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# Assessing the efficacy of artemisinin-based combination therapies (ACTs) against *Plasmodium malariae* and *Plasmodium ovale* infections with low parasite densities: overcoming challenges during molecular analyses

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Master Degree Project in Infection Biology, 45 credits. Spring 2020
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## Abstract

**Background**: Malaria is a major public health issue. Artemisinin-based combination therapies are the WHO recommended treatment for uncomplicated malaria. *Plasmodium malariae* and *Plasmodium ovale* infections are considered underestimated and the effectiveness of artemisinin-based combination treatments against them is poorly documented. The aim of this study was to evaluate the efficacy of dihydroartemisinin-piperaquine against low parasite density *Plasmodium malariae* and *ovale* infections.

**Methods**: DNA was extracted from dried blood spots on filter papers with Chelex®-100 or a column-based extraction method. Species detection and determination was conducted by SYBR Green quantitative PCR targeting the cytochrome b gene (cytb-qPCR) followed by restriction fragment length polymorphism analyses. In total, 241 samples from 53 patients enrolled in a clinical trial were analysed. The obtained molecular data were compared with the microscopy data of the study.

**Results**: Only 69 out of 143 microscopy-positive samples were confirmed as positive by cytb-qPCR. Ninety-three samples were identified as parasite negative by both microscopy and PCR. None of the 36 microscopy-defined coinfections were detected in the molecular analysis. The cytb-qPCR success rate was 72.9% (CI95% 61.4-82.6), 75.0% (CI95% 34.9-96.8) and 14.8% (CI95% 6.9-26.2) for parasite densities above 1000 parasites/ µL, between 600-1000 parasites/ µL and below 600 parasites/ µL, respectively. The observed poor qPCR success rate is most likely due to sample degradation under poor storage conditions.

**Conclusions**: This study highlights the impact on the preservation and quality of *Plasmodium* genomic DNA on dried blood spots, when filter papers are stored for more than 3 years in tropical conditions.

**Key words:** Malaria; *Plasmodium malariae*; *Plasmodium ovale*; low density infections; PCR detection

## List of abbreviations

| | |
|---|---|
| ACTs | Artemisinin-based combination therapies |
| Bp | Base pairs |
| Cytb | Cytochrome b |
| Cq | Quantification cycle |
| $dH_2O$ | Deionized water |
| DNA | Deoxyribonucleic acid |
| DNAses | Deoxyribonucleases |
| FAM | 6-carboxyfluorescein |
| NTC | No template control |
| PCR | Polymerase chain reaction |
| qPCR | Quantitative polymerase chain reaction |
| RDTs | Rapid Diagnostic Tests |
| TAMRA | 6-carboxytetramethyl-rhodamine |
| TBE | Tris/Borate/EDTA |
| VIC | 2-chloro-7phenyl-1,4-dichloro-6-carboxy-fluorescein |

## Popular science summary

### Effective treatments against malaria - or maybe not?

Malaria is one of the three deadliest infectious diseases. Every year millions of people get malaria and hundreds of thousands die from it. Children younger than 5 years old and pregnant women are at the highest risk. The bite of an infected female *Anopheles* mosquito that sucks blood to feed her eggs typically between dawn and dusk, is the way that people get infected with the malaria parasite. The main recommended treatment against malaria is derived from an ancient Chinese herb from the plant *Artemisia annua* that today is used in the form of artemisinin-based combination therapies. Among the six different species of malaria parasites that infect humans, there are two species, *Plasmodium malariae* and *Plasmodium ovale*, which are less studied because they are not as common and deadly as the other species. Consequently, there is poor evidence that artemisinin-based combination therapies are truly effective against these two species.

We aimed to examine if the artemisinin-based combination therapy, dihydroartemisinin-piperaquine, is effective against *Plasmodium malariae* and *Plasmodium ovale.* We collected and analysed 241 samples from 53 patients in Africa, who had been infected with these two species and treated with this specific therapy. A convenient and cheap way to preserve for years the genetic material of the parasite is by collecting few blood drops from infected individuals on suitable filter papers and store them in appropriate conditions. Typically, microscopy is used to see and count the number of the parasites in the blood of patients but nowadays, there are more sensitive molecular methods that can ‘find’ even one parasite in a tidy drop of blood by detecting the DNA of the parasite. Thus, we used a highly sensitive DNA method to detect the presence of the *Plasmodium* parasite and then we determined the *Plasmodium* species by using modified enzymes, which can recognize the DNA of the parasite and ‘cut’ it in different pieces based on the *Plasmodium* species. Finally, we compared our results from the DNA analyses with the microscopy.

Our highly sensitive method was successfully detected and identified the presence of *Plasmodium* parasites in a significantly lower number of samples in comparison to microscopy. A possible explanation could be the DNA damage due to the bad storage of the filter papers in conditions with high temperature and humidity, as these two factors can lead to breakdown of the genetic material throughout the years. Although we were not able to assess the efficacy of the treatment against the two *Plasmodium* species, our study highlights the importance of good storage of the filter papers for further molecular investigation. Future goals include the development of a more sensitive molecular method, capable to overcome the DNA breakage as well as the evaluation of the efficacy of more artemisinin-based combination therapies against *Plasmodium malariae* and *Plasmodium ovale* species.

# 1. Introduction

## 1.1 Public health issue of malaria

Malaria is a life-threatening vector-borne disease that is associated with high morbidity and mortality worldwide. In 2018, WHO estimated 228 million malaria cases and approximately 405.000 malaria deaths in the WHO Regions. Sub-Saharan Africa accounted for 90% of all malaria cases and 94% of the subsequent deaths. Pregnant women, children below 5 years old and people with underlying immune diseases (i.e. HIV/AIDS) are at highest risk of developing severe malaria; deaths in children aged under five accounted for 67% of the global malaria deaths [1]. Rapid diagnosis of the disease, improved surveillance in combination with vector control implementations including the use of insecticide-treated bed nets and indoor residual spraying, have led to an important decline in malaria mortality and transmission. The global effort to achieve the WHO goals for malaria elimination and eradication by 2050 is ongoing and requires adequate funding, strengthened health care systems, expanded strong interventions as well as continuous malaria research and development [2–4].

