PCR based Malaria diagnostics

– Method development and application

Christel Gill Haanshuus

Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
2019

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Thesis for the degree of Philosophiae Doctor (PhD)
at the University of Bergen

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SCIENTIFIC ENVIRONMENT

In addition to the University of Bergen (UiB), the research was mainly conducted in the scientific environment of the Norwegian National Advisory Unit on Tropical Infectious Diseases, Department of Medicine, Haukeland University Hospital, with one part in collaboration with the Benjamin M Pulimood Laboratories for Infection and Inflammation, Department of Medicine Unit 1 and Infectious Diseases, Christian Medical College, Vellore, India. The Norwegian National Advisory Unit on Tropical Infectious Diseases provided the funding of the work.

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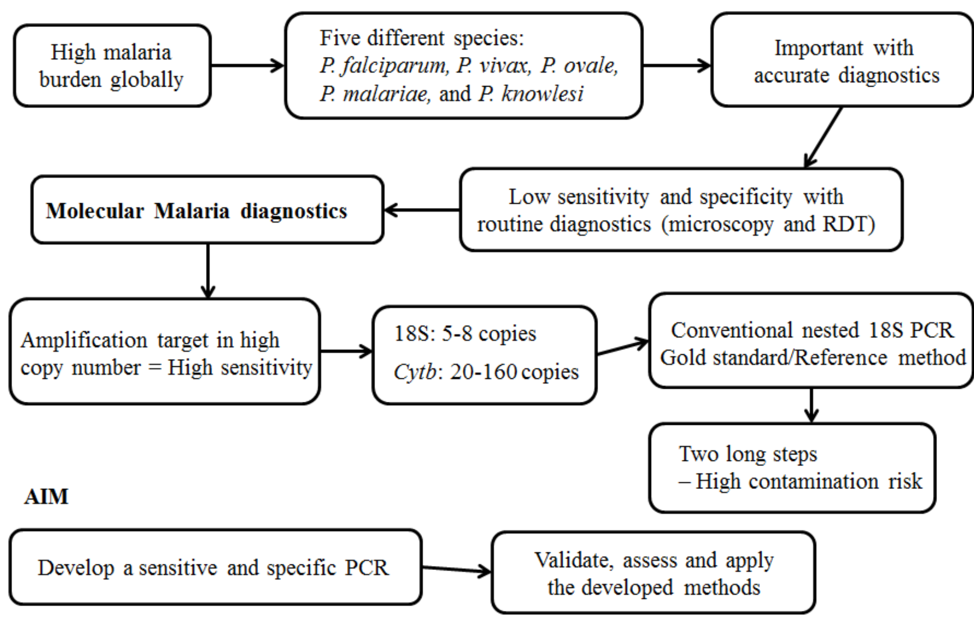
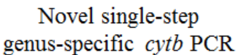
SUMMARY

BACKGROUND



AIM

METHODS



Modified single-step species-specific 18S PCR

Sequencing of genus-specific *cytb* PCR products

RESULTS

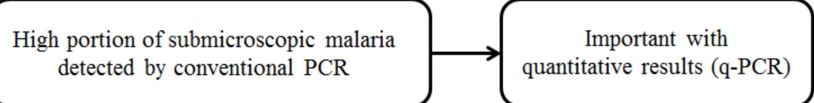
The novel genus-specific *cytb* PCR showed 100% sensitivity and specificity assessed and validated using Norwegian material (N=135)

The modified species-specific 18S PCR showed same performance as reference nested 18S PCR Norwegian material (N=135)

Application of developed PCR methods identified a high malaria prevalence of 19% in a multi-centre hospital-based study Indian material (N=1412)

Species identified by sequencing of genus-specific *cytb* PCR products

BACKGROUND II



AIM II

Convert conventional to real-time PCR

Perform q-PCR

METHODS II

Novel genus-specific *cytb* SYBR PCR

Novel genus-specific *cytb* TaqMan PCR

Sequencing of genus-specific *cytb* SYBR PCR products

RESULTS II

Successful conversion to real-time PCR
The assays assessed and validated using Norwegian material (N=113) and Tanzanian material (N=74)

SYBR more sensitive and specific than TaqMan assay

PCR superior to microscopy and RDT

q-PCR showed a broad range of parasitaemia from very low to high (N=74)

CONCLUSIONS

The genus-specific *cytb* SYBR q-PCR is a good candidate to be used for molecular malaria diagnostics

Sequencing of the genus-specific *cytb* SYBR PCR products can be used as an alternative to species-specific PCR

ABBREVIATIONS

ACT	Artemisinin-based combination therapy
C	Fractional number of cycles
cDNA	Complementary deoxyribonucleic acid
Cox	*Cytochrome c oxidase*
cytb	*Cytochrome b*
DBS	Dried blood spot
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
E	Amplification efficiency
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
G6PD	Glucose-6-phoshpate Dehydrogenase
HRP2	Histamine rich protein 2
ID	Iron Deficiency
IRS	Indoor residual spraying
LAMP	Loop-mediated isothermal amplification
LLIN	Long-lasting insecticidal net
NASBA	Nucleic acid sequence-based amplification
MCA	Melting curve analysis
$MgCl_2$	Magnesium chloride

mRNA	Messenger- ribonucleic acid
pLDH	*Plasmodium* lactate dehydrogenase
p/µl	parasite per microliter
PCR	Polymerase chain reaction
RBC	Red blood cell
RDT	Rapid diagnostic test
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RT-PCR	Reverse transcript polymerase chain reaction
rxn	Reaction
SNP	Single nucleotide polymorphism
t	Threshold
TARE-2	Telomere-associated repetitive element 2
T_m	Melting temperature
*var*ATS	*var* gene acidic terminal sequence
WHO	World Health Organization

CONTENTS

Scientific environment……………………………………………………………3

Acknowledgements……………………………………………………………… 4

Summary…………………………………………………………………………. 6

Abbreviations……………………………………………………………….......... 8

Contents…………………………………………………………………………...10

List of publications………………………………………………………..……….13

Chapter 1: Background…………………………………………………………..14

1.1 Malaria history and epidemiology……………………………………..14

1.2 Diagnostic methods……………………………………………………20

1.2.1 Routine diagnostics……………………………………………20

1.2.2 Molecular diagnostics…………………………………………21

1.3 PCR……………………………………………………………………..22

1.3.1 Extraction of DNA……………………………………………..22

1.3.2 Amplification of purified DNA…………………………………22

1.3.2.1 Optimization of the amplification……………………… 23

1.3.2.2 Amplification target…………………………………… 25

1.3.3 Detection of amplified DNA……………………………………26

1.3.3.1 Conventional PCR……………………………………26

1.3.3.2 Real-time PCR………………………………………..27

1.3.3.3 Quantitative PCR……………………………………28

1.4 Sequencing of PCR products **……………………………………………30**

1.5 Malaria parasitaemia **……………………………………………………..30**

Chapter 2: Rationale of the study……………………………………………… 32

Chapter 3: Aims of the study……………………………………………………33

Chapter 4: Materials and Methods………………………………………………34

4.1 Study design, sample material, and study sites **……………………….. 34**

4.2 DNA extraction and storage **……………………………………………36**

4.3 Conventional PCR **………………………………………………………...37**

4.4 Real-time PCR **……………………………………………………………38**

4.5 Sequencing **……………………………………………………………….. 41**

4.6 Microscopy **……………………………………………………………….. 42**

4.7 RDT **………………………………………………………………………. 42**

4.8 Statistical analysis **……………………………………………………….42**

4.9 Ethical considerations **…………………………………………………….43**

Chapter 5: Summary of results…………………………………………………… 44

5.1 Paper I **……………………………………………………………………. 44**

5.2 Paper II **………………………………………………………………….... 46**

5.3 Paper III **…………………………………………………………………. 47**

Chapter 6: Discussion……………………………………………………………. 50

6.1 Development and optimization of PCR methods **……………………... 50**

6.1.1 Development of a new single-step conventional PCR……….. 50

6.1.2 Modification of a species-specific multiplex 18S PCR……… 54

6.1.3 Conversion of conventional to real-time PCR……………….. 56

6.1.4 Quantitative PCR……………………………………………...58

***6.2 Assessment and application of developed PCR assays*………………. 59**

6.2.1 Assessment of conventional genus- and species-specific PCR.. 59

6.2.2 Application of conventional genus- and species-specific PCR..63

6.2.3 Assessment of cytb SYBR and TaqMan real-time PCR assays.. 66

6.2.4 Application of cytb SYBR PCR focusing on low parasitaemia.. 70

6.2.5 Assessment and application of q-PCR………………………... 73

6.2.6 Other applications of malaria q-PCR…………………………76

Chapter 7: Conclusion……………………………………………………77

Chapter 8: Further recommendations…………………………………..78

References…………………………………………………………………79

Papers I-III

LIST OF PUBLICATIONS

Paper I

Christel G Haanshuus, Stein Christian Mohn, Kristine Mørch, Nina Langeland, Bjørn Blomberg, Kurt Hanevik. **A novel, single-amplification PCR targeting mitochondrial genome highly sensitive and specific in diagnosing malaria among returned travelers in Bergen, Norway**.

Malar J. 2013 Jan 22;12:26.

Paper II

Haanshuus CG, Chandy S, Manoharan A, Vivek R, Mathai D, Xena D, Singh A, Langeland N, Blomberg B, Vasanthan G, Sitaram U, Appasamy J, Nesaraj J, Henry A, Patil S, Alvarez-Uria G, Armstrong L, Mørch K. **A High Malaria Prevalence Identified by PCR among Patients with Acute Undifferentiated Fever in India**. PLoS One. 2016 Jul 7;11(7).

Paper III

Christel Gill Haanshuus, Kristine Mørch, Bjørn Blomberg, Gro Elizabeth Ann Strøm, Nina Langeland, Kurt Hanevik, Stein Christian Mohn. **Assessment of malaria real-time PCR methods and application with focus on low-level parasitaemia**.

PLoS One. 2019 Jul 5;14(7).

1. BACKGROUND

1.1 Malaria history and epidemiology

Malaria is a parasitic vector borne disease, which name derives from the medieval Italian expression *mal aria* meaning 'bad air'. Ancient romans believed that the disease came from the fumes in the swamps. Symptoms of malaria were described in ancient Chinese medical writings as far back as 2700 BC. In modern medicine we know that malaria is caused by a bite from an infected female *Anopheles* mosquito [1].

The protozoan parasite belongs to the genus *Plasmodium* group. In humans five main species are described, *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*. The latter is a zoonotic species; the main hosts are long-/and pig-tailed macaques existing in Southeast Asia, particularly Malaysia [2]. It can be argued for that we have six main species infecting humans due to the recent identified two subspecies of *P. ovale*, *P. ovale curtisi* and *P. ovale wallikeri*. These two distinct species are sympatric, meaning that they co-exist in the same geographical areas [3].

The malaria parasite life cycle is complex and involves various stages in both vector and host (Figure 1). In humans, the sporozoites from a mosquito bite first develop in liver cells, without causing any symptoms. Thereafter the merozoites enter the bloodstream and invade the erythrocytes. *P. vivax* and *P. ovale* have the ability to go into a dormant, hypnozoite, stage in the liver, and if untreated it can cause relapse by invading the bloodstream weeks, or even years later. In the infected red blood cells, trophozoites, the parasites undergo asexual multiplication and form schizonts. Eventually, these schizonts will cause cell rupture and release 8-32 new merozites [4]. The erythrocytic cycle lasts 24-72 hours, depending on the species [5], and in non-immune individuals symptoms can appear from when the bloodstream parasite density exceeds 50-100 parasites per microliter (p/µl) blood. Some of the trophozoites will

differentiate into sexual erythrocytic stages called gametocytes. If the gametocytes are taken up in the blood meal to a female *Anopheles* mosquito, the parasites will undergo a sporogonic cycle in the gut of the mosquito resulting in sporozoites ready for new infections [4].

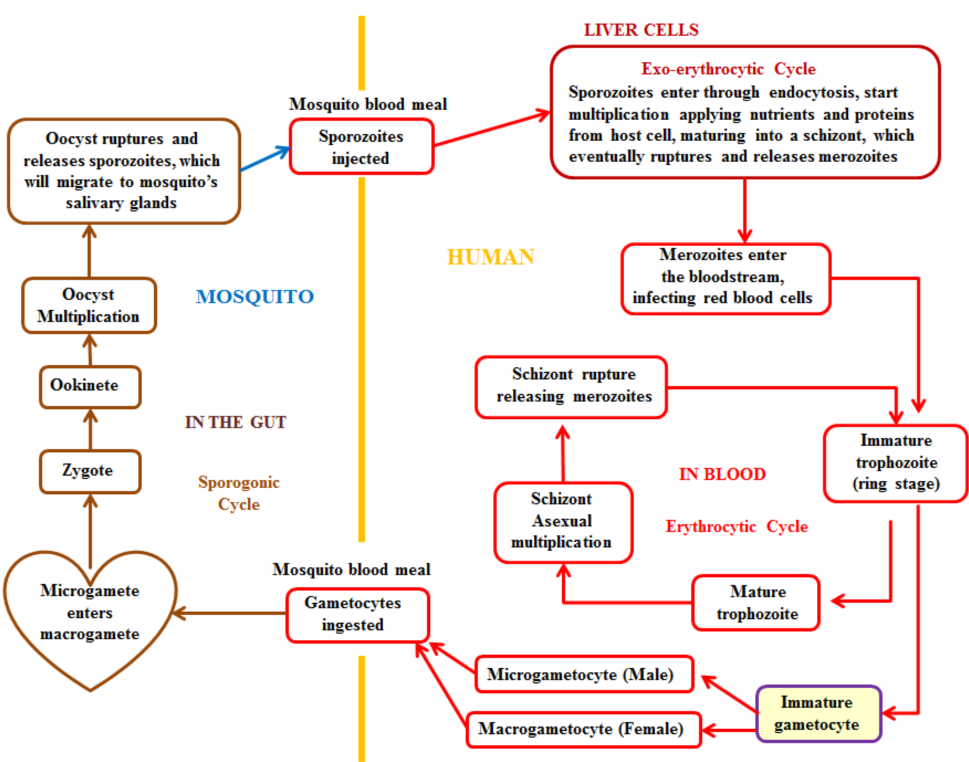
The red blood cells (RBCs) are produced in the bone marrow, and transport oxygen by using haemoglobin. The RBCs use about seven days to mature. Within 120 days they are eaten and recycled by macrophages. Typical male adult density of RBCs is about five million per microliter. The different *Plasmodium* species have not all the same RBC tropism dependent on restricted RBC receptor preferences. *P. vivax*, *P. ovale* and *P. knowlesi* have a cell age tropism to reticulocytes, the young immature RBCs, a stage that lasts for 1-2 days. About 1-6% of the RBCs are reticulocytes, less in adults than in infants. *P. malariae* has a tropism for the oldest RBCs, while, *P. falciparum* has a wide range of receptor preferences, allowing for infection of all RBCs regardless of cell age [6, 7].

It is less understood how some of the trophozoites change their destiny becoming gametocytes, and how this complex sexual stage matures in the host. Studies have shown that immature *P. falciparum* gametocytes migrate to internal host organs such as the spleen and in particular the bone marrow for maturation. Only mature micro- and macrogametocytes are released back into the blood stream. The number of circulating gametocytes is low (~5%) compared to the other parasitic bloodstream stages [8, 9].

Malaria is a tropical disease that can cause acute febrile illness. Symptoms appear 7-18 days after an infective mosquito bite [4]. Almost half of the world's population is at risk of malaria. According to the World Health Organization (WHO) as many as 90 countries and areas have ongoing malaria transmission [10]. The African region is

home for 92% of the global malaria burden's cases and deaths, followed by South-East Asia (5%) and Eastern Mediterranean region (2%). Countries with the largest global burden (80%) are in Sub-Saharan Africa, but also India makes the top 15 list. The recent years the WHO has reported that over 200 million malaria cases, and 400 000 deaths occur every year. It's estimated that children under five years of age account for over 60% of these deaths. Complicated malaria and deaths are mainly attributed to *P. falciparum* [10].

Figure 1: *Plasmodium* parasite life cycle



Among the *Plasmodium* species infecting humans, *P. vivax* is most widely distributed globally [11]. Though, evolution has shown its power in many African countries; individuals with negative Duffy antigen status have a natural protection against *P.*

vivax infections. The Duffy antigen is a chemokine receptor expressed on the surface of RBCs, particularly reticulocytes, which the *P. vivax* parasites use to connect themselves to the RBCs triggering endocytosis [12]. *P. knowlesi* parasites also use this receptor [13], but since this species is zoonotic, the distribution of *P. knowlesi* in humans is limited to the habitants of macaques. Still, *P. knowlesi* is widely distributed in Malaysia, and severe disease and fatal outcomes are reported [14]. Recent research has discovered that *P. vivax* uses an additional receptor on the reticulocytes called Transferrin receptor 1 [15]. Therefore, negative Duffy antigen status does not cover a full *P. vivax* protection [16]. However, this natural protection phenomenon, and the less restricted receptor preference, is thought to be the reason why *P. falciparum* is so highly dominating in the African region. According to the WHO, 99.7% of malaria cases in Africa in 2017 were estimated to be attributed to *P. falciparum* [10]. There are some geographical exceptions in Africa. Ethiopia for instance, is known for its high prevalence of both *P. falciparum* and *P. vivax*. Overall *P. falciparum* is predominating in the regions of South-East Asia (63 %), the Eastern Mediterranean (69%), and the Western Pacific (72%), but countries within these regions have a greater variation in species distribution. There are several geographic areas were *P. vivax* predominates, as well as in the American region (74%) [10]. Some explanations of why *P. vivax* is so widespread, even with the dominance of *P. falciparum*, are the lower blood-stage parasitaemia, fast maturing gametocytes [17], and the ability to establish hypnozoite reservoirs [18]. Despite low parasitaemia, *P. vivax* can cause severe disease that in some circumstances may be life-threating [19].

The neglected species of malaria awareness are *P. ovale* and *P. malariae*; less is known of their receptor preferences, transmission dynamics etc. Both these species are rare and usually cause mild disease. *P. malariae* have a wide global distribution, while *P. ovale* is mostly found in Sub-Saharan Africa and Asia [20-22].

Furthermore, it is not uncommon that the different species are being found as double or triple infections in humans [23].

Other inherited blood conditions such as sickle-cell trait, thalassaemia, glucose-6-phosphate dehydrogenase (G6PD) deficiency, and ovalocytosis, have also emerged through evolution to generate a natural human genetic resistance towards malaria. Paradoxically, all of these conditions can cause severe and fatal illness [10, 24].

Both innate and adaptive (acquired) immunity exist in individuals living in malaria endemic areas, though, these mechanisms are poorly understood. It is suggested that maternal immunoglobulin (infants), and natural killer cells among others are involved in the primary line defence against the hosts' first encounter with a malaria infection (innate immunity) [25]. The adaptive immunity involves the immune memory cells' ability to recognize previous malaria infections. Acquired malaria immunity is described by three pathways; anti-disease and anti-parasite immunity, affecting parasite density and disease severity and morbidity, and premunition, that protects against new infections by maintaining low-level parasitaemia. The acquired immunity is age-dependent, affected by number of infectious bites, and lost within months when leaving endemic areas, or living in areas where transmission ceases [26].

Several studies have investigated malaria related anaemia, a severe and life-threatening condition, especially among young children. A malaria infection leads to a high RBC loss. However, it has been shown that iron deficiency (ID) is associated with reduced parasite growth and suppression of malaria superinfections (infections by more than one genotype) [27, 28]. The parasite is highly dependent on the availability of iron. ID might be a result of the hosts' attempt to starve the parasites from iron supply, although the parasites as well benefit from inhibition of superinfections ensuring transmission of their clones [29, 30]. Studies show conflicting results regarding a possible association of iron supplements in anaemic children and increased malaria risk [31, 32]. A hormone that can cause ID is hepcidin; when up-regulated the enzyme indirectly promotes rest in diet iron uptake, directs iron away from the liver, and triggers macrophages to store their iron. With low iron level in circulation the

RBC replacement by the bone marrow is slow. In typical anaemia hepcidin is suppressed, and the bone marrow is stimulated to increase RBC production [30, 33]. Studies have shown an up-regulation of hepcidin in malaria infections, though in general hepcidin levels are high during inflammations [34]. Therefore levels of ferritin and transferrin saturation might be better markers to describe associations between ID and malaria protection [35].

There are numerous commercial drugs against malaria. Chloroquine was developed in 1934, is a synthetic derivate from Quinine extracted from the South American Cinchona tree, and was a very effective and widely used malaria drug for several years, until its effectiveness declined due to drug resistance [36]. In the later years artemisinin-based combination therapies (ACTs) are recommended by WHO as first-line treatment regime against uncomplicated malaria [37]. For severe *P. falciparum* infection, it is recommended to give artesunate intravenously. Artemisinin is isolated from sweet wormwood, a herb employed in Chinese traditional medicine. It has already been reports on decline in artemisinin drug effectiveness [38, 39]. Worldwide research on artemisinin resistance is ongoing. Primaquine prevents *P. vivax* and *P. ovale* relapses [40]. It is also the only malaria drug with an effective gametocytocidal activity against mature transmissible *P. falciparum* gametocytes [41]. However, primaquine can trigger haemolysis in individuals with G6PD deficiency [42].

In addition to treatment, some drugs are used for malaria prophylaxis in travellers. The most applied ones are atovoquone-proguanil (Malarone), doxycycline and mefloquine (Lariam). In endemic areas use of long-lasting insecticidal nets (LLINs) and/or indoor residual spraying (IRS) are effective malaria control methods. Over 20 different vaccine-projects are being evaluated in clinical or preclinical trials, however due to the complexity of the malaria parasite and its life cycle there is currently no commercially malaria vaccine with full coverage available. Mosquirix has been on the market since

2015, but gives far from 100% coverage, although protects against malaria together with other control methods [10].

1.2 Diagnostic methods

There are several methods which can be used to diagnose malaria. The methods vary in sensitivity and specificity, as well as turnover time and the need for skilled personnel, technical equipment and extensive facilities. Some methods are used in point of care diagnosis, while others are more suitable as a reference method or research.

1.2.1 Routine diagnostics

Light microscopy of Giemsa-stained blood smear is the traditional routine method for diagnosing malaria. The method has a short turnover time, though requires a suitable microscope, and skilled personnel. Microscopy allows for species identification and quantification of parasitaemia, however over- and under-diagnosis is not uncommon, and species might be incorrectly identified [43, 44].

Malaria rapid diagnostic tests (RDTs) are based on immunochromatographic methodology, and detect specific antigens produced by malaria parasites in the blood [45]. The different antigens are histidine-rich protein 2 (HRP2), which is specific to *P. falciparum*, *Plasmodium* lactate dehydrogenase (pLDH), which can be panspecific or specific to either *P. falciparum* or *P. vivax*, and aldolase, which is panspecific. The different RDTs can either detect one of these antigens, or a combination. Commercially available RDTs differ widely in sensitivity and specificity [46]. However, the tests are quick and easy to perform, and recommended as routine method if microscopy is not available or as a supplement to microscopy.

1.2.2 Molecular diagnostics

The limitation of both microscopy and RDT in detecting malaria is low sensitivity and specificity [47-49]. Therefore, during the last decades a variety of molecular techniques have been introduced for malaria diagnostics, such as enzyme-linked immunosorbent assay (ELISA) [50], restriction fragment length polymorphism (RFLP) [51], nucleic acid sequence-based amplification (NASBA) [52], polymerase chain reaction (PCR) [53], and loop-mediated isothermal amplification (LAMP) [54]. The downsides of molecular techniques are the long turnover time, and the requirement of skilled personnel, technical equipment and extensive facilities. Consequently, within malaria diagnostics these methods are mostly applied for surveillance and research purposes.

The techniques PCR and LAMP have been the more popular methods within malaria molecular diagnostics. The first malaria PCR was described in 1990 [55], while the first malaria LAMP was described in 2006 [56]. Both techniques are based on the use of short oligoes, commonly called primers, to amplify a specific target sequence from a few to millions of copies for detection. PCR applies two primers, while LAMP uses six. The main goal with these highly sensitive and specific methods is to make them as suitable as possible for malaria diagnostics under field conditions. Here, the LAMP technique might have an upper hand. In theory the target sequence can be amplified by sole use of a heating block and the amplified product can be detected by direct staining [56]. However, for accurate LAMP sensitivity and specificity, more advanced equipment is required. Nevertheless, the main challenges for both LAMP and PCR, to achieve high sensitivity and specificity, is the need for extracted DNA as material, and the high contamination risk during the amplification process.

1.3 PCR

PCR is a technique that is performed in three stages; extraction of deoxyribonucleic acid (DNA) from material, amplification of a specific DNA sequence, and detection of the amplified product (Figure 2).

1.3.1 Extraction of DNA

Purification of DNA can be executed in several different ways. One of the more applied methods is the use of spin columns, where DNA specifically binds to a silica-gel membrane, while contaminates are washed away [57]. Another method is to use a chelex matrix that binds to PCR inhibitors, like divalent cations and proteins, and the DNA remains in the supernatant [58].

In performing malaria PCR, the choice of sample material is important, both to achieve high sensitivity and to be user-friendly to collect. Typically, the blood is either collected in ethylenediaminetetraacetic acid (EDTA) tubes or as a finger prick on filter papers. Blood on filter papers, commonly called dried blood spots (DBS), are far easier to store and transport than in EDTA tubes, and therefore this collection method is increasingly applied in field studies [59]. EDTA whole blood should be stored on -20°C as soon as possible, while filter papers should be stored dry and in the dark at room temperature [60]. However, the small blood volume (~50 µl) obtained on filter papers, limits the sensitivity of the PCR results [60, 61].

1.3.2 Amplification of purified DNA

The PCR amplification of a specific DNA sequence is executed by three temperature steps by a thermocycler machine (Figure 2B). The first step is denaturation, often at temperatures 95-98°C, where the double-stranded DNA is separated. The next step is annealing, often at temperatures 50-65°C, where two primers bind specifically to the

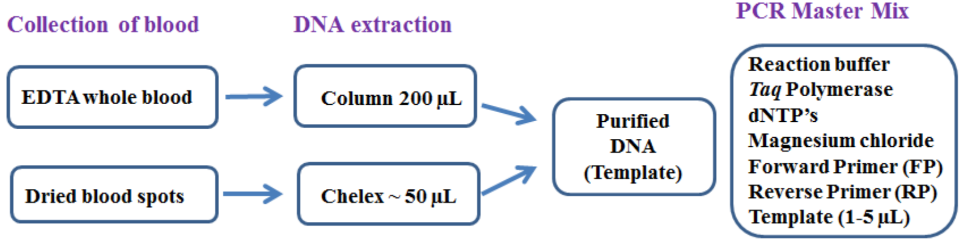
DNA, enclosing the chosen target sequence. The last step is elongation, often at a temperature of 72°C, where a thermostable DNA polymerase in the presence of free deoxynucleoside triphosphates (dNTPs) and magnesium chloride ($MgCl_2$), synthesises new DNA strands complementary to the strands defined by the primers. These three steps are then repeated in cycles 20-50 times, ending up with millions of copies of the target sequence [62].

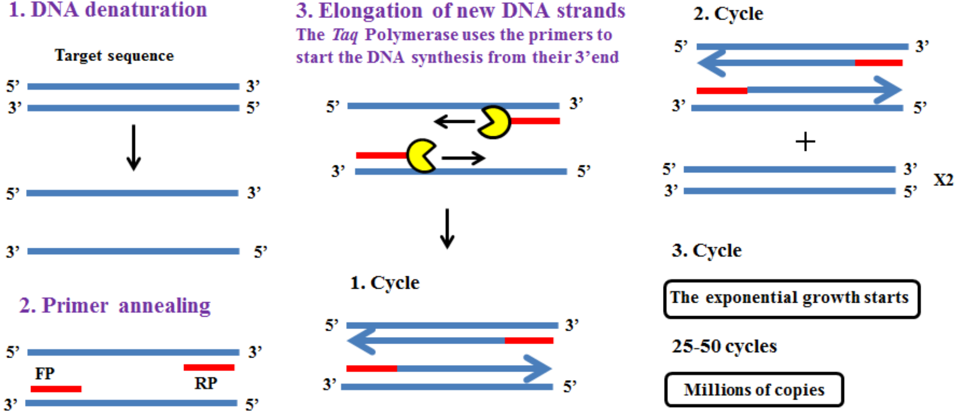
There are different types of PCR amplification. For example, reverse transcription PCR (RT-PCR) is a technique that can detect gene expression/ribonucleic acid (RNA) transcripts levels by primers binding to complementary DNA (cDNA) transcripts from messenger-RNA (mRNA) [63, 64]. Another type is nested PCR (Figure 3), where the target DNA undergoes two separated amplification processes with two different sets of primers. In the second run the amplified product from the first PCR is used as the template, and the second pair of primers encloses an inner sequence of this first product [65]. This nested design makes the method highly sensitive and specific, but also increases the risk of accumulation of PCR products in the laboratory environment. Multiplex PCR amplifies two or more different DNA sequences simultaneously in one amplification reaction. The technique uses multiple primer sets/pairs, one for each amplification target. This process requires that all the primers in the master mix can work at the same annealing temperature [66]. However, multiplex PCR often have low sensitivity and specificity due to primer competition and unspecific primer binding/duplexes [67].

