control.

Supporting Information

Figure S1. Proportion of gametocyte carriers (gray) among *P. falciparum* (upper panel) and *P. vivax* (lower panel) infections separated into three copy number categories for 18S rDNA detected by qPCR or 18S rRNA transcripts detected by qRT-PCR. Sample size of both groups within a category is indicated by numbers within the bars.
(EPS)

Table S1. Primer and probe sequences.
(DOC)

Table S2. PCR profiles and reaction mixes.
(DOC)

Table S3. Limit of detection and amplification efficiencies of all molecular markers determined with control plasmids.
(DOC)

Table S4. Median density of *P. falciparum* and *P. vivax* parasite s/µl detected by microscopy, qPCR or qRT-PCR in samples from 315 children from PNG.
(DOC)

Table S5. Overall parasite prevalence derived from combined results of DNA- and RNA-based detection methods in study population (n=315) and distribution of mixed species co-infections in parasite positive samples.
(DOC)

Author Contributions

Analyzed the data: RW IF. Wrote the manuscript: IF. Conceived the field study: IM. Designed molecular work: IF. Extracted nucleic acids from various blood sampling strategies: RW FM SJ. Developed and validated quantification of parasite stages: RW. Conducted and supervised sample collection in the field: IB LR PS. Supported test development and provided materials for assay validation: HPB.

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Supplementary Table S1: Primer and probe sequences.

A. Generic qPCR and qRT-PCR assays (target: conserved regions in 18S rRNA)		
Species	**Primer**	**Sequence (5' -> 3')**
Plasmodium sp.	**QMAL_fw**	TTA GAT TGC TTC CTT CAG TRC CTT ATG*
	QMAL_rev	TGT TGA GTC AAA TTA AGC CGC AA
	QMAL_probe	**FAM-**TCA ATT CTT TTA ACT TTC TCG CTT GCG CGA **–BHQ**
B. Species-specific qPCRs and qRT-PCR assays		
Species	**Primer**	**Sequence (5' -> 3')**
P. falciparum **(DNA)**	**Pf_S18S_fw**	TAT TGC TTT TGA GAG GTT TTG TTA CTT TG
	Pf_S18S_rev	ACC TCT GAC ATC TGA ATA CGA ATG C
	Pf_S18S_probe	FAM-ACG GGT AGT CAT GAT TGA GTT-MGB-BHQ
P. falciparum **(RNA)**	**Pf_A18S_fw**	TCC GAT AAC GAA CGA GAT CTT AAC
	Pf_A18S_rev	ATG TAT AGT TAC CTA TGT TCA ATT TCA
	PF_A18S_probe	FAM-TAG CGG CGA GTA CAC TAT A-MGB-BHQ
P. vivax **(DNA & RNA)**	**Pv_18S_fw**	GCT TTG TAA TTG GAA TGA TGG GAA T
	Pv_18S_rev	ATG CGC ACA AAG TCG ATA CGA AG
	Pv_18S_probe	HEX-AGC AAC GCT TCT AGC TTA -MGB-BHQ
P. malariae **(DNA & RNA)**	same primers and probe as in ref. 24	
P. ovale **(DNA & RNA)**	same primers and probe as in ref. 24	
C. Gametocyte-specific *pfs 25* and *pvs25* qRT-PCR		
Species	**Primer**	**Sequence (5'>3')**
P. falciparum	**pfs25_fw**	GAA ATC CCG TTT CAT ACG CTT G
	pfs25_rev	AGT TTT AAC AGG ATT GCT TGT ATC TAA
	pfs25_probe	HEX-TGT AAG AAT GTA ACT TGT GGT AAC GGT-BHQ1
P. vivax	**pvs25_fw**	ACA CTT GTG TGC TTG ATG TAT GTC
	pvs25_rev	ACT TTG CCA ATA GCA CAT GAG CAA
	pvs25_probe	FAM-TGC ATT GTT GAG TAC CTC TCG GAA-BHQ1

* wobble R = A/G

Supplementary Table S2: PCR profiles and reaction mixes.

A. qPCR			
qPCR Reaction mix[1]			
Total volume 12 µL	1X gene expression master mix[2]		
	800 nM primer mix		
	200 nM probe[2]		
	2µl of RNA		
qPCR Thermo profile[3]			
Stage	**Step**	**Temperature**	**Time**
Holding	UDG	50°C	2 minutes
Holding	Activation of AmpliTaq polymerase	95°C	10 minutes
Cycling (45x)	Denature	95°C	15 seconds
	Anneal/Extend	58°C	1 minute
B. qRT-PCR: one-tube protocol using the TaqMan® RNA-to-C_T™ *1-Step* Kit[2]			
qRT-PCR Reaction mix [1]			
Total volume 12.5 µL	1X RT-to-CT master mix[2]		
	800 nM primer mix		
	200 nM probe[2]		
	2µl of RNA		
	0.3 µl of Taqman RT enzyme mix (ArrayScript™ UP Reverse Transcriptase and RNase Inhibitor)		
qRT-PCR Thermo profile[3]			
Stage	**Step**	**Temperature**	**Time**
Holding	Reverse transcription	48°C	15 minutes
Holding	Activation of AmpliTaq polymerase	95°C	10 minutes
Cycling (45x)	Denature	95°C	15 seconds
	Anneal/Extend	58°C	1 minute

[1] Reaction mix was prepared on a template-free bench wiped with 2.5M hypochlorite solution. Prepared master mix was added to the reaction plate before transfer to PCR cabinet for template addition. Applied Biosystems MicroAmp® 0.1ml Fast Optical 96-Well Reaction Plate was used for both qPCR and qRT-PCR.

[2] Life Technologies Applied Biosystems, Zug, Switzerland

[3]The GENEX standard thermo profile of StepOnePlus Real-Time PCR system (Applied Biosystems) was modified for both qPCR and qRT-PCR. A maximum of 45 cycles of amplification was set. And all samples with Ct value ≤45 were considered positive.

Supplementary Table S3: Limit of detection and amplification efficiencies of all molecular markers determined with control plasmids.

Assay	**Limit of detection** copy number /µl*	**Amplification efficiencies**
Generic 18S rRNA	1	96.5
P. falciparum 18S rRNA (S-type)	1	82.5
P. vivax 18S rRNA	3	82.2
P. malariae 18S rRNA	1	99.9
P. ovale 18S rRNA	1	92.8
pfs25	1	95.2
pvs25	0.5	92.0

*determined by serial dilution in quintuplicate of control plasmids (PCR template = insert of control plasmid).

Supplementary Table S4. Median density of *P. falciparum* and *P. vivax* parasites/µL detected by microscopy, qPCR or qRT-PCR in samples from 315 children from PNG

	Quantification method of asexual *Plasmodium* stages		
	Light microscopy	**DNA-based approach**	**RNA-based approach**
***P. falciparum* quantification** median [1st quartile, 3rd quartile]	1071 [285, 3839]	350 [24, 1898]	1297 [164, 36904]
***P. vivax* quantification** median [1st quartile, 3rd quartile]	106 [69, 330]	8 [6, 13]	98 [66, 160]

Supplementary Table S5: Overall parasite prevalence derived from combined results of DNA- and RNA-based detection methods in study population (n=315) and distribution of mixed species co-infections in parasite positive samples.

Assay / marker gene	**Positivity**[1]
Plasmodium generic assay	171/315 (54.3%)
Pf single infection	32/171 (18.7%)
Pv single infection	79/171 (46.2%)
Pm single infection	1/171 (0.6%)
Po single infection	0/171 (0%)
Pf + Pv infection	36/171 (21.1%)
Pf + Pm infection	3/171 (1.8%)
Pf + Po infection	0/171 (0%)
Pv + Pm infection	2/171 (1.2%)
Pv + Po infection	0/171 (0%)
Pf + Pv + Pm infection	1/171 (0.6%)
Pf + Pv + Po infection	6/171 (3.5%)
Pf + Pv + Pm + Po infection	0/171 (0%)
missed species typing[2]	11/171 (6.4%)
any *Pf*	78/171 (45.6%)
any *Pv*	124/171 (72.5%)
any *Pm*	7/171 (4.1%)
any *Po*	6/171 (3.5%)

[1]The slight discrepancy to those prevalence rates given in Table 1 derives from very few samples positive by DNA-based detection, but negative by RNA-based detection. Accordingly, the summary result given here shows a slightly higher positivity.

[2]Eleven samples were positive for the *Plasmodium* genus-specific assay, but were negative in all species-specific assays. All but one of these 11 samples derived from RNA-based detection and were characterized by very low copy number (<10 transcripts). These samples must be considered false positive. Due to highly abundant 18S rRNA transcripts in each cell, a low level of aerosol derived contamination is possible. In principle, this issues can be addressed by introducing a cut-off (e.g. for *P. falciparum* 18S rRNA qRT-PCR we in fact applied a cut-off of 10 copies/µl extracted RNA). But our use of the generic assay for screening for all parasite species did not permit application of a stringent cut-off, which according to the occurrence of very high parasite densities would be oriented at *P. falciparum*. Cut off application to *P. vivax* would lead to exclusion of some very low density infections. In consequence, the true parasite prevalence by our generic assay most probably is slightly lower, i.e. excluding the 11 potentially false positive samples and thus amounting to 160/315 (50.8%).

Supplementary Figure

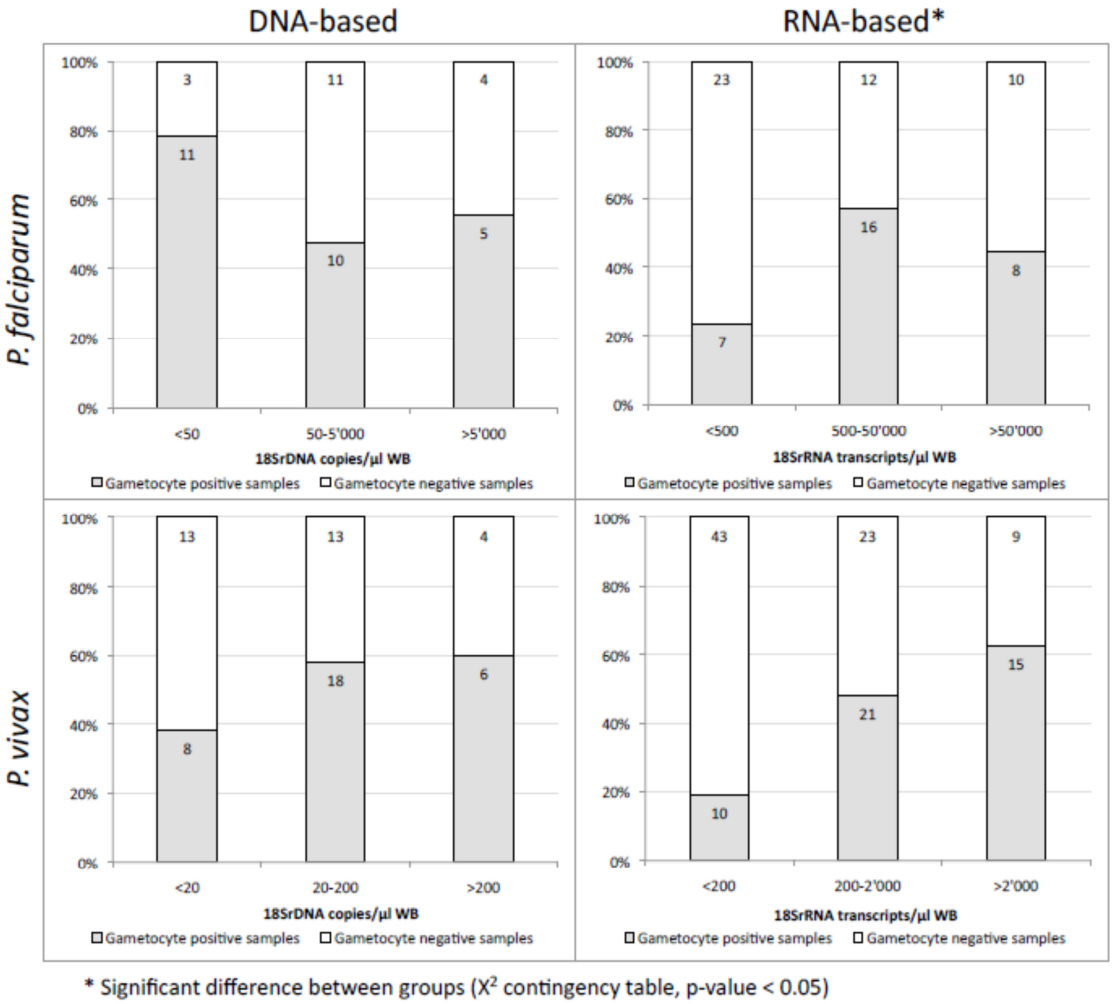

Figure S1. Proportion of gametocyte carriers (gray) among *P. falciparum* (upper panel) and *P. vivax* (lower panel) infections separated into three copy number categories for 18S rDNA detected by qPCR or 18S rRNA transcripts detected by qRT-PCR. Sample size of both groups within a category is indicated by numbers within the bars.

General discussion

The thesis aimed to gauge the extent of underestimation of *P. falciparum* parasite and gametocyte prevalence rates by routine diagnostic tools (RDT and LM) using molecular diagnostic assays as a gold standard. Furthermore, the study estimated the prevalence of submicroscopic infections and attempted to evaluate the local differences in the occurrence of submicroscopic infections at different endemic sites in Tanzania. Two novel qPCR assays for increasing test sensitivity beyond classical PCR were developed in the SwissTPH Molecular laboratory, a subset of the field samples from Tanzania were used to validate these novel assays and compared the results to the routine qPCR assay. Furthermore, the study utilized the gained experience and techniques from the pilot study in Papua New Guinea (PNG), whose aim was to evaluate and improve various methods of RNA sampling strategies for gametocyte detection. These methods were applied in this study to allow for gametocyte detection from community samples in Tanzania.