## 1.2 The *Plasmodium* parasite lifecycle and malaria disease

Malaria is caused by a unicellular apicomplexan parasite of the genus *Plasmodium*. There are over 100 *Plasmodium* parasites but only six are known to infect human: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale curtisi*, *Plasmodium ovale wallikeri* and *Plasmodium knowlesi*. *P. falciparum* and *P. vivax* are the most widespread and dangerous species. *P. falciparum* is the predominant species in Sub-Saharan Africa, Eastern Mediterranean and Western pacific, usually causes high levels of parasitaemia and is associated with severe or complicated malaria that can lead to death whereas *P. vivax* infections are mainly in South-East Asia and Latin America [5]. *P. malariae* is present in all WHO Regions and *P. ovale* is common in Africa and some islands in the Western Pacific Region. Both are related with low prevalence and uncomplicated malaria causing low levels of parasitaemia and they can be found as coinfections with other *Plasmodium* species, mainly *P. falciparum* and *P. vivax* [6,7]. *P. knowlesi* is mainly present in South-East Asia, its main natural reservoir is the South-East Asian macaque monkeys and is the main source of malaria in the Borneo island [1,8].

The lifecycle of *Plasmodium* is complex and includes an arthropod vector (i.e. mosquitoes) and a vertebrate host (Figure 1). The parasite is transmitted to human by an infected female *Anopheles* mosquito, which inoculates the *Plasmodium* sporozoites into the human skin. In the liver (liver stage), sporozoites multiply inside the invaded hepatic cells releasing merozoites (daughter cells), which then attack the red blood cells (RBCs). In the RBCs (blood stage), the asexual multiplication of the merozoites takes place through four subsequent stages (ring stage; trophozoites; schizonts; merozoites or gametocytes) before the rupture of the infected erythrocytes and the release of approximately $10^8$-$10^{12}$ daughter merozoites [9,10]. Based on the *Plasmodium* species, the duration of the blood stage can be varied resulting in different frequency of the fever peaks. *P. knowlesi* merozoites need 24 hours to multiply in the RBCs, *P. falciparum*, *P. vivax* and *P. ovale* merozoites multiplication takes approximately 48 hours while 72 hours are needed for *P. malariae* merozoites (tertian fever). Furthermore, a unique characteristic of *P. ovale* and *P. vivax* is the hypnozoites (dormant forms of the parasites) in the liver that can stay inactive for weeks to years and are associated with latency and relapse malaria [11]. The host immune reaction to the massive rupture of the RBCs cause the symptoms of the disease, which in the case of uncomplicated malaria are ‘flu-like symptoms’ including fever, chills, sweating, headache, body ache and nausea. In the case of *P. falciparum* infection, if the symptoms are left untreated or underestimated, they can rapidly progress to severe malaria followed by high fever, drowsiness, jaundice, anaemia, cerebral malaria, renal failure and death [5].

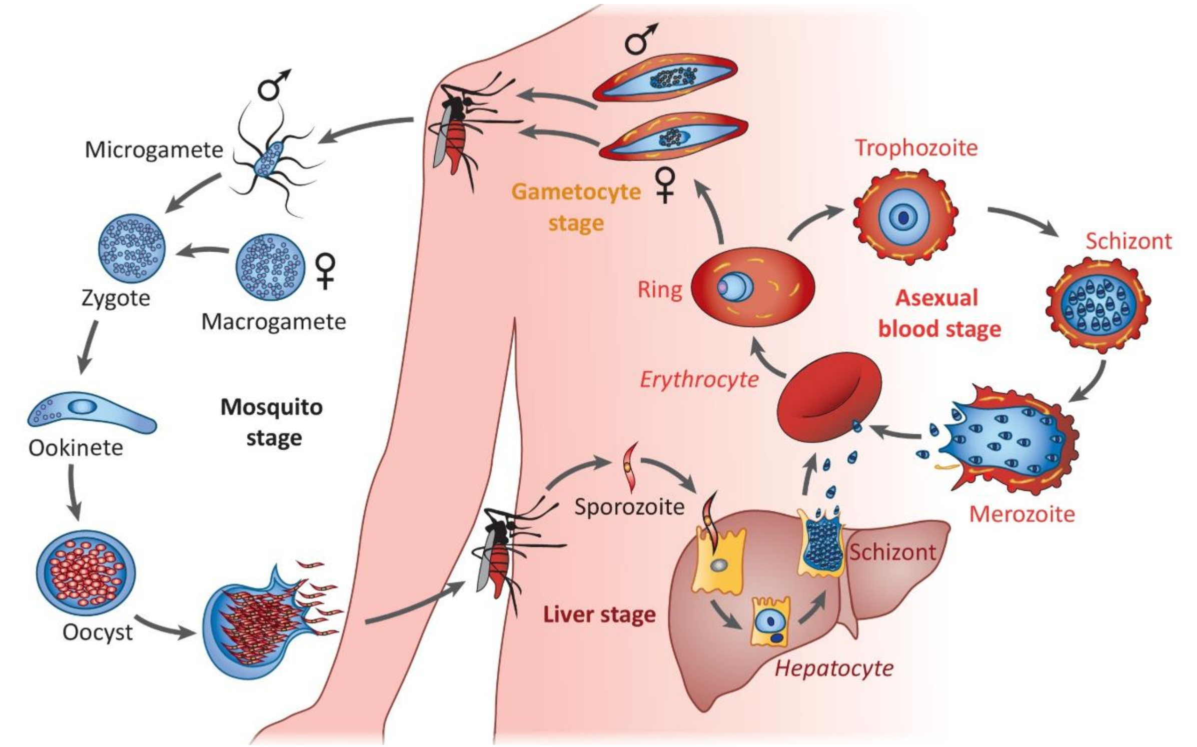


**Figure 1: The lifecycle of *Plasmodium*.** The developmental stages in the human host and the female *Anopheles* mosquito vector are illustrated. Source: Maier *et al.* (2019) *[12]*.