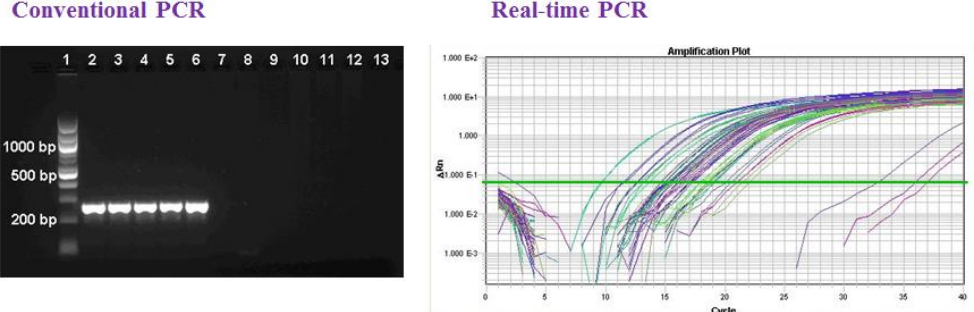
1.3.2.1 Optimization of the amplification

Because primers and target sequence are unique for each specific amplification process, optimization is often needed, or at least beneficial. A perfect balance between specificity and sensitivity is the goal. With low specificity other products than the wanted target sequence are also amplified, while, with low sensitivity the amplified product can be difficult to detect [68].

Figure 2: The principle of malaria PCR-based method







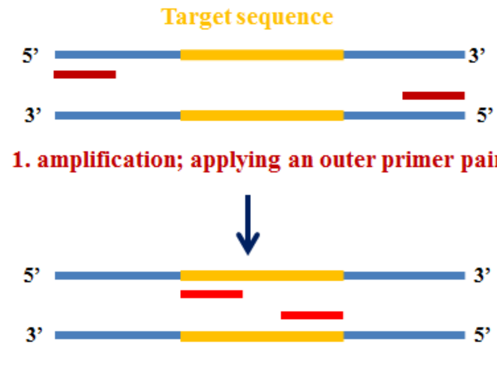
Melting temperature (T_m) is the temperature where one half of a given DNA duplex will dissociate to become single stranded. As an example, primer T_m indicates the primer-template stability at a given temperature. If the annealing temperature is considerably lower than the T_m, the primer will bind strongly, but with an increased risk of unspecific binding. If the annealing temperature is considerable higher than the T_m, there is an increased risk that the primer will not bind at all, resulting in lower sensitivity. Therefore, when deciding the annealing temperature, there is room for optimization, as a difference of only one or two degrees can have an impact [69]. There is also room for optimization regarding the running-time for each temperature step, especially for the annealing and the elongation step. The time for each step must be long enough to successfully match the primers with the correct template sequence, and amplify the product, but if the running-times are too long, unwanted products may also be amplified [68]. The DNA polymerases used in PCR require divalent cations for their activity. Commercial PCR master mix kits usually contain $MgCl_2$. However, adding extra $MgCl_2$ can have a positive impact on PCR efficiency, and the balance between specificity and sensitivity [70]. Other elements to optimize include the amount of template, and the primer concentration [68].

1.3.2.2 Amplification target

To detect low concentration DNA in the blood, the choice of amplification target is of importance [71]. For instance, the genome of a *Plasmodium* parasite has certain genes existing in multiple copies, meaning that the starting point for amplification will be different depending on the choice of DNA target. PCR is an exponential reaction, and the number of sequences the primers can bind to in the first cycle, will impact the total yield. It is also important that the DNA target is a part of a conserved gene, so that binding sites for the primers always will be the same and independent of parasite origin. The first malaria PCR methods used chromosomal small subunit ribosomal RNA 18S locus as target gene, which typically exists in five to eight copies in *P. falciparum* [72]. Thereafter, there was a switch of focus to the conserved *Plasmodium* mitochondrial genome, existing in ~20-160 copies depending on the development

stage [73, 74]. A typical target on the mitochondrial genome is the *cytochrome b* gene (*cytb*) [75]. In 2015 a publication introduced two chromosomal subtelomeric targets; the telomere-associated repetitive element 2 (TARE-2), and the *var* gene acidic terminal sequence (*var*ATS). TARE-2 exists in ~250-280 copies, due to 10 to 12 repeat units presented at 24 of 28 subtelomeric sequences, and the *var*ATS exists in 59 copies, and encodes the *P. falciparum* erythrocyte membrane protein 1 [76].

Figure 3: Principle of nested PCR



1.3.3 Detection of amplified DNA

1.3.3.1 Conventional PCR

The conventional way of detecting an amplified PCR product is by agarose gel electrophoresis, where negative charged DNA molecules migrate through the gel from a negative towards a positive charged field. Similar sized DNA molecules will travel through the gel at the same speed and form a band. By including a standardized DNA ladder it is then possible to interpret the size of the product (Figure 2C). The DNA bands are visualized in UV-light when DNA is dyed either with ethidium bromide or GelRed [77]. However, the technique is time-consuming, and creates a risk of contaminating lab environment with amplified products.

1.3.3.2 Real-time PCR

With real-time PCR the third stage of conventional DNA detection is incorporated into the amplification stage. An advanced thermal cycler monitors directly the amplification of target DNA for each cycle of the PCR process (Figure 2C), by including in the master mix either non-specific dyes that intercalate with double-stranded DNA, or sequence-specific DNA oligoes that are labelled with a fluorescent reporter [78, 79].

Free in a solution non-specific dyes exhibit only low fluorescence, and this background is subtracted during computer analysis. But when bound to double helix molecules formed from single-stranded DNA in the amplification step, the emitted light from the bound dye increases by over a 1000-fold [80]. Examples of these DNA-binding dyes are SYBR Green, EvaGreen, SYTO, and LCGreen. SYBR Green will inhibit PCR if used in high concentration. The others do not inhibit PCR, and can therefore be used in saturated concentrations ensuring that all vacant binding sites are bound by dye [81]. However, the low cost and user-friendly SYBR Green has traditionally been the more widely applied intercalating dye for real-time PCR methods (Figure 4A) [82]. A disadvantage with the non-specific dyes is that they bind to any double-stranded DNA, lowering the specificity of the PCR. Optimization is therefore important; the specificity relies on a high PCR efficiency, and concentration of specific amplified DNA outshining unspecific binding [83].

A labelled sequence-specific DNA oligo used for real-time PCR can be designed in various different ways depending on how the reporter molecule (fluorophore) becomes activated. Many act as a third oligo, commonly called a probe. Examples of labelled oligoes types are hydrolysis (TaqMan) probes, molecular beacons, dual hybridization probes, eclipse probes, scorpions PCR primers, LUX PCR primers, and QZyme PCR primers [79]. Typically the principle is that the fluorophore does not convey a signal when close to a quencher molecule, but when separated from the quencher during

amplification, the fluorophore will release a fluorescence signal for detection [79]. The TaqMan probes are the most applied labelled oligoes used for real-time PCR [84], named after the videogame Pac-Man as during amplification the *Taq* Polymerase eats up the sequence-specific bound probe releasing the fluorophore from the quencher (Figure 4C).

When designing primers for real-time PCR, it is important to consider the length of the PCR product. This is in contrast to conventional PCR, where this is not as crucial as long as the length is within a standardized DNA ladder. Ideal amplicon length for real-time assays is 50-150 base pair (bp). Short amplicons give higher PCR efficiency due to high stability, low variation, and high precision of the amplification process [78, 79]. High efficiency is essential since real-time technique is sensitive to background noise. With conventional PCR the visual amplicon band can be distinguished and confirmed positive from other unspecific products by size. A hydrolysis probe will release only one single fluorophore for each amplicon, while the fluorescent signal applying intercalating dye will be stronger with more dye incorporated in the longer amplicons [85]. In addition, using intercalating dyes a melting curve analysis (MCA) can be performed by an additional step in the real-time PCR software program. Because each specific amplification product have their own distinct melting curve, MCA can identify false positives and primer dimers. The analysis is based on the different amplicons having different melting temperatures, and dissociation characteristics are monitored by heating of double-stranded DNA (Figure 4B) [86].

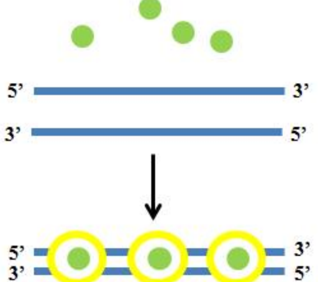
1.3.3.3 Quantitative PCR

In contrast to conventional PCR, the direct detection by real-time allows for quantitative results (q-PCR). The main numbers given by real-time PCR software are cycle threshold (C_t) values, which is inversely proportional to the amount of target DNA in each sample, meaning that low C_t values indicate high parasitaemia, and vice versa. A more accurate way to perform q-PCR is to include a known dilution series of

Figure 4: Principle of real-time PCR

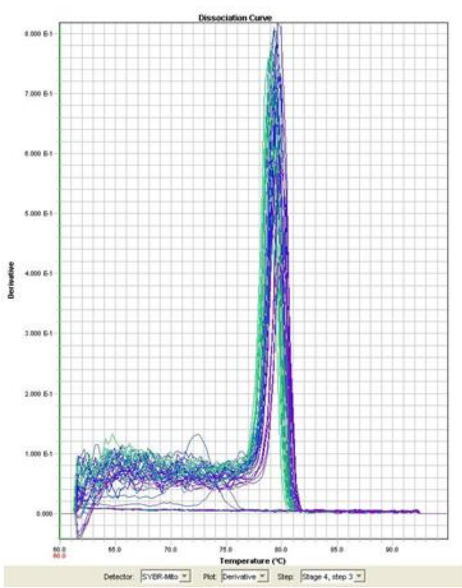
A. SYBR Green

Free in solution; the SYBR Green molecules emit low fluorescence

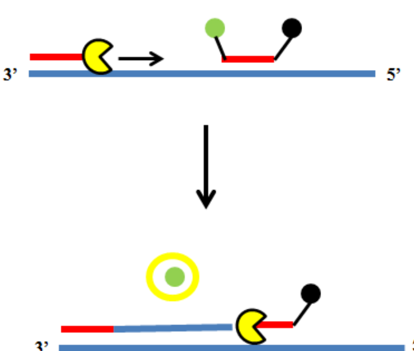


Bound to double-stranded DNA; high intensity of fluorescence signal

B. Melting Curve Analysis



C. TaqMan Probe



Oligo (oligonucleotide)/Primer
A short DNA sequence of typically 18-22 bases, complementary to a given sequence on the amplification target.

***Taq* Polymerase**
In addition to perform DNA synthesis, the enzyme also has 5’-3’ exonuclease activity (cleaving nucleotides). No proof reading function. Can replicate a 1000 base pairs strand of DNA in less than 10 seconds at 72 °C.

Fluorophore molecule
Emitting a strong fluorescence signal when NOT close to a quencher molecule.

Quencher molecule
In close proximity, a quencher will decrease the fluorescence intensity emitted from a fluorophore molecule.

TaqMan probe
A primer with a fluorophore molecule attached to its 5’end, and a quencher molecule attached to its 3’end.

target DNA, that will serve as a standard curve to determine the number of target copies per volume in the sample [87]. The dilution series can either be made from a sample with known number of parasites in known parasite stage or even more exact, a sample with customized plasmid including the target sequence [63, 84, 88]. An optimal standard curve should include at least four clear positive dots covering the whole parasitaemia range of your sample material [89]. The slope value of the standard curve gives the amplification efficiency (E). A value of -3.32 indicates a 100% PCR efficiency [87].

1.4 Sequencing of PCR products

The nucleotide order of a PCR product can be determined by DNA sequencing. There are developed different techniques to sequence a DNA fragment, and most commonly applied is the Sanger sequencing, a chain termination method [90]. The process is advanced, and requires extensive machines and facilities. In malaria diagnostics and research, sequencing is useful for confirming positive PCR products and determining the species and strains [91]. In drug resistance surveillance and research, sequencing is a necessity; resistance is often associated with specific point mutations on relevant parasitic genes that can be identified by sequencing. Certain point mutations on the *P. falciparum pfcrt* gene are associated with Chloroquine resistance [92], and widely reported. While specific point mutations on the *P. falciparum* Kelch 13 propeller domain are associated with artemisinin resistance [38], and reported in malaria cases from South-East Asia [93]. However, until now these mutations are not found in Africa [94], so the surveillance and research on artemisinin resistance is ongoing.

1.5 Malaria parasitaemia

Malaria PCR can detect extremely low densities of target DNA in blood, and compared to microscopy and RDT this technique is superior in sensitivity and specificity [48, 95, 96]. A sensitive PCR will detect a broad range of parasitaemia

levels, also asymptomatic malaria [97-99]. In general low-level parasitaemia may be due to early infection (primary or recurrent), non-*falciparum* infection, premunition, or remains of parasites and denatured DNA after infection clearance by the immune system and antimalarial treatment [100, 101]. Gametocytes are less affected by drugs and the immune system, and circulate in the blood for weeks after ended parasite life cycle pending transmission [102]. A sensitive PCR can be positive for malaria 3-4 days before microscopy, and thereby detect infection earlier [103]. Dependent by amplification target different PCR assays may vary in sensitivity and interpretation of q-PCR results with the various *Plasmodium* blood-stages; from the influence of sequestration in peripheral circulation, to the number of gene copies in the chromosomal and mitochondrial genome [72-74]. In both malaria diagnostics and research it is an advantage to implement q-PCR in order to increase knowledge about disease severity, epidemiology and transmission dynamics [104, 105].

2. RATIONALE OF THE STUDY

Microscopy has historically been the number one method to diagnose malaria, and still is, but has its challenges in accurate sensitivity and specificity. For many years the conventional nested 18S PCR published by Snounou in 1991 was regarded as the gold standard for molecular malaria diagnostics. The nested design provided high sensitivity and specificity. However, this two-step method is time-consuming and increases the risk for contamination. Therefor the need for a competing single-step PCR assay was prominent. A way to compete with the high sensitivity of nested PCR is to apply another amplification target with a higher copy number than the standard target 18S gene.

As PCR became increasingly applied as reference method in malaria research studies, there were reports on PCR being a sensitive enough method to not only detect clinical cases, but also a high portion of asymptomatic malaria compared to microscopy. In recent years, the detection of low parasitaemia has been a hot topic within malaria research; in order to increase knowledge of diagnosing acute fever patients, malaria epidemiology, transmission, and elimination surveillance. To achieve this knowledge, conventional PCR is insufficient, while real-time q-PCR points itself out to be a highly relevant technique for this purpose. The challenge also includes how to interpret malaria q-PCR results.

3. AIMS OF THE STUDY

3.1 Primary aims:

1. To develop a new robust and user-friendly PCR method.

2. To assess the PCR method on relevant clinical material.

3.2 Secondary aims:

1. To validate and compare the developed conventional PCR assay(s) with a reference nested PCR method (Paper I).

2. To compare PCR with routine diagnostics (Paper I, II and III).

3. To determine the malaria prevalence among fever patients in community hospitals in India (Paper II).

4. To optimize and validate the conversion from conventional to real-time PCR (Paper III).

5. To compare the application of different amplification targets (Paper I and III), and the use of platform, SYBR Green versus Taq Man probe (Paper III).

7. To compare the sensitivity of the novel PCR method to different relevant malaria real-time PCR assays (Paper III).

8. To assess application of q-PCR using *P. falciparum* positive Tanzanian field samples (Paper III).

4. MATERIALS AND METHODS

4.1 Study design, sample material, and study sites

Paper I and III were mainly methodological studies, while the work done in Paper II was part of a multi-centre, observational, cross sectional study.

A detailed overview of the patient and reference materials, positive/negative controls and plasmids applied in the three papers is given in Figure 5.

Figure 5A: Patient material applied in papers I, II, and III.

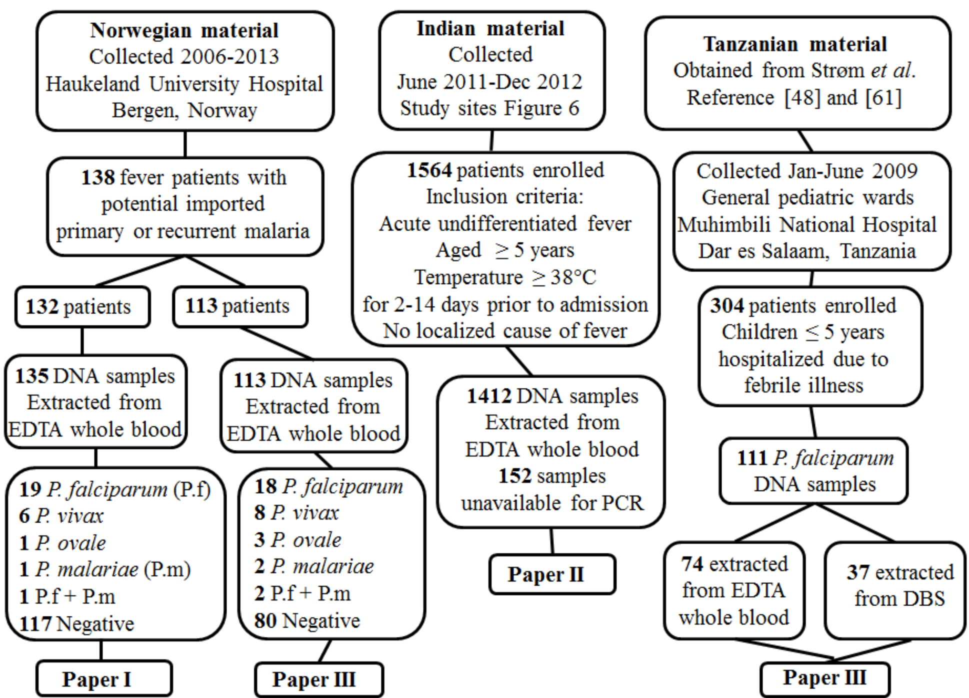
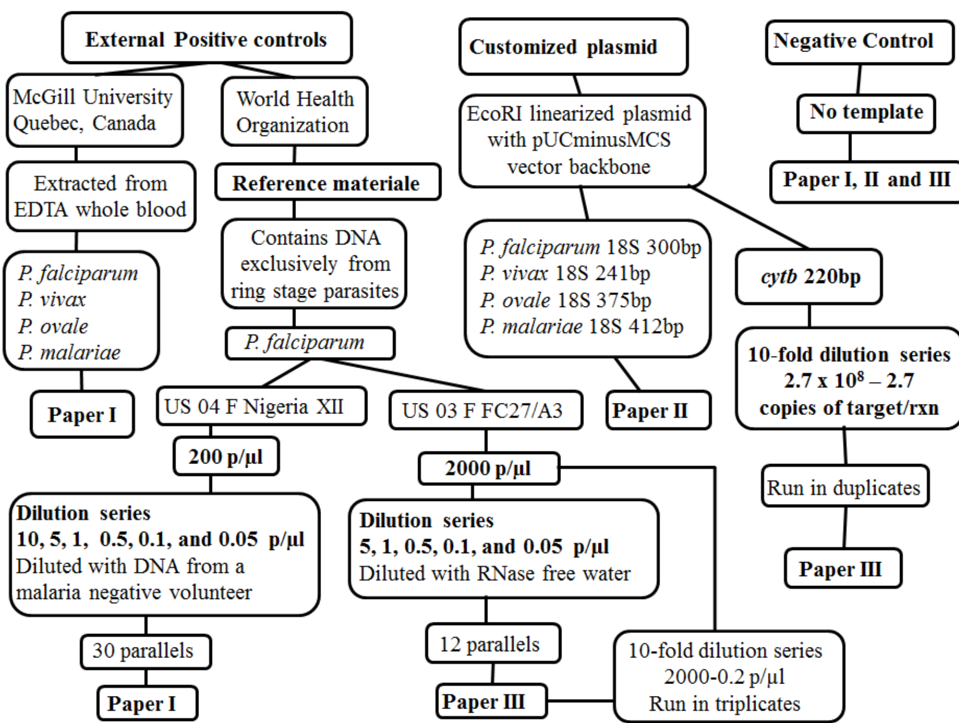


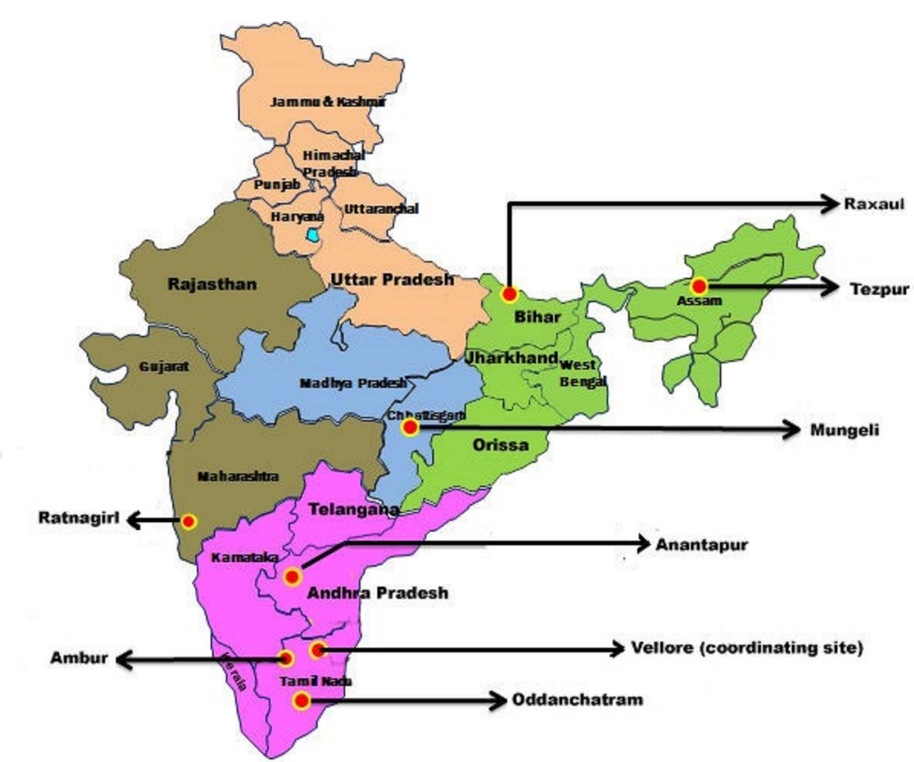
Figure 5B: Controls, reference material, and plasmids



The study sites in Paper II (Figure 6) were secondary level community hospitals with 100 to 500 beds, located in Tezpur (Assam, North East India), Raxaul (Bihar, East India), Mungeli (Chhattisgarh, Central India), Ratnagiri (Maharashtra, West India), Anantapur (Andhra Pradesh, South India), Oddanchatram and Ambur (Tamil Nadu, South India).

The Benjamin M Pulimood Laboratories for Infection and Inflammation, Department of Medicine Unit 1 and Infectious Diseases, Christian Medical College, Vellore, India served as study coordinating centre and reference laboratory.

Figure 6: Study sites in Paper II.



4.2 DNA extraction and storage

All the three papers applied DNA extracted from 200 µl EDTA whole blood material. Both blood and extracted DNA material was stored at -20°C prior to application. The DNA was purified using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In addition, Paper III applied DNA extracted from filter papers which had been purified using Chelex-100 Molecular Biology Grade Resin (Bio-Rad Laboratories, Hercules, CA, USA) as previously described [61]. All the DNA material applied in Paper III had been long-term stored at -80°C after short-term storage at -20°C.

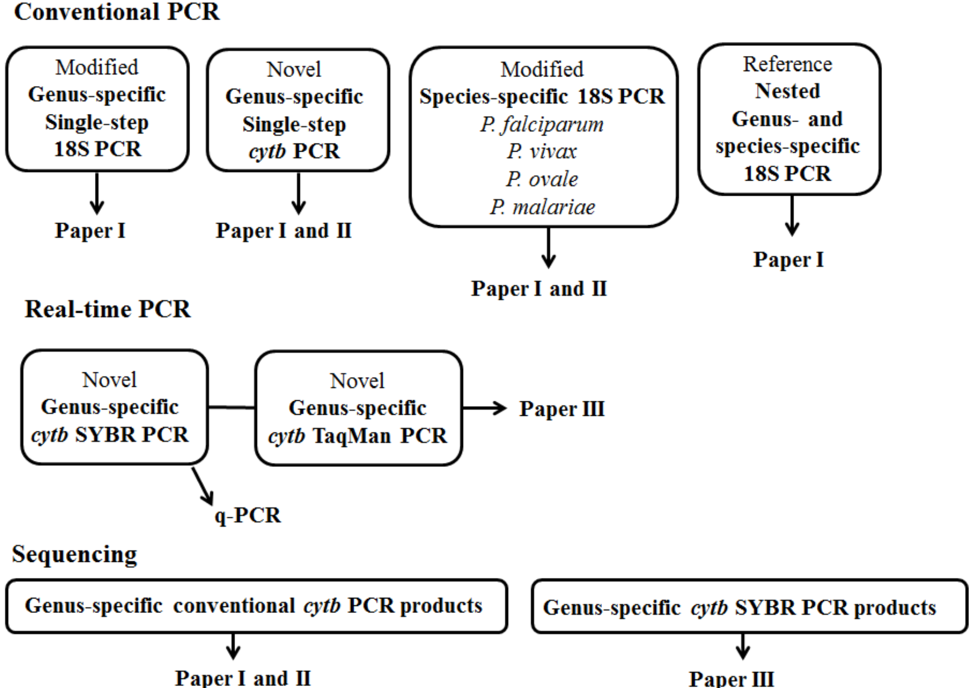
4.3 Conventional PCR

Three developed/modified PCR assays, two genus-specific and one species-specific, were assessed in Paper I. The genus-/ and species-specific nested 18S PCR as described by Singh *et al.* [53], was included as a reference method.

A detailed overview and description of methods and primers is given in Figure 7, and Table 1 and 2.

Notification; the modified genus-specific 18S PCR is not further discussed in this thesis due to focus on the novel genus-specific *cytb* PCR that is applied in all the projects.

Figure 7: A flowchart of PCR methods applied in papers I, II, III.



All reaction mixtures contained 2 µl of DNA template and 12.5 µl 2X HotStarTaq Master Mix (Qiagen) at a total volume of 25 µl. The amplifications were performed by using GeneAmp PCR System 9700 (Applied Biosystems, Carlsbad, CA, USA), and the PCR products were analysed by electrophoresis using 2% SeaKem agarose gel (Lonza, Rockland, ME, USA) with 1X GelRed (Biotium, Hayward, CA, USA).

In Paper II all samples were screened for presence of *Plasmodium* DNA by the novel genus-specific *cytb* PCR developed in Paper I. In case of discordant results between PCR, RDT or routine microscopy, the samples were retested by the genus-specific PCR from the extraction step as a quality control. A malaria infection was confirmed if two or all of the three PCR parallels were positive.

All the genus-specific *cytb* PCR positive samples were further analysed by the modified species-specific 18S PCR protocol also assessed in Paper I. Samples negative by the species-specific PCR, were repeated by the genus-specific PCR, and the PCR products were thereby sequenced for species identification as described in section 4.5. Amplifications were done on AB Applied Biosystem veriti 96 well Thermal cycler (Applied Biosystems), and products detected by electrophoresis on a 2% SeaKem agarose gel (Lonza) stained with ethidium bromide.