Correct diagnosis of parasite infection remains a challenge in many endemic areas. Chapter 2 of the thesis enlightens the evaluation of the performance of classical malaria diagnostic tools in community samples from Tanzania. In general the performance of LM in parasite detection of our community samples was poor. LM detected up to sevenfold lower parasite prevalence rates compared to our molecular assay. The detection limit of LM is known to be low, of about 50-100 parasites/µL blood (Moody et al. 2002). Since the study used community samples most of which were asymptomatic individuals harbouring low parasite densities explains in part the low performance of LM. Other factors such as slide preparation methods, loss of parasites during staining of the slides and erroneous reading of thick smears may also have contributed to the low performance of LM. Other studies in malaria endemic areas have documented similar lower prevalence rates where LM recorded almost 2- 10 folder lower prevalence rates than PCR (Rantala 2010; Li et al. 2014). Overall, by using LM community prevalence rates were greatly underestimated. LM was able to identified identified almost half of the total infections identified by PCR. With such low performance of LM in *P. falciparum* infection detection, it is imperative to apply more sensitive diagnostics for quality control of LM in epidemiological surveys in order to provide most precise information to malaria control efforts.

Interestingly, our LM-determined prevalence rates in the surveyed sites are in line with the most recent national malaria indicator (MIS) surveys (THMIS 2013). The MIS results are national representative data on malaria endemicity obtained from community sampling of children less than five years. The use of molecular assay, revealed the poor performance of LM in parasite detection in community samples, and yet our data is in line with the national MIS data, this suggests that the data from MIS could be largely underestimated since it relies solely on classical diagnostics.

The performance of LM in detecting other P*lasmodium* species was extremely low, in fact only 1/2046 samples collected from the 6 sites was *P. malariae* positive by LM. This data is consistent with other LM results from other field study in Tanzania were the prevalence of *P. malariae* and *P. ovale* was 0.3% each (Tarimo et al. 2001). By 2008 the prevalence of *P. malariae* and *P. ovale* was roughly, 0.5% each (Mboera et al. 2008). Molecular typing to confirm the presence of other *Plasmodium* species in our study was not performed, hence it is difficult to rely on LM results alone.

The use of RDT in parasite detection out-performed LM in field samples. RDT detected more than half of all *P. falciparum* parasites identified by molecular tools. Despite the higher performance of RDT over LM, a number the RDT-positive samples were not confirmed by molecular tools or LM. Most of the unconfirmed RDT positives were from the high endemic areas (Kilombero and Rufiji) indicating the presence of circulating parasite antigens as result of constant exposure to infections. The histidine rich protein-2 (HRP-2) is a commonly used *P. falciparum* antigen in RDTs. The HRP-2 antigen may be detected in human blood even after the parasite has been cleared. Persistence of HRP-2 antigens is known to occur for as long as four weeks after parasite clearance (Mayxay et al. 2001). These circulating antigens may confound point prevalence results because it includes even individuals who have cleared parasitaemia. On the other hand, the ability of RDT to detect recent cleared infections that are no longer detectable by LM or PCR, adds to our understanding of transmission level of an area. The performance of RDT in the field was challenged by the lack of appropriate quality control during our surveys. We could only perform lot testing in laboratory with *P. falciparum* positive blood however, this was not possible during the field

surveys. It has been reported that most RDTs have variations even within batches, hence the batch testing is essential (Mouatcho and Goldring 2013). The integrity of RDTs may be compromised during handling and storage in high temperatures and humid conditions which are bound to occur in field settings (McMorrow et al. 2008; WHO 2014).

In general LM and RDTs are sufficient for malaria diagnosis and remain the standard tools for the diagnosis of malaria in many health centres. In fact, Tanzania is currently scaling-up the use of RDTs in health centres for parasite detection before treatment. So far the use of RDT has shown to reduce over-treatment with antimalarial in health facilities in Tanzania (Masanja et al. 2012). However, the suboptimal performance of RDT and even more for LM in infection detection of asymptomatic community samples, urge for the use of highly sensitive molecular diagnostic techniques to obtain more precise prevalence data.

The application of molecular qPCR assay for parasite detection in all samples from our community surveys yielded higher prevalence rates than those by classical diagnostics. With detection limit of 0.34 parasites/µL blood, the higher efficiency in parasite detection compared to classical diagnostic tools was expected. At sites of low and moderate endemic settings the molecularly determined *P. falciparum* prevalence rates were more or less similar with the classical diagnostics, suggesting that LM, RDT and PCR were equally sensitive and that parasite densities were high enough to be captured by LM. At higher endemic site, the discrepancy between the classical tools and PCR was more pronounced. LM detected less than half of the total infections identified by PCR. The use of molecular parasite detection recorded high submicroscopic prevalence of about 52.7% in Tanzania. These submicroscopic infections were common in adults. Higher submicroscopic prevalences were also confirmed by other studies conducted in Tanzania (Shekalaghe et al. 2007; Manjurano et al. 2011). Evidence from this study and from other studies conducted in Tanzania so far suggest that both microscopic-positive and submicroscopic infections were rare in low endemic areas.

The fact that submicroscopic infection was prevalence in all endemic sites suggest that infections were well controlled both in low and high endemic sites, unlike the meta- analysis by Okell et al in 2012 which indicated that submicroscopic infections were more prevalent in low transmission settings. The very few parasite positive samples obtained at the low and moderate endemic sites impede a firm conclusion on the parasite densities and occurrence of submicroscopic prevalence in the low and moderate endemic sites. For instance, at the

low endemic site, Iringa the only 2/329 parasite-positive individuals could have recently been to other endemic areas where they were likely to be infected.

The relevance of these submicroscopic infections in transmission remains largely unclear. A few Studies on mosquito feeding experiments have indicated that submicroscopic infections contribute frequently to infecting mosquitoes (Schneider et al. 2006).Others studies have indicated submicroscopic infections can infect mosquitoes at a rate 10 times lower than microscopy positive blood samples (Coleman et al. 2004). These few studies suggests that the high submicroscopic infections of about 52.7% recorded in Tanzania have a sizeable contribution to mosquito infection and can play a role in propagating transmission in Tanzania. Moreover, other factors such as host age, asexual parasite densities and gametocyte maturity also seem to play a role in mosquito infections (Churcher et al. 2013).

This study also aimed to detect gametocytes in Tanzanian communities using classical diagnostics, LM and molecular diagnostics. Gametocytes are the parasite specific stages for propagating transmission. Knowledge on the occurrence and quantification of gametocytes in Tanzania communities is essential to evaluate control efforts especially those that aim on transmission reduction. Our results revealed low gametocyte prevalence rates by LM. The general gametocyte prevalence in all 6 sites was less than 1% which is more than 15fold lower than that recorded by qRT-PCR. It is known that at most 1% of the asexual parasites commit to the sexual cycle, hence the low prevalence rate of gametocyte was expected. However our qRT-PCR assay revealed much higher prevalence rates compared to LM. Our results indicate that 96% of the identified gametocytes carriers were submicroscopic. This vast underestimation of gametocyte prevalence by LM is alarming since most epidemiological studies rely on LM, control efforts and stakeholders using such underrated data are greatly hampered. Moreover, the high prevalence of submicroscopic gametocytaemia remains a challenge to malaria control efforts since even submicroscopic gametocytaemia could infect mosquitoes and contribute to onwards transmission.

The study evaluated the two newly developed *P. falciparum* DNA-based qPCR assays at the Swiss TTPH molecular parasitology laboratory. These *TARE-2* and *varATS* assays were validated by community samples from Rufiji, a high endemic area in Tanzania. With a detection limit 10 times higher than the routinely used *18S rRNA* qPCR, these assays

captured the low density infections that were missed even by our routine qPCR. The parasite prevalence estimates of Rufiji were greatly improved. A gain in parasite prevalence was 8.6% by the *varATS* and 9% by *TARE-2* assay. This gain in prevalence suggests that the sensitivity of the diagnostic tool plays a major role in precision of prevalence data. In the context of Tanzania, these methods designed to detect low density infections are most useful in areas of low endemic settings such as Iringa or Zanzibar Islands. In such low endemic areas the prime goal is elimination and thus the priority is to capture all infections unlike in high endemic areas such as Rufiji where, roughly more than half the population is infected.

RNA sampling methods were evaluated in another study from Papua New Guinea. The study aimed to improve blood sampling techniques for RNA preservation in the field for subsequent gametocyte detection. RNA for gametocyte detection was fruitful yielded from whole blood preserved in RNA protect reagent and from 3MM Whatman® filter paper stored in TRIzol® reagent. The RNA protect® sampling method detected 2-fold higher gametocyte prevalence than the filter paper/TRIzol® preservation method by qRT-PCR targeting transcripts of the gametocyte-specific expressed marker *pfs25*. The findings from this pilot project indicated the delay of RNA preservation during field sampling and further storage of samples for 6 months prior to extraction yielded robust RNA for gametocyte detection. Furthermore, the RNA was uncompromised after being stored at -80C for two years. The study also emphasized on the importance proper handling of RNA during extraction and storage in order to limit false positives results through potential aerosols (low level of signal caused by airborne templates).

Lessons learned from this pilot study were implemented to improve sampling for gametocyte detection during community surveys in Tanzania. As most field studies, this study obtained samples from remote areas and faced storage and transport challenges. Despite some delay of several hours in blood sample preservation using RNA protect® gametocyte detection from the yielded RNA was fruitful. Since the results from the previous study indicated that whole blood preserved in RNAprotect® resulted in 2 fold higher prevalence rates, samples from all sites were collected and preserved in RNAprotect®. An exception was made in the Kilombero survey performed in 2011 and Dar Es Salaam survey of 2013 where due to some technical and logistic issues we sampled blood spots onto the 3MM Whatman® filter paper/TRIzol® reagent. However, the study was able to detect gametocytes in both preservation methods.

Study outlook

Although the study revealed high submicroscopic prevalence rates in Tanzania, an unanswered question would be to what extent these submicroscopic carriers contribute to transmission. Studies on mosquito feeding experiments suggest from an equal chance between microscopic positive and submicroscopic infections in infecting mosquitoes (Schneider et al. 2006). Other studies have indicated young children due to their high parasitaemia are the most efficient transmitters (Drakeley et al. 2003). However, the absolute contribution of these submicroscopic infections in Tanzania, which are more common in adults, remains to be evaluated. Data generated from this study cannot answer the question. However, a future study on mosquito feeding experiments can evaluate the proportion of mosquito infected by submicroscopic carriers in comparison to individuals who are parasite carriers identified by light microscopy. Mosquito feeding assays will not only evaluate the extent infectious reservoir in asymptomatic humans but also will determine the importance of these submicroscopic infections in the current elimination era.

This study used light microscopy to identify *P. malariae* and *P. ovale* in addition to identifying *P. falciparum* species in Tanzania. Only 1 *P. malariae* positive sample in over 2000 samples collected from all sites was identified by LM. Since the performance of LM in our study was poor, molecular techniques could be used to identify other non-*falciparum* species in our samples from Tanzania. Since the documented prevalence rates of *P. malariae* and *P. ovale* are very low, <1%, sample pooling experiments to save both cost and time could be done. This information together with *P. falciparum* prevalence data generated will provide more insightful malaria burden information on the *Plasmodium* species prevalence and their distribution in Tanzania.

Study shortfalls and limitations

Measurement of axillary temperature was not included in the study design. The WHO classifies a clinical episode with body temperature >37.5°C and parasitemia >2500parasites/µL. Due to lack of temperature records we could not define the proportion of symptomatic individuals in the surveyed areas.

Furthermore, information on geographical location of the surveyed households was not collected during the study surveys. Geographical positioning system (GPS) would allow mapping of the malaria prevalence data, spatial modelling and the prediction areas of increased risk of malaria also known as hot spots of transmission. Furthermore geographical data cold enable contact tracing studies in the surveyed areas. Other necessary information on bed net ownership and use was not incorporated in our surveys. These data could assist in multivariate analyses to predict other malaria risk factors in different communities.

Sampling methods for RNA requires almost immediate preservation and refrigeration in order to preserve the integrity of RNA. This was difficult to attain in field settings where samples would stay at ambient temperatures up to 7 days before being transferred to the Ifakara Health Institute (IHI) laboratory for RNA preservation and refrigeration. This may have contributed to suboptimal yields of RNA in some samples.

Conclusions

The study has demonstrated for the first time multi-site molecular surveillance data on *P. falciparum* prevalence rates in Tanzania. This reliable data could serve a basis for planning, monitoring and evaluating malaria control efforts. High *P. falciparum* parasite and gametocyte prevalence rates, with high variability in endemic sites were recorded. These prevalence rates are higher than the currently documented national indicator surveys. This information has consequences for malaria control efforts, one of which is based on identifying all infected individuals, and must be acknowledged by all stakeholders.

The study further shows that the use of light microscopy and rapid diagnostic tests in detecting *P. falciparum* infections in community samples greatly underestimated the estimates of infection burden in our study. Thus, these imperfect malaria diagnostics do not provide sufficient accuracy to inform control efforts and are likely to jeopardize malaria control efforts.

Using molecular tools the study confirmed high submicroscopic parasitemia and gametocytemia in Tanzania. If our survey relied solely on LM, over 50% of parasites carriers and 96% gametocyte carriers would have been missed. These results justify the use

molecular tools even in a subset of samples to reliably estimate the infection burden especially in areas of high submicroscopic parasitemia. The occurrence of submicroscopic carriage was prevalent in all endemic settings without a pattern of predominance at low transmission intensity as reported elsewhere. These results can inform control strategies for more targeted interventions towards asymptomatic individuals, mostly adults with primarily submicroscopic infections, whose contribution to transmission cannot be ignored.

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Appendix

During the course of this thesis contributions were made to the following manuscripts published in “Antimicrobial Agents and Chemotherapy” and “Tropical Medicine and International Health” the studies aimed to assess the effectiveness of intermittent preventive treatment during pregnancy (IPTp) in two areas with different malaria transmission intensities in Tanzania. The second applied PCR technique to distinguish between recrudescent and new infections in a study which evaluated the clinical response of Artemether-Lumefantrine in pregnant and non-pregnant women with uncomplicated *Plasmodium falciparum* malaria in Tanzania

These studies are not directly linked to the topic of my thesis, however I contributed in laboratory analysis of the samples.