## 1.3 Malaria diagnosis

### 1.3.1 Malaria diagnosis in health care facilities and clinical trial sites

Microscopy is still considered the ‘gold-standard’ for malaria diagnosis and WHO recommends the microscopic examination and/or the use of Rapid Diagnostic Tests (RDTs), which are based on malaria antigen detection, as reference for the confirmation of suspected malaria prior to administering treatment [13]. Although these two techniques are valuable for the rapid and on-site detection of malaria, their limitations including the need for highly skilled microscopists, the lack of parasite quantification and identification of all species in the case of RDTs, as well as the relatively high detection limit of both methods (i.e. 10-100 and 100 parasite/ μL, respectively), have a big impact on malaria surveillance and epidemiology [14]. Furthermore, the detection of low-density malaria cases in countries with low levels of transmission is crucial for assessment of the interventions and the actual disease burden [15].

### 1.3.2 Molecular tools in malaria diagnosis

Molecular tools in malaria diagnosis have tremendously increased the sensitivity and accuracy of the parasite detection and identification, the last decades. Polymerase chain reaction (PCR) is considered the most sensitive molecular tool for malaria diagnosis and research. The highly conserved *Plasmodium* 18S ribosomal RNA (rRNA) gene and the cytochrome b gene are widely used as targets for amplification with species-specific and/or genus-specific primers in nested, multiplex, real-time and digital PCR with the former to be considered as the gold-method for *Plasmodium* species detection and differentiation. Having a detection limit of almost 100-fold lower (0.004-10 p/ μL) than both microscopy and RDTs, PCR provides accurate data on *Plasmodium* species determination even in low-density samples, can identify mixed infections, allows analysis of genetic variations and is crucial in assessing the effectiveness of antimalarial treatments [16]. Great efforts have been made to improve the availability of molecular and conventional diagnostic tools including new targets for PCR amplification and RDTs, lowering the limit of detection for microscopy and cell-based assays for the detection of malaria that highlight the importance of accurate and reliable tools to achieve a malaria-free world [17].

## 1.4 Malaria treatment

### 1.4.1 Artemisinin-based combination therapies

In 2001 WHO recommended the use of artemisinin-based combination therapies (ACTs) as first-line treatment for non-complicated malaria worldwide. In the ACTs, an artemisinin derivative and a partner drug are combined to achieve rapid parasite clearance and increase the effectiveness of the two drugs by reducing the risk of resistance development. More specifically, the artemisinin component rapidly reduces the number of the circulating parasites in the blood (parasite biomass) and the longer acting partner drug ensures complete parasite clearance, the prevention of a recurrent episode (cure) and resistance development against the artemisinin component [18]. The artemisinin derivatives are quickly eliminated from the body, whereas the partner drugs can be stable for a longer period [19]. In regards to the difference mode of actions, there are drugs that target almost all the developmental stages of the parasite inside the human body [20,21].

The selection and duration of treatment is dependent on the *Plasmodium* species, the epidemiological data, the transmission rate and the population group to be treated. There are five available ACTs for non-complicated *P. falciparum* infections for a recommended 3-day treatment: artemether-lumefantrine, artesunate-amodiaquine, artesunate-mefloquine, artesunate-sulfodaxine-pyrimethamine and dihydroartemisinin-piperaquine. For the treatment of *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi* infections, chloroquine or one of the five ACTs as for *P. falciparum* -in case of chloroquine resistance- are recommended. In the case of *P. vivax* and *P. ovale* infections both the circulating asexual parasites (blood stage) and the hypnozoites (liver stage) should be eliminated (radical cure) to prevent recurrent and relapse malaria, respectively. The use of primaquine, the only available hypnozoitocidal drug, is highly recommended by WHO after the initial treatment with chloroquine or ACTs. Furthermore, primaquine has gametocytocidal effects and is also used to reduce transmission in the regions with low prevalence of *P. falciparum*, *P. malariae* and *P. knowlesi* malaria [18,21].

### 1.4.2 Development of resistance against ACTs

Although the use of ACTs prevents the development of resistance against the artemisinin derivative, the prolonged presence of the partner drug in the body in low concentrations could trigger the development of increased partner drug tolerance and resistance [22]. Resistant parasites to artemisinin components and different partner drugs have been recorded during the last decade since ACTs have been extensively used as first-line treatment. The mechanism of resistance development usually involves single nucleotide polymorphisms (SNPs) in specific *Plasmodium* genes or changes in the gene’s copy number. Although *P. falciparum* and *P. vivax* resistant parasites to the used ACTs have been already detected, *P. ovale* and *P. malariae* seems to be sensitive to the chloroquine/ ACTs treatment [23,24]. The time required to eliminate the parasites from the body after the selected treatment, i.e. parasite clearance rate, is an effective way to evaluate the efficacy of the treatment and can also act as a first indicator of resistance development [25].

### 1.4.3 Therapeutic efficacy studies

The limited number of available antimalarial drugs and particularly, artemisinin derivatives raises high concerns about *Plasmodium* resistance development. WHO recommends the continuous ACTs evaluation by implementing *in vivo* therapeutic efficacy studies, which can provide timely information about the parasite's response to the drugs. In therapeutic efficacy studies, finger-prick blood samples are collected every 12 hours for the first 3 days after the treatment initiation, then, weekly until a defined follow-up period (28 or 42 days) and examined by microscopy to assess the parasite's sensitivity to the artemisinin derivative (parasite clearance rate) and to partner drug (radical cure rate), respectively. In case of microscopically detected recurrent parasitaemia within the follow-up period, PCR-genotyping is used to distinguish between a new or recrudescence infection, with the later to be considered as treatment failure. One important limitation of the therapeutic efficacy studies is the high reliance on the microscopy examination. Having a relatively high detection limit (10-100 parasites/ μL) compared to molecular methods such as PCR (less than 1 parasite/ μL), microscopy is inappropriate to detect infections with parasite densities below its detection limit resulting in underestimation of low-density infections and consequently misleading ACTs efficacy [26,27].