4.4 Real-time PCR

In Paper III the conventional *cytb* PCR developed and applied in papers I and II was converted into two real-time assays, one using SYBR Green and one TaqMan probe.

A detailed overview and description of methods and primers is given in Figure 7, and Table 1 and 2.

Table 1: Description of methods applied in papers I, II, and III.

Method	Primers	Primer conc.	$MgCl_2$ conc. *	PCR-Program
Modified **Genus-specific Single-step 18S PCR** (1200 bp)	F: rPLU 6 R: rPLU 5	1 µM	4 mM	Step 1, 95°C for 15 min; step 2, denaturation at 95°C for 10 sec; step 3, annealing at 63°C for 10 sec; step 4, extension at 72°C for 75 sec; steps 2-4 repeated 50 times; and step 5, 72°C for 10 min
Novel **Genus-specific Single-step *cytb* PCR** (220 bp)	F: PgMt19 F3 R: PgMt19 B3	1 µM	4 mM	Step 1, 95°C for 15 min; step 2, denaturation at 95°C for 10 sec; step 3, annealing at 62°C for 10 sec; step 4, extension at 72°C for 15 sec; steps 2-4 repeated 50 times; and step 5, 72°C for 10 min
Modified **Species-specific 18S PCR**				
P. falciparum (300 bp)	F: *P. falciparum*	1 µM	2 mM	Step 1, 95°C for 15 min; step 2, denaturation at 95°C for 10 sec; step 3, annealing at 65°C for 10 sec; step 4, extension at 72°C for 30 sec; steps 2-4 repeated 45 times; and step 5, 72°C for 10 min
P. vivax (241 bp)	F: *P. vivax*	1 µM	1 mM	
P. ovale (375 bp)	F: *P. ovale*	0.6 µM	4 mM	
P. malariae (412 bp)	F: *P. malariae*	1 µM	2 mM	
	R: Species-specific	1/ 0.6 µM		
Novel **Genus-specific *cytb* SYBR** (220 bp)	F: PgMt19 F3 R: PgMt19 B3	600 nM	1 mM	Step 1, 50°C for 2 min; step 2, 95°C for 10 min; step 3, denaturation at 95°C for 15 sec; step 4, annealing at 59°C for 50 sec; and step 5, amplification at 72°C for 10 sec, steps 3-5 repeated 45 times
Novel **Genus-specific *cytb* TaqMan** (220 bp)	F: PgMt19 F3 R: PgMt19 B3 PgMt(28)-Probe	600 nM 200 nM	1 mM	Step 1, 50°C for 2 min; step 2, 95°C for 10 min; step 3, denaturation at 95°C for 15 sec; step 4, annealing at 59°C for 50 sec; and step 5, amplification at 72°C for 10 sec, steps 3-5 repeated 45 times

* New England BioLabs, Ipswich, MA, USA.

Table 2: Description of primers applied in papers I, II and III.

Primer	Sequence	Length (bp)	GC (%)	Published by
rPLU 6 forward	5′-tta aaa ttg cag tta aaa cg	20	25	[65]
rPLU 5 reverse	5′-cct gtt gtt gcc tta aac ttc	21	43	[65]
PgMt19 F3 forward	5′-tcg ctt cta acg gtg aac	18	50	[108]
PgMt19 B3 forward	5′-aat tga tag tat cag cta tcc ata g	25	32	[108]
***P. falciparum* forward**	5′-aac aga cgg gta gtc atg att gag	24	46	[66]
***P. vivax* forward**	5′-gag cgt tca aag caa aca ga	23	39	Paper I
***P. ovale* forward**	5′-ctg ttc ttt gca ttc ctt atg c	22	41	[66]
***P. malariae* forward**	5′-cgt taa gaa taa acg cca agc g	22	41	[66]
Species-specific reverse	5′-gta tct gat cgt ctt cac tcc c	22	50	[66]
PgMt(28)-Probe	6-FAM-ctt cta aca ttc cac ttg ctt ata act g-BHQ-1	28	36	Paper III

The primers were obtained from Eurogentec (Seraing, Belgium), analysed using Oligo v6 primer analysis software (Molecular Biology Insights, Cascade, CO, USA), the sequence alignment editor software BioEdit v7 (Tom Hall, Carlsbad, CA, USA), and tested with Basic Local Alignment Search Tool (BLAST, National Center for Biotechnology Information, Bethesda, MD, USA).

A sensitivity comparison including five relevant real-time PCR assays (details given in Table 3) was also performed in Paper III. The included assays were applied as previously published except from a few fixed parameters as described below.

All the real-time assays run in Paper III applied 2 µl DNA template, and 12,5 µl SYBR Select Master Mix/ TaqMan Universal Master Mix II, with UNG (Applied Biosystems), at a total volume of 25 µl. The amplifications were performed using ABI Prism 7900HT Sequence Detection System (Applied Biosystems) with the threshold automatically set. For the SYBR assays melting curve analysis was included given by the program SDS 2.3 (Applied Biosystems).

Quantitative PCR was performed applying customized plasmid with *cytb* 220 bp insert (OriGene Technologies, Rockville, MD, USA) in a 10-fold dilution series, range 2.7 x 10^8 – 2.7 copies of target DNA/reaction (rxn).

Table 3: Characteristics of the five included real-time PCR assays

	Method	Specificity	Target genome	Target gene	Amplicon length	Platform	Year	Reference
1	**Lefterova**	Pan	Chrom	18S rRNA	317 bp	SYBR	2015	[91]
2	**Xu**	Pan	Mito	*cytb*	430 bp	SYBR	2015	[51]
3	**Farrugia**	Pan	Mito	*cytb*	203 bp	TaqMan	2011	[75]
4	**Hofmann**	P.f	Chrom	TARE-2	93 bp	SYBR	2015	[76]
5	**Hofmann**	P.f	Chrom	*var*ATS	65 bp	TaqMan	2015	[76]

Abbreviations: P.f, *Plasmodium falciparum*; Chrom, chromosomal; Mito, mitochondrial; *cytb*, cytochrome *b* gene; telomere-associated repetitive element 2, TARE-2; *var* gene acidic terminal sequence, *var*ATS.

4.5 Sequencing

All the three papers present species-specific PCR results obtained by sequencing. For Paper I all positive products by the developed genus-specific *cytb* conventional PCR were sequenced in both directions using the primers PgMt19 F3&B3. The PCR products were purified with ExoSAP-IT (USB Corporation, Cleveland, OH, USA) according to the manufacturer's instructions, prior to applying the following cycle conditions using GeneAmp PCR System 9700 (Applied Biosystems): step 1, 96°C for 10 sec; step 2, 62°C for 5 sec; step 3, 60°C for 4 min; steps 1-3 repeated 27 times. The reaction mixture contained 1 µl BigDye v1.1 (Applied Biosystems), 2 µl sequencing buffer 5X (Applied Biosystems), 0.5 µM primer, and 1 µl template to a total volume of 10 µl. The sequences were obtained with the ABI PRISM 3730 DNA Analyzer (Applied Biosystems), and the BioEdit v7 was used prior to sequence identification by BLAST. For Paper II only positive genus-specific PCR products that were negative by the species-specific PCR were sequenced in one direction completing the species identification, while for Paper III all the genus-specific *cytb* SYBR PCR positive results among the Norwegian material were sequenced in one direction.

4.6 Microscopy

All the three papers compared PCR and microscopy results. The microscopy results presented in papers I and II were part of routine work-up according to hospital procedures, while the microscopy results presented in Paper III were obtained from thin blood research slides which had been stained with Giemsa 5% for 20 min after 30-sec fixation with 100% methanol.

4.7 RDT

Papers II and III compared RDT data with PCR results.

For Paper II the RDT ParaHIT-Total Ver. 1.0 Device 55IC204-10 (Span Diagnostics Ltd, Surat, India) was applied on EDTA whole blood stored at -20°C following manufacturer's instructions. The test detects *P. falciparum* specific HRP2 and aldolase antigen of pan-malaria species (*P. falciparum*, *P. malariae*, *P. vivax*, and *P. ovale*). A red band in the 'Pf' region alone indicates that the sample is reactive for *P. falciparum* (usually in case of low parasitaemia), while red bands in both 'Pf' and 'Pan' region indicate either single infection by *P. falciparum* or a mixed infection of *P. falciparum* with one of the other species. Appearance of a red band in the 'Pan' region alone indicates that the sample is reactive for infection by a non-*falciparum* species.

In Paper III we used RDT data produced for the study by Strøm *et al.* [48]. The RDT applied was the First Response Malaria Ag pLDH/HRP2 Combo card on EDTA whole blood stored at -20°C following manufacturer's instructions. In two separate regions the test detects HRP2 for *P. falciparum* and pLDH for pan-malaria species (*P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*).

4.8 Statistical analysis

In Paper I we compared the different PCR methods by applying prtest command using Stata 11 (Stata Corp, College Station, TX, USA). The differences in sensitivity and

specificity with 95% confidence intervals (95% CI) were calculated, and non-inferior of the test was considered proved if the upper boundary of the 95% CI of the sensitivity difference was less than a predefined delta of 5%.

In Paper II confidence intervals for tests' sensitivities, specificities, and positive and negative predictive values were calculated using the cii command in Stata 14, and presented as exact 95% confidence intervals.

In Paper III both the statistical univariate and the multivariate logistic regression analysis were performed applying IBM SPSS Statistics version 24 (SPSS Inc., IBM Company). For the univariate analysis the data were organized into categorical variables for 2x2 cross-tabulation analysis, and assessed with effect estimates (odds ratio) with corresponding confidence intervals, as well as Chi-squared test, or Fisher's exact test if few observations. For the multivariate analysis the p-values were calculated by the Likelihood ratio test using SPSS and QuickCalcs (GraphPad Software). In the regression model, all variables with p-value <0.1 from the univariate analysis, excluding variables with extensive numbers of missing values, were included, and the model was evaluated by the Hosmer and Lemeshow Test, and by residuals and Cook distances analysis. For both the univariate and the multivariate analysis, a correlation was regarded as statistically significant if p-value was < 0.05.

4.9 Ethical considerations

Both the projects presented in Paper I and III were approved by the Regional Committee for Ethics in Medical Research (REK) in Bergen, Norway (No.2011/942 (Paper I), No.2015/886 and 2016/584 (Paper III)), while the project presented in Paper II was a collaboration between Haukeland University Hospital and Christian Medical College, approved by both REK (2010/2271-5) and the Institutional Research Board at Christian Medical College, Vellore, Tamil Nadu (No. 7242 dated 11th of August 2010). Written, informed consent was obtained from the patients.

5. SUMMARY OF RESULTS

Flowcharts over the main results from papers I, II and III are given in Figure 8.

5.1 Paper I

A novel, single-amplification PCR targeting mitochondrial genome highly sensitive and specific in diagnosing malaria among returned travellers in Bergen, Norway

Christel G Haanshuus, Stein Christian Mohn, Kristine Mørch, Nina Langeland, Bjørn Blomberg, Kurt Hanevik

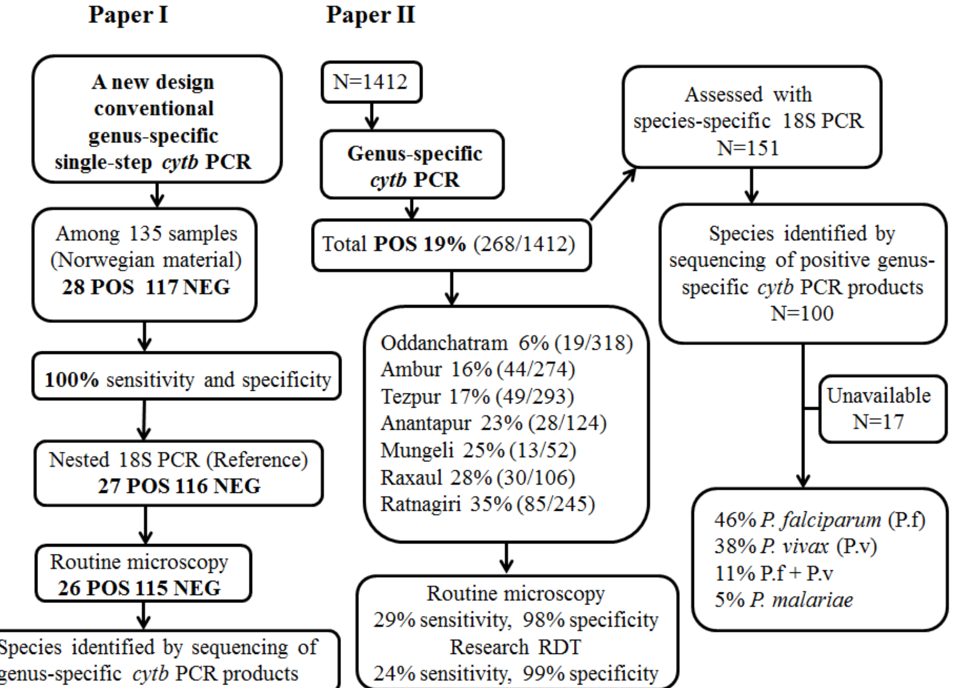
Malar J. 2013 Jan 22;12:26.

***Plasmodium* genus-specific detection:** In this study we design a novel genus-specific PCR that for positive samples amplifies in one step a 220 bp long amplicon by using a target sequence on the mitochondrial *cytb* gene. The *cytb* PCR showed a high sensitivity and specificity compared to routine microscopy and reference nested 18S PCR. Among a Norwegian sample collection (N=135) from fever patients with potential imported malaria, a consensus of true malaria positive samples were defined by at least two out of three methods positive, 21% (28/135). The *cytb* PCR had a 100% sensitivity (28/28), nested 18S PCR 96% (27/28), and microscopy 93% (26/28). A dilution of 0.5 ring stage parasite/µl (reference material) run in 30 parallels differentiated the two highly sensitive PCR methods; *cytb* PCR 97% (29/30) and nested 18S PCR 87% (26/30). Both methods detected as low as 0.05 p/µl, but not consistently.

***Plasmodium* species-specific detection:** In addition to developing a genus-specific *cytb* PCR, we also modified and optimized a species-specific single-step 18S PCR, which was 100% sensitive and specific compared to species-specific nested 18S PCR. Furthermore, the 28 positive genus-specific *cytb* PCR products were sequenced to confirm true positivity, revealing six single nucleotide polymorphisms (SNPs) and one

insert/deletion allowing for species determination of the 28 sequences. Overall there were seven samples (six patients) with discordant results between the different methods. Routine microscopy overlooked one *P. malariae*, and one *P. vivax* infection. One recurrent (relapse) *P. vivax* infection was inconclusive by microscopy, while two single infections, one *P. ovale* and one *P. vivax*, were interpreted by microscopy as double infections with *P. falciparum*. In one double *P. falciparum* and *P. malariae* infection, microscopy under-diagnosed the *P. malariae*. Sequencing of the *cytb* PCR products also missed the *P. malariae* in the double infection. One recurrent (recrudescence) *P. falciparum* infection was positive by microscopy; one ring stage parasite was detected. Positive as well by the *cytb* PCR sequencing, while none of the 18S PCR assays detected this infection. The discordant results showed how important it is to include PCR as a reference method for microscopy.

Figure 8A: An overview of the main results in papers I and II.



5.2 Paper II

A High Malaria Prevalence Identified by PCR among Patients with Acute Undifferentiated Fever in India

Haanshuus CG, Chandy S, Manoharan A, Vivek R, Mathai D, Xena D, Singh A, Langeland N, Blomberg B, Vasanthan G, Sitaram U, Appasamy J, Nesaraj J, Henry A, Patil S, Alvarez-Uria G, Armstrong L, Mørch K

PLoS One. 2016 Jul 7;11(7).

***Plasmodium* genus-specific prevalence:** This study was a part of a large multicentre study investigating causes of acute undifferentiated fever (N=1564). Seven community hospitals in six states of India were included, and 1412 samples were available for malaria PCR testing. In total 19% (268/1412) was positive by genus-specific *cytb* PCR. The seven sites had a prevalence as following; Oddanchatram (South India) 6% (19/318), Ambur (South) 16% (44/274), Tezpur (North-East) 17% (49/293), Anantapur (South) 23% (28/124), Mungeli (Central) 25% (13/52), Raxaul (East) 28% (30/106), and Ratnagiri (West) 35% (85/245).

***Plasmodium* species-specific prevalence:** Among the 268 genus-specific *cytb* PCR positives, 251 samples were available for species-specific 18S PCR. Overall, 46% *P. falciparum* and 38% *P. vivax* single infections were detected, while *P. falciparum* and *P. vivax* double infection was found in 11%. Mungeli (85%), Raxaul (79%) and Anantapur (52%) had a predominance of *P. falciparum*, while Ambur (55%) had a *P. vivax* predominance. Tezpur, Ratnagiri and Oddanchatram had a more equal distribution of *P. falciparum* and *P. vivax*. *P. malariae* was found in 5% of the malaria cases, and all the sites except Mungeli and Raxaul detected *P. malariae* infections. *P. ovale* was not found in any site.

Performance of routine microscopy and RDT compared to PCR: Sensitivity by microscopy (N=1168) was 29% (66/228), and by RDT (N=1407) 24% (65/268). The specificity was high for both methods; 98% (918/940) by microscopy, and 99% (1129/1139) by RDT. Only 5% (2/43) were negative by PCR among both microscopy

and RDT positives. From confirmed PCR positive samples, microscopy found a malaria prevalence of 6%, and RDT 5%. The number of false positive blood slides by microscopy was 22. However, 18 of these were from the same site, indicating a high specificity of routine microscopy in six of the seven hospitals. At species level, routine microscopy had a tendency to misdiagnose *P. falciparum* as *P. vivax*, while there was a high specificity in correctly identifying *P. vivax* infections. None of the *P. malariae* infections were found by microscopy. Among the RDT positives, seven *P. falciparum* infections were misclassified as non-*falciparum*. Neither did RDT detect any of the *P. malariae* single infections (N=9).

5.3 Paper III

Assessment of malaria real-time PCR methods and application with focus on low-level parasitaemia

Christel Gill Haanshuus, Kristine Mørch, Bjørn Blomberg, Gro Elizabeth Ann Strøm, Nina Langeland, Kurt Hanevik, Stein Christian Mohn

PLoS One. 2019 Jul 5;14(7).

Conversion from *cytb* conventional to real-time PCR: By optimization, the genus-specific *cytb* conventional PCR (Paper I) was successfully converted to a real-time PCR assessed by using reference material, a Norwegian collection (N=113) and Tanzanian field material (N=111). Applying SYBR Green to detect positives showed a higher sensitivity and specificity than using TaqMan probe. The sequencing of *cytb* SYBR 220 bp amplicons (N=33) confirmed that these real-time PCR products gave high-quality sequences of full length, and correctly identified the species at the same level as its conventional counterpart method (Paper I).

Sensitivity comparison of seven real-time PCR assays: Applying low dilutions of *P. falciparum* reference material (5-0.05 p/µl) run in 12 parallels establishing reproducibility with low-level parasitaemia; this study *cytb* SYBR PCR, Xu *et al*. *cytb* SYBR PCR, and Hofmann *et al*. *var*ATS TaqMan PCR showed highest sensitivity

among the seven real-time assays. Furthermore, by applying the collection of *P. falciparum* positive Tanzanian field samples (N=111), the *var*ATS TaqMan PCR was slightly more sensitive than this study *cytb* SYBR PCR, 86% (96/111) versus 82% (91/111).

Application and assessment of q-PCR: To present an alternative and relevant application of q-PCR data, the association between low-level parasitaemia identified by the *cytb* SYBR q-PCR and clinical and demographic factors (N=74), were evaluated applying cross tabulation and multiple logistic regression analysis. The results are presented in Figure 8C.

Figure 8B: An overview of results in Paper III.

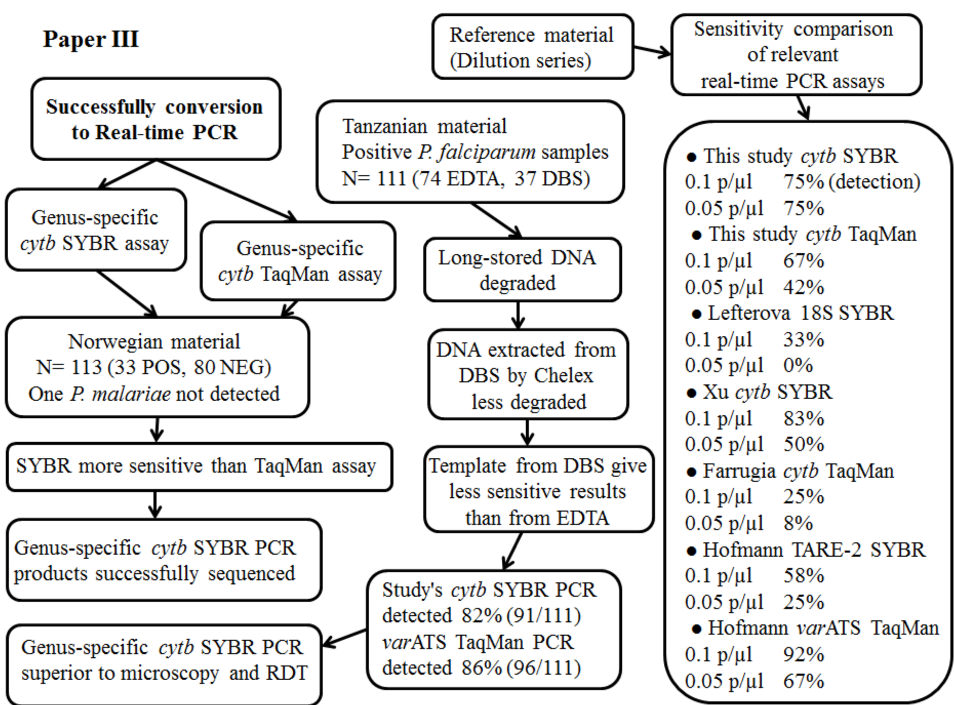
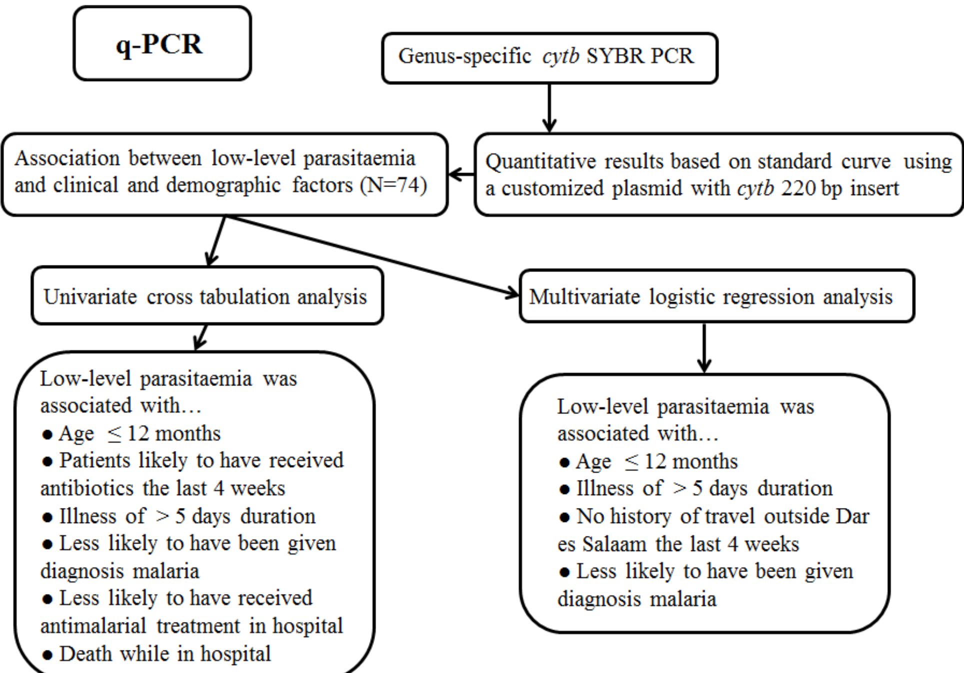


Figure 8C: Main results from the q-PCR assessment in Paper III



6. DISCUSSION

6.1 Development and optimization of PCR methods

6.1.1 Development of a new single-step conventional PCR (Paper I)

At the time this study started the gold standard for malaria PCR was considered to be the genus- and species-specific nested 18S PCR published by Singh *et al.* in 1999, modified from the nested PCR presented by Snounou *et al.* in 1993 [53, 65]. A highly sensitive and specific method due to the nested design, but compared to a single-step PCR, twice as costly, long turnover time, and risk of technical errors and contamination. Amplification targets on chromosomal small subunit ribosomal RNA 18S locus are expected to be in 5-8 copies per parasite nucleus [72]. A target with a high copy number should give a higher sensitivity [71]. For example with 18S, if the blood concentration was one ring-stage p/µl, using 2 µl template gives the exponential reaction a starting point of 10-16 copies. Theoretically, one single copy of target DNA is enough for detection, statistically, a positive amplification needs a minimum of three copies/rxn for reproducible results, but practically, about 10 copies/rxn is of the lower limit [106, 107].

The single-step malaria PCR developed for Paper I applied two of the six LAMP primers published by Polley *et. al* [108]. This LAMP was genus-specific, targeting all the human *Plasmodium* species. Although the various *Plasmodium* species have distinct differences in morphology, execution of the erythrocytic cycle etc., their genomes are nearly identical, especially for conserved genes. The amplification target was a sequence on the mitochondrial *cytb* gene, which encodes the main subunit of a transmembrane complex involved in electron transport, pumping proton to generate ATP. The 6 kb mitochondrial genome consists of only three genes in one copy each, *Cytochrome c oxidase* subunit 1 (*Cox I*), *Cox III* and *cytb*, but one mitochondrion

harbours about 20 copies of the genome [73]. Ring-stage parasites have one mitochondrion [74]. However, gametocytes are reported to have up to eight fold higher quantity of the mitochondrial genome, ~160 copies [74, 109, 110], probably because the sexual gametocytes have a higher demand for energy and are more metabolic active than the asexual stages [74]. Only the female gametes inherit the mitochondrial genome, and the sex ratio in the blood circulation is in favour of the female gametes [111, 112].

When designing primers for PCR method development, it is important that the two primers have suitable T_m. Factors as the length, GC-content, next neighbour phenomenon, and salt concentration, have an effect on the primer T_m [113]. The two LAMP primers chosen to design a new single-step PCR had the following T_m; 59°C (18 bases, 50% GC) and 60°C (25 bases, 32% GC), calculated by the program Oligo (Molecular Biology Insights). Since it was only one degree separating the two primers, they had a close affinity to the primer sites at the same annealing temperature, which is a great advantage. Optimal T_m of primers is 60-64°C, so the T_m of the chosen primers were on the lower limit of optimal T_m. However, in the optimization process it was still room for testing high annealing temperatures to adjust for higher specificity avoiding unspecific primer binding, and lower temperatures to adjust for higher sensitivity. During a long optimization process, an annealing temperature interval from 56-66°C was tested.

The two LAMP primers had a distance between each other enclosing an amplicon of 220 bp, which is a perfect amplicon size for conventional PCR. With short amplicons the elongation time can be reduced, and amplification precision increases, but 220 bp was still long enough to give a strong visible band on an agarose gel within a 100 bp DNA ladder. The *cytb* PCR program was designed with many cycles (50) for high sensitivity, but with short time-steps for high precision (10-15 sec). Because of the many cycles, the annealing temperature that gave the best results was 62°C. The high

annealing temperature and the short time-steps allowed for further increasing the sensitivity by using high concentrations of primers (1 µM) and additional $MgCl_2$ (4 mM) in the master mix. $MgCl_2$ increases the activity of the polymerase, although, excess concentration can lead to the enzyme having a lower fidelity [114]. The end result of the optimization was a robust PCR program suited for the chosen primers, giving a highly sensitive and specific amplification of target sequence applying only one amplification step.