Tropical Medicine and International Health doi:10.1111/tmi.12349

VOLUME 19 NO 9 PP 1048–1056 SEPTEMBER 2014

Effectiveness of intermittent preventive treatment with sulfadoxine–pyrimethamine during pregnancy on placental malaria, maternal anaemia and birthweight in areas with high and low malaria transmission intensity in Tanzania

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Abstract

OBJECTIVE To assess the effectiveness of IPTp in two areas with different malaria transmission intensities.

METHODS Prospective observational study recruiting pregnant women in two health facilities in areas with high and low malaria transmission intensities. A structured questionnaire was used for interview. Maternal clinic cards and medical logs were assessed to determine drug intake. Placental parasitaemia was screened using both light microscopy and real-time quantitative PCR.

RESULTS Of 350 pregnant women were recruited and screened for placental parasitaemia, 175 from each area. Prevalence of placental parasitaemia was 16.6% (CI 11.4–22.9) in the high transmission area and 2.3% (CI 0.6–5.7) in the low transmission area. Being primigravida and residing in a high transmission area were significant risk factors for placental malaria (OR 2.4; CI 1.1–5.0; $P = 0.025$) and (OR 9.4; CI 3.2–27.7; $P < 0.001$), respectively. IPTp was associated with a lower risk of placental malaria (OR 0.3; CI 0.1–1.0; $P = 0.044$); the effect was more pronounced in the high transmission area (OR 0.2; CI 0.06–0.7; $P = 0.015$) than in the low transmission area (OR 0.4; CI 0.04–4.5; $P = 0.478$). IPTp use was not associated with reduced risk of maternal anaemia or low birthweight, regardless of transmission intensity. The number needed to treat (NNT) was four (CI 2–6) women in the high transmission area and 33 (20–50) in the low transmission area to prevent one case of placental malaria.

CONCLUSION IPTp may have an effect on lowering the risk of placental malaria in areas of high transmission, but this effect did not translate into a benefit on risks of maternal anaemia or low birthweight. The NNT needs to be considered, and weighted against that of other protective measures, eventually targeting areas which are above a certain threshold of malaria transmission to maximise the benefit.

keywords IPTp-SP, placental malaria, anaemia, low birthweight

Introduction

Pregnancy-associated malaria (PAM) is an important cause of maternal and neonatal morbidity such as severe maternal anaemia, intrauterine growth retardation, low birthweight (LBW), premature delivery, intra-uterine death and stillbirth, and can lead to maternal or neonatal mortality (Desai *et al.* 2007). Apart from malaria, anaemia in pregnancy may be secondary to iron, folate and vitamin B deficiency, sickle cell diseases, HIV or helminthic, which may also lead to LBW (Savage *et al.* 2007).

The placenta is a preferred site for *Plasmodium falciparum* to accumulate and cause placental insufficiency due to sequestration, with deleterious consequences for both mother and foetus (Brabin *et al.* 2004). Placental parasitaemia, maternal anaemia and LBW are more frequent in areas with stable than unstable malaria transmission because of the considerably higher level of acquired malaria immunity among women (Nosten *et al.* 2004).

Use of at least two doses of sulfadoxine–pyrimethamine (IPTp-SP) after quickening for intermittent preventive treatment during pregnancy has long been recommended by WHO for malaria control (WHO 2004). The IPTp regimen has recently been modified: it is now suggested that the first IPTp-SP dose to be administered as early as possible during second trimester and that each SP dose should be given at least a month apart until delivery (WHO 2012). There is a growing concern that IPTp-SP effectiveness may be jeopardised by the high degree of resistance of *P. falciparum* to SP, which is a major concern in East Africa and hence questions the viability of IPTp-SP use in this area (Menendez *et al.* 2008; Feng *et al.* 2010; Harrington *et al.* 2011). However, a meta-analysis by Kayentao *et al.* (2013) supports the beneficial effects of three or more doses of IPTp in reducing the risk of LBW.

With the reported significant decline of malaria in most areas of East Africa including Tanzania (O'meara *et al.* 2010; D'acremont *et al.* 2011), it becomes essential to evaluate the benefit of routine IPTp-SP, especially in areas with low malaria transmission. This may assist decision-making on the relevance of advocating universal IPTp in areas where SP adverse reactions may outweigh the benefit of the treatment. Little is known regarding effectiveness of IPTp-SP over other preventive measures for PAM morbidity in areas with low malaria transmission. The aim of this study was to assess the effect of IPTp on placental malaria, PAM morbidity and birth outcomes in areas with high and low malaria transmission intensity.

Methods

Study area

This observational study was carried out in Moshi municipal in northeastern Tanzania and in Rufiji District in the eastern, coastal area of the country. Moshi is a low malaria transmission area with a malaria prevalence of 1.0%, whereas Rufiji is a moderate to high malaria transmission area with prevalence of 20.8% (TACAIDS 2013). The prevalence of *P. falciparum* dihydropteroate synthase (Pfdhps) gene 581G mutation was 56% in infected malaria cases, according to an evaluation conducted 6 years ago in Tanga, a region adjacent to the two study areas (Alifrangis *et al.* 2009). This same area had a day 14 SP treatment failure rate as high of 68% among children (Gesase *et al.* 2009). Mawenzi Hospital in Moshi and Kibiti Health Centre (HC) in Rufiji were involved to recruit study participants. In 2012, malaria accounted for 4% of the total outpatient cases in Mawenzi Hospital and 51% in Kibiti Health Centre (MOH 2013). Both facilities are publicly owned and provide free antenatal care and delivery services. The standard of care and capacity to handle obstetric emergences in the two facilities are similar.

Study design

A prospective study was conducted from July to October 2012, enrolling pregnant women who came for delivery in Mawenzi Hospital and Kibiti HC obstetric wards. The selection criteria for recruiting participants were as follows: residency in the study area for at least 1 year, gestational age of 28 weeks and presence of up-to-date medical information. Cases of multiple pregnancies and severe conditions such as eclampsia were excluded. A structured questionnaire was used to interview women. Information from the mother's medical registry and Reproductive and Child Health (RCH) clinic card was used to verify and complement the generated information. In case of discrepancies on IPTp-SP status between RCH log and what the participant had reported, the participant's information was considered the truth after further interview to verify specifications of the reported medicine. Other recorded information included social-demographic characteristics, parity, ITNs use, history of malaria illness during pregnancy, use of haematinics and anthelmintic drugs. Gestational age was estimated based on the date of last normal menstrual period and compared with the estimated fundal height recorded during the first RCH booking. Low birthweight was defined as below 2500 g, preterm birth as gestational age below 37 weeks and maternal anaemia as a haemoglobin level below 11 g/dl. Placental malaria infection was defined as parasite positive results based on either blood slide smear reading or polymerase chain reaction (PCR) results.

Sample collection and examination

Placental blood was collected within 1 h after delivery. An incision was made on at least three sites of placenta on the maternal side, where accumulated intervillous blood was collected using a blunt syringe. About two drops of collected blood (about 100 μl) were spotted onto a 3MM® Whatman filter paper, air dried and preserved in plastic zipped locked bags for PCR genotyping. Filter papers were then transferred to the laboratory, and DNA was extracted using the Chelex® method (Plowe *et al.* 1995). The DNA was stored at −20 °C until further use. Malaria parasite positivity was determined by quantitative real-time PCR targeting the *P. falciparum* S-type gene as described by Wampfler *et al.* (2013).

Thick and thin blood smears were also prepared and stained with Giemsa. Blood slides were examined independently by two experienced laboratory technicians using a light microscope. Discrepant findings were reviewed by a third technician until consensus on positivity was reached. Parasites in thick film fields were counted per 200 leukocytes, and the parasite count was multiplied by a factor of 40 to give parasites per μl of blood.

Statistical analysis

Data analysis was performed using STATA® 12.0 (Stata Corporation, College Station, TX, USA). Numerical variables were summarised into median and range. Categorical variables were summarised using cross-tabulation to estimate different proportion. The primary outcome was the proportion with placental malaria. The bivariable models included IPTp, maternal age, gravidity, transmission intensity level, history of malaria illness in pregnancy, HIV status and ITNs as explanatory variables. Variables associated with the outcome having a $P < 0.2$ in the bivariable model were retained in the final adjusted logistic regression model to estimate odds ratio (OR) and 95% confidence intervals (CI). The same method was performed for secondary outcomes, that is maternal anaemia and LBW.

The absolute risk reduction (ARR) was calculated to estimate difference between the risk of placental malaria in IPTp-SP exposure and non-exposure women. Number needed to treat (NNT) was estimated based on ARR (Schechtman 2002).

Ethics

Ethical approval for the study was granted by the Kilimanjaro Christian Medical University College (KCMUCo) Research Ethics Committee. Written informed consent was obtained from all participants.

Results

A total of 350 pregnant women were recruited and screened for placenta parasitaemia, 175 from the high malaria transmission area (Rufiji) and 175 from the low malaria transmission area (Moshi). There were no early maternal deaths or referrals to a tertiary health facility during the study period. At the time of recruitment, the mean maternal age was 25.2 years (standard deviation [SD] 6.9) and the mean gestational age 37.2 (SD 2.2) weeks. 319 (91%) recruited women reported to have used one or more dose of IPTp. The median (interquartile range [IQ]) gestational age when the first IPTp dose was administered was 24 weeks (19–32), and the median (IQ) gestational age at the second administration of IPTp dose was 30 weeks (24–36). The participants' main characteristics are presented in Table 1.

Prevalence of placental malaria and associated factors

Prevalence of placental malaria was 16.6% (29/175) in the high transmission area and 2.3% (4/175) in the low transmission area. In high malaria transmission area, the prevalence of placenta malaria was 8% (14/175) by light microscopy and 15.4% (27/175) by PCR, whereas in low transmission area, it was 1.1% (2/175) and 1.7% (3/175), respectively.

Table 1 Characteristics of study participants from the Moshi and Rufiji study sites

Characteristics	Moshi (n = 175) n (%)	Rufiji (n = 175) n (%)	Total 350 (%)
Age (years)			
<20	35 (20.0)	50 (28.6)	85 (24.3)
20–35	126 (72.0)	104 (59.4)	230 (65.7)
>35	14 (8.0)	21 (12.0)	35 (10.0)
Gravidity			
Primigravida	73 (41.7)	58 (33.1)	131 (37.4)
Multigravida	102 (58.3)	117 (66.9)	219 (62.6)
IPTp use			
Not at all	19 (10.9)	12 (6.9)	31 (8.9)
Single dose	66 (37.7)	72 (41.1)	138 (39.4)
≥Two doses	90 (51.4)	91 (52.0)	181 (51.7)
ITNs use			
Yes	161 (92.0)	170 (97.1)	331 (94.6)
No	14 (8.0)	5 (2.9)	19 (5.4)
HIV status			
Positive	8 (4.6)	4 (2.3)	12 (3.4)
Negative	160 (91.4)	171 (97.7)	331 (94.6)
No results	7 (4.0)	0 (0.0)	7 (2.0)
Haemoglobin level (g/dl)*	11.1 (1.7)	10.5 (1.4)	10.7 (1.6)
Parasite density (count/μl)*	18 (14.1)	269.9 (336.5)	238.4 (324.9)
Pregnancy outcome			
Birth outcome			
Live birth	172 (98.3)	172 (98.3)	344 (98.3)
Stillbirth	3 (1.7)	3 (1.7)	6 (1.7)
Birthweight			
≥2500 g	168 (96.0)	164 (93.7)	332 (94.9)
<2500 g	7 (4.0)	11 (6.3)	18 (5.1)
Gestational age at birth			
Term	149 (85.1)	150 (85.7)	174 (49.7)
Preterm	26 (14.9)	25 (14.3)	176 (50.3)

*Haemoglobin level and placenta parasite density presented in mean (SD).

Details of variables associated with placental malaria by bivariable and logistic regression model are shown in Table 2. Women living in high transmission areas were nine times more likely to have placental malaria than those living in low transmission areas (adjusted OR 9.4; CI 3.2–27.7; $P < 0.001$). Primigravidae were twice as likely to have placental malaria as multigravidae (adjusted OR 2.4; CI 1.1–5.0; $P = 0.025$). There was no evidence of the association between placental malaria and HIV status or history of malaria during pregnancy.

Effectiveness of IPTp on placental malaria

In the high transmission area, of the 163/175 (93.1%) women who reported to have used at least one dose of IPTP, twenty-four (14.7%) were found to have placental malaria, while 139 (85.3%) had no placental malaria. Among the 12 of 175 (6.9%) who reported not to have used IPTp during their pregnancy, five (41.7%) were found to have placental malaria. In the low transmission area, of the 156/175 (89%) women who reported to have used at least one dose of IPTp, three (2.0%) were found to have placental malaria. Among the 19 (10.9%) who reported not to have used IPTp, one had placental malaria. In multivariate analysis, one dose or more of IPTp had 80% protective efficacy against placental malaria in the high transmission area (adjusted OR 0.2; CI 0.06–0.7; $P = 0.015$), and 60% protective efficacy in the low transmission area (adjusted OR 0.4; CI 0.04–4.5; $P = 0.478$) (Table 3a,b). There was no significant relationship between the number of IPTp doses taken and the prevalence of placental malaria in either high or low transmission area (Figure 1).