## 1.5 Reference study

Between October 2011 and February 2016, the West African Network for Clinical Trials of Antimalarial Drugs (WANECAM) [28] longitudinal study was conducted in Mali, Guinea and Burkina Faso (Sub-Saharan Africa). The primary aim of the study was to evaluate the efficacy and safety of second-line ACTs dihydroartemisinin-piperaquine and a new registered artesunate-pyronaridine in comparison to first-line ACTs artesunate-amodiaquine and artemether-lumefantrine when used for the treatment of repeated clinical malaria episodes for more than two years in West Africa. In total, 4.710 patients with symptoms of uncomplicated malaria were selected, enrolled and randomised to the four different treatment arms (dihydroartemisinin-piperaquine, artesunate-pyronaridine, artemether-lumefantrine, artesunate-amodiaquine). The inclusion/ exclusion criteria and the randomization of the treatment arms are extensively described in the publication [28].

Based on the microscopic examination, the WANECAM study showed that dihydroartemisinin-piperaquine and artesunate-pyronaridine had as high efficacy against *Plasmodium falciparum* as the two first-line ACTs in west Africa (Table 1). The efficacy of dihydroartemisinin-piperaquine and artesunate-pyronaridine, for non-falciparum species, was also equal to artemether-lumefantrine and artesunate-amodiaquine (i.e. greater than 99% at day 28 and greater than 84.6% at day 42), highlighting their use as competent alternatives for the repeated malaria treatment in the region. Surprisingly, artemether-lumefantrine treatment at day 42 had low efficacy against *Plasmodium malariae* and *Plasmodium ovale* infections (75% and 62.5%, respectively).

*Plasmodium malariae* and *Plasmodium ovale* are generally sensitive to ACTs and chloroquine treatment worldwide with no indication of resistance development; only one study has reported *Plasmodium malariae* resistance to chloroquine, so far [29–32]. This is the only study to date that highlights the decreased efficacy of the first-line ACT artemether-lumefantrine against *Plasmodium malariae* and *ovale* infections in West Africa. Further investigation and confirmation of the microscopic data is warranted in order to further assess the efficacy of ACTs in low density *Plasmodium malariae* and *Plasmodium ovale* infections in Sub-Saharan Africa.

**Table 1: Summary of the efficacy of the four ACTs against *P. falciparum*, *P. malariae* and *P. ovale* infections**

| | **Dihydroartemisinin-piperaquine** | **Artesunate-pyronaridine** | **Artemether-lumefantrine** | **Artesunate-amodiaquine** |
|---|---|---|---|---|
| **Efficacy** | | | | |
| • **PCR-adjusted ACPR*** | | | | |
| *P. falciparum* | | | | |
| Day 28 | > 99.6% | > 99.5% | > 98.2% | > 99.1% |
| Day 42 | > 97.7% | > 98.5% | > 97.7% | > 98.6% |
| • **PCR-unadjusted ACPR** | | | | |
| *P. falciparum* | | | | |
| Day 28 | > 98.5% | > 94.5% | > 78.9% | > 87.3% |
| Day 42 | > 93.1% | > 73.2% | > 62.8% | > 68.4% |
| *P. malariae* | | | | |
| Day 28 | > 99.9% | > 99.9% | > 99.9% | > 98.6% |
| Day 42 | > 98.7% | > 87.1% | > 75% | > 85.1% |
| *P. ovale* | | | | |
| Day 28 | > 99.9% | > 99.9% | > 99.9% | > 99.9% |
| Day 42 | > 99.9% | > 84.6% | > 62.5% | > 99.9% |
| **Total number of patients** | 1340 | 1342 | 967 | 1061 |

*ACPR: Adequate clinical and parasitological response

## 2. Aim

The aim of this project was to verify the microscopic results using a highly sensitive PCR-based method and to further assess the parasite clearance after ACT treatment in order to confirm the efficacy of ACTs against *Plasmodium malariae* and *Plasmodium ovale* infections. However, we were unable to address this aim due to various challenges encountered during the molecular analyses as will be described in the results section.

## 3. Materials and Methods

### 3.1 Collection of field samples

For the aim of this thesis, samples collected during the WANECAM I study in 2011-2016 in Bougoula and Maferenya centres in Mali and Guinea were analysed. Finger prick blood samples on Whatman 3MM filter paper (GE Healthcare, Buckinghamshire, UK) were collected by the medical staff in the study centres at day 0 prior to treatment, every 12 ± 2 hours after the time 0 (time when the first dose was administered) for 72 hours or until two sequential samples were microscopically parasite-negative as well as at days 7, 14, 21, 28, 35, 42 and 63 [28]. All samples were stored in sealed plastic bags within an envelope without desiccants at room temperature in the trial sites. Samples with confirmed *P. malariae* and *P. ovale* mono- or co-infections, determined by microscopy that had been treated with dihydroartemisinin-piperaquine were included in the molecular analyses of this study. For these patients, the filter papers from all treated episodes as well as from the recurrent episodes were selected for analysis. The selected timepoints for every patient were: i) day 0 until the first timepoint in which the blood sample evaluated as parasite-free on microscopy examination, ii) day 42 and/or day 63 and iii) day 0 of recurrent episodes. In total 241 selected samples from 53 patients were collected from the study site in Bougoula in February 2019. Samples were organised by patient reference number (ID) and placed in plastic bags with desiccant and transported to Karolinska Institutet, Sweden for molecular analysis. Five of the 241 samples were missing microscopy data and were excluded from the molecular analyses.