The short amplicon length also contributed to that the PCR products easily could be sequenced, giving high-quality sequences confirming true positives. The sequencing of the 28 positives using BLAST revealed six SNPs and one insert/deletion, which could be used to distinguish between the four species *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. The latter species had as much as a four SNPs difference from the other species, while *P. falciparum* and *P. vivax* had two SNPs. *P. ovale* had an extra T-nucleotide in its sequence, which none of the other species had. We did not have the expertise to investigate if these SNPs and the extra nucleotide had any effect on the reading frame for the protein synthesis of the *cytb* gene, nor had the opportunity to evaluate further if the SNPs are universal in different strains of the species. This sequencing technique has later been applied in other malaria studies [48, 115, 116], without detecting any deviations to the concerned SNPs. In the Paper I project we used both forward and reverse primers to sequence the products, but the sequences had such high quality that with the other publications the sequencing was only performed in one direction, cutting the cost in half.

A weakness of the sequencing technique is the detection of mixed infections. In theory, a mixed infection has the potential to be detected by sequencing due to visible double (or triple) peaks were the SNPs are located, but this depends upon that both variants of the PCR product are presented in relatively equal quantity. In our Norwegian material we had only one double infection with *P. falciparum* and *P.*

malariae. In this case we could not identify any double peaks, probably due to the *P. falciparum* predominance compared to low-level *P. malariae* parasitaemia. Looking at the results from Paper II, where sequencing was performed on positive *cytb* PCR samples, which the species-specific 18S PCR failed to detect, we found cases showing double peaks equivalent to mixed infection of *P. falciparum* and *P. vivax*. Contamination could not be ruled out, but ambiguous samples were sequenced twice for quality assurance.

The sequencing could potentially also identify *P. knowlesi* infections. *P. knowlesi* is genetically very alike *P. vivax*, and for our 220 bp amplicon, only one SNP differentiate these two species. Therefore, this SNP's position needs to be evaluated carefully to not misinterpret a *P. knowlesi* as a *P. vivax* and vice versa. Because the spread of *P. knowlesi* is restricted to specific areas, the species can often be excluded based on information of possible infection site. Applying the sequencing method in the Paper II-project showed distinct peaks in the SNP's positions even with very low-level parasitaemia simplifying the interpretation of the sequences. The method has proven itself to be a powerful tool to be used for species determination when species-specific 18S PCR is not sensitive enough to detect low-level parasitaemia or with ambiguous results by species-specific PCR.

Relevant competing conventional PCR methods targeting the mitochondrial genome, are Cunha *et al.* 2009 applying *P. falciparum*-specific *cox III* target (single-step PCR) showing a 100% sensitivity compared to microscopy [117], Steenkeste *et al.* 2010 targeting genus-specific *cytb* (nested PCR) showed similar sensitivity and specificity compared to nested 18S PCR [118], Isozumi *et al.* 2014 targeting genus-specific *cox III* (conventional PCR) detected 10-50% more cases compared to standard 18S PCR [119], and Echeverry *et al.* 2016 applying genus-specific *cox III* direct single-step PCR detected three more cases out of 21 than nested 18S PCR [120]. The Steenkeste genus-

specific PCR products can be species identified by using restriction fragment length polymorphism-denaturing high performance liquid chromatography (RFLP-dHPLC).

6.1.2 Modification of a species-specific multiplex 18S PCR (Paper I)

A major challenge with species-specific malaria PCR are the genetical similarities in conserved genes which might result in unspecific primer binding and reduced specificity. Applying conserved genes as PCR target is important; the target has to be specific to the relevant species, but also work on all variant of strains from different geographically areas. The different *Plasmodium* species have almost identical sequences for conserved genes with often only sporadic SNPs separating the sequences.

In 2003 a species-specific multiplex 18S PCR was published by Padley *et al.* [66]. This multiplex PCR targeted the four species *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*, by applying a common reverse primer and species-specific forward primers. However, as with all multiplex PCR assays; the effect of competing primers and reactions is always an issue. Therefore, we decided to modify the multiplex PCR by amplifying target sequences in separated reactions instead of one reaction. This increased cost, logistics and working-hours, but reduced the risk of cross-bindings due to similar primer sites. The product size of the four different amplicons was also quite alike making it difficult to interpret the multiplex results on agarose gels. Between *P. vivax* and *P. falciparum* amplicons there was only a 24 bp difference, and a difference of 37 bp between *P. ovale* and *P. malariae*. Nevertheless, running the species-specific primers in separate reactions, we still experienced cross-binding of the *P. vivax* primer to high parasitaemia *P. falciparum* infections. We therefore decided to design a new *P. vivax* primer, which did not bind unspecific to *P. falciparum* even with 20% parasitaemia.

The six hour multiplex PCR program consisted of 43 cycles with extreme long time-steps; 45 sec denaturation, 90 sec annealing, and 5 min elongation time. The long time-steps increases the sensitivity, but also the risk of cross-bindings. We went for another strategy when designing a common PCR program for all the four reactions; 45 cycles with 10 sec denaturation and annealing steps, and 30 sec elongation (two hour running time). In addition we went for a high annealing temperature 65°C versus 60°C in the original program (the species-specific primer T_m values varied from 62°C to 69°C). When performing species-specific malaria PCR a high specificity is a must, more than achieving high sensitivity. However, since each primer pair was in separated reactions, we found a balance between sensitivity and specificity by individually adjusting for primer concentration and additional $MgCl_2$. The end result was a modified species-specific 18S PCR which showed to be as sensitive and specific as the species-specific nested 18S PCR [53]. After being assessed in Paper I, our species-specific PCR has been applied for identifying species prevalence in three other publications [48, 115, 116].

Relevant competing species-specific PCR methods are Rougemont *et al.* 2004 a real-time 18S PCR applying four different species-specific probes [121], Mangold *et al.* 2005 a real-time SYBR 18S PCR using MCA to differentiate between four species [122], Chew *et al.* 2012 a single-step hexaplex conventional 18S PCR targeting all five species [123], Kamau *et al.* 2013 a duplex (*P. falciparum* and *P. vivax*) real-time TaqMan 18S PCR [124], and Reller *et al.* 2013 a multiplex real-time TaqMan PCR targeting 18S (*P. falciparum*), AMA1 (*P. vivax*) and plasmepsin (*P. ovale*, *P. malariae*, and *P. knowlesi*) [125]. All of these methods varied in sensitivity and specificity depending on species or if the infection was single or mixed. Two newer methods are Saito *et al.* 2018, a multiplex single-tube nested PCR targeting mitochondrial *cox III*, where detection is performed by single-stranded tag hybridization chromatographic printed-array strip with a 89% sensitivity and 100% specificity compared to standard nested PCR [126], and Komaki-Yasuda *et al.* 2018, a

nested 18S PCR detecting all five species using a fast PCR enzyme reducing reaction time with results highly consistent with microscopy [127].

6.1.3 Conversion of conventional to real-time PCR (Paper III)

During this study period there was a change of focus on malaria PCR. In the beginning the attention was on presenting a sensitive and specific PCR as a reference method for microscopy and RDT. The performance of routine diagnostics was questionable, and there was a need for a more accurate method which could reveal true answers. However, as PCR methods were more and more included in malaria research studies, it was discovered that PCR detects on average twice as many malaria infections as microscopy [98]. Our genus-specific *cytb* conventional PCR was applied in studies from Tanzania [48] and India (Paper II), where the PCR detected as much as 72% (55/76, N=304) and 71% (162/228, N=1168) submicroscopic malaria respectively. Consequently also our focus went to question what this portion of submicroscopic low-level parasitaemia actually represents, and if the quantitative results could differentiate between clinical cases and asymptomatic malaria.

Therefore to keep up with the ongoing change of focus on malaria PCR, we needed to convert our conventional *cytb* PCR into a real-time q-PCR. The amplicon size of 220 bp is on the higher limit of what is optimal for real-time PCR; usually the amplicon size is 50-150 bp. One of the differences between real-time and conventional PCR is that the Ct values are detected in the exponential phase of the amplification, while the conventional PCR shows the product from the end-point plateau phase. Precise detection in the exponential phase is reliant upon high PCR efficiency with low variation and high precision. Short amplicons have less risk of errors than longer amplicons. However, our amplicon size of 220 bp was still within the limits of conversion to real-time PCR, especially when considering the advantage of sequencing the genus-specific PCR product for species identification.

A real-time PCR program is less adjustable than a conventional PCR program, due to the detection of targets during the exponential phase versus detecting an end-point result. A standard real-time program is mostly used; 40 cycles of 95°C for 15 sec, and 60°C for 1 min, no elongation step. The *Taq* polymerase still has a high activity at 60°C, so the real-time PCR principle is that the whole amplification process occurs at the annealing stage. The primers bind, and then the polymerase immediately starts the elongation step, preferably amplifying a short sequence. For every cycle the amount of amplified target is registered, and a positive sample will separate itself from negatives by an increased fluorescence signal above the automatically generated threshold.

It is recommended that with long amplicons the elongation step at 72°C is included. We followed this recommendation, and the sensitivity was increased by including a 10 sec elongation step. However, we still needed to adjust for an even higher sensitivity ensuring detection of low-level parasitaemia by lowering the annealing temperature with one degree, and apply 45 cycles instead of only 40. To balance the high sensitivity with also achieving high specificity, we reduced the primer concentration (600 nM) and additional $MgCl_2$ (1 mM) compared to the conventional PCR assay. Our conventional *cytb* PCR was successfully converted to both a SYBR and a TaqMan probe real-time assay. Regarding design of a TaqMan probe, the T_m of probes should be 6-8°C above the T_m of the primers, since it is important that the probe has attached to the target sequence before the primers bind and initiate Taq polymerase activity. Our probe had a T_m of 5-6°C above the primers (28 bp, 36% GC).

Relevant competing real-time PCR methods targeting the mitochondrial genome are Farrugia *et al.* 2011 applying TaqMan probe targeting *cytb* [75], and Xu *et al.* 2015 applying SYBR green and also targeting *cytb* [51]. Relevant competing real-time PCR methods using other multi-copy targets are Lefterova *et al.* 2015 applying SYBR targeting 18S [91], and Hofmann *et al.* 2015 applying subtelomeric targets presenting

one SYBR TARE-2 and one TaqMan *var*ATS assay [76]. All of these methods were included in the sensitivity comparison in Paper III.

6.1.4 Quantitative PCR (Paper III)

The obvious difference between conventional and real-time PCR is the technique of detecting the PCR products. With real-time PCR, not only is the detection incorporated into the amplification stage, but real-time registrations also allows for quantitative values. C_t results present the fractional number of cycles (C) required to generate the fluorescence signal exceeding an arbitrary threshold (t), meaning that the fluorescence is not a direct measurement of the number of amplicons of the last cycle, but cumulatively reflects the fluorescence generated during all previous cycles. The threshold is typically automatically set by the software based on the volume of background signals. Since the background varies, the threshold also varies, not only between different PCR assays, but also between different runs using same template and assay. Therefore, comparing C_t values between assays and runs may be misleading.

Although C_t values give a perception of quantity, the values do not portray the level of parasitaemia. High C_t values reflect low levels of target DNA in the template, and vice versa. For precise quantification of parasitaemia in malaria positive samples, a standard curve should be applied, preferably using a customized plasmid for highest accuracy. With a customized plasmid you will be able to calculate the exact number of copies per reaction (rxn). In clinical parasitology, the parasitaemia is often given by percentage (microscopy) or p/µl. However, since PCR detects DNA, and more precisely, the number of target sequences in the template, it can be complicated to directly convert the number of target copies/µl over to p/µl due to the complexity of the malaria parasite lifecycle. Firstly, it depends on the chosen amplification target. Chromosomal targets will have the same copy number per parasite nucleus, but since some of the parasite stages harbour more than one nucleus (Figure 1), it will not be

theoretically correct to present the results as p/µl. A few publications have started to use the definition parasite nucleus/µl. With amplification targets on the mitochondrial genome the definition of parasitaemia is even more complicated, because some of the different parasite stages might not harbour the same number of mitochondrion clusters [74, 110]. Therefore, with malaria q-PCR the most precise way to present parasitaemia will be by the definition copies/rxn. We have in our papers used p/µl when presenting results applying reference material. The reference material consisted exclusive of ring-stage parasites, and this parasite stage harbours only one nucleus and one mitochondrion. But for clinical samples there will most likely be a mix of several parasite stages. For symptomatic *P. falciparum* infections microscopy mainly detects ring-stage parasites, however, there will often also be a low density of the other stages merozoites, trophozoites, schizonts and gametocytes which PCR will detect. For instance, molecular studies have shown that most individuals with asexual parasites have as well submicroscopic gametocyte carriage [128]. In addition, the definition p/µl give an impression that the results reflect the level of live parasites, while PCR results might also present residual DNA (further discussed in sections 6.2.1 and 6.2.4). In section 6.2.3-5 some of the results given in copies/rxn, are as well presented in p/µl for better understanding of quantity. The conversion is calculated by first dividing the number of copies/rxn with 2 due to the use of 2 µl template, than secondly dividing the number with 20 as one mitochondrion have 20 copies of the mitochondrial genome, giving an approximate quantity of p/µl, although not reflecting possible target variance due to different parasite stages or residual DNA.

6.2 Assessment and application of developed PCR assays

6.2.1 Assessment of conventional genus- and species-specific PCR (Paper I)

The novel genus-specific single-step *cytb* PCR, and the modified species-specific 18S PCR, were assessed using a Norwegian clinical material consisting of 135 samples of suspected imported malaria. The patients (N=132) had been diagnosed with malaria in

26 cases by routine microscopy. Since PCR was reported to be more sensitive and specific than microscopy, we decided to evaluate a malaria positive sample as a consensus of all the method results. At least two of the methods were needed to detect the same result to conclude true positives/negatives. The consensus revealed 28 true malaria positives and 107 negatives. The assessment of the developed PCR assays showed that these methods were more sensitive and specific than microscopy. Seven samples (six patients) had discordant results between the different methods. Microscopy overlooked one *P. malariae* and one *P. vivax* primary infections detected by PCR. Typical clinical malaria cases where PCR is more sensitive than microscopy are early (primary or recurrent) infections as well as non-falciparum infections.

P. malariae infections almost always have low parasitaemia, and can therefore be challenging to detect. The low parasitaemia is due to several factors as 72 hour erythrocyte cycle, low number of merozoites per ruptured schizont, on average eight, and preference for older erythrocytes [21, 129]. Although individuals with a *P. malariae* infection might experience symptomatic malaria characteristics as periodic chills and fever patterns, many of the *P. malariae* cases have the potential to go several months or even years without knowing their parasitic status, contributing to sustaining a worldwide distribution of *P. malariae* [5, 21]. However, a portion of the *P. malariae* cases end up in mixed infections [22, 23], and are therefore treated as a consequence of routine diagnostics detecting *P. falciparum*/*P. vivax* infections.

The *P. vivax* infection overlooked by microscopy was positive two days later, and the patient was diagnosed with severe malaria and treated. That PCR detected the primary sample may indicate low parasitaemia due to early infection. PCR is reported to detect malaria on average 3.5 days prior to the ability of microscopy to detect the infection [103]. The species interpretation for this sample was inconclusive by microscopy. Since the patient had been in contact with macaques on a trip to New Guinea, *P. knowlesi* was suspected, but neither *P. falciparum* nor *P. vivax* was excluded as the

causing species. *P. knowlesi* is challenging to diagnose by microscopy. Firstly, low practical experience due to the rarity of *P. knowlesi* has to be expected. Secondly, *P. knowlesi* share the similar receptor affinities as *P. vivax*, and have a preference for reticulocytes. Early trophozoites morphologically resemble those of *P. falciparum*, and *P. knowlesi* have the ability to achieve high parasitaemia due to a 24 hour erythrocyte cycle producing up to 16 merozoites per schizont. Mature trophozoites, schizonts and gametocytes of *P. knowlesi*, are morphological similar to those of *P. malariae* [129, 130]. Before PCR and sequencing discovered the fifth human *Plasmodium* species in studies from Malaysia, *P. knowlesi* infections were believed to have been misinterpreted as *P. malariae* [2]. Within four weeks later the same patient was admitted with recurrent malaria still with inconclusive diagnosis of species by microscopy. As mentioned *P. vivax* and *P. ovale* have the ability to go into a dormant stage in the liver, which can cause recurrent infection (relapse) weeks later if not given hypnozoite-eradicating treatment.

Next discordant results found in Paper I; two PCR confirmed single non-falciparum infections (one *P. ovale* and one *P. vivax*) were over-diagnosed as double infections with *P. falciparum* by microscopy. The two cases were reported to have at least 1% parasitaemia. With high parasitaemia, *P. falciparum* is suspected for a good reason; the species has no age preference for the red blood cells, and produces as much as ~20-30 merozoites per schizont in a 48 hour erythrocyte cycle. In comparison *P. vivax* has a 48 hour erythrocyte cycle producing 12-24 merozoites per schizont, and *P. ovale* has a ~48-50 hour cycle producing 6-14 merozoites, both with preference to reticulocytes [5, 129]. If we look at the real-time SYBR PCR results performed on the Norwegian material (Paper III), we found that on average the C_t value for *P. falciparum* infections was 19 (N=17), for *P. vivax* 20 (N=8), and for *P. ovale* 22 (N=3). This can be expected based on the different species' life-cycle ability to induce high parasitaemia. However, individual factors also affect the level of parasitaemia. The Norwegian material is a collection of travellers without any natural acquired immunity against malaria.

The last discordant result to be discussed is the recurrent (recrudescence) *P. falciparum* case, positive by both microscopy and the *cytb* PCR. Microscopy detected one single ring-stage parasite, while among the PCR methods applied only the *cytb* PCR detected the infection. We argued in Paper I that the reason why the 18S PCR assays did not detect the infection, could be due to the lower copy number of target sequence. Our theory was that since the patient had been diagnosed with *P. falciparum* two weeks prior, a submicroscopic level of gametocytes may have developed in the meantime. Maturation of *P. falciparum* gametocytes takes 7-10 days [131]. The high copy number of the mitochondrial genome in gametocytes, about 16-32 times higher than an 18S target [72-74], could give an explanation to why the 18S PCR assays failed to detect when the *cytb* PCR did not. The theory of *cytb* PCR detection of submicroscopic gametocytaemia is plausible. Mature gametocytes in the blood stream do not cause clinical symptoms in the human host. Neither the immune system, nor antimalarial drugs expect primaquine, have an effect on the gametocytes, hence they can circulate fairly undisturbed in the blood post-treatment, awaiting transmission [41, 100, 102]. Why the immune system does not directly attack the gametocytes is a mystery [132], although a rapid asexual parasite clearance by an effective immune and antimalarial drug response may have some indirect gametocidal effect on the level of mature gametocytes and their longevity [133]. Of course, we cannot rule out that the DNA detected was from parasite residuals rather than live parasites. The residuals can be sequestered in organs like the spleen and liver. It is reported that a macrophage eats up an infected RBC within 30 min [134], and that the phagocytic system has the potential to dispose up to 40-80% of the total RBC mass in a few days [135], but accumulated macrophages/parasite residuals in organs might take longer time to be cleared from the body.

This thorough discussion regarding the discordant results found in Paper I is meant to emphasize the challenges routine microscopy encounters in diagnosing malaria, especially in determining the species. PCR has the ability to give more definitive answers, and should be recognized as a gold standard for detecting malaria. But PCR

methodology has its weaknesses; PCR results can obscure the diagnosing of cause of fever due to the portion of possible asymptomatic malaria detected by PCR. One thing is to interpret PCR results from returned travellers, another is to interpret the scale of parasitaemias found in endemic areas where premunition exists. In general, PCR is a too slow method for point-of-care regarding severe malaria cases. However, PCR can provide useful confirmation of true positives and species determination preventing misdiagnosis and subsequently mistreatment of malaria cases, as well as support education on practical microscopy skills.

6.2.2 Application of conventional genus- and species-specific PCR (Paper II)

Due to its high sensitivity and specificity the conventional genus-specific *cytb* PCR (Paper I) was chosen as gold standard for determination of the malaria prevalence among samples collected in the large multicentre study investigating causes of acute undifferentiated fever in India. In literature from India at the time of study and publication, there were to our knowledge no previous hospital based malaria prevalence studies using PCR from the relevant study areas. Therefore, it was difficult to predict malaria PCR prevalence from the study sites. Studies reporting malaria prevalence by microscopy among hospital fever patients could be potential misleading due to the questionable accuracy of microscopy. However, microscopy prevalence might give a good indication of clinical symptomatic malaria in endemic areas. Blood parasite levels as low as 50 p/µl can induce symptoms depending on immunity [136]. Microscopy has lowest detection limit of 4-20 p/µl under optimal conditions, but in field settings the limit is 50-100 p/µl [137].

Since this was a hospital based study, the malaria prevalence given by PCR is not a measurement of the prevalence in the community/site. The study investigated the cause of acute undifferentiated fever, and PCR detected a high portion of submicroscopic malaria. Therefore the PCR prevalence might give an indication of what level of malaria burden is in the community, and not only the level of hospital

based clinical malaria. The PCR results showed high malaria prevalence in all the seven sites, in total 19% (268/1412), and apart from Oddanchatram (South India) with a prevalence of 6%, the six other sites had as high as 16% to 35% prevalence. The proportion of asymptomatic malaria among these numbers is unknown, but suspected to be fairly high because of the level of undetected malaria by microscopy and RDT. The level of submicroscopic malaria was as high as 71%. Potential bias to the high PCR prevalence might be contamination; however all genus-specific PCR results with discordancy with either microscopy or RDT were re-tested from the DNA extraction step. While potential bias to the distribution of malaria across the sites might be factors as patients having contracted malaria in another area than where they were hospitalised, and a selective inclusion of patients and samples in the favour of clinically suspected malaria. In addition, the differences between the hospitals in bed capacity, and enrolment of patients/samples to the study, may obscure the results. In epidemiological studies it's common to adjust for missing patients/samples in regards to what was expected to be enrolled based on sample size calculations. This was not done in this study, and these factors may be included as potential bias to the presented distribution of malaria across the sites.

In regards to distribution of asymptomatic malaria, the proportion might be high in endemic regions due to premunition. But level of gametocytaemias is reported to be increased in areas of low transmission intensity [99, 105, 138, 139]. A large microscopy based study from India investigating the prevalence of *P. falciparum* gametocytaemia from several site all over India reported highest prevalence in West India [140]. Ratnagiri (West India) had the highest prevalence in our study.

India has historically been considered as a *P. vivax* dominating country, however the last decades there has been a shift in species distribution towards *P. falciparum*, which is suspected being attributed to chloroquine resistance in *P. falciparum* [141, 142]. Our study found 46% single *P. falciparum* versus 38% single *P. vivax* infections. Predominance of *P. falciparum* is expected among hospitalized malaria patients who are more prone to have severe disease, although 71% of the PCR positives were

submicroscopic malaria. The species results from routine microscopy showed a slight tendency to misdiagnose *P.falciparum* infections as *P. vivax*, while diagnosing *P. vivax* had a high specificity. Therefore, it cannot be ruled out that prehistory of predominating *P. vivax* areas affect the judgement of species determination, pointing out the importance of including PCR as quality assurance of malaria routine diagnostics. PCR also identified 11 *P. malariae* infections, which were not detected by microscopy. *P. malariae* infections are almost always in low parasite density, and consequently difficult for less sensitive methods to detect. PCR is a good tool to give awareness of the distribution for also the more neglected species [23, 143].

The performance of the RDT in this study showed an unexpected low sensitivity, detecting only 24% (65/268) of the PCR positive malaria samples, but a high specificity of 99% (1129/1139). Studies comparing microscopy and RDT show discordant results, some find RDT more sensitive detecting malaria than microscopy, others find the opposite result [47, 48, 144-146]. According to Wongsrichanalai *et al.* the sensitivity of RDT can be as low as 5-15 p/µl [137], but the WHO's large evaluation of RDTs found that the different commercial RDTs differ widely in sensitivity and specificity [46]. Our study's RDT ParaHIT-Total device detects 200 *P. falciparum* p/µl with a detection score of 85% [46]. Since the conventional *cytb* PCR detects 0.5 p/µl with 97% (29/30) sensitivity, the low detection rate of the RDT was not so unexpected after all.

The application of stored frozen EDTA blood as material is a possible bias to the low RDT sensitivity. Furthermore, the device has two regions, one detecting *P. falciparum* specific HRP2, and one detecting aldolase antigen of pan-malaria species. The sensitivity/specificity of the two regions is independent of each other. It seems like the 'Pf' region was less sensitive than the 'pan' region, and that the 'pan' was less specific than the 'Pf' region; among the RDT negatives >50% were PCR positive for *P. falciparum*, while among RDT positives seven *P. falciparum* infections were misidentified as non-*falciparum*.

One of the conclusions in Paper II was that patients with low parasitaemia might serve as a reservoir for transmission rather that suffering from clinical malaria, pointing out the need for quantitative PCR malaria results. The 71% detection of submicroscopic malaria was not expected, but emphasises the necessity to further increase our awareness towards low-level parasitaemia and asymptomatic malaria. The portion of asymptomatic malaria was unknown in this study, and obscured the interpretation of the PCR results regarding diagnosing possible alternative causes of fever. We asked ourselves the question if q-PCR malaria results could have contributed to a higher level of knowledge around the boundary between clinical cases and asymptomatic malaria.

6.2.3 Assessment of *cytb* SYBR and TaqMan real-time PCR assays (Paper III)

The conventional *cytb* PCR was successfully converted to real-time applying both SYBR Green and TaqMan probe. The Norwegian clinical material (33 positives and 80 negatives) was used to assess the two real-time assays. Two out of the previous positives from 2013 were negative by the real-time assays; the recrudescent *P. falciparum* sample, and the microscopy overlooked *P. malariae* sample. We argued in Paper III that the lower sensitivities by the real-time assays were due to degradation of long termed frozen DNA, as indicated by conventional *cytb* PCR reanalysis of stored samples (Figure 3 in Paper III). However, the conventional PCR still detected the *P. malariae* sample, which can be due to the difference of five cycles in the PCR programs. The conventional PCR program has 50 contra 45 cycles in the real-time PCR program. Although we cannot rule out that the *cytb* real-time assays have slightly lower sensitivity towards detecting *P. malariae* infections than its conventional counterpart. To resolve this ambiguity a larger collection of *P. malariae* infections would have been needed. Otherwise, regarding the possible degraded DNA, the *cytb* real-time assays seemed to have as high sensitivity and specificity as its conventional counterpart.

Applying SYBR, a longer amplicon can be beneficial. In contrast to probes, which release only a single fluorophore for each amplicon, SYBR will with longer amplicons generate a stronger signal increasing the sensitivity as more dye is incorporated [85]. The *cytb* SYBR assay (amplicon 220 bp) seemed to have an advantage in sensitivity compared to using probe in this study as shown in Paper III. Furthermore, since SYBR binds unspecifically to all double-stranded DNA, it is important to have a high PCR efficiency so that the concentration of specific amplified DNA outshines the unspecific binding of SYBR [83]. The specificity of the SYBR assay in Paper III was 100%. Surprisingly, the probe assay showed one unspecific binding (C_t value 41) when assessing 80 negatives from the Norwegian material. Overall in Paper III the SYBR assay was favoured over using probe.

By applying exclusively *P. falciparum* ring-stage reference material, the *cytb* real-time assays were compared in sensitivity with five other relevant real-time PCR methods. The conventional *cytb* PCR had shown a very high sensitivity, submicroscopic level above 70% [48, 115]. However, the amplicon length (220 bp) is on the upper limit of successful conversion to real-time PCR. To ensure that our optimized *cytb* real-time assays could compete with other malaria real-time PCR methods reported to be ultra-sensitive, and to investigate the effect of platform and amplification target, five relevant assays were included in a sensitivity assessment (Table 3) [51, 75, 76, 91]. The methods were executed carefully according to their publications. To compare the assays, three parameters were chosen as common denominators for all the methods; master mix kit, reaction volume, and volume of template. Commercial master mix kits differ slightly in functionality, and are often suited to the different brands of real-time machines. The master mix kits used in Paper III were suited for real-time machine by Applied. The reaction volume chosen was 25 µl as used for the conventional *cytb* PCR in Paper I and II. Commonly applied reaction volume is 10-50 µl. The volume of template was chosen to be 2 µl, as previously used for the conventional *cytb* PCR. Typically applied volume of template is 1-5 µl.