NNT with IPTp to prevent placental malaria

In the high transmission area, 24 of 163 women who used IPTp had placental malaria *vs.* five of 12 women who did not use IPTp. This gives an ARR of 27% for women to have placental malaria after using at least one dose of IPTp. Thus, in the high malaria transmission area, four (CI 2–6) pregnant women need to be treated with IPTp to prevent one case of placental malaria (NNT=1/0.27 = 3.7). By stratifying for gravidity, 14 of 54 primigravidae who used IPTp in the same transmission area had placental malaria *vs.* two of four primigravidae who did not use IPTp. Among multigravidae, 10 of 109 who used IPTp had placental malaria *vs.* three of eight women who did not use IPTp. The ARR in primigravida in this area was 24% (0.5–0.26 × 100) and 29%

Table 2 Strength of association between placental malaria and other factors

	Placental malaria					
Variable	Yes 33 (%)	No 317 (%)	Crude OR (95% CI)	*P*	Adjusted OR* (95% CI)	*P*†
Age (years)						
<25	14 (42)	146 (46)	0.9 (0.4–1.8)	0.860	2.5 (0.7–9.5)	0.164
≥25	19 (58)	171 (54)				
Gravidity						
Primigravidae	17 (52)	114 (36)	1.9 (0.9–3.9)	0.083	2.4 (1.1–5.0)	0.025
Multigravidae	16 (48)	203 (64)				
Transmission						
High	29 (88)	146 (46)	8.5 (2.9–24.7)	<0.001	9.4 (3.2–27.7)	<0.001
Low	4 (12)	171 (54)				
History of malaria						
Yes	6 (18)	62 (20)	0.9 (0.4–2.3)	0.849	1.1 (0.4–2.9)	0.846
No	27 (82)	255 (80)				
HIV status‡						
Positive	1 (3)	11 (4)	0.9 (0.1–7.0)	0.904	1.2 (0.1–11.2)	0.883
Negative	31 (97)	300 (96)				
ITNs use						
Yes	33	317	–	–	–	–
No	0	0				

*Adjusted for gravidity and area of malaria transmission.
†Estimated from the logistic regression model.
‡Seven women had no HIV results.

Table 3 IPTp use in relation to placenta malaria, maternal anaemia and low birthweight in (a) high (b) low malaria transmission areas

Variable	Frequency		Crude OR (95% CI)	*P*	Adjusted OR* (95% CI)	*P*†
	Yes	No				
(a)						
Placental malaria	29 (%)	146 (%)				
IPTp use						
Yes	24 (82.8)	139 (95.2)	0.2 (0.1–0.8)	0.023	0.2 (0.1–0.7)	0.015
No	5 (17.2)	7 (4.8)				
Maternal anaemia‡	81 (%)	53 (%)				
IPTp use						
Yes	76 (93.8)	49 (92.5)	1.2 (0.3–4.8)	0.756	1.2 (0.3–4.9)	0.755
No	5 (6.2)	4 (7.5)				
Low birthweight	11 (%)	164 (%)				
IPTp use						
Yes	9 (81.8)	154 (93.9)	0.3 (0.1–1.5)	0.146	0.3 (0.1–1.5)	0.146
No	2 (18.2)	10 (6.1)				
(b)						
Placental malaria	4 (%)	171 (%)				
IPTp use						
Yes	3 (75.5)	153 (89.5)	0.4 (0.03–3.6)	0.378	0.4 (0.04–4.5)	0.478
No	1 (25.5)	18 (10.5)				
Maternal anaemia§	39 (%)	50 (%)				
IPTp use						
Yes	36 (92.3)	42 (84.0)	2.3 (0.7–9.3)	0.247	2.6 (0.6–10.7)	0.191
No	3 (7.7)	8 (16.0)				
Low birthweight	7 (%)	168 (%)				
IPTp use						
Yes	6 (85.7)	150 (89.3)	0.7 (0.1–6.3)	0.767	0.7 (0.1–6.4)	0.757
No	1 (14.3)	18 (10.7)				

*Adjusted for gravidity.
†Estimated from the logistic regression model.
‡No haemoglobin level information in 41 women.
§No haemoglobin level information in 86 women.

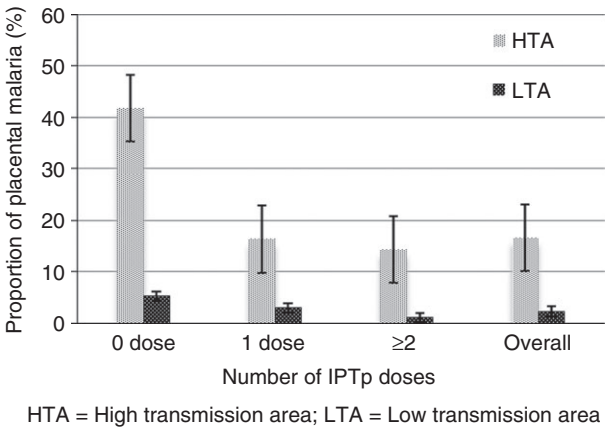

Figure 1 Prevalence of placental malaria in relation to IPTp doses taken in low and high malaria transmission areas. HTA, high transmission area; LTA, low transmission area.

(0.38–0.09 × 100) in multigravidae. Therefore, four (CI 2–4) primigravidae need to be treated in the high transmission area with at least one dose of IPTp to prevent one case of placental malaria (NNT = 1/0.24 = 4.2) *vs.* three (CI 3–11) for multigravidae (NNT = 1/0.29 = 3.4).

In the low transmission area, three of 156 women who used at least one dose of IPTp had placental malaria *vs.* one of 19 women who did not use IPTp. This gives an ARR of 3% (0.05–0.02 × 100). Therefore, in the low transmission area, 33 (CI 20–50) pregnant women need to be treated with at least a single dose of IPTp to prevent one case of placental malaria (NNT = 1/0.03 = 33.3). One of 69 primigravidae in the same transmission area had placental malaria, whereas there was none among those who did not use IPTp at all. For multigravidae in this transmission area, two of 87 who used IPTp had placental malaria *vs.* one of 15 of who did not use IPTp. The ARR in primigravidae in this area was less

than 0 and for multigravidae, 4% (0.067–0.023 × 100). Therefore, 25 (CI 14–50) multigravidae need to be treated in the low transmission area with at least a single dose of IPTp to prevent one case of placental malaria (NNT = 1/0.04 = 25).

IPTp and maternal anaemia

A total of 223/350 (63.7%) study women had their haemoglobin concentration measured before delivery. The prevalence of maternal anaemia in the high transmission area was 60.4% (81/134; CI 51.6–68.8) and 43.8% (39/89; CI 33.3–54.7) in the low transmission area. Living in an area of high malaria transmission was associated with a significantly higher risk of maternal anaemia than living in low endemic area (adjusted OR 1.8; CI 1.0–3.2; $P = 0.036$) (Table 4). The prevalence of maternal anaemia in the high transmission area among women who used at least one dose of IPTp was 61% (76/125), similar to the 56% (5/9) among those who did not use IPTp (adjusted OR 1.2; CI 0.3–4.8; $P = 0.755$) (Table 3a). In the low transmission area, prevalence of anaemia was 46.2% (36/78) among women who used at least one dose of IPTp *vs.* 27.3% (3/11) among those who did not (adjusted OR 2.6; CI 0.6–10.7; $P = 0.191$) (Table 3b). Among other explanatory variables, placental malaria, gravidity, history of malaria infection during pregnancy, HIV status, anthelminthic and use of iron and folate supplementation at least a month during pregnancy period had no statistically significant effect on maternal anaemia (Table 4).

IPTp and low birthweight

The prevalence of LBW in the high transmission area was 6.3% (11/175; CI 3.2–11.0) *vs.* 4% (7/175; CI 1.6–8.1) in low transmission area (adjusted OR 1.7; CI 0.6–4.5; $P = 0.293$) (Table 5). The prevalence of LBW in the high transmission area among women who used at least one dose of IPTp was 5.5% (9/163) *vs.* 16.7% (2/12) for those who did not (adjusted OR 0.3; CI 0.1–1.5; $P = 0.146$) (Table 3a). The prevalence of LBW in the low transmission area among women who used at least one dose of IPTp was 3.8% (6/156) *vs.* 5.3% (1/19) for

Table 4 Strength of association between maternal anaemia and other factors

	Anaemia					
Variable	Yes 120 (%)	No 103 (%)	Crude RR (95% CI)	*P*	Adjusted RR* (95% CI)	*P*†
Placental malaria						
Infected	20 (16.7)	10 (9.7)	1.9 (0.8–4.2)	0.133	1.5 (0.6–3.4)	0.362
Not infected	100 (83.3)	93 (90.3)				
Gravidity						
Primigravidae	42 (35.0)	44 (42.7)	0.7 (0.4–1.2)	0.238	0.7 (0.4–1.3)	0.261
Multigravidae	78 (65.0)	59 (57.3)				
Transmission						
High	81 (67.5)	53 (51.5)	2.0 (1.1–3.4)	0.015	1.8 (1.0–3.2)	0.036
Low	39 (32.5)	50 (48.5)				
History of malaria						
Yes	24 (20.0)	19 (18.4)	1.1 (0.6–2.1)	0.769	1.1 (0.6–2.2)	0.737
No	96 (80.0)	84 (81.6)				
HIV status‡						
Positive	4 (3.4)	4 (3.9)	0.9 (0.2–3.5)	0.835	0.9 (0.2–3.9)	0.912
Negative	115 (96.6)	99 (96.1)				
Iron and folates§						
Yes	105 (88.2)	91 (89.2)	0.8 (0.4–2.1)	0.819	0.8 (0.3–1.8)	0.552
No	14 (11.8)	11 (10.8)				
Anthelminthic						
Yes	108 (90.0)	89 (86.4)	0.3 (0.4–1.2)	0.406	1.1 (0.5–2.6)	0.804
No	12 (10.0)	14 (13.6)				

*Adjusted for placental malaria and transmission intensity.
†Estimated from the logistic regression model.
‡One woman had no HIV result.
§Missing iron and folate use information in two women.

Table 5 Strength of association between low birthweight and other risk factors

Risk factor	Low birthweight: Yes 18 (%)	Low birthweight: No 332 (%)	Crude RR (95% CI)	*P*	Adjusted RR* (95% CI)	*P*†
Placental malaria						
Infected	3 (16.7)	30 (9.0)	2.0 (0.6–7.4)	0.290	2.1 (0.6–7.5)	0.279
Not infected	15 (83.3)	302 (91.0)				
Gravidity						
Primigravida	7 (38.9)	124 (37.3)	1.1 (0.4–2.8)	0.895	1.1 (0.4–3.1)	0.809
Multigravida	11 (61.1)	208 (62.7)				
Transmission						
High	11 (61.1)	164 (49.4)	1.6 (0.6–4.3)	0.337	1.7 (0.6–4.5)	0.293
Low	7 (38.9)	168 (50.6)				
History of malaria						
Yes	6 (33.3)	62 (18.7)	2.2 (0.8–6.0)	0.134	2.2 (0.8–6.1)	0.130
No	12 (66.7)	270 (81.3)				

*Adjusted for history of malaria.
†Estimated from the logistic regression model.

those who did not (adjusted OR 0.7; CI 0.1–6.4; $P = 0.757$) (Table 3b). Among other explanatory variables, placental malaria, gravidity, history of malaria infection during pregnancy, HIV status, anthelminthic and use of iron and folate supplementation at least a month during pregnancy period had no statistically significant effect on the risk of LBW (Table 5).

Discussion

To our knowledge, the present study is the first to evaluate the effectiveness of IPTp in relation to placental malaria in areas with different malaria transmission intensities and high parasite resistance to SP. The evaluation takes into account other malaria preventive measures such as ITNs, and other preventive measures against maternal anaemia such as routine anthelminthic, iron and folate supplementation. The study responded to a call from WHO that emphasised the importance of enhanced regular monitoring of IPTp effectiveness (WHO 2012).

Overall 9% prevalence of placental malaria in this study corresponds to 8% prevalence which was observed 9 years ago in Ifakara, another part of the country (Kabanywanyi *et al.* 2008). It shows that malaria in pregnancy is still an important health issue in Tanzania that needs to be addressed by effective preventive measures. Self-reporting of ITNs use by 95% of pregnant women is encouraging considering the reported ITNs efficacy of 23% against placental parasitaemia, 33% against miscarriage/stillbirth and 23% against LBW according to a systematic reviews of randomised trials (Gamble *et al.* 2007). The possibility of overestimating ITNs use in the present study cannot be excluded due to self-reporting as the participant may try to please the interviewer. In the present study, all women with placental malaria reported to have used ITNs, which precludes any effectiveness calculation. Our assessment of IPTp effectiveness applies thus only in condition of full ITN coverage.

Use of IPTp was associated with protection against placental malaria in all study areas. The study findings agree with previous studies and reviewed evidence of IPTp to reduce the risk of placental malaria (Hommerich *et al.* 2007; Kayentao *et al.* 2013). There was no IPTp dose-dependent relationship to reduced risk of placental malaria. The latter is opposite to what was reported by Kayentao *et al.* (2013) in a systematic review that risk of placental infection decreases with increased number of IPTp doses. However, our study was not powered to determine the effect of increasing dose on placental malaria. The time interval between the last IPTp administrated dose and screening for placental malaria may be a stronger determinant for detecting parasitaemia rather than the cumulative number of doses a woman received during her pregnancy. This is essential to consider, particularly in high transmission areas, where the daily likelihood for a woman to have infectious mosquito bites is higher.

Due to the observed significant risk of having placental malaria when living in a high transmission area, the value for money of IPTp was much higher in areas of high transmission intensity, with only one-eighth of the number of women treated with at least one dose of IPTp required to avert on case of placental malaria compared to the low transmission area. Because of the known

higher risk of placental malaria in primigravidae, we attempted to estimate the NNT in this group *vs.* the multigravida ones in both areas, but the small sample size and uneven representation of the exposure groups, both being potential biases of the study, did not allow meaningful results. In view of the importance to determine NNT in other malaria interventions in pregnancy such as ITN use, 18 and 50 pregnant women are the NNT with ITN to prevent at least one placental malaria case and an LBW case, respectively, as observed in a randomised trial of ITN in high malaria transmission area in Kenya (ter Kuile *et al.* 2003). The NNT should be more regularly used as criterion to prioritise interventions, especially so in moderate to low transmission areas. This estimation is essential in an era of declining malaria.