### 3.2 Blood slide microscopy

Thin and thick Giemsa stained blood smears were prepared and analysed at the study sites, in accordance with WHO guidelines [33]. Parasitaemia was quantified by standard approximation method (40 * number of parasites per 200 white blood cells). If no sexual or asexual parasites found in 2.500 WBCs or 200 high-power fields, the sample was declared as parasite negative. Two independent, trained microscopists, masked to the selected treatment, were assigned to examine the blood smears and a third microscopist was assigned in case of contradictory results from the first two examiners.

### 3.3 Laboratory controls for PCR detection of Plasmodium species

Positive and negative controls were used as reference for the *Plasmodium* species detection and identification by PCR. 3D7 *P. falciparum* laboratory cultured parasites were used as *P. falciparum* positive controls, while blood samples from patients diagnosed with *P. malariae, P. ovale* and *P. vivax* infections at the Karolinska University Hospital, Sweden were served as *P. malariae, P. ovale* and *P. vivax* positive controls. Parasitaemia was defined as described above. Positive controls for all four *Plasmodium* species with parasite densities of 10 parasites/ µL and 1 parasite/ µL were generated by dilution in parasite negative whole blood. 30 µL of each control was spotted onto Whatman 3MM paper (GE Healthcare, Buckinghamshire, UK), left to air-dry and kept at room temperature. After DNA extraction, dilutions in distilled water ($dH_2O$) were made for the positive controls to reach the equivalence of 5 parasites/ µL. Parasite negative whole blood from healthy individuals was also spotted onto filter paper and extracted together with the samples as a negative control. A no template control (NTC) of only deionized water was added during the PCR.

### 3.4 Sample preparation for DNA extraction

For the control samples, two or three 3 mm punches were cut using paper punchers. Field samples were cut using a scissor to achieve the equivalent amount of blood as in the three 3mm control punches i.e. approximately 3 pieces of 3x3 mm with blood saturated on both sides. If the filter papers were not saturated, more pieces were used to ensure comparable amount of blood between field and control samples. Both the scissors and paper punchers were rinsed with tap water following with 70% and 90% ethanol and air-dried before cutting to avoid cross-contamination between samples. The paper discs and punches from each sample were collected in 1.5 ml microfuge tubes for DNA extraction. Each piece/punch of filter paper was estimated to have approximately 3-5 µL of blood if the filter paper was saturated on both sides.

### 3.5 DNA extraction from the cut filter papers

#### 3.5.1 Chelex®-100 DNA extraction

Genomic DNA was extracted using the Chelex®-100 method [34]. In brief, the paper discs were incubated in 800 µL of 0.2% saponin (Sigma-Aldrich, St Louis, MO, USA) in phosphate-buffered saline (PBS; Gibco, Paisley, UK) for 5 minutes on shaker at room temperature followed by 30 minutes incubation in 800 μL of PBS under the same conditions. After removal of PBS, 100 μL of 10% Chelex®-100 (Bio-Rad, Hercules, CA, USA) in deionized water (Sigma-Aldrich, St Louis, MO, USA) was added to the paper discs, which were incubated at 95 °C for 10 minutes and centrifuged for 3 minutes at 12000 rpm. Finally, the supernatant containing genomic DNA was isolated after 5 minutes centrifugation at 12000 rpm. Extracted DNA was stored at 4 °C for short term or at -20 °C for long term storage.

### 3.5.2 QIAamp DNA mini kit extraction

Genomic DNA was extracted using the QIAamp DNA mini kit (Qiagen Gmbh, Germany) according to the manufacturer's instructions and the protocol 'DNA purification from dried blot spots' [35]. In the final step the genomic DNA was incubated for 5 minutes at room temperature with 100 μL or 150 μL elution buffer before the final centrifugation. For samples, which were unsuccessfully extracted with Chelex®-100 boiling method, the QIAamp mini kit was used to re-extract the genomic DNA from these paper discs if they were available. Extracted DNA was stored as above.

## 3.6 Quantitative PCR for *Plasmodium* species detection

### 3.6.1 Cytb quantitative PCR (cytb-qPCR)

A SYBR Green real-time PCR method targeting the *Plasmodium* conserved cytochrome b gene (cytb-qPCR), followed by restriction fragment length polymorphism [36] was used for parasite detection and species identification. Briefly, 5 μL of extracted DNA was used as a template in a final volume of 20 or 50 μL PCR reaction with 1X SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) and 0.25 μM of forward (PlasMtF) and reverse (PlasMtR) primers, respectively (Table 1). Increasing the DNA template to 8 μL and 12.5 μL was also assessed in a final volume of 50 μL as above. Samples were run first in duplicates or triplicates based on parasite densities (PCR final volume 20 μL). If negative in the initial assessments, the cytb-qPCR was repeated in triplicates in a higher PCR reaction volume (50 μL). *P. falciparum, P. malariae* and *P. ovale* positive controls and negative controls were included in each plate of samples. The thermal profile for this qPCR was: 95 °C for 4 minutes, 45 cycles at 95 °C for 15 seconds and 60 °C for 90 seconds and took place in the CFX96 Touch™ Real Time PCR Detection System (Bio-Rad, Hercules, CA). The signal acquisition was obtained at the end of each cycle using SYBR Green as fluorophore, although PCR positivity was defined as the presence of PCR amplicons after gel-electrophoresis in 1.5% agarose gels stained with RedGel (Biotinum Inc, Hayward, CA, USA).

### 3.6.2 18S rRNA probe-based quantitative PCR (18S-qPCR_K)

An additional probe-based real-time PCR targeting the highly conserved *Plasmodium* 18S ribosomal RNA was used for *Plasmodium* detection [37]. Briefly, 5 μL of extracted DNA was used as template in a final volume of 20 μL with 1X SsoAdvanced™ Universal Probes Supermix (Bio-Rad, Hercules, CA, USA), 0.25 μM of forward (KamauF) and reverse (KamauR) primers and 0.125 μM of 5'-FAM-labelled KamauProbe (Table 1). All samples were run in duplicates, with positive and negative controls included. The thermal profile for this qPCR was: 95 °C for 3 minutes, 45 cycles at 95 °C for 15 seconds and 60 °C for 60 seconds and took place in the CFX96 Touch™ machine. PCR positivity was defined as exponential DNA amplification with a Cq value below 40.