The five included methods performed applicable even with the three fixed parameters. Only two of the methods had a slighter lower E-value than reported, but this could be due to the limitation of the reference material severing as standard curve. The reference material gave only four dilutions (2000-2 µl). A broader range in the dilution series could have provided more accurate E-values. Applying a customized plasmid is optimal, but expensive. We cannot rule out that the included methods were negatively influenced by applying the fixed parameters. However, a good robust PCR method should be applicable under variable conditions, and adapt to different real-time PCR machines and routine environments. Overall, the methods performed as expected in line with what was reported from their publications. The choice of amplification target appeared to have an impact on sensitivity, with 18S as the weakest target and possibly *var*ATS as the strongest. Other factors such as amplicon length, binding-affinity of the primers, and stability of target sequence, might also have an influence on the sensitivity of the PCR. In a clinical setting all the assays will be sufficient. It is merely the detection of low-level parasitaemia that separates these highly sensitive PCR assays.

A dilemma in Paper III was the concern around setting a cut-off for positivity. Since primers in negative samples have a tendency to bind unspecific during a PCR program with many cycles, it is common to set a cut-off to avoid the risk of presenting false positives. With conventional PCR the end product is presented on a gel, and unspecific products can be revealed by observing incorrect sized bands. A probe will bind as a third oligo and therefore increase specificity greatly, but still unspecific binding and primer-diming can occur, which may be difficult to distinguish from true positives without sequencing the product, especially when the cycle number is high. The sensitivity for SYBR is in principle lower than for probe assays due to the unspecific binding to all double-stranded DNA. However, with SYBR you have the ability to perform MCA which is a powerful tool to reveal false positives. Since the sensitivity comparison using the reference material showed that several of the assays detected target DNA for dilutions 0.1 and 0.05 µl with an average C_t value of around 40, we

decided that setting a C_t cut-off for positivity would restrict the detection of low-level parasitaemia which was our focusing in Paper III. Regarding the Tanzanian material, which consisted of confirmed positive samples, we cannot rule out that amplifications late in the PCR programs are unspecific bindings rather than true positives. For the two probe assays assessed, we regarded all amplifications with curve in the exponential phase as positives, while with the SYBR assay, we thoroughly investigated each melting curve, and some late cycle amplifications were interpreted as false positive amplifications and concluded negative. Therefore, with focus on detecting low-level parasitaemia, we experienced that applying SYBR was much more practical and beneficial than using probe.

The Tanzanian material consisted of 111 *P. falciparum* positive DNA samples confirmed by the conventional *cytb* PCR and the species-specific 18S PCR/sequencing in 2013 [48, 61]. The DNA had been long term stored on -80°C, but moved to -20°C during the present study (Paper III). Possible degradation of the stored DNA was suspected. Analysed by four PCR methods, eight samples of low parasitaemia were no longer reproducibly positive, but only one sample had none of the parallels detected by any of the methods. A positive sample was defined by at least two out of three parallels positive. DNA samples are regarded as a fairly stable material if kept in a freezer, and are often used in methodology assessments [147], although, it has been debated if freezing and thawing of samples can have a degrading effect on the DNA [148]. DNA degradation will not be notable for samples with a high amount of target sequences, but for samples with only a few copies of target, degradation may have an impact, and the samples will be positive in a randomly/non-consistently detection trend depending on the actual amount of target in the template (2 µl). Some samples might also have been more prone to degradation than others due to a varied number of defrosting.

Because of the degraded DNA the Tanzanian material could be applied as a sensitivity assessment of the three real-time PCR methods, our two *cytb* assays and the *var*ATS assay. The results showed a broad range of parasitaemias, and the *var*ATS method was slightly more sensitive in detecting the low-level parasitaemia samples than the two *cytb* PCR methods, most likely due to the different number of target copies. The *var* gene is situated on the chromosomal genome and exists in about 59 copies [76]. For ring-stage parasites, and possibly other asexual stages, the *var*ATS target will be about three times more sensitive than *cytb* target based on copies. Whereas with the gametocytes the mitochondrial genome may be in as much as 160 copies, and then the *cytb* target will be at least twice as sensitive as the *var*ATS target. Because many of the children in the Tanzanian material had been given antimalarial drugs within four weeks prior to admission (62%), gametocytaemia was suspected in the low-level parasitaemia samples. However, since the *var*ATS assay showed highest sensitivity, we speculated in Paper III that many of the low-level parasitaemias were mostly asexual parasite detections, rather than gametocytaemia. Even though the patients were young children, we cannot rule out that malaria with low parasitaemia was due to innate immunity or premunition. For example low parasitaemia was associated with children under one year. Antibodies from mother given trough birth or breastmilk might give young children a protection against developing malaria from infectious bites [149, 150]. Furthermore, malaria superinfections are rarely seen in young and semi-immune children compared to older children and adults, which might be because young children have a high level of asexual erythrocytic parasites protecting them from new malaria infections [30].

6.2.4 Application of *cytb* SYBR PCR focusing on low parasitaemia (Paper III)

To investigate the challenges in applying DNA extracted from different materials, we compared the *cytb* SYBR PCR results using DNA from 37 patients, where blood had been collected and DNA extracted in two ways (EDTA whole blood/Qiagen column and DBS/Chelex). Among the original 74 conventional PCR positives using DNA extracted from EDTA blood, only 59% (44/74) were positive using DNA from DBS

material [48, 61]. This underlined the lower sensitivity applying DBS; none of the very low-level parasitaemias were expected to be detected by using DBS material. Not so surprisingly considering the great difference in blood volume for the two diverse collection methods; 200 µl EDTA blood versus 50 µl DBS blood, and as reported by Strøm *et al.* only six punches (~25 µl blood) are being used in the Chelex extraction method [151]. Looking at the *cytb* SYBR PCR results (N=37), applying DBS is fairly sensitive; DBS detected down to 25 target copies/rxn, corresponding to ~ 0.5 p/µl. In comparison, in the q-PCR assessment (N=74) we defined low parasitaemia as ≤ 1000 copies/rxn, and using EDTA material detected down to 1 copy/rxn, corresponding to 0.025 p/µl. Therefore, applying DBS material is applicable for detecting clinical cases, but will miss cases in asymptomatic malaria research.

Regarding the two C_t trends from the results EDTA versus DBS template, they showed a constant difference of 4-9 cycles (for 31 out of 37 samples the difference was 7-9 cycles), which once again underline the lower sensitivity using DBS, although the consistent trends also show how accurate and robust PCR truly is, even applying stored DNA. Furthermore, looking at the results from the sensitivity assessment of the previously PCR positive Tanzanian field material (N=111), DNA extracted from DBS showed a higher stability with only a few previously positive samples being negative compared to several false negatives by applying whole blood DNA. This can be due to the fact that ion-exchange resin Chelex solution inhibits DNA degradation by chelating metal ions, which otherwise may catalyze breakdown of DNA [152].

Plasma may also be used for malaria real-time PCR. It is shown an association between high parasitaemia and severe malaria applying plasma as material. However, using plasma has its limitations due to general lower sensitivity than applying whole blood [153, 154].

The conventional PCR results from Paper I and II defined PCR as superior to microscopy and RDT in sensitivity and specificity, and the real-time SYBR PCR results in Paper III showed the same. Looking at the *cytb* SYBR PCR results applying the Tanzanian *P. falciparum* positive material (N=74), microscopy showed a low sensitivity of 32% (24/74). Among the microscopy positives the sample with lowest number of copies/rxn by the SYBR PCR had as much as 15300 copies/rxn, corresponding to 380 p/µl. The RDT had higher sensitivity than microscopy detecting 53% (35/66) positives. Among the RDT positives four of the samples were defined in Paper III as low parasitaemia, and the samples with lowest number of copies/rxn had 25 copies, corresponding to 0.6 p/µl. For 31 RDT positives the parasitaemia was ≥ 95 p/µl. Among the RDT negatives (N=31) all of them were regarded as low parasitaemias (≤ 15 p/µl).

These three diagnostic methods, microscopy, RDT and PCR, have very distinct approaches to identifying malaria. Some of the variance in sensitivity and specificity can be explained having knowledge and understanding about what the methods actually detect. Live parasites are directly visualized by microscopy, and therefore this method will mostly identify symptomatic malaria infections of high parasitaemia. Microscopy has the potential to detect gametocytes [140], but this parasite stage is for an unknown reason always in a low density in the blood [132]. RDT detects antigens, which are proteins produced by live parasites, but can be retained in circulation after parasite clearance. Because of this, RDT is reported to potentially be positive as long as up to two months post-treatment. A systematic review of publications on persistent positivity of RDTs concluded that for HPR2 antigen 50% of the tests were still positive by day 15, and 5% positive after 36 days. For the Plasmodium lactate dehydrogenase antigen, 50% were positive by day 2, and 5% after 10 days. In young children 50% of the tests were still positive by day 11, and 5% after 31 days, while, for adults there was a persistent positivity in 50% by day 4, and 5% after 19 days [155]. The review paper did not describe RDTs using aldolase, such as the RDT applied in

Paper II. False RDT positives can also occur, it has been reported that false positives are associated with patients expressing a high level of rheumatoid factor [156].

PCR detects target DNA derived from infected RBCs, free merozoites, and parasite residuals sequestered in immune cells or free in the blood circulation. This means that PCR has the potential to detect low density of malaria DNA produced by premunition, early (primary or recurrent) infections, non-*falciparum* infections, dormant stages, gametocytes, persistent clones, and destroyed parasites. In endemic areas residents can have repeated and low-level asymptomatic parasitaemia in the blood, controlled by acquired immunity, protecting them from further infections (premunition). The portion of low-level parasitaemia detected by real-time PCR caused by premunition is unknown, but immunity and prevalence of asymptomatic malaria in an area is related to level of transmission. Gametocytes can circulate for weeks and months post-treatment, while untreated chronic malaria infections may also persist at low densities for months, even years [24, 101]. There is also evidence from studies that asexual blood stage *P. falciparum* parasites may become temporarily inert or dormant subsequently surviving therapeutic concentrations of anti-malarial drugs, and that these low density dormant forms can be detected by PCR, but not microscopy [136].

It is unknown for how long DNA residuals will circulate in the blood after parasite clearance, but it is probably depends on level of parasitaemia and the effectiveness of drug-treatment and immune response. The spleen has a major function in removing malaria parasites from the circulation. A phenomenon called pitting involves the spleen removing intra-erythrocytic parasite particles without destroying the red blood cell and sending the cells back out in circulation [157]. In an endemic setting collected blood will represent a dynamic possibility of PCR positivity due to the many factors causing parasite DNA to be present in the blood.

6.2.5 Assessment and application of q-PCR (Paper III)

An ideal application of malaria q-PCR would be to be able to differentiate between clinical symptomatic malaria and asymptomatic malaria. However, this is a dream scenario. Unfortunately it is not possible to define a cut-off for several reasons. Examples of clinical low-level parasitaemias are early and non-*falciparum* infections. Circulating gametocytes are reported to not be harmful to the host, but are responsible for the transmission reservoir. There has been developed real-time PCR methods detecting mRNA specifically produced by gametocytes, but mRNA material is expensive and challenging to collect and extract. Special sample tubes with RNase activity are needed to preserve the mRNA efficiently [64, 158]. In general malaria real-time DNA-based PCR technology has not come further (yet) than to show quantitative results; one can only speculate about the reasons behind low-level parasitaemia. It can also be debated if all asymptomatic malaria should be treated in regards to premunition and further spread of drug resistance.

One of our objectives in Paper III was to present an alternative and relevant application of q-PCR data investigating the association between low parasitaemia and clinical and demographic factors (N=74). Since the samples had been collected in order to perform malaria PCR, but for a different purpose and application (Strøm *et al.* investigating challenges in diagnosing paediatric malaria [48]), the collection may not have been optimal for this q-PCR approach; small sample size, limited age interval (children ≤ 5 years), as well as few clinical parameters. Another limitation to the q-PCR assessment was the possible degradation of the stored DNA, which resulted in several of the previously PCR confirmed positive samples were negative by present *cytb* SYBR PCR. Regarding the patient data, we decided that it was correct to also include q-PCR results with value zero in the assessment.

The association between the q-PCR results and included patient data were evaluated applying cross tabulation (univariate) and multiple logistic regression (multivariate)

analysis. We decided to categorize the q-PCR values into the categories ≤ 1000 copies/rxn (N=37, range 0-953, C_t value ≥ 27) defined as low parasitaemia, and > 1000 copies/rxn (N=37, range 3769-1.6x10^7, Ct value ≤ 24) defined as high parasitaemia. This categorization was based on a thorough evaluation of the different frequency data, for example it was a gap of three cycles between low and high parasitaemia distributions. The median value was 2360 copies/rxn (N=74). A 1000 copies/rxn is a considerable low limit corresponding to only 25 p/µl. However, we wanted the low parasitaemia to represent submicroscopic malaria; the range of high parasitaemia started at 3770 copies/rxn, corresponding to ~100 p/µl, which under optimal conditions an experienced microscopist would be able to detect. Except for two samples, all of the high parasitaemia samples had more than 10 000 copies/rxn corresponding to > 250 p/µl. In this study microscopy had the most sensitive detection at C_t value 23 (15300 copies/rxn, 380 p/µl).

Both the univariate and multivariate analysis found a significant correlation between age ≤ 12 months and low parasitaemia. As mentioned, antibodies from mother and in breastmilk may give some protection in the children's first months of life [149, 150], which may be an explanation to this association. Furthermore, in the univariate, but not in the multivariate analysis, low parasitaemia was significantly associated with death in hospital. Severe *P. falciparum* infection kills hundreds of thousands people every year, especially young children. Malaria is regarded as one of the most prominent infectious diseases worldwide, with highest burden in Africa, and high malaria parasitaemia is known to be a risk factor for death. In our material (N=74) 15 of the children died in hospital, and 11 of these were in the category low parasitaemia. The explanation to the significance found in the univariate analysis can be all from over-diagnosed malaria, to missed opportunity of early malaria treatment, or to an unknown effect of a recent malaria episode. Regarding acute undifferentiated febrile illness in malaria endemic areas, the awareness towards malaria being the cause of fever is high, and may obscure the diagnosis of other severe blood stream infections. Low parasitaemia was associated with the children being less frequently given the

diagnosis malaria, and less frequently given antimalarial treatment in hospital. Furthermore, the low density DNA detected by PCR could stem from a recently treated malaria episode; young children are reported to have an increased risk of death due to anaemia in the subsequently months after having been hospitalized with malaria [159, 160]. A malaria infection causes a great loss of red blood cells in the host. In malaria endemic areas iron deficiency is suspected to give young children a natural protection against malaria, but subsequently may cause macrophages to retain their iron, which potentially can have an effect on the behaviour of macrophage-tropic and iron-requiring pathogens such as non-typhoidal *Salmonella*, tuberculosis and HIV-1 [30].

6.2.6 Other applications of malaria q-PCR

The WHO has identified 21 countries that can be able to eliminate malaria by 2020 [10]. To survey malaria control and elimination strategies, applying ultra-sensitive and specific screening tools such as the *cytb* SYBR q-PCR method are of importance. Ways of controlling and eliminating malaria can be using LLITNs, IRS, or mass drug administration (MDA), where all members of a defined population are given anti-malarial treatment. There are different variations of MDA. The treatment can target a small high-risk area such as a household, village, or hot-spot, or target high-risk populations such as pregnant women or infants/children. In mass/focal screening and treatment programs (M/FSAT), all members of a defined population are tested for malaria and those with a positive result, and/or members in close proximity to the positive case, are treated [161]. The effect and long-term results of the different elimination strategies are necessary to follow up by using sensitive and specific methods detecting all levels of parasitaemias. Other settings were PCR-based detection of low-level parasitaemia are of importance, are malaria screening and surveillance of blood-bank and donor-transplantation material [162], persistent *Plasmodium* clones [163], clinical vaccine trials [164], drug resistant trends [165], and multiplicity of *Plasmodium* infections [166].

7. CONCLUSION

PCR is an important tool in malaria diagnostics, epidemiology, and surveillance research. The technology is highly sensitive and specific, and is superior to microscopy and RTD. However, there is still need to further improve the performance of malaria PCR techniques. The different PCR methods show variances in sensitivity and specificity, as well as in practicality and application. The choice of amplification target has an impact on sensitivity. The previously standard 18S target showed low sensitivity compared to new relevant targets. The chromosomal *P. falciparum*-specific *var*ATS target showed a very high sensitivity, as did the mitochondrial genus-specific *cytb* target, which is particularly sensitive towards gametocytes. The high portion of submicroscopic cases found in endemic areas may be asymptomatic carriage of malaria, and can obscure the diagnosis of other severe blood stream infections causing acute undifferentiated febrile illness.

Real-time technology has several advantages over conventional PCR, although it is a more expensive method. Quantitative results give valuable information, and increases awareness and knowledge about the level of parasitaemia, especially submicroscopic malaria. It is not possible to set a cut-off between clinical and asymptomatic cases based on only q-PCR results. By testing several real-time PCR assays, applying both SYBR and TaqMan methodology, it was advantageous to use an intercalating fluorescence dye suitable for MCA rather than labelled hydrolysis probes. MCA helped to distinguish between true low-level malaria positives and unspecific binding or primer-dimers. The highly sensitive, specific and user-friendly *cytb* SYBR q-PCR developed in this study can be a useful tool in epidemiological and surveillance research, as well as for clinical malaria diagnostics. The method is genus-specific, which is an advantage in large screening projects. Among ambiguous samples, and in settings where species-specific PCR fails to detect low-level parasitaemia, confirmation and species identification by sequencing of the genus-specific real-time PCR products can be used as a contingency.

8. FURTHER RECOMMENDATIONS

● Implement PCR as a quality assurance of malaria routine diagnostics.

● Include q-PCR in epidemiological studies.

● Stored DNA should be applied with caution, and DNA extracted from whole blood is preferable. Further investigation of material for the *cytb* SYBR PCR method is direct PCR methodology [167], and pooling of template [168].

● PCR should be applied to give awareness to the neglected *P. malariae* species, which is often missed by microscopy.

● Investigate further if the SNPs found in the *cytb* amplicon are universal in different strains of the species.

● The alternative q-PCR approach presented in this study should be applied in larger, prospective studies employing comprehensive diagnostics for the major relevant differential diagnosis of acute undifferentiated febrile illness, and include tuberculosis, HIV and iron status.

● An unknown portion of the low parasitaemias is probably due to gametocytes, and methods detecting mRNA specifically produced by gametocytes could be relevant to increase knowledge of transmission dynamics.

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I

METHODOLOGY Open Access

A novel, single-amplification PCR targeting mitochondrial genome highly sensitive and specific in diagnosing malaria among returned travellers in Bergen, Norway

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Abstract

Background: Nested PCR is a commonly used technique in diagnosis of malaria owing to its high sensitivity and specificity. However, it is time-consuming, open to considerable risk of contamination and has low cost-efficiency. Using amplification targets presented in multiple copies, such as rRNA 18S, or mitochondrial targets with an even higher copy number, might increase sensitivity.

Methods: The sensitivity and specificity of two newly designed *Plasmodium* genus-specific single-round amplification PCR programmes, based on previously published primers targeting 18S and mitochondrial genome, were compared with a widely used nested 18S PCR. Analyses of dilution series from *Plasmodium falciparum* reference material were performed, as well as retrospective analyses of 135 blood samples, evaluated by routine microscopy, from 132 fever patients with potential imported malaria. Sequencing of the 220 bp mitochondrial PCR products was performed.

Results: At the threshold dilution 0.5 parasites/μl, the sensitivity of the mitochondrial PCR was 97% (29/30 parallels), that of the single-round 18S PCR 93% and the reference nested 18S PCR 87%. All three assays detected as low as 0.05 p/μl, though not consistently. In the patient cohort, malaria was diagnosed in 21% (28/135) samples, defined as positive by at least two methods. Both single-round amplification assays identified all malaria positives diagnosed by nested PCR that had sensitivity of 96% (27/28). The mitochondrial PCR detected one additional sample, also positive by microscopy, and was the only method with 100% sensitivity (28/28). The sensitivity and specificity of the mitochondrial PCR were statistically non-inferior to that of the reference nested PCR. Microscopy missed two infections detected by all PCR assays. Sequencing of the genus-specific mitochondrial PCR products revealed different single nucleotide polymorphisms which allowed species identification of the 28 sequences with following distribution; 20 *P. falciparum*, six *Plasmodium vivax*, one *Plasmodium ovale* and one *Plasmodium malariae*.

Conclusions: In this study, design of PCR programmes with suitable parameters and optimization resulted in simpler and faster single-round amplification assays. Both sensitivity and specificity of the novel mitochondrial PCR was 100% and proved non-inferior to that of the reference nested PCR. Sequencing of genus-specific mitochondrial PCR products could be used for species determination.

Keywords: Malaria, Diagnostics, PCR, Amplification, Sequencing, Mitochondrial DNA, 18S, Sensitivity, Gametocytes, Returned travellers

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Background

More than three billion people world-wide are exposed to malaria, resulting in an estimated 200 million malaria cases and 1.2 million deaths in 2010 [1]. Routine diagnosis is usually done by microscopy and/or rapid diagnostic antigen detection tests, and may be influenced by factors such as the technologists' level of experience, and equipment quality [2,3]. Several molecular techniques have been developed, including polymerase chain reaction (PCR), with the aim of increasing the sensitivity and specificity [4,5].

Malaria PCR was first introduced in 1990 [6], and subsequently has evolved with new amplification methods such as Real-Time PCR and loop-mediated isothermal amplification (LAMP) [7,8]. Both the latter methods have advantages over conventional PCR in turnaround time and in practical use as the detection step is incorporated into the amplification step. Nevertheless, the nested PCR originally described by Snounou *et al.* in 1993 [9] and improved in 1999 [10], remains commonly used and often regarded as a gold standard/reference method. Nested PCR exhibits high sensitivity and specificity due to two amplification steps, but is time-consuming, open to considerable risk of contamination and has low cost-efficiency [11-16].

High sensitivity has also been achieved by using amplification targets existing in multiple copies in the *Plasmodium* genome [17]. A common target is the conserved small subunit ribosomal RNA 18S locus [9,18,19] which in the *Plasmodium falciparum* chromosomal genome exists in five to eight copies depending on the strain [20]. Snounou *et al.* [9] reported a sensitivity of one to 10 parasites per microlitre (p/μl) of blood using nested PCR with 18S as the target gene. Polley *et al.* [21] reported a sensitivity of 5 p/μl introducing a LAMP method using an amplification target on the 6 kb mitochondrial genome. In comparison, a LAMP assay employing primers targeting 18S had a sensitivity limited to approximately 100 copies of the gene for *P. falciparum* [19]. Early ring stage *P. falciparum* parasites typically have one mitochondrial organelle, which contains about 20 copies of the 6 kb genome, while mature gametocytes have as much as four to eight mitochondrial organelles [22,23]. Although one would expect assays using mitochondrial targets to show higher sensitivity given the higher copy number, PCR-based methods targeting 18S are commonly the methods of choice [17,24].

The main aim of this study was to design practical single-round amplification *Plasmodium* genus-specific PCR assays, based on previously described primers targeting the 18S locus [9] and the mitochondrial genome [21], with sensitivity and specificity non-inferior to nested PCR [10]. Comparisons were performed using reference material and samples from a cohort of fever patients with potential imported malaria in Bergen, Norway.

Methods

Patient materials, positive controls and reference sample

The patient material used in this study had been collected between 2006 and 2011 at Haukeland University Hospital, Bergen, Norway. It included 135 whole blood samples from a cohort of 132 fever patients with potential imported primary or recurrent malaria. As part of the routine work-up these samples had been previously analysed for malaria parasites on Giemsa-stained, thin and thick slides by experienced microscopists. The routine microscopy results and clinical information were collected retrospectively from patient files.

External DNA controls extracted from *P. falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae* supplied by the Centre for Tropical Diseases, McGill University (Quebec, Canada) [25] were used in the validation of the genus-/ and species-specific PCR assays in the study. In addition, an external reference sample of *P. falciparum*, US 04 F Nigeria XII (World Health Organization, Geneva, Switzerland), was used to examine the sensitivity of the genus-specific PCR assays. *P. falciparum* was the only cultivated species accessible. The reference material contained exclusively ring stage parasites in a concentration of 200 p/μl. Employing template from the reference material together with extracted DNA from blood of a Norwegian malaria negative volunteer, a combination of two 10-folds dilutions series were prepared giving the following series: 10 p/μl, 5 p/μl, 1 p/μl, 0.5 p/μl, 0.1 p/μl, 0.05 p/μl, and 0.001 p/μl.

From all blood samples DNA was extracted using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Both blood and extracted DNA material was stored at -20°C prior to application.

PCR methods

Three PCR assays, two genus-specific and one species-specific, were assessed in the cohort of patient samples described above. The genus-/ and species-specific nested 18S PCR as described by Singh *et al.* [10] was included as a reference method. In each PCR assay the reaction mixtures contained 2 μl of DNA template and 12.5 μl 2X HotStarTaq Master Mix (Qiagen) at a total volume of 25 μl. The amplifications were performed by using GeneAmp PCR System 9700 (Applied Biosystems, Carlsbad, CA, USA), and the PCR products were analysed by electrophoresis using 2% SeaKem® agarose gel (Lonza, Rockland, ME, USA) with 1X GelRed™ (Biotium, Hayward, CA, USA). Concentrations of primers and additional $MgCl_2$ were optimized for each assay examined as described below.

One genus-specific PCR assay employed primers rPLU 6 and rPLU 5 (Table 1) targeting 18S. The amplification conditions were modified and optimized from the original nested [9] to a single-round amplification assay with cycle parameters as follows: step 1, 95°C for 15 min; step 2, denaturation at 95°C for 10 sec; step 3, annealing at 63°C for 10 sec; step 4, extension at 72°C for 75 sec; steps 2-4 repeated 50 times; and step 5, 72°C for 10 min.

The other genus-specific assay was a new single-round amplification PCR using primers PgMt19 F3 and PgMt19 B3 (Table 1), targeting the mitochondrial genome and previously employed in a LAMP assay [21]. The primers were analysed using Oligo v6 primer analysis software (Molecular Biology Insights, Cascade, CO, USA), and tested with Basic Local Alignment Search Tool (BLAST, National Center for Biotechnology Information, Bethesda, MD, USA) before being considered suitable for use in a conventional PCR assay. Subsequently, the following amplification conditions were designed: step 1, 95°C for 15 min; step 2, denaturation at 95°C for 10 sec; step 3, annealing at 62°C for 10 sec; step 4, extension at 72°C for 15 sec; steps 2-4 repeated 50 times; and step 5, 72°C for 10 min. Reaction mixture for both assays contained 250 nM of each primer, and additionally 4 mM $MgCl_2$ (New England BioLabs, Ipswich, MA, USA).

The *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae* species-specific PCR protocol was employed on all genus-specific PCR positive samples applying primers targeting 18S previously published by Padley *et al.* [26]. As opposed to the original multiplex assay, each sample was analyzed in four separate reaction mixtures to avoid difficulties in species interpretation due to similar product sizes. Only a few single nucleotide polymorphisms (SNPs) distinguish the different forward primers' hybridization sites. In order to avoid non-specific cross-binding between *P. falciparum* and *P. vivax* samples, a new *P. vivax* forward primer (Table 1) was designed using Oligo v6, the sequence alignment editor software BioEdit v7 (Tom Hall, Carlsbad, CA, USA) and BLAST. The amplification conditions were modified and amplification time substantially reduced from six to two hours. The new cycling parameters were: step 1, 95°C for 15 min; step 2, denaturation at 95°C for 10 sec; step 3, annealing at 65°C for 10 sec; step 4, extension at 72°C for 30 sec; steps 2-4 repeated 45 times; and step 5, 72°C for 10 min. The reaction mixtures contained additional $MgCl_2$ which was optimized as follows: 4 mM for *P. ovale*, 2 mM for *P. falciparum* and *P. malariae*, and 1 mM for *P. vivax*. The primer concentrations were 250 nM with an exception of 150 nM for *P. ovale*.

Sequencing

For quality assurance purposes, all PCR products from genus-specific positive samples using primers PgMt19 F3&B3 (Table 1) were sequenced in both directions. The PCR products were purified with ExoSAP-IT® (USB Corporation, Cleveland, OH, USA) according to the manufacturer's instructions, prior to the following cycle conditions being applied using GeneAmp PCR System 9700 (Applied Biosystems): step 1, 96°C for 10 sec; step 2, 62°C for 5 sec; step 3, 60°C for 4 min; steps 1-3 repeated 27 times. Each reaction mixture contained 1 μl BigDye v1.1 (Applied Biosystems), 2 μl sequencing buffer 5X (Applied Biosystems), 0.5 μM primer, and 1 μl template at a total volume of 10 μl. The sequences were obtained with the ABI PRISM® 3730 DNA Analyzer (Applied Biosystems). BioEdit v7 was used prior to sequence identification using BLAST.