The primary aim of malaria preventive measures in pregnancy is to prevent deleterious effects of malaria in woman and the baby. The usual concept is that IPTp reduces maternal parasitaemia and hence maternal anaemia, placental malaria and LBW. IPTp effectiveness was challenged by Harrington *et al.* (2011) who conducted a study in Muheza, Tanzania, an area known to have high SP resistance, where IPTp neither lowered the risk of placental malaria nor had reduced maternal anaemia or LBW. A further challenging finding was reported by Gutman *et al.* (2013) in Malawi, which showed that IPTp did not reduce the frequency of placental malaria but was associated with improved birth outcomes. We found that IPTp did reduce placental malaria, but had no effect on maternal anaemia. For LBW, there was still a beneficial but insignificant effect, probably due to the low rate in both study areas. Women who delivered at home may have had an adverse outcome, but our study had the limitation of recruiting at delivery. However, inconsistences of IPTp effectiveness on improving maternal anaemia and LBW have also been reported in a systematic review by McClure *et al.* (2013). It is likely that malaria is only one of the important contributors of maternal anaemia and LBW in developing countries. Hence it will be essential in the coming years to monitor the changes in maternal malaria morbidity and the dynamic of LBW rates in areas of declining malaria transmission to better understand the roles of malaria, malnutrition, other infections and social-economic factors on maternal and baby outcomes.

Conclusion

The study shows that IPTp was associated with a lower rate of placental malaria, but this effect did not translate into protection against maternal anaemia and low birthweight. The NNT may suggest IPTp as an appropriate malaria control intervention, at least in areas with high level of malaria transmission. IPTp benefit is questionable in areas of low transmission. The NNT should be regularly evaluated in different level of malaria transmission and parasite resistance, different geographic settings and on both mother and infant outcomes to best maximise benefit at reasonable costs.

Acknowledgements

We sincerely thank the pregnant women for their cooperation and all staff of Mawezi Regional Hospital and Kibiti HC who were involved in the study. Special thanks go to Athumani Mzuyu, Fadhili Mwakitete and Bakari Kissa of Rufiji HDSS, Jackson Thomas and Happy Mkali of IHI Bagamoyo Clinical Laboratory, and Alutu Masokoto of KCMC Teaching Hospital in Moshi.

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AAC Accepts, published online ahead of print on 27 May 2014
Antimicrob. Agents Chemother. doi:10.1128/AAC.02595-14

Population pharmacokinetics and clinical response of artemether-lumefantrine in pregnant and non-pregnant women with uncomplicated *Plasmodium falciparum* malaria in Tanzania

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Running title: PK of arthemether-lumefantrine in pregnancy

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Abstract

Background: Artemether-Lumefantrine (AL) is the first line treatment for uncomplicated malaria in second and third trimester of pregnancy. Its efficacy has recently been challenged in pregnancy due to altered pharmacokinetic (PK) properties in this vulnerable group. The aim of this study was to determine the PK profile of AL in pregnant and non-pregnant women and assess their therapeutic outcome.

Methods: Thirty-three pregnant women and 22 non-pregnant women with malaria were treated with AL (80/480mg) twice daily for 3 days. All patients provided five venous plasma samples for drug quantification at random times over 7 days. Inter- and intra-individual variability was assessed and covariates effects quantified using a nonlinear mixed-effect modeling approach (NONMEM®).

Results: A one-compartment model with first-order absorption and elimination with linear metabolism from drug to metabolite fitted the data best for both arthemether (AM), lumefantrine (LF) and their metabolites. Pregnancy status and diarrhea showed a significant influence on LF PK. Lumefantrine relative bioavailability and metabolism rate into desmethyl-lumefantrine were respectively 34% lower and 78% higher in pregnant women than in non-pregnant patients. Overall PCR-uncorrected treatment failure was 18% in pregnant women and 5% in non-pregnant women (OR = 4.04; p value 0.22). A high median day 7 lumefantrine concentration was significantly associated with adequate clinical and parasitological response (p = 0.03).

Conclusion: The observed reduction in lumefantrine relative bioavailability in pregnant women may explain the higher treatment failure in this group, mostly due to lower post-treatment prophylaxis. Hence, a modified treatment regimen of malaria in pregnancy should be considered.

47 **Background**

48 Malaria in pregnancy is a major public health problem, which is associated with high maternal
49 and perinatal mortality in tropical and subtropical regions (1). Pregnant women are at increased
50 risk of clinical malaria compared to non-pregnant women because of the associated
51 immunological and hormonal changes in pregnancy (2). Substantial direct risks to pregnant
52 women include severe maternal anaemia, and those affecting the baby are intra-uterine growth
53 retardation, intra-uterine death, stillbirth, premature delivery, low birth-weight, and perinatal and
54 neonatal morbidity and mortality (3). Because of all this, malaria in pregnancy should be treated
55 effectively.

56 Artemether-lumefantrine (AL) (20mg and 120mg, respectively) is one of the most popular and
57 efficacious fixed dose artemisinin-based combination therapies (ACT) against *Plasmodium*
58 *falciparum*. It is currently available at a subsidized cost in most malaria endemic countries. AL
59 has proved to be non-inferior to quinine in East Africa for the treatment of *P falciparum*
60 infection in second and third trimester of pregnancy (4). ACTs are recommended by the World
61 Health Organization (WHO) as the first line treatment for uncomplicated malaria in the second
62 and third trimester of pregnancy (5). Unfortunately, general inter-individual variability on drug
63 absorption, distribution to different compartments of the body and tissues, plasma binding
64 proteins, rate of metabolism, enterohepatic recirculation, and excretion may be associated with
65 changes in bioavailability of a drug and consequently may affect the therapeutic efficacy (6).

66 Pregnancy has been reported to affect the efficacy of some drugs, including antimalarials. This is
67 due to physiological changes which lower drug absorption, speed up drug clearance and increase
68 body fluid volume of distribution (7-9). Elevation of estrogens, progesterone, cortisol and
69 prolactin hormones during pregnancy have been linked to altered metabolic activity of several

hepatic cytochrome P450 enzymes. For instance, catalytic activity of CYP3A4, CYP2C9 and CYP2A6 enzymes increases during pregnancy (10, 11), and these enzymes are responsible for lumefantrine and artemether metabolism (12, 13). Hence, it is expected that significant alteration of the pharmacokinetics (PK) of most antimalarial drugs during pregnancy occurs, which may be associated with lower drug concentrations and lower antimalarial cure rate, especially in advanced pregnancy (14-16). A higher treatment failure rate has indeed been observed in pregnant women when compared to non-pregnant ones living in the same area (16). Several PK studies on artemether (AM), lumefantrine (LF) and their respective metabolites, dihydroartemisinin (DHA) and debutyl-lumefantrine (DLF) have demonstrated low plasma concentration of these drugs in pregnant women compared to non-pregnant adults. However, most of these studies included healthy male adult volunteers as a comparative group rather than female malaria patients (16-19). Because of various determinants of PK and therapeutic outcome, it is essential to have a comparative population of non-pregnant women of the same study area with the same disease.

An important concern during the course of AL treatment is to achieve adequate residual LF level after complete elimination of AM and DHA so that it may clear all residual malaria parasite (9). Therefore, day 7 LF concentration level has been proposed as a good indicator of AL effectiveness (20, 21). Recent pharmacokinetics study of AL in Cambodia and Tanzania reported that the targeted day 7 LF concentration was also not achieved in a significant number of non-pregnant adult patients. In Tanzania, 35% of samples had LF concentration below the cut-off value of 175 ng/ml at day 7 (22). In pregnancy, whereby host antiparasite immunity is somehow compromised (2), a higher day 7 venous concentration of LF may be required than what has previously been proposed in studies involving non-pregnant adult patients i.e. a cut-off values of

175 ng/ml or 280 ng/ml in order to achieve effective therapeutic outcome and 600 ng/ml for maximal efficacy (23, 24). Some predictive models have suggested that a twice-daily regimen of AL for 5 days would be preferable in later pregnancy in order to achieve sufficient drug concentration in plasma (19). Increasing the duration of AL administration is indeed expected to increase the residual LF levels in the subsequent post-treatment cycle so as to reduce chances of recrudescence (22). This should be interpreted with caution because extending the duration of treatment regimen may possibly lead to lower adherence. Doubling the dose might be another option but actually it may not be appropriate because absorption of LF is dose-limited (25).

The aim of the present study was to characterize the PK profile of AL and their metabolites, to determine their variability and to identify factors that might explain variations in drugs and metabolites levels in pregnant (second and third trimester of pregnancy) and non-pregnant women of the same area, and to assess cure rate and parasitological clearance in these two groups. The model developed for lumefantrine was used to simulate day 7 concentrations under standard and alternative dosage regimens and quantify the percentages of pregnant and non-pregnant women having concentrations below different proposed cut-off thresholds.

Material and methods

Study design and procedures

This study was conducted in Rufiji district, within a Coastal region in Eastern Tanzania. The asexual parasitaemia prevalence is 14% and *Plasmodium falciparum* is the predominant species (26). The study was carried out at Kibiti health center from April to September 2012. Approval for the study was granted by two independent ethical review bodies; (i) Research Ethics Committee of Ifakara Health Institute (IHI) and (ii) National Institute for Medical Research (NIMR) Ethical Committee. All women signed an informed consent prior to enrolment.

Pregnant and non-pregnant women diagnosed with uncomplicated malaria were recruited from either out-patient department or Reproductive and Child Health (RCH) clinic. Inclusion criteria were women aged 18 year and above, resident of Rufiji study area, pregnant during their second and third trimester, and having signs or symptoms suggestive of uncomplicated malaria with fever (axillary temperature $\geq 37.5^0$C) or history of fever for the past 24 hours, *P falciparum* detected by microscopy, and hemoglobin level ≥ 7 g/dl. Exclusion criteria were known allergy to AL or quinine, history of renal, liver or heart problem, hyperparasitaemia above 200,000/μL, reported intake of any antimalarial within the past 28 days, unable to take oral medication, and vomiting the medication within 1 hour of first dose intake. The same criteria applied to non-pregnant women (control group) that were recruited concurrently during the same study period after informed consent. A full medical history including concomitant illness and concomitant medication was recorded. Clinical examination on the day of enrollment was performed by an experienced physician. Patients were also seen by the clinician during follow up visits at day 1, 2, 3, 7, 14, 28 and 42 whereby axillary temperature was measured as well as evaluation of malaria related symptoms (5). Gestational age was determined from the estimated first day of the last normal menstrual period and compared to clinical examination of a fundal height. In case of any discrepancy, gestational age was recalculated from the estimated age at first RCH visit.

Drug regimen

Enrolled participants received four tablets of AL (Coartem® Novartis Pharma AG, Basel; 20 mg AM and 120 mg LF) over the course of 3 days at 0, 8, 24, 36, 48 and 60 hours. Each dose was administered with 200 ml of milk containing 4.5 g of fat because of the associated increase in LF bioavailability when taken with a fat rich meal (27). All patients were asked to come back to the health center for each drug administration and observed for one hour after dose intake. None of

the patients was admitted during the course of AL treatment but one pregnant woman who developed severe malaria at day 1 was admitted and the treatment was changed to intravenous quinine. A limited number of patients were administered drug at home by the study's clinician or field assistant, specifically for those who had difficulties to come to the clinic at scheduled times for observed drug administration.

Blood samples

To determine AM, DHA, LF and DLF concentration, 2 ml of venous blood sample was drawn from the patient at random times between 8 and 11 am on day 0, 1, 2, 3 and 7. The schedule for sample collections agrees with WHO recommendation for lumefantrine concentration measurement, but is suboptimal for artemether due to practical difficulties for patients to attend all requested time points (21). Day 0 blood sample was collected before starting the medication as a baseline so as to determine the presence of any antimalarial in patient's plasma prior to treatment due to intake of non-declared drugs (28, 29). The blood samples in an EDTA vacutainer® tube were centrifuged at 2,000 x g for 5 minutes and the plasma stored in cryotubes. Samples were kept at -25^{0}C for at most 6 weeks before transferred to Ifakara Health Institute (IHI) Bagamoyo clinical laboratory for temporary storage at -80^{0}C. It si known that the storage of plasma samples for bioassay of artemether, lumefantrine and their metabolites at -20^{0}C for 8 months does not affect drug concentration (21). All samples were packed in dry ice and then shipped to clinical pharmacology laboratory of the University Hospital in Lausanne, Switzerland, to perform the drug bioassay.

To estimate the parasite density and clearance rate, capillary blood from a finger prick was taken at day 0, 3, 7, 14, 28 and 42. Samples were collected on blood slide Giemsa stained thick and thin blood smear were examined by two different experienced microscopists using light

microscopy. Parasite in thick film fields were counted per 200 leukocytes and the parasite count was multiplied by a factor of 40 to give parasites per µl of blood. Approximately 50µl of finger pricked blood was spotted onto Whatman® filter paper cards (3MM). DNA was extracted from Whatman® filter paper cards by Chelex method (30). In order to differentiate between recrudescence and new infection, samples were genotyped by the most polymorphic marker the merozoite surface protein 2 (*MSP 2*) and the amplicons were visualized in a 2% agarose gel as described elsewhere (31).

Drug assay

Plasma concentrations of AM, DHA, LF and DLF were determined using a validated liquid chromatography-tandem mass spectrometry method (LC-MS/MS) (32). The presence of 10 other antimalarial drugs and metabolites i.e. artesunate, amodiaquine, *N*-desethyl-amodiaquine, piperaquine, pyronaridine, mefloquine, chloroquine, pyrimethamine and sulfadoxine were also assessed at the same time. This is a standard procedure on how LC-MS/MS operates and it helps to ensure that the malaria outcome that was observed was due to AL intake, and not to any other residual antimalarial. The assay is precise (3.1% - 12.6% for inter-day variation coefficient) and sensitive (0.15 – 3.0 ng/dl for lower limit quantification [LOQ] of basic or neutral antimalarial and 0.75 – 5 ng/dl for artemisinin derivatives).