### 3.6.3 HumTubB probe-based quantitative PCR (HumTubB-qPCR_HumTubB)

A modified version of a probe-based quantitative PCR [38] was used for the detection of human DNA. Briefly, 5 μL of extracted DNA was used as template in a final volume of 20 μL with 1X SsoAdvanced™ Universal Probes Supermix (2X), 0.25 μM of forward (HumTuBBF) and reverse (HumTuBBR) primers and 0.125 μM of 5’-VIC labelled HumTuBBprobe (Table 1). All samples were run in duplicates, with positive and negative controls included. The thermal profile for this qPCR was: 95 °C for 3 minutes, 45 cycles at 95 °C for 15 seconds and 60 °C for 60 seconds and took place in the CFX96 Touch™ machine. PCR positivity was defined as exponential DNA amplification with a Cq value below 40.

**Table 2: Sequences of used primers and probes for Plasmodium species detection.**

| *Name of primers/ probe* | *Sequence of primers/ probes* |
|---|---|
| *PlasMtF* | 5’-TGG TAG CAC AAA TCC TTT AGG G-3’ |
| *PlasMtR* | 5’-TGG TAA TTG ACA TCC AAT CC-3’ |
| *KamauF* | 5’-GCT CTT TCT TGA TTT CTT GGA TG-3’ |
| *KamauR* | 5′-AGC AGG TTA AGA TCT CGT TCG-3’ |
| *KamauProbe* | 5’-6-FAM-ATG GCC GTT TTT AGT TCG TG-TARMA-3’ |
| *HumTuBBF* | 5’-AAG GAG GTC GAT GAG CAG AT-3’ |
| *HumTuBBR* | 5’-GCT GTC TTG ACA TTG TTG GG-3’ |
| *HumTuBBprobe* | 5’-VIC-TTA ACG TGC AGA ACA AGA ACA GCA GCT -TAMRA-3’ |

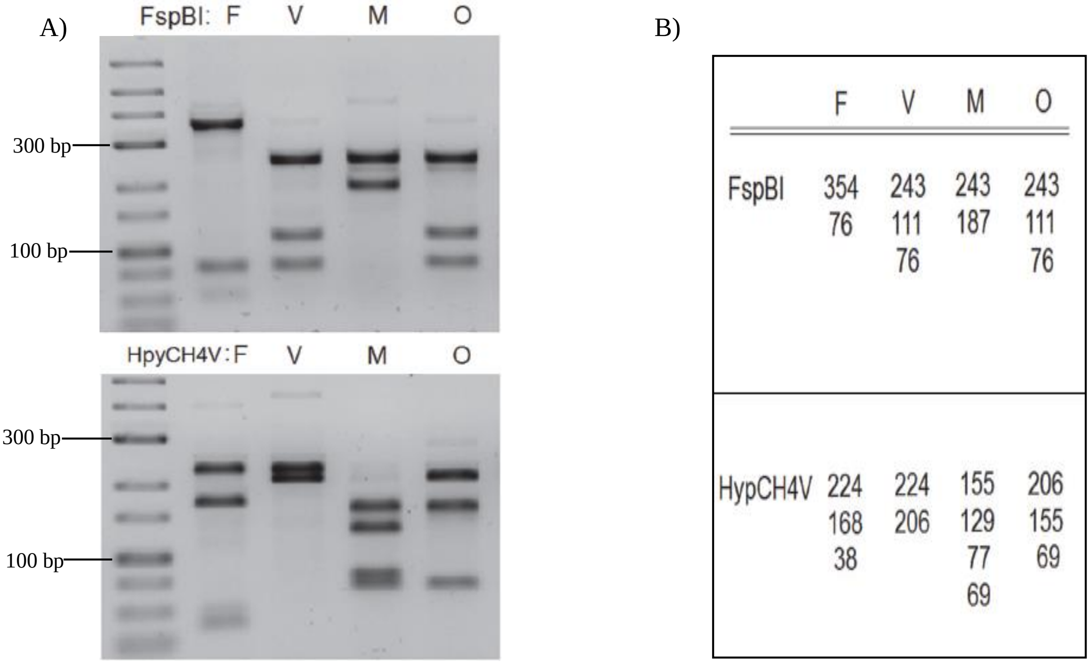
*All primers and probes were from ThermoFisher Scientific, Waltham, MA, USA

### 3.7 Gel electrophoresis

The visualization of the cytb-qPCR and RFLP reaction products was done in 1.5% and 2% UltraPure™ agarose gels (ThermoFisher Scientific, Waltham, MA, USA) in 1X TBE buffer (ThermoFisher Scientific, Waltham, MA, USA), respectively and stained with GelRed™ Nucleic Acid Gel stain. The cytb-qPCR and RFLP products were mixed with loading dye (Promega, WI, USA) prior to electrophoresis and 3 μL of GeneRuler™ Low Range DNA Ladder (ThermoFisher Scientific, Waltham, MA, USA) in loading dye was used as DNA ruler. A UV transilluminator or a ChemiDoc™ Imagining System (Bio-Rad, Hercules, CA, USA) with the ImageLab™ Software were utilized for the final visualization and imaging of the gel.