Statistical methods

Proportions were compared applying prtest command using Stata 11 (Stata Corp, College Station, Texas, USA), and differences in sensitivity and specificity with 95% confidence intervals (95% CI) were calculated. Non-inferior of the test was considered proved if the upper boundary of the 95% CI of the sensitivity difference was less than a predefined delta of 5%.

Table 1 Primers applied for the amplifications and sequencing examined in this study

Primer[1]	Sequence	Published by
rPLU 6 forward	5′-tta aaa ttg cag tta aaa cg	Snounou *et al.* [9]
rPLU 5 reverse	5′-cct gtt gtt gcc tta aac ttc	Snounou *et al.* [9]
PgMt19 F3 forward	5′-tcg ctt cta acg gtg aac	Polley *et al.* [21]
PgMt19 B3 reverse	5′-aat tga tag tat cag cta tcc ata g	Polley *et al.* [21]
***Plasmodium falciparum* forward**	5′-aac aga cgg gta gtc atg att gag	Padley *et al.* [26]
***Plasmodium vivax* forward**	5′-gag cgt tca aag caa aca ga	This study
***Plasmodium ovale* forward**	5′-ctg ttc ttt gca ttc ctt atg c	Padley *et al.* [26]
***Plasmodium malariae* forward**	5′-cgt taa gaa taa acg cca agc g	Padley *et al.* [26]
Species-specific reverse	5′-gta tct gat cgt ctt cac tcc c	Padley *et al.* [26]

[1] The primers were obtained from Eurogentec (Seraing, Belgium).

Ethics

The study was approved by the Regional Committee for Ethics in Medical Research (No.2011/942).

Results

The sensitivity of detection was examined for the three genus-specific amplification assays; the new single-round amplification mitochondrial PCR employing primers PgMt19 F3&B3 [21], the modified single-round amplification 18S PCR employing primers rPLU 6&5 [9], and nested 18S PCR [10] as the reference method. Based on 30 parallels of the described dilution series, the 0.5 p/μl dilution proved to be the threshold detection level for all three assays (Figure 1). The mitochondrial PCR detected 0.5 p/μl with 97% sensitivity (29/30), while the modified 18S PCR and nested 18S PCR detected 0.5 p/μl with 93% (28/30) and 87% (26/30) sensitivity, respectively. At the threshold detection level of 0.5 p/μl, the sensitivity of the mitochondrial PCR, was statistically non-inferior to that of the reference nested 18S PCR, as the upper boundary of the 95% CI (-23.8% to 3.8%) of the sensitivity difference (10.0%) was less than the predefined delta of 5%. Although, the sensitivity of the modified 18S PCR also was higher than that of the reference nested 18S PCR in absolute numbers, statistical non-inferiority could not be proved in this sample size (sensitivity difference -6.7%, 95% CI -21.8% to 8.4%). All three assays detected positive DNA as low as 0.05 p/μl, though not consistently so by any method.

After having determined sensitivity of detection in standardized material, the 135 patient samples (132 patients) were screened for the presence of malaria by the three genus-specific PCR assays. Among these samples, 21% (28/135) were defined as malaria positive by at least two of the methods among microscopy and the three different genus-specific PCR assays. The new mitochondrial PCR was 100% sensitive detecting all 28 positives. Both the modified 18S PCR and the nested 18S PCR detected 27 of the positives corresponding to a sensitivity of 96%. Routine microscopy detected 26 of the positives corresponding to a sensitivity of 93% (Table 2). The mitochondrial PCR detected one positive sample not detected by any of the two 18S PCR assays, which was also positive by microscopy. Two positive samples not detected by microscopy were detected by all three PCR assays (Table 2). The sensitivity of the mitochondrial PCR was statistically non-inferior to that of the reference 18S PCR, as the upper boundary of the 95% CI (-10.4% to 3.3%) of the sensitivity difference (-3.6%) was less than the predefined delta of 5%. Although the sensitivity of the modified 18S PCR was equal to that of the reference nested 18S PCR in absolute numbers, statistical non-inferiority could not be proved in this patient cohort (sensitivity difference 0%, 95% CI -9.7% to 9.7%).

None of the malaria-negative patients had false positive tests by any of the diagnostic methods, thus all the tests had 100% specificity in this cohort. The specificity of both the mitochondrial PCR and the modified 18S PCR were statistically non-inferior to that of the reference nested 18S PCR (specificity difference 0.0%, 95% CI 0.0 to 0.0%).

The 28 malaria positive samples were all further examined by two species-specific 18S amplification assays; an assay modified from a multiplex PCR [26], and species-specific nested PCR assay [10] as the reference method. The first assay, which was modified from a multiplex into four separate reactions with a new *P. vivax* forward primer, as described above, showed no cross-bindings between the new *P. vivax* primer and *P. falciparum* positive samples (Figure 2). Previously observations showed that the original *P. vivax* forward primer unspecifically cross-bonded with *P. falciparum* positive samples, especially when the parasitaemia was high.

Sequencing of the genus-specific mitochondrial PCR products was performed giving high-quality sequences of full length. When the sequences were run through

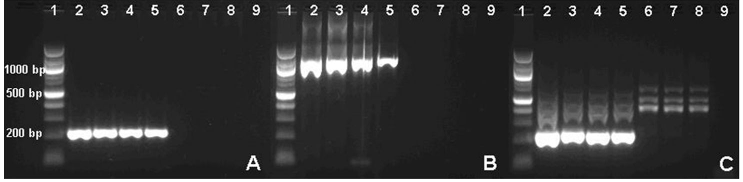

Figure 1 Sensitivity of three different *Plasmodium* genus PCR protocols. A dilution series, run in 30 parallels, was prepared from a 200 p/μl stock of *Plasmodium falciparum*, US 04 F Nigeria XII. The 2% agarose gel picture shows a typical parallel for each assay; new single-round amplification mitochondrial PCR employing primers PgMt19 F3 & B3 [21] (**A**), modified single-round amplification 18S PCR employing primers rPLU 6 & 5 [9] (**B**), and gold standard genus-specific nested 18S PCR [10] (**C**). The product sizes are 220 base pair (bp), 1200 bp, and 250 bp, respectively. Lane 1 = 100 bp DNA Ladder (New England BioLabs), lane 2 = 10 p/μl, 3 = 5 p/μl, 4 = 1 p/μl, 5 = 0.5 p/μl, 6 = 0.1 p/μl, 7 = 0.05 p/μl, 8 = 0.001 p/μl, and 9 = no template. The new mitochondrial PCR had more defined bands than the modified 18S PCR, and especially the nested 18S PCR.

Table 2 Genus-specific results from a cohort of 132 fever patients with potential imported malaria

Samples (n = 135)	New single-round amplification mitochondrial (PCR*PgMt19 F3 & B3* [21])	Modified single-round amplification 18S PCR *(rPLU 6 & 5* [9])	Genus-specific nested 18S PCR [10]	Microscopy
Positive	28	27	27	26
Negative	107	108	108	109

BLAST the results showed two to six SNPs and one insert/deletion which allowed species determination in all the 28 sequences, revealing 20 *P. falciparum*, six *P. vivax*, one *P. ovale*, and one *P. malariae* sequences (Figure 3, Table 3). Both species-specific 18S PCR assays identified 18 *P. falciparum*, six *P. vivax*, one *P. ovale*, and one *P. malariae* single infections, and one double infection of *P. falciparum* and *P. malariae* (Table 3). While the routine microscopy had identified 20 *P. falciparum*, three *P.m vivax*, and one *Plasmodium knowlesi*/inconclusive single infections, and two samples with *P. falciparum* in double infection with *P. ovale* and *P. vivax*, respectively (Table 3).

In seven samples (six patients) the different methods had discordant results (Table 4). Based on the combined results from routine microscopy, all PCR assays and sequencing, these samples were evaluated giving the following interpretations; sequencing as a diagnostic method missed *Plasmodium malariae* in a double infection of *P. falciparum* and *P. malariae*, all 18S PCR assays (genus-/and species-specific) missed one *P. falciparum* positive sample detected by both the new mitochondrial PCR and microscopy, and routine microscopy missed one *P. malariae* and one *P. vivax* single infection as well as *P. malariae* in a double infection of *P. falciparum* and *P. malariae*. Microscopy incorrectly evaluated two single infections as double infections with *P. falciparum*, and incorrectly evaluated a *P. vivax* as *P. knowlesi* infection (Table 4).

Figure 2 No cross-binding reactions applying new *Plasmodium vivax* forward primer in modified species-specific 18S PCR [26]. The original *Plasmodium vivax* primer from a multiplex PCR [26] cross-bonded with *Plasmodium falciparum* when applied in the modified species-specific 18S PCR [26]. The new *Plasmodium vivax* (Pv) primer was cross tested against different *Plasmodium falciparum* (Pf) patient samples with diverse levels of high parasitaemia. The results showed on a 2% agarose gel. Lane 1 = 100 bp DNA Ladder, lanes 2-6 = Five different positive Pv patient samples, lane 7 = 2% Pf, lane 8 = 2-3% Pf, lane 9 = 7% Pf, lane 10 = 7-10% Pf, lane 11 = 10-15% Pf, lane 12 = 20% Pf, and lane 13 = No template. The product size of Pv is 241 bp.

Discussion

The two *Plasmodium* genus-specific single-round amplification PCR assays presented in this study were as follows: a new mitochondrial PCR employing primers PgMt19 F3&B3 [21], and a modified 18S PCR employing primers rPLU 6&5 [9]. By designing single-round amplification assays, the cost-efficiency, turnaround time, contamination risk, and the possibility of technical errors are considerably reduced compared with the nested PCR reference method [10]. Previously published 18S primers, rPLU3&4 [10], were also considered for single-round amplification because of short product size (250 bp *versus* 1200 bp), but discarded due to high annealing temperatures, 71 and 75°C (contra 63 and 64°C for rPLU6&5). Snounou and Singh [9,10] reported that the 18S primers showed reduced sensitivity in single-round PCR amplifications, however this study shows that single-round amplifications have the same sensitivity and specificity as nested PCR given suitable parameters and optimisation. The new PCR programs were designed with a high number of cycles and high concentration of additional magnesium chloride, so that the sensitivity of nested PCR was maintained. Furthermore, due to the quick cycles in the designed programs and the high annealing temperatures, also the specificity of nested PCR was maintained despite the high number of cycles. The results from the dilution series showed that both the single-round amplification assays detected a higher number of parallels with positive DNA at the threshold dilution level of 0.5 p/μl compared to the nested PCR reference method. In the patient material the two single-round assays correctly identified all the positives and negatives found by the nested PCR, with the exception of one sample positive only by mitochondrial PCR and microscopy. Statistically the sensitivity of the mitochondrial PCR was non-inferior to that of the reference nested 18S PCR in this patient cohort as well as in the dilution series (at the threshold 0.5p/ μl dilution). Although the sensitivity of the modified 18S PCR could not be proven to be statistically non-inferior to nested PCR in this limited sample size, the test was

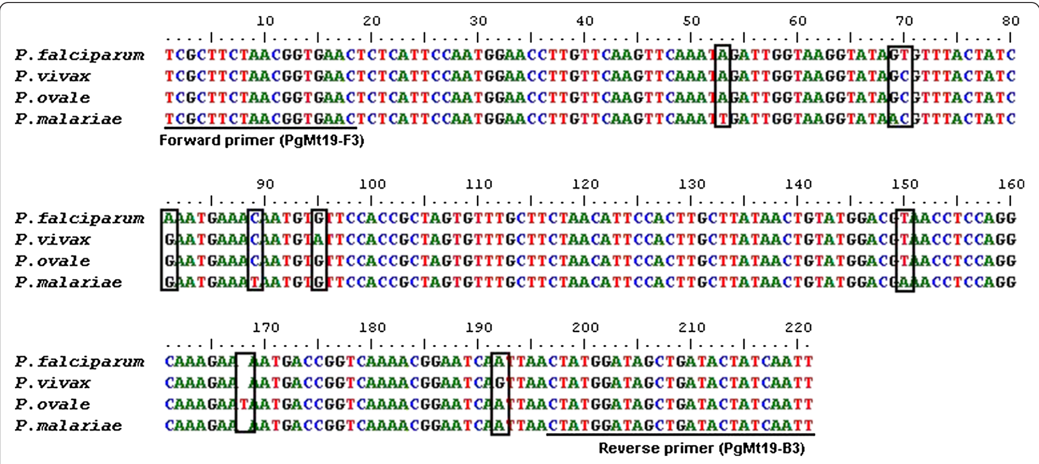

Figure 3 Sequencing of *Plasmodium* genus-specific mitochondrial PCR products reveals polymorphisms consequently identifying malaria species. Sequencing of the genus-specific mitochondrial PCR products, employing primers PgMt19 F3&B3 [21], gave high-quality sequences of full length. Run through BLAST the results showed that the four species were distinguished by two to six single nucleotide polymorphisms and one insert/deletion consequently allowing sequencing to be used as an alternative species-specific determination method in our cohort.

equal to or better than the nested PCR in absolute numbers. The specificities of both single-round amplification assays were statistically non-inferior to that of the nested PCR.

In *Plasmodium* parasites the mitochondrial genome is presented in a higher copy number than 18S, especially in gametocytes. While the copy number of 18S locus varies depending on strains, and not on the stages of the parasite since it is located on the chromosomal genome

Table 3 Species-specific results among malaria genus positive samples

Samples (n = 28)	Sequencing *PgMt19 F3 & B3* [21]	Modified species-specific 18S PCR [26]	Species-specific nested 18S PCR [10]	Microscopy
18	Pf	Pf	Pf	Pf
1	Pf	Pf + Pm	Pf + Pm	Pf
1	Pf	Negative	Negative	Pf
3	Pv	Pv	Pv	Pv
1	Pv	Pv	Pv	Negative
1	Pv	Pv	Pv	Pf + Pv
1	Pv	Pv[1]	Pv[1]	Pk[2]
1	Po	Po	Po	Pf + Po
1	Pm	Pm	Pm	Negative

Abbreviations: Pf, *Plasmodium falciparum*; Pv, *Plasmodium vivax*; Po, *Plasmodium ovale*; Pm, *Plasmodium malariae*; Pk, *Plasmodium knowlesi*.
[1] *Plasmodium knowlesi* primers were not included in the assay.
[2] Evaluated as inconclusive species by microscopy, but with emphasis on possible *Plasmodium knowlesi* infection.

[20], the multiple mitochondrial genome is located in the mitochondrial organelles which can varies in number through the different parasite's stages [22,23]. The results from the dilution series, containing ring stage parasites exclusively, showed the same threshold detection level for all three genus-specific PCR assays, though the mitochondrial PCR had highest sensitivity on this detection level. In ring stage parasites the copy number of the mitochondrial genome is approximately three to four times higher than the copy number of the 18S locus. Compared to 18S PCR, the mitochondrial PCR detected one more sample among the patient material, and notably none of the four 18S PCR assays (two genus-/ and two species-specific) detected this sample. The patient had recrudescence following primary *P. falciparum* infection two weeks earlier. Maturation of *P. falciparum* gametocytes takes eight to 10 days [27,28]. Although microscopy detected one single ring stage parasite, the difference in sensitivity in this sample is possibly due to submicroscopic gametocytaemia; the copy number of the mitochondrial genome in a gametocyte is approximately 10 to 32 times higher than the copy number of the 18S locus.

Due to short product size, 220 bp, the amplification time with the new mitochondrial PCR programme takes one-and-a-half hours less than the modified 18S PCR programme, and three hours less than nested 18S PCR [10]. Another advantage of the short product size, was that the mitochondrial PCR products could be easily full-length sequenced. As an alternative to species-specific PCR, the SNPs found in the sequences allowed

Table 4 Seven samples (six patients) with discordant results between the different methods

Patient	Microscopy	Parasitaemia by microscopy	18S PCR assays	Mitochondrial PCR/sequencing	Primary/ recurrent infection	Place of infection	Interpretation
P 1	Negative	0[1]	Pm	Pm	Primary	Ghana	Pm overlooked by microscopy
P 2	Negative[2]	0[1]	Pv	Pv	Primary	New Guinea	Pv overlooked by microscopy
P 2	Pf, Pv or Pk	1%	Pv	Pv	Recurrent (Relapse)	New Guinea	Inconclusive/ incorrect diagnosis by microscopy
P 3	Pf + Po	<1%	Po	Po	Recurrent (Relapse)	Uganda	Over-diagnosed mixed infection by microscopy
P 4	Pf	<1%	Pf + Pm	Pf	Primary	Liberia	Under-diagnosed mixed infection by microscopy/sequencing
P 5	Pf + Pv	1%	Pv	Pv	Primary[3]	SEA or CA	Over-diagnosed mixed infection by microscopy
P 6	Pf [4]	<1%	Negative	Pf	Recurrent (Recrudescence)	Guinea	Un-detected low Pf parasitaemia by 18S PCR

Abbreviations: Pf, *Plasmodium falciparum*; Pv, *Plasmodium vivax*; Po, *Plasmodium ovale*; Pm, *Plasmodium malariae*; Pk, *Plasmodium knowlesi*; SEA, South east Asia; CA, Central America.
[1] Not detected any parasites.
[2] Positive microscopy two days later (1% parasitaemia) and then the patient was diagnosed with severe malaria and treatment initiated.
[3] Not given hypnozoite-eradicating treatment and re-admitted four weeks later with relapse of *Plasmodium vivax*.
[4] Only one parasite detected.

for specific species determination (Figure 3). However the validity of the SNPs warrants further investigation to evaluate if the SNPs are universal in different strains of the species. All of the 28 sequences correctly identified species compared to the species confirmed by either the species-specific 18S PCR assays or microscopy. However, sequencing only identified *P. falciparum* species in a double infection with *P. malariae*, which was detected by both the species-specific 18S PCR assays. This can be explained by *P. falciparum* predominance due to its ability to induce high parasitaemia. Despite missing the less numerous species in mixed infections, sequencing of the mitochondrial PCR products can be useful in species diagnosis. Compared to a species-specific multiplex PCR, the sequencing method avoids unwanted cross-bindings due to similar primers' hybridization sites, difficulties in interpreting results due to similar product sizes, and lower sensitivity due to competing primers. Due to high quality sequences this method only requires one reaction for every positive sample to perform species determination, compared to four/five in non-multiplex species-specific PCR assays. Sequencing of positive mitochondrial PCR products also allows for identification of species where species-specific 18S PCR assays may fail to detect low parasitaemia, as shown in the sample with recrudescence in this patient cohort. The simple and rapid mitochondrial PCR assay, with the advantages of high sensitivity and that species-specific sequencing is possible in positive samples, might therefore have a value in screening purposes, especially in large scale epidemiologic and surveillance studies as well as in diagnostics.

Conclusions

In this study two simple and rapid single-round amplification assays for detection of malaria were described. The novel mitochondrial PCR was the only method with 100% sensitivity in this patient cohort, and both its sensitivity and specificity was statistically non-inferior to that of the reference 18S nested PCR [10]. The method may be of particular value in samples of low parasitaemia/ gametocytaemia and in large-scale studies. Sequencing of the genus-specific mitochondrial PCR products or developing the assay to real-time PCR could be an alternative to species-specific PCR in species determination.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CGH designed, developed and optimized the PCR programs, performed the analyses, and wrote the first draft of the manuscript. SCM contributed in the development of the PCR methods. KM contributed in providing the clinical data, and in the clinical interpretations. NL initiated the collection of the patient material. BB performed the statistical analysis. KH was involved in including the reference material. All authors contributed to planning of the study, interpretation of the results, revision of the manuscript, and approved the final version.

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II

RESEARCH ARTICLE

A High Malaria Prevalence Identified by PCR among Patients with Acute Undifferentiated Fever in India

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Abstract

Background

Approximately one million malaria cases were reported in India in 2015, based on microscopy. This study aims to assess the malaria prevalence among hospitalised fever patients in India identified by PCR, and to evaluate the performance of routine diagnostic methods.

Methods

During June 2011-December 2012, patients admitted with acute undifferentiated fever to seven secondary level community hospitals in Assam (Tezpur), Bihar (Raxaul), Chhattisgarh (Mungeli), Maharashtra (Ratnagiri), Andhra Pradesh (Anantapur) and Tamil Nadu (Oddanchatram and Ambur) were included. The malaria prevalence was assessed by polymerase chain reaction (PCR), routine microscopy, and a rapid diagnostic test (RDT) with PCR as a reference method.

Results

The malaria prevalence by PCR was 19% (268/1412) ranging from 6% (Oddanchatram, South India) to 35% (Ratnagiri, West India). Among malaria positive patients *P. falciparum* single infection was detected in 46%, while 38% had *P. vivax*, 11% mixed infections with *P. falciparum* and *P. vivax*, and 5% *P. malariae*. Compared to PCR, microscopy had sensitivity of 29% and specificity of 98%, while the RDT had sensitivity of 24% and specificity of 99%.

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

Conclusions

High malaria prevalence was identified by PCR in this cohort. Routine diagnostic methods had low sensitivity compared to PCR. The results suggest that malaria is underdiagnosed in rural India. However, low parasitaemia controlled by immunity may constitute a proportion of PCR positive cases, which calls for awareness of the fact that other pathogens could be responsible for the febrile disease in submicroscopic malaria.

Introduction

Malaria is one of the leading infectious causes of morbidity and death. The World Health Organization (WHO) estimated 214 million malaria cases and 438,000 malaria deaths globally in 2015 [1]. In India 1,102,205 malaria cases and 561 deaths were reported in 2015 based on microscopy and rapid diagnostic tests (RDT) [1]. Less than two malaria cases per thousand individuals per year (Annual Parasite Index (API)) is reported in most parts of India, lowest in South-, North- and West, and highest in Central-, East- and North-East [2]. However, data from malaria surveillance are uncertain since a majority of the population live in poverty in rural areas and has limited access to diagnostic services, and recent studies have shown that malaria prevalence and case fatality is underreported by surveillance systems [2, 3].

Routine malaria diagnostic methods have several limitations compared to polymerase chain reaction (PCR). Accurate microscopy requires skilled personnel and high quality technical equipment [4], and commercially available RDTs differ widely in sensitivity and specificity [5]. Both methods fail to detect low-level parasitaemia, and are inferior in optimal species identification [4, 5]. In principle, PCR can detect parasitaemia as low as one gene copy, and allows differentiation of all five *Plasmodium* species [6, 7]. In point of care diagnosis, PCR cannot replace the traditional diagnostic methods, as the technique is relatively costly, resource-demanding and time-consuming. However, using PCR as a reference method provides more accurate information about prevalence and species distribution [8, 9]. Furthermore, within the variety of PCR protocols, the choice of gene target influences the sensitivity of PCR. Targeting the mitochondrial genome yield higher sensitivity than the common 18S gene due to a higher number of gene copies per parasite [10, 11].

The primary objective of this study was to assess the proportion of malaria infections among patients with acute undifferentiated fever, presenting to secondary level community hospitals at multiple sites across India, using a highly sensitive and specific PCR targeting mitochondrial DNA as a reference method [11]. The secondary objective was to evaluate the sensitivity, specificity and species-specificity of routine microscopy and RDT compared to PCR.

Materials and Methods

Study design and population

The present work was part of a multi-centre, observational, cross sectional study, investigating the proportion of acute undifferentiated fever attributable to different infections. From June 2011 to December 2012, 1564 patients admitted to seven hospitals in rural or semi-urban areas in six different states of India, were enrolled prospectively and consecutively. Inclusion criteria was inpatients aged ≥5 years with temperature ≥38°C for 2–14 days prior to admission, with no localized causes of fever. Patients received health care according to routines at the participating hospitals, and no additional interventions were performed as part of the study.

Study sites

The study sites were located in Tezpur (Assam, North East India), Raxaul (Bihar, East India), Mungeli (Chhattisgarh, Central India), Ratnagiri (Maharashtra, West India), Anantapur (Andhra Pradesh, South India), Oddanchatram and Ambur (Tamil Nadu, South India). Fig 1 shows the locations of the study sites. These are secondary level community hospitals with 100 to 500 beds. The rainy seasons vary between the sites, as outlined in the result section presenting seasonal variations. The Benjamin M Pulimood Laboratories for Infection and Inflammation, Department of Medicine Unit 1 and Infectious Diseases, Christian Medical College, Vellore, India served as study coordinating centre and reference laboratory.

Study procedures

Blood (0.5–1 ml and 3–5 ml from paediatric and adult patients, respectively) was drawn into EDTA tubes and stored at -20°C at the study sites before they were bulk shipped on dry ice to the coordinating centre, where molecular and antigen testing for malaria was performed. Test analyses were performed blinded from other test results. Peripheral blood smears were prepared and examined for malaria parasites at the study sites as part of the routine microscopy work-up according to the hospitals procedures. Technicians were trained and involved in routine smear examination at the respective sites, and were retrained at the reference laboratory during the start of the study. Quality control slides were sent to the sites and reported back to the reference laboratory in a satisfactory manner.

DNA for PCR analysis was extracted from 200 μl whole blood using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and stored at -20°C prior to application. All samples were screened for presence of *Plasmodium* DNA by a genus-specific pan-malaria PCR assay targeting the mitochondrial genome. The assay was done as previously described [11], but with a primer concentration of 1μM. In case of discordant results between PCR, RDT or routine microscopy, the samples were retested by the

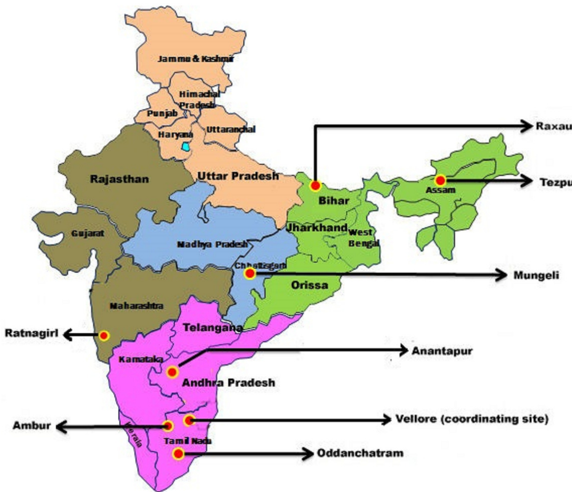

Fig 1. Location of seven community hospitals in six states of India participating in the study.

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genus-specific PCR from the extraction step as a quality control. A malaria infection was confirmed if two or all of the three repeat PCRs were positive.

All genus-specific PCR positive samples were further analysed by a species-specific PCR targeting the 18S of *P. falciparum*, *P. vivax* and *P. malaria*. A modified version of the original protocol by Padley et al. [12] was used as described previously [11]. Samples negative by the species-specific PCR, were repeated by the genus-specific PCR and the PCR products were thereby sequenced for species identification as described previously [11]. The sequencing could potentially identify all five species including *P. ovale* and *P. knowlesi*.

Amplifications were done on AB Applied Biosystem veriti 96 well Thermal cycler (Applied Biosystems, Carlsbad, CA, USA), and products detected by electrophoresis on a 2% SeaKem agarose gel (Lonza, Rocland, ME, USA) stained with ethidium bromide.

The EDTA-blood samples, stored at -20°C, were also tested with the RDT ParaHIT-Total Ver. 1.0 Device 55IC204-10 (Span Diagnostics Ltd, Surat, India) at the reference laboratory following manufacturer's instructions. It detects *P. falciparum* specific Histidine-Rich-Protein II and aldolase antigen of pan-malaria species (*P. falciparum*, *P. malariae*, *P. vivax*, *and P. ovale*). The test card has two regions, 'Pf' and 'Pan'. A red band in the 'Pf' region alone indicates that the sample is reactive for *P. falciparum* (usually in case of low parasitaemia). Red bands in both 'Pf' and 'Pan' region indicate either single infection by *P. falciparum* or a mixed infection of *P. falciparum* with *P. vivax*, *P. ovale* or *P. malariae*. Appearance of a red band in the 'Pan' region alone indicates that the sample is reactive for infection by a malaria species other than *P. falciparum*. The RDT kits were quality checked using known positive and negative controls.