The bioassays were carried out at the Laboratory of clinical pharmacology of the Lausanne University Hospital, which takes part in the quality control system of the worldwide antimalarial resistance network (WWARN).

Efficacy assessment

AL efficacy was determined by cure rate and parasitological clearance. The definition of treatment response was according to WHO recommendations on the methods for surveillance of antimalarial drug efficacy (33). Treatment response was thus classified into early treatment failure (ETF), late clinical failure (LCF), late parasitological failure (LPF) and adequate clinical and parasitological response (ACPR). Participants who developed either clinical or parasitological failure as defined above received quinine 10 mg/kg of body weight three times a day for 7 days, according to standard treatment guidelines (34).

Pharmacokinetic analysis

Drugs and their metabolites were modeled using the NONMEM computer program version 7.2 (NM-TRAN version II) (35)with the PsN-Toolkit version 3.5.3 (36). The program uses mixed (fixed and random) effects regression to estimate population means and variances of the pharmacokinetic parameters and to identify factors that influence them.

Structural model. One and two-compartment models with first-order absorption and elimination and linear metabolization to DLF and DHA were compared to describe, respectively, LF and AM pharmacokinetics with an additional compartment used to characterize metabolite data. Sequential and simultaneous parent-metabolite modeling methods were used for LF/DLF and AM/DHA, respectively. The final estimated parameters were drug and metabolite systemic clearances (CL and CL_{met}), volume of distribution of the central compartment (V_C) and metabolism rate constant from the drug to the metabolite compartment (K_{23}). Owing to identifiability problems, the volume of distributions of DLF and DHA could not be estimated and were assumed to be equal to those of LF and AM, respectively. Because of the limited number of measurements in the absorption phase, the absorption rate constants (K_a) could not be adequately estimated and were thus fixed to 0.7 and 0.54 h^{-1} to achieve AM and LF peak plasma

206 concentrations, respectively, 2 h and 6-8 h after drug intake (37). Finally, the known pre-
207 systemic conversion of AM into DHA was modeled estimating the fraction of the AM dose
208 directly converted into the metabolite using $1-F_1$, with $F_1 = 1$ representing AM relative
209 bioavailability. Since the drugs were given orally, these parameters represent apparent values. In
210 case the analysis of baseline plasma samples showed non-zero concentration of the drugs
211 (suggesting that AL was previously taken), a factor (F_0) was introduced in the model in order to
212 estimate the residual doses from previous treatments.

213 **Statistical model.** Inter-patient variability of all the PK parameters was described by exponential
214 errors following a log-normal distribution, as illustrated by the equation $\theta_j=\theta\cdot\exp(\eta_j)$, where θ_j is
215 the pharmacokinetic parameter associated with the j^{th} individual, θ is the average population
216 value, and η_j is the j^{th} individual component of the inter-patient random effect, an independent,
217 normally distributed variable with mean 0 and variance ω^2. In order to constrain individual F_1 to
218 vary between 0 and 1, a logit function (logit F_1) was used. Correlations between PK parameters
219 were also investigated. Finally, proportional, additive and combined proportional-additive error
220 models were compared to describe the intrapatient (residual) variability for both drug and
221 metabolite. The correlation between drug and metabolite concentration measurements was tested
222 using the NONMEM® L2 item.

223 **Covariate model.** Available covariates were: pregnancy status, body weight, body mass index
224 (BMI), age, gestational age and diarrhea. The covariate analysis was performed using a stepwise
225 insertion/deletion approach. Visual inspection of the correlation between post hoc individual
226 estimates of the PK parameters and the available patients' characteristics was conducted at first.
227 During the forward selection, potential covariates influencing the kinetic parameters were
228 sequentially incorporated in the model and retained if statistical significance was achieved in

NONMEM® ($p<0.05$). Backward deletion was performed once the model including all the significant factors was built. It consisted of removing the covariates one at time, starting from the most insignificant one, until no further non-significant deterioration of the model was observed ($p<0.01$). The typical value of the pharmacokinetic parameters θ was modeled to depend linearly on the covariate X (continuous covariates centered on the population median; dichotomous variables coded as 0 and 1) using $\theta=\theta_a \cdot (1+\theta_b \cdot X)$, where θ_a is the mean estimate and θ_b is the relative deviation of the mean due to the X covariate. Body weight (BW) effect was alternatively modeled using the allometric function $\theta=\theta_a \cdot \left(\frac{BW}{MBW}\right)^{\theta_c}$, where MBW is the median population BW and θ_c was fixed to literature values, i.e. 0.75 for CL and 1 for V. Linear and allometric functions were then compared to identify the model describing at best the relationships between BW and the pharmacokinetic parameters.

Selection of the model and parameter estimation. Drugs and metabolites were fitted by use of the first-order conditional (FOCE) method with interaction using the subroutine ADVAN5. Concentrations below the quantification limit (BQL) of the assay were treated using the M3 method described by Beal as implemented in the paper of Ahn et al (38, 39). Nevertheless, when using the L2 function, BQL data were replaced by LOQ/2 and handled with the M6 approach (39). The log likelihood ratio test, based on differences in the OFV value (ΔOFV) provided by NONMEM®, was employed to discriminate between hierarchical models. Since a ΔOFV between any two models approximates a χ^2 distribution, a change of the objective function was considered statistically significant if it exceeded 3.84 ($p < 0.05$) for 1 additional parameter in model-building and covariate forward-addition steps or 6.63 ($p < 0.01$) in covariate backward elimination. The Akaike information criterion (AIC) was used to compare non-nested models. Shrinkage was also examined. Additional criteria for model selection were diagnostic goodness-

of-fit plots, precision of pharmacokinetic parameters estimates, and the reduction of the parameters inter-patient variability.

Validation of the model. The stability of the final model was assessed by means of the bootstrap method implemented in PsN, generating two-thousand datasets by re-sampling from the original dataset. Mean parameters values with their 95% confidential interval ($CI_{95\%}$) were derived and compared with the final pharmacokinetic model estimates. Model validation was performed by visual predictive checks (VPC), simulating data for 1000 individuals based on the final model and generating 2.5^{th}, 50^{th} and 97.5^{th} percentiles. The observed concentrations were plotted against the 95% prediction interval ($PI_{95\%}$) of the simulated dataset at each time point and visually compared. Figures were generated with GraphPad Prism® (Version 6.00 for Windows, GraphPad Software, San Diego California USA, http://www.graphpad.com/).

Model-based stimulation for LF. The concentration-time profiles of LF in 1000 individuals receiving two different regimens of 6 doses over 3 days (at 0, 8, 24, 36, 48 and 60 h) and 5 days (at 0, 8, 24, 48, 72 and 96 h.) were derived by simulations based on the final model including inter-patient variability. Day 7 predicted median concentrations with their $PI_{95\%}$ for pregnant and non-pregnant women were derived. In addition, these simulations allowed quantifying the percentages of pregnant and non-pregnant women having a day 7 concentrations below different proposed cut-off thresholds of 175 ng/ml, 280 ng/ml and 600 ng/ml associated with treatment efficacy (24, 40, 41).

Other statistical analyses

The relation of the outcome variable (treatment failure) and explanatory variables were tested using a t-test for continuous variables (predicted LF day 7 concentrations, gestation age, baseline

parasite count and BMI) and Pearson chi-square for categorical variable (pregnancy status, residual antimalarial and diarrhoea). A p-value below 0.05 was considered statistically significant. All the statistical analyses were performed using STATA® 12.0 (Stata Corporation, College Station, Texas, USA).

Results

Demographic and clinical parameters. Thirty-five pregnant women and 22 non-pregnant women with acute *Plasmodium falciparum* malaria were enrolled in the study from 23rd April to 5th September 2012. Two of the enrolled pregnant women were withdrawn from the study at day 2 and 7 because they refused to continue participating in the study. Two (9.1%) non-pregnant women were lost for follow-up at day 42. None of the pregnant women were lost for follow up. Baseline characteristics of pregnant and non-pregnant women are presented in *Table 1*. Two pregnant women presented with diarrhea at the day of enrollment and throughout the course of treatment. None of the study participants vomited the drug. All participants had normal physical condition on examination with no history of any chronic disease or smoking. Twenty-six women (14 pregnant and 12 non-pregnant) reported to have taken paracetamol before enrollment. The median gestational age among pregnant women was 27 (range: 14 – 37) weeks with relatively equal numbers of women in the second and third trimester of pregnancy. No participant during the study period had miscarriage or stillbirth, or any other severe adverse drug reaction.

Residual antimalarial. Blood samples from all 57 recruited participants in the study were screened to determine the presence of any antimalarial drugs prior to initiation of malaria treatment. Fifty-five (96.5%) had at least one antimalarial in their plasma: 89.5% (29 pregnant and 22 non-pregnant) of participants had plasma LF above the LOQ but the drug concentration was generally low with the average of 37.3 ng/ml. Other antimalarial drugs which were detected

were DLF 8 (14.0%), AM 4 (7%), DHA 0 (0.0%), sulfadoxine 14 (24.6%), pyrimethamine 11 (19.3%) and quinine 1 (1.8%). Summarized statistics are shown in *Table 2*. Out of 14 participants detected with sulfadoxine, 13 were pregnant with a median baseline parasitaemia of 72086 (range 3920 – 198080) counts/µL [*Figure 1*]. Sulfadoxine concentration persisted at relatively constant concentration throughout the first 7 days of monitoring plasma drug levels.

Population pharmacokinetic analysis

A total of 265 LF, 263 DLF, 146 AM, and 98 DHA plasma concentrations were included in the analysis. Twenty-five percent (n=37) AM, 7% (n=7) DHA and 2% (n=4) DLF concentrations were below the respective LOQs. The median (range) of samples available per study subject was 5 (4 – 5) for LF, 4 (3 – 5) for DLF, 3 (1 – 5) for AM and 2 (1 – 4) for DHA.

Artemether

AM and DHA pharmacokinetics were best described using a one-compartment model with first-order absorption from the gastrointestinal tract and linear metabolism to DHA, including pre-systemic conversion into the metabolite. Elimination of both compounds was modeled using a first-order process. The few basal AM concentrations did not allow estimating a residual dose from previous treatments. Inclusion of an inter-patient variability on V_c, CL_M, K_{23} or F_1 in addition to AM CL did not improve description of the data ($\Delta OFV \geq -1.9$, $p \geq 0.17$). A mixed error model best described residual intra-patient variability for AM and a proportional one for DHA. No correlations between the drug and the metabolite concentrations could be identified. Structural model shrinkages lower than 15% were found for all the inter- and intra-individual variability. Our results show that 21% of the AM dose is converted pre-systemically into DHA. None of the available covariates significantly affected AM or DHA pharmacokinetics ($\Delta OFV \leq 3.0$, $p \geq 0.08$). Although non-significant, an increase of 37% in drug CL in pregnant women

compared to non-pregnant ones was however observed (ΔOFV = -1, p = 0.32). The final model parameters' estimates and bootstrap evaluations are given in *Table 3*. The model was considered reliable since the obtained parameter estimates laid within the bootstrap $CI_{95\%}$. VPC graphs of AM and DHA are shown in *Figure 2A*.

Lumefantrine

A one-compartment model with first-order absorption and elimination was retained to depict LF data. A two-compartment model did not improve the model fit (ΔOFV = -0.1, p = 0.75). Average dose from previous treatment (F_0) was estimated to be 3.2 mg with a large inter-individual variability (ΔOFV = -40, $p = 2.5 \cdot 10^{-10}$). In addition to CL, an inter-patient variability on V_c (ΔOFV = -23, $p = 1.6 \cdot 10^{-6}$) and a correlation between CL and V_c improved significantly the fit (ΔOFV = -117, $p = 2.9 \cdot 10^{-27}$). The assignment of an inter-patient variability on LF bioavailability F_1 (fixed to 1) accounting for the correlation between CL and V_c and their variability resulted in additional improvement of the model fit (ΔOFV = -9.5, $p = 8.7 \cdot 10^{-3}$). Metabolite concentrations were included in the model using a supplementary compartment with linear metabolism from the LF central compartment. The addition of an inter-individual variability on K_{23} improved significantly the description of the data (ΔOFV = -41, $p = 1.5 \cdot 10^{-10}$), while no enhancement was observed when assigning variability on CL_M (ΔOFV = -0.02, p = 0.89). Residual intra-patient variability was best described using a proportional and mixed error model for LF and DLF, respectively. The model was further improved by including a correlation between drug and metabolite concentrations (ΔOFV = -85, $p = 3.0 \cdot 10^{-20}$). Structural model shrinkages for the inter- and intra-individual variability were all estimated to be lower than 15%.

In univariable analyses, pregnancy and diarrhea were identified as significant covariates for both F_1 (ΔOFV = -5.1, p = 0.024 and ΔOFV = -15, $p = 1.1 \cdot 10^{-4}$) and K_{23} (ΔOFV = -13, $p = 3.1 \cdot 10^{-4}$

and ΔOFV = -4, p = 0.045). None of the remaining covariates influenced LF and DLF pharmacokinetics (ΔOFV ≥ -1.4, p ≥ 0.24). Multivariable combination of the significant covariates showed an additive influence of pregnancy and diarrhea on F_1 and pregnancy on K_{23} (ΔOFV = -33, p = $3.2 \cdot 10^{-7}$ with respect to the model without covariates). Our results show that relative bioavailability is 34% lower and metabolism rate 78% higher in pregnant women compared to non-pregnant patients. A decrease of 83% in F_1 was observed in women with diarrhea as compared to those who had no diarrhea. *Table 3* illustrates the final model parameters' estimates together with their bootstrap evaluations. The model was considered reliable since the obtained parameter estimates laid within the bootstrap $CI_{95\%}$. *Figure 2B* shows the concentration time-plots of LF and DLF for pregnant and non-pregnant women included in the analysis with average population predictions and 95% intervals.