### 3.8 Restriction fragment length polymorphism (RFLP) analysis

The cytb-qPCR products identified as positive by gel electrophoresis were digested with FspBI restriction enzyme (ThermoFisher Scientific, Waltham, MA, USA). Briefly, 5 μL of cytb-qPCR product were digested in a final volume of 25 μL with 1X Tango buffer and 10 Units of FspBI enzyme (ThermoFisher Scientific). After 16 hours digestion at 37 °C, in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA), the RFLP products were run in 2% UltraPure™ agarose gel stained with RedGel until sufficient separation of the DNA fragments. In case of unclear pattern recognition, especially for potential *P. vivax* and *P. ovale* infections, 5 μL of the cytb-qPCR products were digested in a final volume 25 μL with 1X of Cutsmart® buffer and 5 Units of HpyCH4V restriction enzyme (New England Biolabs, Hitchin, UK) for 16 hours at 37 $^{o}$C. The gel electrophoresis of the cytb-qPCR RFLP products, the analysis of the gel and the species determination were done as described above. Strong PCR amplicons were diluted in $dH_2O$ before adding to the RFLP reaction. Positive controls were used as pattern reference for comparison with the unknown samples (Figure 2).



|  | F | V | M | O |
|---|---|---|---|---|
| FspBI | 354<br>76 | 243<br>111<br>76 | 243<br>187 | 243<br>111<br>76 |
| HypCH4V | 224<br>168<br>38 | 224<br>206 | 155<br>129<br>77<br>69 | 206<br>155<br>69 |

**Figure 2: RFLP analysis and determination of the major four *Plasmodium* species.** A) The digestion patterns of the 430 bp cytb-qPCR product using FspBI (top) and HpyCH4V (bottom) restriction enzymes. Ladder: GeneRuler™ Low Range DNA Ladder, B) The lengths (in bp) of the digested product with FspBI and HpyCH4V enzymes for the four major *Plasmodium* species. F= *P. falciparum*; V= *P. vivax*; M= *P. malariae*; O= *P. ovale*. Source: Xu *et al.,* 2015 *[36]*

### 3.9 Measurement of DNA concentration

A NanoDrop™ 2000/ 2000c Spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) was utilized to measure the concentration of QIAamp extracted genomic DNA eluted in 150 μL elution buffer in both field and control samples following manufacturer's instructions.

### 3.10 Statistical analysis

The 95% confidence interval (CI95%) was calculated for cytb-qPCR success rates. The level of agreement between the microscopy and cytb-qPCR results was evaluated by Cohen's kappa (κ) analysis. Statistical analyses were conducted using Excel and IBM SPSS Statistics.

### 3.11 Ethical considerations

The WANECAM I clinical trial (trial registration number: ACTR201105000286876) was performed in accordance with the Declaration of Helsinki and ethical approval was obtained from the local ethics committees in Mali, Burkina Faso and Guinea. For all the participants, a written informed consent was provided prior to their enrolment in the study. In the case of children, a written consent was obtained by their parents/guardians. Ethical approval for the molecular analysis carried out at Karolinska Institutet, was obtained from the Regional Ethics Review Board in Stockholm, Sweden (Dnr: 2017/499-32).

# 4. Results

## 4.1 Optimization of the starting material for detection of low-density *Plasmodium* infections

Previous work in the research group showed that the Chelex®-100 boiling method provides the highest sensitivity when extracting low density samples [39]. Therefore, the optimal number of punches required for the detection of low-density *P. malariae* and *P. ovale* infections with Chelex®-100 method was investigated using positive controls. The cytb-qPCR success rate ranged between 16.7-55.6% when DNA was extracted from 2 punches and 87.5-94.4% when DNA was extracted from 3 punches for the detection of the lowest 1 p/ µL parasite density controls (Figure 3). The cytb-qPCR success rate was 100% for the detection of *P. malariae* and *P. ovale* parasite densities 5 and 10 p/ µL for 2 and 3 punches. The cytb-qPCR reproducibility was typically 1-2 positives/triplicate for 1 p/ µL controls and 2-3 positives/triplicate for controls with parasite densities 5 p/ µL and 10 p/ µL.

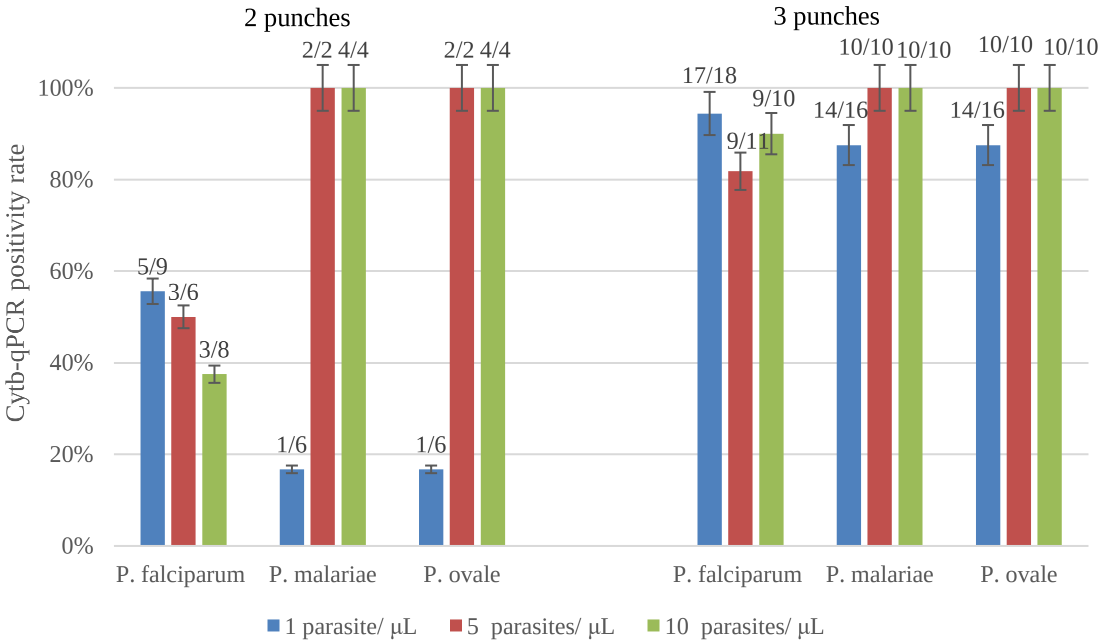


**Figure 3: Comparison of cytb-qPCR repeatability between DNA extraction from 2 punches and 3 punches.** Schematic representation of the cytb-qPCR positivity rate when Chelex®-100 extraction method was used to extract DNA from positive controls using 2 or 3 punches. The PCR positivity is indicated as n/N over each column. n= number of positives; N= total repeats.