Statistical analysis

Confidence intervals for tests' sensitivities, specificities, positive and negative predictive values were calculated using the cii command in Stata 14 (StataCorp, College Station, TX, USA), and presented as exact 95% confidence intervals.

Ethical approval

The study was approved by the Institutional Research Board at Christian Medical College, Vellore, Tamil Nadu (No. 7242 dated 11th of August 2010) and by the Regional Ethics Committee of Norway (2010/2271-5). Written, informed consent was obtained from the patients.

Results

Among patients enrolled in the acute fever study (N = 1564), samples from 1412 patients were available for malaria PCR testing, and these patients were included in the analyses. Fig 2 shows a flowchart of the investigations performed based on results and samples available. Among the 1412 patients, 815 (58%) were men, and 584 (41%) women. Mean (median) age was 34 (32) years, and 177 (13%) patients were $\leq$ 14 years old.

Prevalence of malaria by PCR, all species

Genus-specific PCR identified a malaria prevalence of 19% (268/1412) as shown in Table 1. The prevalence varied from 6% (19/318) in Oddanchatram in South India to 35% (85/245) in Ratnagiri in West India.

Seasonal variations in malaria prevalence is shown in Fig 3. Increased number of PCR positive cases were seen during or short after the rainy seasons at most of the sites.

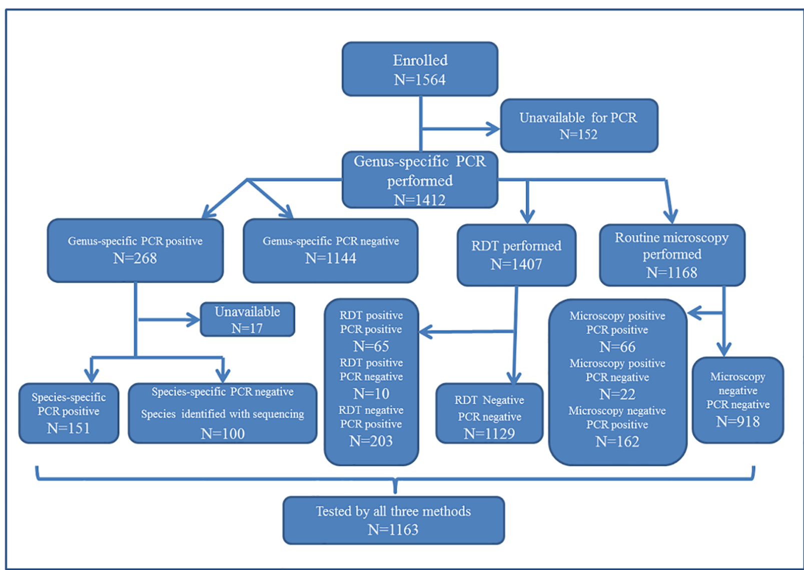

Fig 2. Flowchart showing investigations performed based on results and samples available.

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Species distribution by PCR

Species determination by species-specific PCR or sequencing was performed on samples from 251 patients (Fig 2). Overall, *P. falciparum* single infection was detected in 46%, while 38% had *P. vivax* single infections, and 11% had mixed infections with *P. falciparum* and *P. vivax*. Species distribution at the sites is presented in Table 2. *P. falciparum* dominated in Mungeli (Central India), Raxaul (East India) and Anantapur (South India). In Ambur (South India), *P. vivax* accounted for the majority. In Ratnagiri (West India), Oddanchatram (South India) and Tezpur (North-East India) the difference in *P. vivax* and *P. falciparum* distribution was less prominent. Mixed infections with *P. falciparum* and *P. vivax* were detected in a high number of

Table 1. Malaria prevalence by PCR among patients admitted with acute undifferentiated fever to seven community hospitals across India, (N = 1412).

Sites	N*	Malaria PCR positives	
		N	(%)
Total	**1412**	268	19
Ratnagiri (West India)	**245**	85	35
Raxaul (East India)	**106**	30	28
Mungeli (Central India)	**52**	13	25
Anantapur (South India)	**124**	28	23
Tezpur (North-East India)	**293**	49	17
Ambur (South India)	**274**	44	16
Oddanchatram (South India)	**318**	19	6

*Total number of samples examined with malaria genus-specific PCR.

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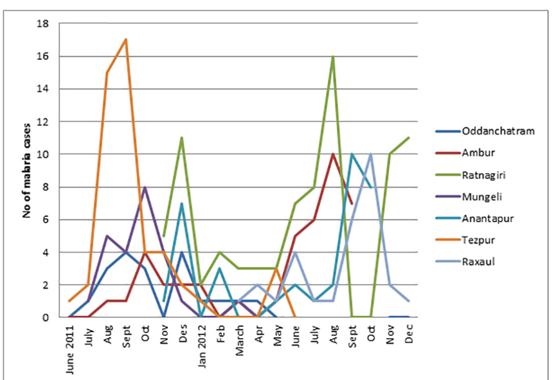

Fig 3. Seasonal variation among malaria PCR positive cases. The sites have the following rainy seasons: Oddanchatram; June to December, with peak monsoon from October to December. Ambur; June to December, with a peak monsoon from October to December. Ratnagiri; June to November. Mungeli; June to September, or early October. Anantapur; Dry climate, but rainy season from May to October, with its peak in September. Tezpur; April to September, with peak monsoon in July and August. Raxaul; July to September, with peak monsoon in August.

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patients in Anantapur, Ambur, and Tezpur. *P. malariae* was found at all sites except Mungeli and Raxaul. *P. ovale* was not detected in any of the patients.

Performance of routine microscopy and RDT compared to PCR

Among the 1412 samples analysed by PCR, 1168 samples were analysed with routine microscopy and 1407 with RDT (Fig 2).

The total malaria prevalence detected by microscopy and confirmed positive by PCR was 6% (66/1168); at Ratnagiri 12% (29/267), Raxaul 2% (2/100), Mungeli 28% (2/5), Anantapur 8% (9/113), Tezpur 5% (14/276), Ambur 6% (7/117) and Oddanchatram 1% (3/318). Among these malaria patients, 92% (61/66, missing values 1/66) were adults ($\geq$ 14 years old), and among PCR positive patients with negative microscopy, 85% (137/162, missing values 10/162)

Table 2. Malaria species distribution by PCR, (N = 251).

Sites	N*	P.f		P.v		P.f+P.v		P.m		P.f+P.m		P.v+P.m	
		N	(%)	N	(%)	N	(%)	N	(%)	N	(%)	N	(%)
Total	**251**	116	(46)	96	(38)	27	(11)	9	(4)	2	(1)	1	(0.4)
Mungeli	**13**	11	(85)	1	(8)	1	(8)	0		0		0	
Raxaul	**19**	15	(79)	4	(21)	0		0		0		0	
Anantapur	**27**	14	(52)	7	(26)	5	(19)	1	(4)	0		0	
Tezpur	**44**	20	(46)	15	(34)	6	(14)	3	(7)	0		0	
Ratnagiri	**85**	37	(44)	39	(46)	7	(8)	0		1	(1)	1	(1)
Oddanchatram	**19**	8	(42)	6	(32)	1	(5)	3	(16)	1	(5)	0	
Ambur	**44**	11	(25)	24	(55)	7	(16)	2	(5)	0		0	

Abbreviations: P.f, *Plasmodium falciparum*; P.v, *Plasmodium vivax*; P.m, *Plasmodium malariae*.

*Total number of malaria genus-specific PCR positive samples available for species determination by either species-specific PCR or sequencing.

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Table 3. Performance of routine microscopy and RDT compared to PCR.

	Routine microscopy	RDT
	(N = 1168)	(N = 1407)
	Percentage, 95% CI, n/total	Percentage, 95% CI, n/total
Sensitivity	29% (23%-35%), 66/228	24% (19%-30%), 65/268
Specificity	98% (96%-99%), 918/940	99% (98%-100%), 1129/1139
Positive predictive value	75% (65%-84%), 66/88	87% (77%-93%), 65/75
Negative predictive value	85% (83%-87%), 918/1080	85% (83%-87%), 1129/1332

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were adults. Compared to PCR the sensitivity of routine microscopy was 29% (Table 3). Among false positive blood slides, 18/22 was from one site, indicating high specificity of routine microscopy in six of the seven hospitals.

Compared to PCR the sensitivity of the RDT was as low as 24%, while only 1% (10/1407) was false positive (Table 3). The total prevalence of malaria detected by RDT confirmed positive by PCR was 5% (65/1407); at Ratnagiri 7% (17/242), Raxaul 3% (3/106), Mungeli 12% (6/52), Anantapur 7% (9/124), Tezpur 5% (16/292), Ambur 4% (11/274) and Oddanchatram 1% (3/317).

The species concordance between PCR and routine microscopy is presented in Table 4. Microscopy was more likely to correctly identify *P. vivax* than *P. falciparum*. None of the *P. malariae* infections were detected by microscopy.

Table 5 presents species identification by RDT compared to PCR. Only one mixed infection was detected and RDT misidentified seven *P. falciparum* malaria infections as non-*falciparum*. None of the *P. malariae* single infections were detected by RDT.

S1 Table shows a comparison of the concordance among the three methods (N = 1163). Negative RDT among patients positive both by routine microscopy and PCR, was found in 38% (25/66), while negative microscopy among those positive both by RDT and PCR was found in 31% (18/59). Only 5% (2/43) were negative by PCR among those positive both by microscopy and RDT.

Table 4. Malaria species concordance between PCR and routine microscopy results, (N = 1168).

			Routine microscopy					
			P.f	P.v	P.f+P.v	P.o	Positive[1]	Neg
			(N = 26)	(N = 50)	(N = 5)	(N = 1)	(N = 6)	(N = 1080)
PCR	**P.f**	**(N = 100)**	14	8	1	1	1	75
	P.v	**(N = 81)**	3	21	3	0	2	52
	P.f+P.v	**(N = 20)**	1	4	1	0	1	13
	P.m	**(N = 8)**	0	0	0	0	0	8
	P.f+P.m	**(N = 2)**	0	0	0	0	0	2
	P.v+P.m	**(N = 1)**	0	1	0	0	0	0
	Positive[1]	**(N = 16)**	2	1	0	0	1	12
	Negative	**(N = 940)**	6	15	0	0	1	918

Abbreviations: P.f, *Plasmodium falciparum*; P.v, *Plasmodium vivax*; P.o, *Plasmodium ovale*; P.m, *Plasmodium malariae*.

Data shown for all patients examined by both PCR and microscopy.

[1]Species identification not available.

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Table 5. Malaria species concordance between PCR and RDT results, (N = 1407).

			RDT				
			P.f	Pan	P.f+Pan	Positive[2]	Neg
			(N = 9)	(N = 37)	(N = 27)	(N = 2)	(N = 1332)
PCR	P.f	(N = 116)	6	3	19	0	88
	P.v	(N = 96)	0	23	2	0	71
	P.f+P.v	(N = 27)	0	4	1	0	22
	P.m	(N = 9)	0	0	0	0	9
	P.f+P.m	(N = 2)	0	0	0	0	2
	P.v+P.m	(N = 1)	0	1	0	0	0
	Positive[1]	(N = 17)	1	3	2	0	11
	Negative	(N = 1144)	2	3	3	2	1129

Abbreviations: P.f, *Plasmodium falciparum*; P.v, *Plasmodium vivax*; P.m, *Plasmodium malariae*.

Data shown for all patients examined with both PCR and RDT.

[1]Species identification not available.

[2]Species identification not recorded.

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Discussion

In this study, among hospitalized patients with acute undifferentiated fever in six states of India, we report a malaria prevalence as high as 19% using a sensitive PCR [11], compared to a prevalence of 6% identified by routine microscopy confirmed by positive PCR.

There are to our knowledge no previous hospital based malaria prevalence studies using PCR from the areas in the present study, and only a limited number of microscopy based reports. Two studies from Maharashtra (West India) among hospitalised fever patients reported a malaria prevalence of 10% (44/448) and 12% (144/1197) [13, 14], while the present study found a prevalence of 35% in Ratnagiri, Maharashtra. From Assam (North-East India) one multicentre study reported a malaria prevalence of 30% (97/324) among hospitalised fever patients [15], which is higher than the prevalence of 17% found in Tezpur, Assam. No hospital based studies are available from Chhattisgarh (Central India) where we found a prevalence of 25% in Mungeli, however, high malaria prevalence is reported in community based surveys from the neighbouring states Madhya Pradesh (Central India) and Orissa (East India) [16–18]. High prevalence, 16% and 23%, was found in the southern sites Ambur (Tamil Nadu) and Anantapur (Andhra Pradesh) in line with a study from a tertiary care hospital in Tamil Nadu reporting a malaria prevalence of 17% among fever patients [19].

During the last 30 years there has been an increasing incidence of *P. falciparum* compared to *P. vivax* in India, which has been attributed to chloroquine resistance in *P. falciparum* [20, 21]. Our findings supported this trend; PCR identified 46% single *P. falciparum* versus 38% single *P. vivax* infections. Predominance of *P. falciparum* is reported in West-, Central-, East- and North East India, and predominance of *P. vivax* in North- and South India [1], partly in line with the present study, where Ambur (Tamil Nadu) had the highest proportion of *P. vivax* (55%), and predominance of *P. falciparum* was found in the sites in Andhra Pradesh, Bihar, Chhattisgarh and Assam. However, also in Oddanchatram in Tamil Nadu the proportion of *P. falciparum* was higher than of *P. vivax*. Predominance of *P. falciparum* is expected among hospitalized malaria patients who are more prone to have severe disease, and species distribution in this study is not representative for malaria in the community population. However, the finding of *P. falciparum* predominance in the majority of the sites underlines the importance of considering *P. falciparum* aetiology in febrile patients in all parts of India. Furthermore, a high

proportion of double infections (12%) were detected as supported by previous PCR studies in India [8, 9, 22].

The sensitivity detecting malaria infections by routine microscopy in the present study was only 29%, supported by several other field studies reporting low sensitivity of routine microscopy compared to PCR [23–25]. One study using PCR among fever patients in Orissa in India, reported a malaria prevalence of 81% by PCR compared to 43% by microscopy [8]. In a review of studies comparing PCR and microscopy, PCR detected on average twice as many malaria infections [26]. The low sensitivity by microscopy can be due to suboptimal staining, poor quality and inadequately maintained microscopes, and microscopists who are insufficiently trained or fatigued by high workload. However, even under optimal conditions microscopy-based diagnosis does not achieve the low detection limits that PCR-based methods yield. The PCR applied in the present study has a sensitivity of at least 0.5 parasites/μl [11], and an experienced person in a reference laboratory would not be expected to detect parasitaemia lower than 50 parasites/μl by microscopy [4]. Furthermore, the low species-specificity of microscopy compared to PCR in the present study is supported by similar findings in previous PCR studies from India [8, 9, 22].

In the present study RDT had a sensitivity as low as 24% compared to PCR, and >50% of the PCR positives which were negative by RDT were *P. falciparum*. The RDT ParaHIT-Total Device (Span Diagnostics) was chosen as it was widely used in routine diagnostics, easily available, reasonably priced, and could be stored at room temperature (25°C). In 2014 a study from India reported 70% sensitivity of this RDT compared to microscopy in detecting *P. falciparum* [27]. The sensitivity of the *P. falciparum* specific RDT ParaHIT *f* (Span Diagnostics) compared to microscopy ranged from 11% and 30%, respectively, in two Tanzanian studies [28, 29], to 85% in an Indian study [30]. According to the WHO's evaluation of RDTs, the ParaHIT-Total Device detect 200 *P. falciparum* parasites/μl with a detection score of 85% [5], supporting that malaria with low parasitaemia probably constitute a proportion of the cases detected by PCR in this cohort.

The low sensitivity of microscopy and RDT compared to PCR may have two potential causes with impact on case management: True low sensitivity of routine diagnostics or asymptomatic parasitaemia in patients with other infections. Challenges regarding routine diagnostics in resource poor settings has been described above. A challenge using PCR in clinical diagnosis is that the method potentially also detects low parasitaemia in semi-immune individuals and not the true cause of fever. The pathogen actually causing the febrile disease may thereby be overlooked. In the present study, submicroscopic malaria was detected by PCR in 71% (162/228), and the level of asymptomatic malaria, and another infection responsible for the febrile disease, among these is unknown. Submicroscopic and asymptomatic malaria is not restricted to high endemic regions; increased prevalence has been reported from areas of low transmission intensity [31–34], and varying prevalence of asymptomatic malaria from different parts of India has been reported. Based on microscopy, two studies in tribal populations in Eastern India reported a prevalence of asymptomatic malaria of 8% and 25% respectively [35, 36], while two studies among pregnant women attending antenatal clinics in Jharkhand (East India) and Chhattisgarh (Central India) reported prevalence of asymptomatic parasitaemia as low as 1% (21/1985) and 0.5% (12/2457) respectively [37, 38]. Submicroscopic malaria is reported to be more common in adults due to the effect of acquired immunity [31, 39, 40]. The proportion of malaria identified only by PCR was not higher among adults than children in the present study.

In addition to the probability of parasitaemia controlled by immunity, a proportion of the patients in this cohort potentially suffering from a nonmalarial cause of fever, could have positive malaria PCR explained by other mechanisms. PCR may remain positive for several weeks

after effective malaria treatment, which is probably explained by residual asexual parasites and/or gametocytaemia [33]. The mitochondrial PCR is particularly sensitive in detecting gametocytes because this parasite stage harbour up to eight mitochondria organelles, compared to the ring stage which harbours only one organelle [41]. In order to further elucidate the clinical impact of PCR positive malaria, quantitative real-time PCR, and gametocyte specific reverse transcriptase PCR, are methods that can be performed [42, 43]. In clinical practice in malaria endemic areas, other causes of febrile disease, such as bacterial sepsis, should be ruled out also when malaria parasites are detected, especially when there is low parasitaemia.

Although there is a risk for over-diagnosing malaria as the cause of febrile disease by PCR, the fact that the method detects low density parasitaemia, undetected by microscopy and RDT, underlines the need for accessible molecular tools for better diagnosis and estimations of the true malaria burden in India. Further, in control and elimination strategies, PCR is essential for detection of submicroscopic and asymptomatic malaria which contribute to the infectious reservoir of the disease [31, 33, 39, 40].

A potential limitation regarding the distribution of malaria across the sites is that a proportion of the patients may have contracted malaria in another area than they were hospitalised. Further, occurrence of selection bias in the inclusion of patients and samples in the favour of clinically suspected malaria cannot be ruled out. Ineffective adherence to routines in a PCR laboratory can result in false positivity due to contamination. Contamination can occur in all steps of the procedure, but it is especially important to limit accumulation of amplification products in the laboratory environment [44]. Substantial effort was given to preparations and training to set the PCR contamination risk to a minimum, with focus on executing the different steps in separate rooms, strict routines for maintaining a sterile environment, correct handling of samples etc. All genus-specific PCR results with discordancy with either RDT or microscopy were re-tested from the DNA extraction step, as a quality control.

Conclusions

This multi-centre study from secondary level community hospitals in six states in India, reports a high prevalence of malaria among patients admitted with acute undifferentiated fever, applying PCR as the reference method. Routine microscopy and RDT had low sensitivity and species-specificity compared to PCR. As PCR is often not available or feasible in routine diagnostics, RDT and microscopy remains the mainstay in work-up of fever patients. The results of this study calls for awareness of the importance of quality assurance of malaria routine diagnostics. In work up of hospitalised febrile patients it should also be taken into consideration that patients with low parasitaemia might serve as a reservoir for transmission rather than suffering from clinical malaria, and other potential causes of fever should be ruled out.

Supporting Information

S1 Dataset. Data on patients and malaria analyses.
(SAV)

S1 Table. The sensitivity and specificity of three diagnostic methods detecting malaria, among the patients were all methods were performed (N = 1163).
(PDF)

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Author Contributions

Conceived and designed the experiments: DM KM CGH AS AM GV NL BB. Performed the experiments: DM SC AM AS GV JA JN AH SP GA LA KM CGH RV DX US. Analyzed the data: CGH SC AM NL BB KM. Wrote the paper: CGH KM SC AM RV DM DX AS NL BB GV US JA JN AH SP GA LA.

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III

RESEARCH ARTICLE

Assessment of malaria real-time PCR methods and application with focus on low-level parasitaemia

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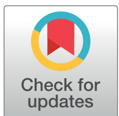

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Abstract

In epidemiological surveys and surveillance the application of molecular tools is essential in detecting submicroscopic malaria. A genus-specific conventional cytochrome *b* (*cytb*) PCR has shown high sensitivity in field studies, detecting 70% submicroscopic malaria. The main objective of this study was to assess the conversion from conventional to real-time PCR testing both SYBR and probe protocols, and including quantitative (q) PCR. The protocols were assessed applying well-defined clinical patient material consisting of 33 positive and 80 negative samples. Sequencing of positive PCR products was performed. In addition, a sensitivity comparison of real-time PCR methods was done by including five relevant assays investigating the effect of amplification target and platform. Sensitivity was further examined using field material consisting of 111 *P.falciparum* positive samples from Tanzanian children (< 5 years), as well as using related patient data to assess the application of q-PCR with focus on low-level parasitaemia. Both the *cytb* SYBR and probe PCR protocols showed as high sensitivity and specificity as their conventional counterpart, except missing one *P. malariae* sample. The SYBR protocol was more sensitive and specific than using probe. Overall, choice of amplification target applied is relevant for achieving ultra-sensitivity, and using intercalating fluorescence dye rather than labelled hydrolysis probes is favourable. Application of q-PCR analysis in field projects is important for the awareness and understanding of low-level parasitaemia. For use in clinical diagnosis and epidemiological studies the highly sensitive and user-friendly *cytb* SYBR q-PCR method is a relevant tool. The genus-specific method has the advantage that species identification by sequencing can be performed as an alternative to species-specific PCR.

Introduction

Malaria is a major threat to global health with half the world's population being at risk of getting infected. In 2016 there were 216 million malaria cases causing almost half a million deaths,

particularly among children in Sub-Saharan Africa and India [1]. The World Health Organisation has identified 21 countries that may be able to eliminate malaria by 2020 [1]. Healthy, asymptomatic humans carrying malaria parasites in the blood represent a reservoir for transmission [2], and are a key challenge to the elimination of malaria [3, 4]. Polymerase chain reaction (PCR) methods have gained a strong foothold in research and epidemiology during the last two decades. PCR has shown to be superior in sensitivity and specificity compared to microscopy and rapid diagnostic tests (RDT) [5–8]. However, there is still need to further improve the performance of malaria PCR techniques and to develop quantitative methods for field studies, to address the issue of asymptomatic carriage of malaria, and to increase our understanding of malaria epidemiology which may help improve control and elimination strategies [4, 6].

Visualizing conventional PCR products by gel electrophoresis is time-consuming and resource-demanding, and increases the risk of contamination. With real-time PCR the detection step is incorporated into the amplification; for each cycle, target DNA is directly detected, usually by either intercalating SYBR green, or fluorescence labelled hydrolysis probes [9, 10]. The direct detection allows for quantitative results (q-PCR), presented either by standard cycle threshold (C_t) values, which is inversely proportional to the amount of target DNA in the sample, or even more exact quantitation by applying a known dilution series of target DNA (for example customized plasmid) which serves as a standard curve to determine the number of target copies per volume in the sample. Thus, q-PCR could be a highly valuable tool to study low-level malaria parasitaemia [10, 11].

During the last decade, there has been a focus on the use of amplification targets with a high copy number in the malaria genome to increase the sensitivity of the PCR. Examples of these multi-targets are chromosomal small subunit ribosomal RNA 18S locus, cytochrome b gene (*cytb*) on the mitochondrial genome, the chromosomal subtelomeric targets telomere-associated repetitive element 2 (TARE-2) and *var* gene acidic terminal sequence (*var*ATS). The first malaria PCR methods were based on the 18S target, which typically exists in five to eight copies in *P. falciparum* [12]. Subsequently, mitochondrial targets, such as *cytb*, emerged since the genome is conserved and exists in about 20–160 copies depending on the development stage [13, 14]. In 2015 two new multi-targets were reported; TARE-2, which exists in about 250–280 copies due to 10 to 12 repeat units presented at 24 of 28 subtelomeric sequences, and *var*ATS which exists in about 59 copies and is encoding the *P. falciparum* erythrocyte membrane protein 1 [15].

A genus-specific *cytb* conventional PCR [16] has proved to be highly sensitive and specific in field studies [5, 17]. The main objective of the present study was to develop a robust screening tool by converting the conventional PCR to a real-time PCR, evaluate two different fluorescent dyes SYBR green and TaqMan probe, and to assess the assays using well-defined patient materials from clinical and field collections [5, 16, 18]. Secondary objectives were to compare relevant real-time methods in order to investigate the effect the choice of platform (SYBR versus probe) and amplification target has on sensitivity, and to implement q-PCR analysis on patient data from field material with focus on low-level parasitaemia.

Materials and methods

Samples

All sample material applied in this study was stored DNA (-20/80˚C), which had been extracted either from whole blood using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, or from filter paper applying Chelex-100

Molecular Biology Grade Resin (Bio-Rad Laboratories, Hercules, CA, USA) as previously described [19].

The clinical patient material, used to assess the sensitivity and specificity of the designed *cytb* SYBR/TaqMan real-time assays, was a defined collection of 33 confirmed positive (18 *P. falciparum*, eight *P. vivax*, three *P. ovale*, two *P. malariae*, and two *P. falciparum* + *P. malariae*), and 80 confirmed negative samples, from 113 fever patients with potential malaria collected between 2006 and 2013 at Haukeland University Hospital, Bergen, Norway [16]. The consensus on which samples were positive or negative was based on results from previously performed analyses by routine microscopy, the PCR reference method described by Singh *et al.* [20], a genus-specific *cytb* PCR, a species-specific 18S PCR (all conventional), and sequencing [16].

The field material, used to investigate sensitivity and to perform q-PCR, was a defined collection of 111 positive *P.falciparum* DNA samples (74 patients), where 74 were extracted from 200 μl EDTA whole blood, and 37 from filter papers. The DNA was obtained from 304 children who were hospitalized due to febrile illness between January and June 2009 at the general pediatric wards at Muhimbili National Hospital, Dar es Salaam, Tanzania [5, 18]. The samples were confirmed positive by the same genus-specific *cytb*, and species-specific 18S conventional PCR assays (or sequencing) as the Norwegian cohort [16]. Clinical and demographic data, such as age, gender, travel outside Dar es Salaam the last four weeks, referral from other hospitals, any use of antibiotics or antimalarial drugs the last four weeks, length of sickness, other clinical diagnoses, and outcome, were included in the analysis of potential correlation with level of parasitaemia by q-PCR. Details regarding the study population and clinical findings in the Tanzanian field study have been reported previously [5].

Reference material

To compare the sensitivity of seven different real-time PCR methods a reference strain of *P. falciparum* (US 03 F FC27/A3), containing exclusively ring stage parasites in a concentration of 2000 parasite/μl (p/μl), was diluted in RNase-free water (Qiagen) into the following dilutions: 5 p/μl, 1 p/μl, 0.5 p/μl, 0.1 p/μl, and 0.05 p/μl, run in 12 parallels. In addition, a 10-fold dilution series run in triplicates, 2000–0.2 p/μl, was included to assess the amplification efficiency (E) of the real-time assays.

Customized plasmid for q-PCR

To serve as a standard curve for q-PCR, a custom designed EcoRI linearized q-PCR template, with a pUCminusMCS vector backbone and this study's *cytb* amplification target (220 bp) as insert (OriGene Technologies, Rockville, MD, USA), was applied in a 10-fold dilution series, range 2.7 x 10^8–2.7 copies of target DNA/reaction (rxn), run in duplicates.

PCR methods

A conventional single-step genus-specific *cytb* PCR [16] was converted to one SYBR and one TaqMan real-time PCR protocol using the same primers.

To investigate sensitivity differences/trends, and effects the number of copies of the amplification targets may have, five relevant and comparable real-time PCR methods were included. Table 1 shows the characteristics of the real-time PCR assays.

Otherwise, the protocols for the five included real-time PCR methods were carefully followed as previously described [15, 21–23].

For the *cytb* SYBR/TaqMan real-time PCR protocols designed and optimized in this study the following primers were applied: PgMt19 F3 forward (5'-tcg ctt cta acg gtg aac) and PgMt19

Table 1. Characteristics of real-time PCR methods.