Concentration-time simulation of lumefantrine

The day 7 predicted median concentrations of LF after administration of a 6-dose regimen over 3 days were 908 ($PI_{95\%}$: 217 – 3256) ng/ml for pregnant women and 1382 ($PI_{95\%}$: 386 – 5135) ng/ml for non-pregnant women (p = 0.10). While considering the large inter-patient variability in the kinetics of LF, 3% of the pregnant women would have day 7 concentrations below the cut-off value of 175 ng/ml, 9% below 280 ng/ml and 31% below 600 ng/ml. For non-pregnant women, 1% would exhibit day 7 concentrations below the cut-off value of 170 ng/ml, 2% below 280 ng/ml and 15 % below 600 ng/ml. Prolonging the time of drug administration over 5 days among pregnant women would provide median concentrations of 1374 ($PI_{95\%}$ 367 – 5536) ng/ml, with 0.1%, 2% and 16% of patients with concentrations below the cut-off value of 175 ng/ml, 280 ng/ml and 600 ng/ml, respectively [*Figure 3*].

Pharmacodynamics

There were a total of 7 therapeutic failures in the study, 6 (18.2%) pregnant women and 1 (4.5%) non-pregnant woman (OR = 4.04; p = 0.22). Among pregnant women, one developed ETF at day 1. She presented with signs and symptoms suggestive of severe malaria, was admitted and kept on full dose of intravenous quinine. One pregnant women had LCF, presented with fever (body temperature = 38.7^0C) at day 20, blood slide confirmed to have parasitaemia of 10,750 counts/µL. The remaining four pregnant women had LPF, one at day 28 and three at day 42. One non-pregnant woman had LPF at day 28. Hence, the overall PCR uncorrected efficacy of AL in the study was 87%, 82% (6/33) in pregnant women and 95% (1/22) in non-pregnant women. PCR investigation confirmed recrudescent infection in two women, one with ETF and the other with LCF, both pregnant; the remaining 5 (71%) had new infections.

Analysis of day 7 LF concentration was done irrespective of the nature of the failure (new infection or a recrudescence). The mean day 7 plasma concentration was 971 (726 – 1216) ng/ml in pregnant and 1261 (999 – 1522) ng/ml in non-pregnant women (p = 0.109) [*Figure 4A*]. Day 7 LF concentration was significantly lower among women with therapeutic failure than those with ACPR. The mean LF concentration among women with ACPR was 1154 (967 – 1341) ng/ml whereas, for the women with LCF and LPF it was 507 (95 – 919) ng/ml (p = 0.029) [*Figure 4B*]. Twenty percent of study participants had day 7 LF concentrations below 600 ng/ml. Only two patients (33%) out of six among the ones who developed LCF and LPF had day 7 LF concentrations below 600 ng/ml and all were pregnant. Potential predictors of treatment failure in addition to day 7 LF concentration were pregnancy status, gestational age, baseline parasite count, residual antimalarial and BMI and none was statistically significant.

Discussion

388 The study describes the pharmacokinetic properties of AM, LF and their active metabolites DHA
389 and DLF in pregnant and non-pregnant women with malaria. The role of different covariates that
390 could influence AL bioavailability, distribution and clearance in the two groups were carefully
391 analyzed. The study differs from previous reports of population pharmacokinetics of AM and LF
392 in pregnancy (16, 18, 19) by having a comparative group of non-pregnant women with malaria
393 from the same population with relatively similar characteristics.

394 **Prior treatment.** Detectable residual antimalarial among recruited participants was
395 unexpectedly high. This might be explained by uncontrolled prescription of AL, a first line
396 malaria treatment, which is highly available and easily accessible from both private and public
397 facilities (42, 43). Prevalence of residual antimalarial among participants was higher than what
398 was reported five years ago from *in vivo* studies in Ifakara (Tanzania) and Praeh Vihear
399 (Cambodia) which was 74.3% and 50%, respectively (28, 29). Such high prevalence of residual
400 antimalarial levels in this population, particularly LF, is alarming because it can promote
401 emergence and spread of drug resistance parasite. Also, the high residual prevalence of LF,
402 irrespective of pregnancy trimester suggests a considerable AL exposure in the first trimester.
403 There is an urgent need to monitor closely the implementation of standard malaria treatment
404 guideline and discourage self-treatment by not acquiring antimalarial from drug venders without
405 attended and screened for presence of malaria parasitaemia. Significant levels of detected
406 sulfadoxine among pregnant women were probably the result of SP received from RCH clinic for
407 Intermittent Preventive Treatment (IPTp).

408 **Pharmacokinetics.** LF pharmacokinetics is known to exhibit a multi-compartment disposition.
409 The short sampling duration in the present study, however, prevented an appropriate
410 characterization of the drug profound disposition. A simple one-compartment model was thus

411 employed to describe LF concentration-time profile. The important study finding was the lower
412 LF plasma concentration among pregnant patients compared to non-pregnant ones. This is
413 similar to what has been observed in a Thailand study in which the concentration of LF was
414 approximately half that of non-pregnant patients from historical data in the same population (16).
415 The reason for low LF concentration may be due to physiological changes related to pregnancy
416 status which accounts for reduced absorption, expanded volume of distribution, elevated drug
417 metabolism and clearance rate (6). The observed increase in LF metabolism rate among pregnant
418 women is explained by hormonal changes in pregnancy which increases catalytic activity of
419 hepatic enzymes such as CYP3A4, an important enzyme for LF metabolism (11). The design of
420 the study did not allow displaying the effect of reduced absorption on LF bioavailability.

421 Altered bowel condition such as having diarrhea during malaria treatment has a significant effect
422 on drug absorption and consequently lowers drug bioavailability. Increase of gastro-intestinal
423 motility due to diarrhea reduces intestinal transit time of a drug, and this time is important to
424 maximize drug absorption (44). The latter explains why LF concentration, a high lipophilic
425 compound, was 83% lower in women with diarrhea compared to the ones with no diarrhea. It is
426 therefore important to assess for presence of diarrhea in patients and correct dosage regimens
427 accordingly.

428 It is important to study concentration levels of a slowly eliminated partner antimalarial drug such
429 as LF so as to determine minimum parasiticidal concentration (MPC) and minimum inhibitory
430 concentration (MIC) of malaria parasite (20). The observed day 7 median concentration of LF
431 was lower in pregnant than in non-pregnant women. However, the concentration among pregnant
432 women was twofold higher compared to what had been observed in Thai pregnant patients (19).
433 It is also higher than the concentrations previously reported in non-pregnant adults and paediatric

434 patients in Ifakara-Tanzania, Thailand, Cambodia and the Lao People's Democratic Republic
435 (22, 24, 45, 46). Higher day 7 LF levels in the present study may be due to the administration of
436 a standard recommended adult dose of AL with food (5) to all patients regardless of the patient's
437 body weight.

438 The observed higher AM clearance in pregnant as opposed to non-pregnant women, although not
439 statistically significant, could explain the differences in the therapeutic outcome. Little has been
440 done on AM bioassay in relation to its specific role on therapeutic efficacy in pregnancy as
441 opposed to LF. AM can better explain ETF and hence, further studies are encouraged with
442 detailed assessment on AM pharmacokinetics despite of its shorter half-life.

443 The simulations under the standard 6 dose of AL over 3 days schedule show that a non-
444 negligible number of pregnant women would have LF concentrations below various proposed
445 therapeutic threshold targets at day 7. Splitting the same recommended total dose over a 5 day
446 regimen would greatly improve the probability of exhibiting therapeutic drug concentrations.
447 The latter has already been shown in other pharmacokinetics studies (22, 23, 47), but the benefit
448 might be jeopardized by poor adherence to treatment in the prolonged regimen. Hence, a formal
449 assessment of feasibility should be performed.

450 **Pharmacodynamics.** The observed cure rate and parasite clearance in pregnant women was
451 lower compared to that of non-pregnant patients despite having the same median baseline
452 parasitaemia. The observed lower LF concentration at day 7 among the patients with therapeutic
453 failure could be one of the reasons explaining this difference. In order to improve therapeutic
454 efficacy, it is therefore important to consider dose increase or modifying treatment regimen to
455 allow higher day 7 LF concentrations. Day 7 LF concentration above 600 ng/dl was associated

with 100% efficacy among pregnant patients in Thailand (40). The latter was not observed in our study; indeed 3 out of the 5 (60%) pregnant women with LCF or LTF had day 7 LF concentration above 600 ng/ml. This observation suggests that the proposed 600 ng/dl cut-off value better predicts parasite clearance of ongoing infection, rather than occurrence of new infection in the follow-up period. 600 ng/dl LF concentration at day 7 is not high enough to ensure post-treatment prophylaxis effect up to day 42. Indeed, reinfections were not all prevented with a day 7 LF concentration of 600 ng/ml. Partner drugs with longer half-life might offer better protection (20).

Baseline parasitaemia was not an important factor to determine therapeutic response among study participants. Indeed mean baseline parasite count in patients with ACPR was twofold higher compared to the ones with therapeutic failure. This is contrary to what has been reported in previous studies involving pregnant and non-pregnant patients in which patients with higher baseline parasitaemia were more likely to fail treatment (40, 47). However, baseline peripheral parasitaemia in pregnant women usually does not tell much about the actual picture of parasite level that a pregnant woman might have because of parasite sequestration in the placenta (48).

Therapeutic failure rate among pregnant women in our study was much lower than that observed in Thailand in recent AL pharmacodynamics studies whereby therapeutic failure among pregnant patients was more than 30% (19, 40). We have reason to believe that AL is more efficacious in Africa than in Southeast Asia where resistance to other antimalarial drugs such as quinine, mefloquine and artesunate has increased (14, 49).

Conclusion

The current AL treatment regimen in pregnancy is challenged by having low post-treatment prophylactic effect. Pregnancy is an important associated factor for low plasma concentration of LF probably due to reduced drug absorption, elevated drug metabolism and rapid clearance rate. It is therefore important to evaluate new treatment regimens of AL in this vulnerable group that would target higher day 7 LF concentration levels.

Competing interests

The authors declare that they have no competing interests

Acknowledgements

We sincerely thank the patients for their coorperation and all staff involved in the study. Special thanks are given to Sigilbert Mrema, Athumani Mzuyu, Bakari Kissa, Fadhili Mwakitete and Sajidu Ismail of Rufiji Health Demographic Surveillance System (HDSS), and Jackson Thomas and Happy Mkali of IHI Bagamoyo Clinical Laboratory. This work was supported by Ifakara Health Institute (IHI) and European and Developing Countries Trial Partnership (EDCTP) through Malaria in Pregnancy Preventive Alternative Drugs (MiPPAD) project.

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670 **Tables and figures**

671 **Table 1:** Characteristics of study participants with *P falciparum* malaria on the day of enrollment

Characteristics	Pregnant women (n=33) Median (range)	Non-pregnant women (n=22) Median (range)
Age (years)	25 (18 -41)	21.5 (18 -35)
Body weight (Kg)	52 (40 – 80)	48.5 (41 – 79)
Height (cm)	158 (147 – 169)	157 (150 – 174)
BMI	21.8 (16.5 – 30.1)	20.3 (16.4 – 33.3)
Haemoglobin (g/dl)	10.2 (7.1 – 13.3)	13.4 (8 – 15.5)
Temperature (^{0}C)	37.1 (36.0 – 39)	37.2 (36.0 – 39.6)
Parasitaemia (counts/µL)	25,280 (560 – 198,080)	22,280 (560 – 195,680)
Gestation age (weeks)	27 (14 – 37)	NA
**Pregnancy – trimesters*		
Second trimester (%)	17 (52)	NA
Third trimesters (%)	16 (48)	NA

672 *Trimester presented in number (%). NA means not applicable

673 **Table 2:** Plasma concentration of residual antimalarial drugs detected prior to treatment with AL

674 in 57 recruited study patients [ng/ml]

Antimalarial	Patients (%)	Plasma concentration [ng/ml]			
		Mean	Median	Minimum	Maximum
Lumefantrine	51 (89.5)	37.3	24.9	5.4	205.5
Desbutyl-limefantrine	8 (14.0)	2.5	1.5	0.3	6
Artemether	4 (7)	26.4	2.9	0.4	157.3
Sulfadoxine	14 (24.6)	1,334.3	1,298.1	5.3	3,615.6
Pyrimethamine	11 (19.6)	6.9	5.3	1.7	18
Quinine	1 (1.8)	12.3	12.3	12.3	12.3

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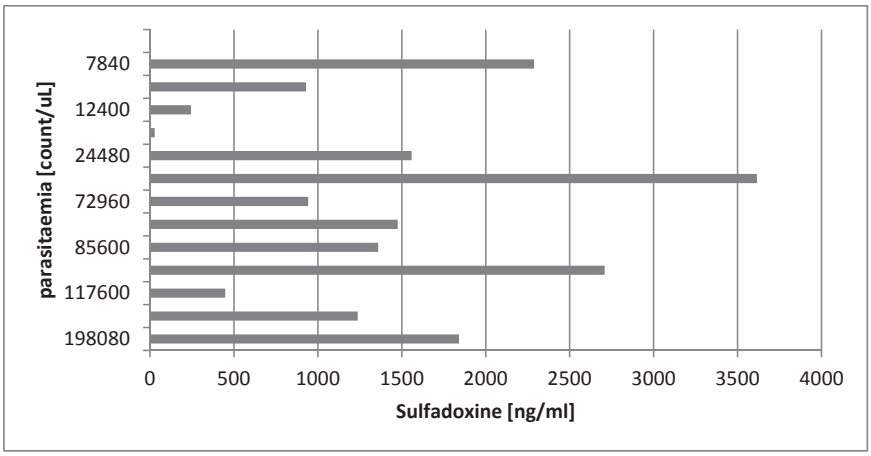
676 **Table 3:** Final population parameter estimates of artemether, lumefantrine and their metabolites

677 and their bootstrap evaluations in 2000 replicates

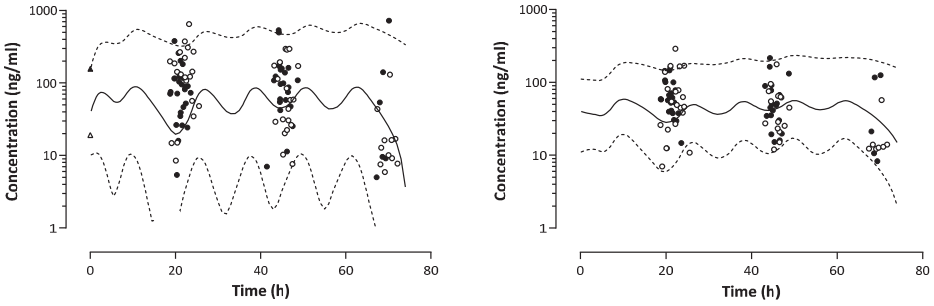
Population pharmacokinetics analysis					**Bootstrap evaluation**			
Parameter	**Estimate**	**SE[a] (%)**	**IIV[b] (%)**	**SE[c] (%)**	**Estimate**	**$CI_{95\%}$[d]**	**IIV[b] (%)**	**$CI_{95\%}$[d]**
Artemether								
CL (L/h)	98	24	99	65	102	69-140	93	66-120
V_c (L)	373	16			354	225-492		
$LogitF_1$	1.4	27			1.5	0.7-2.6		
K_a (h^{-1})	Fixed to 0.70							
V_M (L)	Fixed to V_c							
K_{23} (h^{-1})	0.084				0.088	0.05-0.16		
CL_M(L/h)	71	46			69	38-136		
$\sigma_{prop,AM}$ (CV%)	72	26			69	49-87		
$\sigma_{add,AM}$ (µmol/L)	0.13	7			0.13	0.03-0.20		
$\sigma_{prop,DHA}$ (CV%)	53	14			51	44-59		
Lumefantrine								
CL (L/h)	2.8	12			2.8	2.2-3.6		
V_c (L)	134	14			134	101-174		
F_1	Fixed to 1	65	50				61	43-77
θ_{PregF1}	-0.33	37			-0.31	-(0.52-0.05)		
$\theta_{diarrF1}$	-0.84	15			-0.78	-(0.95-0.44)		
K_a (h^{-1})	Fixed to 0.54							
V_M (L)	Fixed to V_c							
F_0 (mg)	2.7	18	87	46	2.95	1.9-4.4	116	70-164
K_{23} (h^{-1})	$1.6 \cdot 10^{-4}$		46	54	$1.6 \cdot 10^{-4}$	$(1.2\text{-}2.0) \cdot 10^{-4}$	44	31-57
$\theta_{PregK23}$	0.80	32			0.80	0.4-1.3		
CL_M(L/h)	2.6	15			2.6	1.9-3.5		
$\sigma_{prop,LF}$ (CV%)	51	32[e]			51	45-56		
$\sigma_{prop,DLF}$(CV%)	39	40[e]			38	32-44		
Correlation LF/DLF	68	18			67	63-69		
$\sigma_{add,DLF}$(µmol/L)	$4.4 \cdot 10^{-3}$	17[e]			$4.9 \cdot 10^{-3}$	$(3.8\text{-}6.1) \cdot 10^{-3}$		

678 *Abbreviations:* CL: clearance, V_C: central volume of distribution, $logitF_1$: logit F_1
679 expressed as a logit function, k_a: first-order absorption rate constant, V_M: volume of
680 distribution of the metabolite, F_0: residual amount from the previous treatment, k_{23}:
681 metabolism rate constant, CL_{met}: metabolite clearance, σ_{prop}: exponential residual error,
682 σ_{add}: additive residual error, $\theta_{X\ PAR}$: effect of the X covariate on the parameter PAR
683 expressed as $(1- \theta_{X\ PAR}\ X)$.
684 [a] Standard error (S.E.) of the estimate θ_i defined as S.E estimate/estimate, expressed as a
685 percentage
686 [b] Inter-individual variability
687 [c] Standard error (S.E.) of the coefficient of variation or the additive component of the
688 residual error defined as √S.E estimate/estimate, expressed as a percentage
689 [d] 95% confidence interval (C.I.)
690
691 **Figure 1**: Relationship between parasite density at enrollment and plasma residual levels of
692 sulfadoxine prior treatment in 14 pregnant women

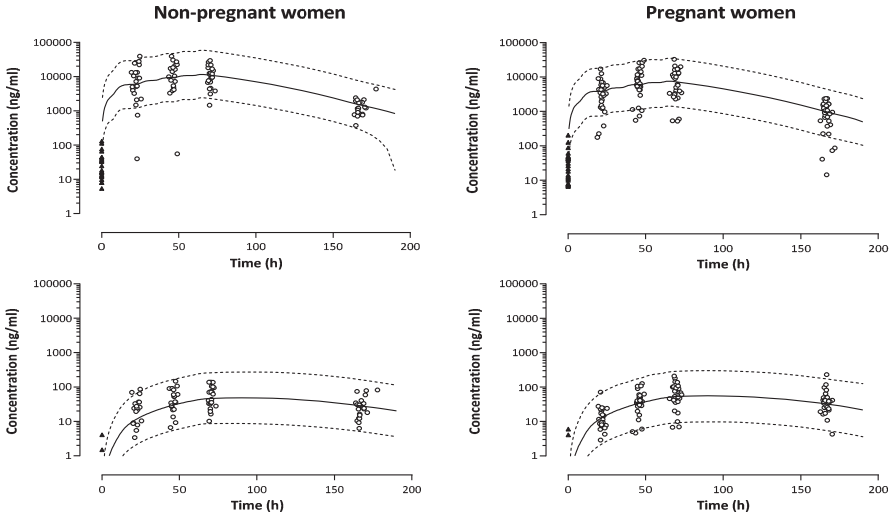


693

694 **Figure 2A:** Observed AM (left panel) and DHA plasma concentrations (right panel). Filled and
695 empty circles represent pregnant and non-pregnant women, respectively. The solid line
696 represents the average predicted concentrations and the dashed lines the 95th prediction intervals.

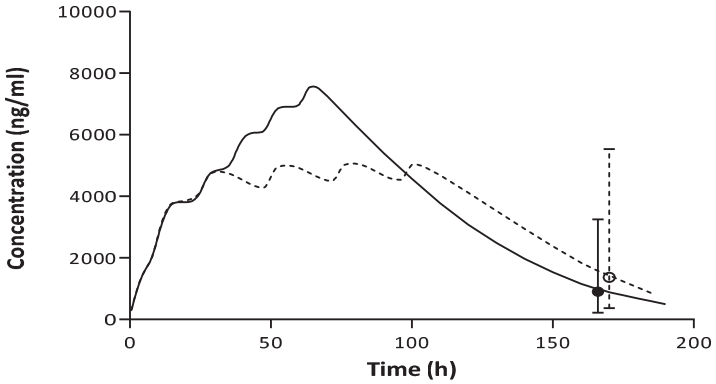


697
698 **Figure 2B:** Observed LF (upper panels) and DLF plasma concentrations (lower panels) in
699 pregnant and non-pregnant women. Triangles residual plasma concentrations of LF and DLF
700 found prior treatment initiation. The solid lines represent the mean population prediction and the
701 dotted lines $PI_{95\%}$.



702
703

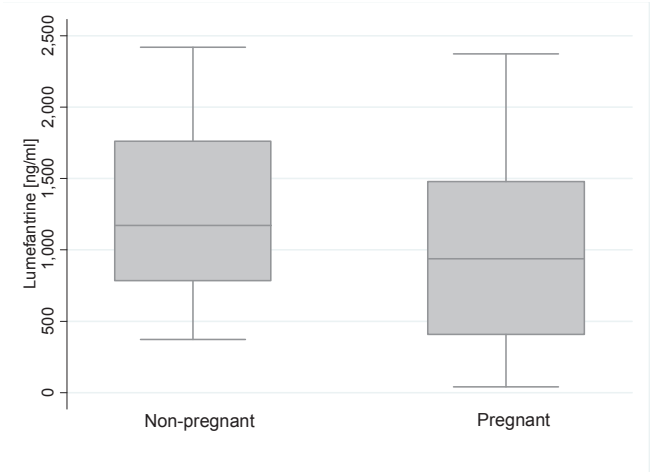
704 **Figure 3:** Predicted median concentration of lumefantrine (LF) after administration of
705 6·480 mg regimen over 3 (continuous line) and 5 days (dotted line) in pregnant women.
706 Day 7 (168h) median predicted concentrations (circles) with their $PI_{95\%}$ are shown for the
707 two dosage regimens.



708

709

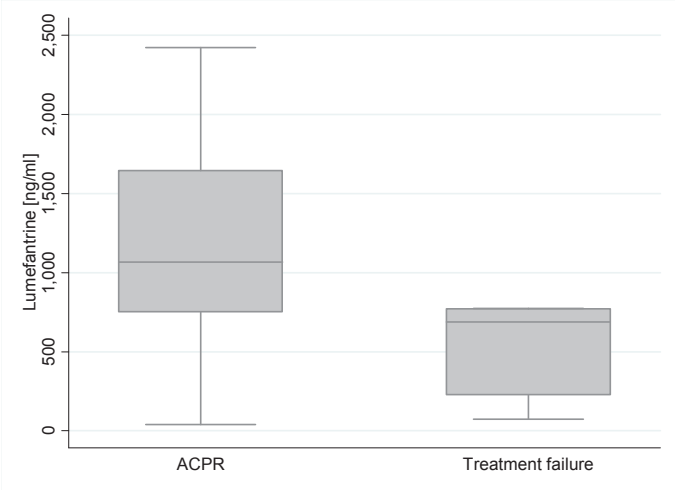
710 **Figure 4A:** Day 7 plasma concentration of lumefantrine in pregnant (n = 32) and non-pregnant
711 (n = 22) study women



712

713 **Figure 4B:** Day 7 plasma concentration of lumefantrine in women with ACPR (n = 48) and

714 those with treatment failure (n = 6) *



715

716 * Day 7 lumefantrine concentration could not be assessed in one woman since a rescue treatment

717 with quinine was given at day 1 because of early treatment failure.

CURRICULUM VITAE

Felista .W. Mwingira

Personal particulars

Sex	Female
Nationality	Tanzanian
Date of birth	5th march, 1981
Marital status	Married
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Education

Sept 2010 - Sept 2014	University of Basel, Swiss Tropical and Public health institute; PhD in microbiology. **Project:** Molecular community surveillance of *Plasmodium falciparum* in 5 sites of different malaria endemicity in Tanzania
2007 – Sept 2010	University of Dar es Salaam- Tanzania Master in Science (Applied zoology), by **thesis**. **Thesis title: "**Assessment of genetic diversity of *Plasmodium falciparum* in sub Saharan Africa**."**
2002 – 2006	University of Dar es Salaam- Bachelor of Science with Education **(Hons)** Majors in Chemistry, Biology and Education
2001 and below:	Primary, ordinary and advanced secondary education

Work experience

May 2006 to 2010	Dar es Salaam University College of Education (DUCE) - a constituent college of the University of Dar es Salaam (UDSM). Tutorial Assistant, Assistant Lecturer, Biological Sciences Department
Duties:	Research, Consultancy and Teaching – (Parasitology, Cell biology and Genetics)
2011	Laboratory training at the SwissTPH molecular parasitology and infectious biology lab.
2012-2013	Field work planning and execution, supervising field team in 5 regions (Dar-es Salaam, coastal region, Iringa, Tanga and Morogoro- Tanzania

Conferences and course attended

Poster presentation: 10th Annual BioMalPar/EVIMalaR Conference: Biology and Pathology of the Malaria- Heidelberg May 2014, Title: **Molecular epidemiology of asymptomatic P*. falciparum* malaria in areas of different malaria endemicity - Tanzania**

DGP 6th Short **Course for Young Parasitologists**- may 2012- Heidelberg Germany,

Statistics for biology students 2011- Geneve – Swiss Institute of Bioinformatics

SCIENTIFIC PUBLICATIONS

1. **Mwingira F**, Nkwengulila G, Schoepflin S, Sumari D, Beck HP, Snounou G, Felger I, Olliaro P, Mugittu K.(2011) *Plasmodium falciparum msp1, msp2 and glurp* allele frequency and diversity in Sub-Saharan Africa. Malar J. 2011 Apr 6;10:79. doi: 10.1186/1475-2875-10-79.

2. Wampfler R*, **Mwingira F*,** Javati S, Robinson L, Betuela I, et al. (2013) Strategies for Detection of *Plasmodium species* Gametocytes. PLoS ONE 8(9): e76316. doi:10.1371/journal.pone.0076316 (*first co-authors)

3. Mosha D., Guidi M., **Mwingira F**., Abdulla S., Mercier T., Decosterd LA., Csajka C. and Genton B. (2014) Population pharmacokinetics and clinical response of artemether-lumefantrine in pregnant and non-pregnant women with uncomplicated *Plasmodium falciparum* malaria in Tanzania. *Antimicrob. Agents Chemother. AAC.02595-14; doi:10.1128/AAC.02595-1*

4. Mosha, D., Chilongola, J., Ndeserua, R., **Mwingira, F.,** & Genton, B. (2014). Effectiveness of intermittent preventive treatment with sulfadoxine-pyrimethamine during pregnancy on placental malaria, maternal anaemia and birthweight in areas with high and low malaria transmission intensity in Tanzania. *Tropical Medicine & International Health : TM & IH*, *00*(00). doi:10.1111/tmi.12349

5. Hofmann N*, **Mwingira F*,** Shekalaghe S, Robinson L, Mueller I, Felger I (2014) Ultra-sensitive detection of *Plasmodium falciparum* by amplification of multi-copy sub-telomeric targets reveals underestimation of parasite prevalence. *PLos Medicine* Submitted

6. **Mwingira F**, Genton B, Kabanywanyi AM and Felger I (2014) Comparison of detection methods for *P. falciparum* prevalence and gametocyte carriage in community survey in Tanzania. *Malaria journal* in press