Methods	Specificity	Target genome	Target gene	No. copy of gene in P.f	Product size	Species identification by sequencing
This study	Pan	Mito	*cytb*	~20–160[a]	220 bp	Yes
Lefterova et al. **2015** [21]	Pan	Chrom	18S rRNA	~5–8[b]	317 bp	Yes
Xu et al. **2015** [22]	Pan	Mito	*cytb*	~20–160[a]	430 bp	No[e]
Farrugia et al. **2011** [23]	Pan	Mito	*cytb*	~20–160[a]	203 bp	No
Hofmann et al. **2015** [15]	P.f	Chrom	TARE-2	~250–280[c]	93 bp	-
Hofmann et al. **2015** [15]	P.f	Chrom	*var*ATS	~59[d]	65 bp	-

Abbreviations: P.f, *Plasmodium falciparum*; Mito, mitochondrial; Chrom, chromosomal; *cytb*, cytochrome *b* gene; telomere-associated repetitive element 2, TARE-2; *var* gene acidic terminal sequence, *var*ATS.

[a] Depending on which stage of the parasite cycle; About 20 copies in early ring stage, and about 80–120 copies in mature gametocytes [13, 14].

[b] Depending on the strain [12].

[c] TARE-2, specific to *P. falciparum*, consists of 10 to 12 repeat units presented at 24 of 28 subtelomeric sequences [15].

[d] The *var* gene family is located primarily in the subtelomeric sequences, and encode the *P. falciparum* erythrocyte membrane protein 1 [15].

[e] Species identification can be performed using restriction fragment length polymorphism analysis of the real-time PCR amplified product [22].

To optimize their comparability, all the real-time assays applied 2 μl DNA template, and 12,5 μl SYBR Select Master Mix/ TaqMan Universal Master Mix II, with UNG (Applied Biosystems, Carlsbad, CA, USA), at a total volume of 25 μl. The amplifications were performed using ABI Prism 7900HT Sequence Detection System (Applied Biosystems), the threshold was automatically set, and for the SYBR assays melting curve analysis was included given by the program SDS 2.3 (Applied Biosystems).

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B3 reverse (5'-aat tga tag tat cag cta tcc ata g), previously published for a loop-mediated isothermal amplification method [24]. The primer concentration was 600 nM of each primer, and 200 nM of the TaqMan probe PgMt(28)-Probe 6-FAM-ctt cta aca ttc cac ttg ctt ata act g-BHQ-1 (Eurogentec, Seraing, Belgium). In addition, the reaction mix contained 1 mM $MgCl_2$ (New England BioLabs, Ipswich, MA, USA). Both protocols used the following cycling parameters; step 1, 50˚C for 2 min; step 2, 95˚C for 10 min; step 3, denaturation at 95˚C for 15 sec; step 4, annealing at 59˚C for 50 sec; and step 5, amplification at 72˚C for 10 sec, steps 3–5 repeated 45 times.

To investigate the quality of the stored DNA, samples of extremely low parasitaemia were reanalysed with the *cytb* conventional PCR (primers PgMt19 F3&B3), run in triplicates, as previously described by Haanshuus *et al.* [16], but with a primer concentration of 1 μM (incorrect concentration given in the publication [16]).

To ensure that real-time PCR products showed the same high quality sequences for species identification as for the *cytb* conventional PCR [16], all positive products amplified by the *cytb* SYBR real-time PCR from the Norwegian material, were sequenced in one direction applying primer PgMt19 F3 as previously described [16].

Statistical analyses

Statistical univariate analysis was performed applying IBM SPSS Statistics version 24 (SPSS Inc., IBM Company). The data were organized into categorical variables for 2x2 cross-tabulation analysis, and assessed with effect estimates (odds ratio) with corresponding confidence intervals, as well as Chi-squared test, or Fisher's exact test if few observations. A correlation was regarded as statistically significant if p-value was < 0.05.

Multivariate logistic regression analysis was also performed applying SPSS. The p-values were calculated by using the Likelihood ratio test, where in addition to SPSS the program QuickCalcs (GraphPad Software) was applied for this purpose. All variables with p-value <0.1 from the univariate analysis, excluding variables with extensive numbers of missing values, were included in

the multivariate regression model. The confirmation that the model fitted the data was evaluated by the Hosmer and Lemeshow Test, and by residuals and Cook distances analysis.

Ethical approval

The study was approved by the Regional Committee for Ethics in Medical Research in Western Norway (No.2015/886 and 2016/584).

Results

All raw data, including threshold, C_t- and quantification values, are given in supporting information S1 Dataset. The clinical and field DNA samples were always run in triplicates, and a positive result was defined as minimum two detections out of three.

Assessment, comparability, and sensitivity of real-time PCR methods

The sensitivity and specificity of the designed and optimized genus-specific *cytb* SYBR and TaqMan PCR protocols were assessed applying the Norwegian clinical material. The real-time assays showed as high sensitivity and specificity as their conventional PCR counterpart [16], except missing one *P. malariae* sample (S1 Table).

Applying *P. falciparum* reference material, the study's two protocols and five relevant real-time PCR methods (characteristics presented in Table 1) were compared to investigate how the choice of platform and amplification target affects the sensitivity (Tables 2 and S2).

Among the five included assays the *var*ATS TaqMan PCR method [15] showed a high sensitivity, and was therefore chosen for further assessment together with the designed *cytb* SYBR/TaqMan PCR protocols, applying *P.falciparum* positive Tanzanian field material (Table 3). Furthermore, the sensitivity of applying whole blood versus that of using filter paper as field material was compared, and the difference in C_t trends for the *cytb* SYBR real-time PCR results are shown in Fig 1. Among the field samples extracted from whole blood, previously obtained research microscopy and RDT results were correlated with positive C_t values of the *cytb* SYBR PCR as presented in Fig 2.

Through the analysis of both the Norwegian and Tanzanian material, several of the previously positive DNA samples turned out negative by the real-time PCR methods. Therefore, the quality of the stored DNA was tested by reanalysing, with the *cytb* conventional PCR [5, 16, 18], all samples which were negative or had a C_t value above 30 by the *cytb* SYBR real-time method. Fig 3 shows a comparison between the conventional and the real-time PCR methods.

Table 2. Sensitivity comparison of real-time PCR methods applying five different dilutions of *P.falciparum* reference material. [a].

	Platform	5 p/μl ($\bar{C}_t$)	1 p/μl ($\bar{C}_t$)	0.5 p/μl ($\bar{C}_t$)	0.1 p/μl ($\bar{C}_t$)	0.05 p/μl ($\bar{C}_t$)
This study_*cytb*	SYBR	12 (30)	12 (33)	12 (34)	9 (36)	9 (38)
This study_*cytb*	TaqMan	12 (30)	12 (32)	12 (33)	8 (35)	5 (37)
Lefterova_18S	SYBR	12 (28)	12 (32)	11 (34)	4 (36)	0
Xu_*cytb*	SYBR	12 (28)	12 (32)	12 (33)	10 (35)	6 (35)
Farrugia_*cytb*	TaqMan	12 (34)	12 (37)	12 (38)	3 (41)	1 (42)
Hofmann_TARE-2	SYBR	12 (33)	12 (37)	12 (38)	7 (40)	3 (39)
Hofmann_*var*ATS	TaqMan	12 (34)	12 (36)	12 (37)	11 (40)	8 (41)

[a] The threshold was automatically set for each assay run, and the C_t values correspond to the threshold. The results are given as the number of positives out of 12 parallels.

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Table 3. Sensitivity assessment of real-time PCR methods applying positive *P. falciparum* Tanzanian field DNA samples (N = 111). [a].

		This study Pan_*cytb*_SYBR	This study Pan_*cytb*_TaqMan	Hofmann et al. *P.f_var*ATS_TaqMan
Samples EDTA[b] (N = 74)	Positives $C_t < 20$	25	20	19
	Positives C_t 20–30	16	19	18
	Positives C_t 31–40	13	15	24
	Positives $C_t > 40$	0	0	0
	Positives total	54	54	61
	Negatives	20	20	13
Samples Filter[c] (N = 37)	Positives $C_t < 20$	0	0	0
	Positives C_t 20–30	27	23	26
	Positives C_t 31–40	10	11	8
	Positives $C_t > 40$	0	0	1
	Positives total	37	34	35
	Negatives	0	3	2

Abbreviations: P.f, *Plasmodium falciparum*

[a] Predefined samples by genus-specific *cytb* conventional PCR, and species-specific 18S PCR (or sequencing) [5, 18]. Data on concluding positivity is given in S1 Dataset.

[b] DNA extracted from 200 μl EDTA whole blood by a spin-column method.

[c] DNA extracted from filter paper (~50 μl blood) by a Chelex-100 method.

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The designed *cytb* SYBR PCR assay showed better results than using TaqMan probe (Tables 2 and 3 and S1 and S2). Therefore this method was chosen for the q-PCR analysis of the Tanzanian field material, and to test the quality of sequencing as species identification method. The sequencing confirmed high-quality sequences of full length, and gave correct species identification, except for only detecting the dominating *P. falciparum* parasitaemia in two double infections. This result was equal to that of applying the conventional PCR version, and the polymorphisms distinguishing the species were previously described [16].

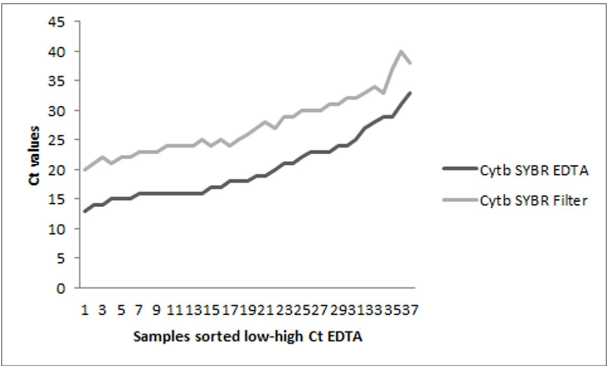

Fig 1. C_t value comparisons of DNA samples extracted from 200 μl EDTA whole blood versus filter paper 50 μl blood (N = 37). Positive *P.falciparum* material from 37 patients had been collected and extracted by two different methods resulting in DNA from whole blood purified by spin-column, and DNA from filter paper purified by Chelex-100 [5, 18]. The two C_t trends showed a constant difference of 4–9 cycles, and for 31 out of 37 samples the difference was 7–9 cycles.

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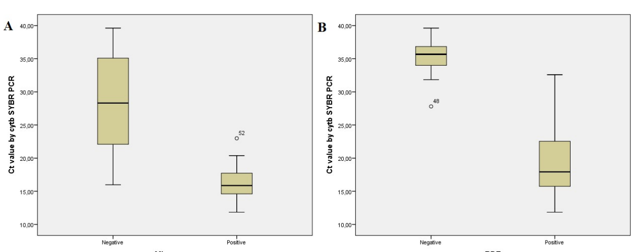

Fig 2. Boxplots showing how research microscopy and RDT results correlate to positive C_t values among Tanzanian field samples. (A) A boxplot showing how research microscopy results negative/positive correlate to positive *cytb* SYBR real-time PCR results (N = 54). (B) A boxplot showing how rapid diagnostic test (RDT) results negative/positive correlates to positive *cytb* SYBR real-time PCR results (N = 47).

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Application and assessment of quantitative PCR field data

To present an alternative and relevant application of q-PCR data in epidemiological studies, the association between low-level parasitaemia identified by the *cytb* SYBR q-PCR results and clinical and demographic factors (among the 74 field samples extracted from whole blood),

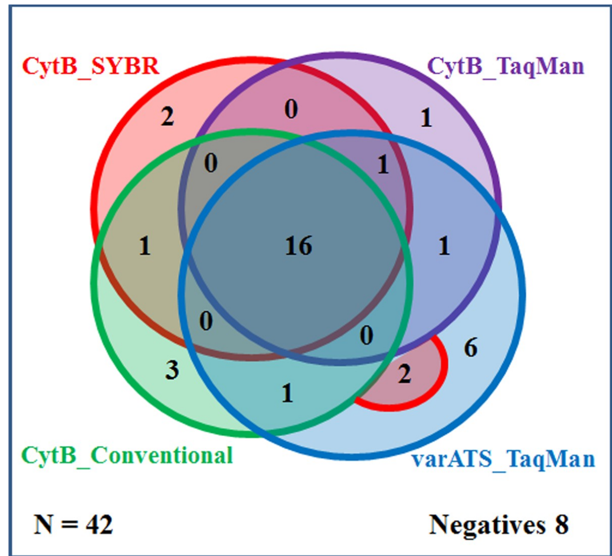

Fig 3. Sensitivity analysis and method comparison of PCR methods applying positive *P.falciparum* field DNA samples with extreme low parasitaemia (N = 42). Extreme low parasitaemia samples that counted negative or C_t value above 30 by *cytb* SYBR real-time PCR were further analyzed. This was done to investigate if the stored DNA had been degraded over time, as well as compare the performance of the four PCR methods. The *var*ATS TaqMan real-time PCR had a higher detection rate for low parasitaemia. However, for all of the methods eight of the previously positive *P. falciparum* samples were now negative. A positive sample was defined by at least two out of three parallels detected, and only one of the eight negatives had no positive detections of parallels by any of the methods. For several of the samples it was a randomly/non-consistently detection trend, depending on the actual amount of amplification target in the template. Detailed data are given in S1 Dataset.

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were evaluated applying cross tabulation and multiple logistic regression analysis. Based on an evaluation of C_t results, frequency data, and what was of low density parasitaemia (S1 and S2 Datasets), the q-PCR values were categorized into the two categories ≤1000 copies/rxn (N = 37, range 0–953, corresponds to C_t value ≥27) defined as low parasitaemia, and > 1000 copies/rxn (N = 37, range 3769–1.6x10^7, corresponds to C_t value ≤24) defined as high parasitaemia. The median value was 2361 copies/rxn. When applying 2 μl template, 1000 copies of the *cytb* target/rxn, corresponds to 3–25 *P. falciparum* p/μl depending on the parasite stage (Table 1). Microscopy had the most sensitive detection at C_t value 23 and 15314 copies/rxn, which corresponds to ~50–380 p/μl. In comparison, according to Hänscheid et al. 1% parasitaemia is measured to be ~50.000 p/μl [25].

In the univariate analysis (Table 4), low-level parasitaemia was associated with age ≤12 months, illness of >5 days duration, and death while in hospital. Patients with low-level parasitaemia were more likely to have received antibiotics the last four weeks, but less likely to have been diagnosed with malaria, and less likely to have received antimalarial treatment in hospital. In the multivariate analysis (S3 Table), low level parasitaemia was associated with age ≤12 months, length of illness >5 days, no history of travel outside Dar es Salaam the last four weeks, and not being diagnosed with malaria. The variables antibiotic and antimalarial treatment in hospital were omitted from the regression model due to a high number of missing values.

Discussion

Several studies report that PCR techniques are superior to microscopy, especially in detecting low density parasitaemia [26–28]. A review of studies comparing PCR and microscopy, found that PCR detects on average twice as many malaria infections as microscopy [29]. The genus-specific *cytb* conventional PCR [16], has previously been applied in studies from Tanzania and India, where the PCR detected as much as 72% (55/76, N = 304) and 71% (162/228, N = 1168) submicroscopic malaria respectively [5, 17]. The conversion and optimization from conventional to real-time PCR, using either SYBR green or TaqMan probe, resulted in similar sensitivity and specificity (S1 Table and Fig 3 and S1 Dataset).

Applying SYBR is less expensive than using probes. Though the specificity of SYBR assays is debated; while probes bind specifically to target DNA as an third oligo, the specificity of SYBR relies on a high efficiency and concentration of specific amplified DNA outshining the unspecific binding of SYBR [30]. Short amplicons give higher efficiencies. In contrast to probes, which release only a single fluorophore for each amplicon, SYBR will with longer amplicons generate a stronger signal increasing the sensitivity as more dye is incorporated [31]. This is in agreement with the observed higher sensitivity of the study's SYBR assay compared to using probe (Tables 2 and 3 and S1 and S1 Datasets). Using SYBR showed 100% specificity, while the TaqMan assay had one unspecific binding at C_t value 41 (S1 Table). The SYBR protocol in this study is recommended compared to using TaqMan probe.

Comparing different real-time PCR methods showed that the assays are remarkably similar in sensitivity (Table 2). The 18S SYBR PCR (Lefterova *et al.* [21]) had the lowest sensitivity, as might be expected since the target gene is in only a few copies (Table 1). Surprisingly, the method using the target in most copies, TARE-2 SYBR PCR (Hofmann *et al.* [15]), was not the most sensitive one. However, this agrees with the authors' report of lower sensitivity than expected, likely due to the degenerate sequence of the TARE-2 repeat units or by the clustered distribution of the repeats at chromosome ends. The three most sensitive assays were the *var*-ATS TaqMan (Hofmann *et al.* [15]), the *cytb* SYBR (Xu *et al.* [22]), and this study's *cytb* SYBR PCR. The *cytb* SYBR assay from Xu *et al.* showed the lowest amplification efficiency (S1 Table), which is in agreement with the authors' report on a secondary structure of the main

Table 4. Univariate cross tabulation analysis of factors associated with low parasitaemia (≤1000 copies/rxn).

	Low parasitaemia ≤ 1000 copies/rxn (%)	High parasitaemia > 1000 copies/rxn (%)	OR (95% CI)	p-value
Age (N = 73)				
≤ 12 months	17 (47.2)	5 (13.5)	5.73 (1.82–18.04)	0.002*
> 12 months	19 (52.8)	32 (86.5)		
Sex (N = 74)				
Male	22 (59.5)	19 (51.4)	1.39 (0.55–3.49)	0.483
Female	15 (40.5)	18 (48.6)		
Travel outside Dar last 4 weeks (N = 63)				
No	19 (65.5)	15 (44.1)	2.41 (0.87–6.69)	0.089
Yes	10 (34.5)	19 (55.9)		
Referral from other hospital (N = 71)				
No	24 (68.6)	29 (80.6)	0.53 (0.18–1.57)	0.246
Yes	11 (19.4)	7 (19.4)		
Antibiotics the last 4 weeks (N = 66)				
No	5 (14.3)	15 (48.4)	0.18 (0.06–0.58)	0.003*
Yes	30 (85.7)	16 (51.6)		
Antimalarials the last 4 weeks (N = 68)				
No	15 (44.1)	11 (32.4)	1.65 (0.62–4.43)	0.318
Yes	19 (55.9)	23 (67.6)		
Length of sickness (N = 72)				
≤ 5 days	16 (43.2)	29 (82.9)	0.16 (0.05–0.47)	0.001*
> 5 days	21 (56.8)	6 (17.1)		
Antibiotic treatment in hospital (N = 74)				
No	2 (5.4)	4 (10.8)	0.47 (0.08–2.75)	0.219
Yes	35 (94.6)	33 (89.2)		
Antimalarial treatment in hospital (N = 74)				
No	8 (21.6)	1 (2.7)	9.93 (1.17–84.04)	0.008*
Yes	29 (78.4)	36 (97.3)		
Given diagnosis malaria (N = 74)				
No	22 (59.5)	10 (27.0)	3.96 (1.49–10.53)	0.005*
Yes	15 (40.5)	27 (73.0)		
Given diagnosis septicaemia (N = 74)				
No	24 (67.6)	32 (86.5)	0.33 (0.10–1.05)	0.053
Yes	12 (32.4)	5 (13.5)		
Length of admission (N = 74)				
≤ 5 days	24 (64.9)	20 (54.1)	1.57 (0.62–4.00)	0.343
> 5 days	13 (35.1)	17 (45.9)		
Outcome (N = 74)				
Dead	11 (29.7)	4 (10.8)	3.49 (1.00–12.24)	0.043*
Alive	26 (70.3)	33 (89.2)		

Abbreviation: OR Odds ratio, 95%CI 95% Confidence Interval, *Dar* Dar es Salaam.

*Significant results (p-value < 0.005).

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amplicon that likely contributes to a competing binding-site for the primers, and can affect optimal sensitivity. This study's assays, the 18S SYBR (Lefterova *et al.*), and the *cytb* SYBR (Xu *et al.*), are genus-specific PCR methods which have the advantage of species identification by

either sequencing or restriction fragment length polymorphism analysis. The TARE-2 and *var*-ATS methods are restricted to *P.falciparum*. In clinical use any of these PCR assays will be sensitive enough; however, in detecting low-level parasitaemia the small differences in sensitivity might be relevant. Furthermore, amplification in late cycles of PCR programs can be due to unspecific binding or primer-dimers. However, it was in this study decided to not set a cut-off for positivity as also low-level parasitaemia has late amplifications (Table 2). Hence the SYBR methods were more beneficial to apply than TaqMan probes focusing in detecting low-level parasitaemia, since SYBR melting curve analysis (MCA) can be performed revealing if late cycle amplifications are unspecific binding or primer-dimers rather than true positives [32].

PCR detects very low densities of target DNA in the blood (S1 and S2 Datasets), and compared to microscopy and RDT, the technique has a superior sensitivity in detecting low-level parasitaemia (Fig 2). Asymptomatic malaria among PCR positives should always be considered [17, 33]. To achieve a low detection limit, the type of material is important. Filter papers are increasingly used in field studies, as the material is easier to collect, store, and transport than EDTA whole blood, but result in lower test sensitivity due to the smaller blood volume obtained, ~50 versus 200 μl (Fig 1) [18]. Target DNA extracted from filter papers had in this study a detection limit of 25 copies/rxn, while DNA from EDTA had 1 copy/rxn. Therefore, applying filter papers gives a high sensitivity, but a substantial portion of the low-level parasitaemia will be missed which might be relevant in surveillance and epidemiological studies.

The *P. falciparum* positive field material extracted from whole blood (N = 74), showed a broad variation in level of parasitaemia (Table 3). There is a great complexity of reasons why malaria DNA detected only by PCR is obtained in the blood stream. Low density of malaria DNA may derive from early (primary or recurrent) infection, non-*falciparum* infection (as indicated in S1 Table where a clinical *P. malariae* infection showed a high C_t value), premunition, or remains of parasites and denatured DNA after infection clearance by the immune system and antimalarial treatment. Also some antibiotics may reduce the parasite level in the blood without fully clearing the infection [34]. Sexual stage parasites (gametocytes) are less affected by antimalarial drugs and the immune system, and thus circulate in the blood for weeks after parasite life cycles are ended awaiting transmission [35, 36]. Untreated chronic low-level parasitaemia might persist for months as parasite clones can circulate for ~200 days [37].

Due to potentially reduced quality of the stored DNA, and low reproducibility for samples with extremely low parasitaemia (Fig 3), the field material (N = 111) was also used for sensitivity assessment of the the designed *cytb* SYBR/TaqMan and the *var*ATS TaqMan PCR methods. Compared to DNA extracted from whole blood, the DNA from filter paper showed high stability with only a few previously positive samples testing negative (Table 3), which may be caused by the ion-exchange resin Chelex solution inhibiting DNA degradation by chelating metal ions that otherwise can catalyze breakdown of DNA [38]. Table 3 also showed that the *var*ATS assay detected five more positive samples than the *cytb* SYBR method. With regards to the two different amplification targets *cytb* versus *var*ATS, *cytb* PCR assays should in theory be twice as sensitive as *var*ATS PCR in detecting gametocytes, while *var*ATS targets should be about three times as sensitive as *cytb* in detecting ring stage parasites (Table 1). Therefore, it can be speculated if a substantial proportion the field samples with low-level parasitaemia are malaria infections dominated by asexual blood stage parasites, rather than gametocytes.

By applying a customized plasmid, quantitation analysis was performed, and the association between degree of parasitaemia defined by q-PCR values (N = 74) and clinical and demographic features was assessed (Tables 3 and S3). The study population was children <5 years. Children have a less developed immune system than adults, though antibodies from their mother and in breastmilk give some protection in their first months of life [39, 40]. Both the univariate and multivariate analysis found a significant correlation between age $\leq$12 months

and low parasitaemia, which could be due to immunity and asymptomatic malaria in this group. Among children age ≤12 months, 37% (7/19) had received antimalarials within four weeks of admission, versus 71% (34/48) among children age >12 months (S2 Dataset). Furthermore, in the univariate analysis, low parasitaemia was significantly associated with death in hospital (Table 3), though not significant in the multivariate analysis (S3 Table). The association is counter-intuitive, as high parasitaemia is known to be a risk factor for death. However, low parasitaemia malaria may be wrongly interpreted as clinical malaria obscuring the diagnosis of other severe blood stream infections. In a previous study from the same hospital, bloodstream-infections caused by multi-resistant Gram-negative bacteria carried case-fatality rates (>70%) more than three-fold that of malaria (≈20%) [41]. The finding that patients with low-level parasitaemia less frequently had been given a diagnosis of malaria, and less frequently had received antimalarial treatment, support that other infections may have been the cause of disease and mortality in these children, or might indicate missed opportunity for treatment of early malaria infection. The DNA detected by PCR can stem from a recent malaria episode for which the patient had already received treatment and recovered from. Indeed, children who have recently been hospitalized with malaria and anemia have substantially increased risk of death in the subsequent months [42, 43]. Among the children 81% (51/63) had received either antibiotics 70% (46/66), antimalarials 62% (42/68), or both 46% (29/63), within four weeks prior to admission (S2 Dataset). A limitation to the q-PCR assessment is the small sample size and the limit age-interval (children <5 years), as well as few clinical parameters. The samples used in the assessment had been collected in order to perform malaria PCR, but for a different purpose and application (Strøm *et al.* investigating challenges in diagnosing paediatric malaria [5]). Therefore, to assess predictors of clinical outcome in low-parasitaemia, this alternative q-PCR approach should be applied in larger, prospective studies employing comprehensive diagnostics for the major relevant differential diagnoses of acute undifferentiated febrile illness, including blood culture and serology for rickettsia and viral infections. Furthermore, since an unknown portion of the low parasitaemias probably is due to gametocytes, methods detecting mRNA specifically produced by gametocytes could be relevant to include increasing knowledge around transmission dynamics [44].

Stored DNA is commonly used in PCR assessments and analysis, since extracted DNA is regarded as a fairly stable material if kept in a freezer [45]. It has been debated if freezing and thawing of samples can have a denaturing effect on the DNA [46]. The quality of the stored DNA in this study was tested by reanalyzing the samples with the *cytb* conventional PCR used in the first analyses of the Tanzanian field material [5]. The results indicated that the DNA might have been degraded over time (Fig 3 and S1 Dataset), which, is a possible bias to the correlations shown in Fig 2 and Table 4. However, Fig 1 demonstrates the accuracy and consistency of PCR, and shows no indication of denatured DNA in samples with high parasitaemia. For samples with extremely low parasitaemia, reproducibility by PCR is challenging (Fig 3 and S1 Dataset). The number of times each sample was defrosted varied due to testing, therefore some samples might have been more prone to degradation than others (S1 Dataset). Since all the 74 samples assessed with q-PCR were previously confirmed positive by PCR (data on concluding positivity is given in S1 Dataset), it was decided in regards to the patient data that also q-PCR results with value zero should be included in the definition of low parasitaemia (≤1000 copies/rxn).

Conclusions

Choice of amplification target applied in real-time malaria PCR is relevant for achieving high sensitivity, especially in detecting low-level parasitaemia. Furthermore, it is advantageous to use an intercalating fluorescence dye suitable for MCA rather than labelled hydrolysis probes.

Application of q-PCR analysis should be included in epidemiological studies, and may increase awareness and understanding of low-level parasitaemia. The highly sensitive, specific, and user-friendly *cytb* SYBR q-PCR developed in this study can be useful in both epidemiological and clinical malaria studies. The method is genus-specific, which is an advantage in large screening projects. Among ambiguous samples, and in settings where species-specific PCR fails to detect low parasitaemia, confirmation and species identification by sequencing of the genus-specific real-time PCR products can be used as a contingency.

Supporting information

S1 Dataset. Raw PCR data.
(XLSX)

S2 Dataset. Patient and q-PCR data.
(SAV)

S1 Table. Sensitivity and specificity assessment of designed real-time PCR protocols applying Norwegian clinical DNA samples (N = 113).
(DOCX)

S2 Table. Amplification efficiencies for real-time PCR methods applying a 10-fold dilution series of reference material.
(DOCX)

S3 Table. Multivariate logistic regression analysis of factors associated with low parasitaemia ($\leq$1000 copies/rxn).
(DOCX)

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