## 4.2 Insufficient *Plasmodium* species detection after Chelex®-100 extraction

After optimising the starting material to 3 punches/pieces for adequate DNA extraction and species detection, analyses of the first 35 field samples were done using Chelex®-100 and the cytb-qPCR RFLP method. The PCR success rate was 39.3% (11/28; CI95% 21.5-59.4) when DNA was extracted with Chelex®-100 method (Figure 4). Only 2/12 microscopically confirmed *P. malariae* monoinfections and no coinfections (*P. falciparum* and *P. malariae*) were identified. There was low agreement between the microscopy and qPCR results (κ= 0.23, p=0.001). Low cytb-qPCR reproducibility was also observed in the qPCR positive samples (1-2 positives/triplicate).

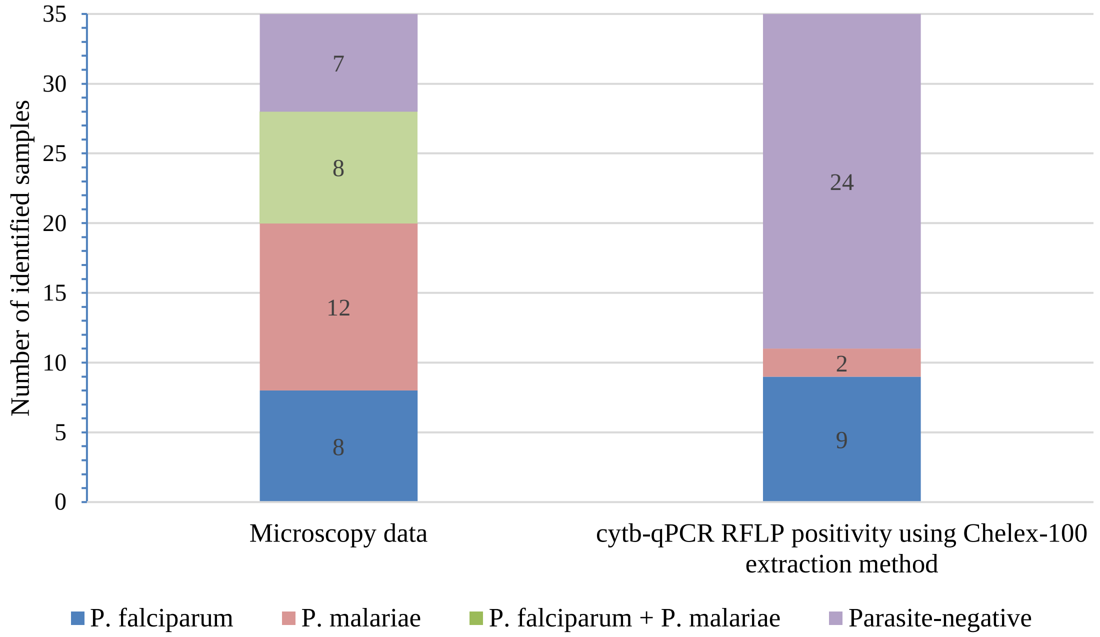


**Figure 4: Plasmodium species detection and identification by cytb-qPCR and RFLP analysis in 35 samples using Chelex®-100 method**. The two columns represent the microscopy data (left column) and the molecular data (right column) as derived from the molecular analysis of these samples. The successfully identified samples are coloured with blue for *P. falciparum* monoinfections, orange for *P. malariae* monoinfections, green for *P. falciparum* and *P malariae* coinfections and purple for samples detected as parasite negative.

### 4.3 Comparison of Chelex®-100 method and QIAamp column-based extraction method

Previous work with high density *P. falciparum* samples from the same clinical trial showed that QIAamp column based extraction performed better in downstream qPCR analysis compared to Chelex®-100 extraction. Thus, the two methods were compared using 25 samples with medium-low parasite densities. The PCR success rate was 15% (3/20; CI95% 3.2-37.9) when DNA was extracted with Chelex®-100 and 55% (11/20; CI95% 31.5-76.9) when DNA was extracted with QIAamp column-based method (Table 3). Only 6.7% (1/15) of the samples with medium-low parasite densities (below 1000 p/ µL) were identified using Chelex®-100 and 40% (6/15) of the samples when QIAamp column-based extraction was used. No coinfections were identified and low cytb-qPCR reproducibility was detected in both methods (1-2 positives/triplicate).

**Table 3: Comparison of two extraction methods with microscopy data.** In total 25 field samples were extracted with both Chelex®-100 and QIAamp DNA mini kit. The results of the cytb-qPCR were compared with the microscopy data.

| Analysed samples | Microscopy | PCR positivity when Chelex®-100 extraction method used | PCR positivity when QIAamp DNA mini kit used |
|---|---|---|---|
| **Parasite-positive by species:** | | | |
| ▪ *P. falciparum* monoinfections | 3 | 2 | 8 |
| ▪ *P. malariae* monoinfections | 12 | 1 | 3 |
| ▪ *P. falciparum* + *P. malariae* coinfections | 5 | 0 | 0 |

| | | | |
|---|---|---|---|
| **Parasite positive by parasite density:** | | | |
| ▪ > 1000 parasite/ µL | 5 | 2 | 5 |
| ▪ 600 -1000 parasite/ µL | 3 | 0 | 3 |
| ▪ < 600 parasite/ µL | 12 | 1 | 3 |
| **Total positive** | 20 | 3 | 11 |
| **Total negative** | 5 | 22 | 14 |

### 4.4 Comparing old and new primers for the cytb-qPCR

Although QIAamp column based extraction increased the PCR positivity in comparison to Chelex®-100, the cytb-qPCR success rate was still lower than expected since it is supposed to be much more sensitive than microscopy. Therefore, the cytb-qPCR RFLP method was evaluated using a set of new and old primers. Three *P. falciparum* field samples with medium-low parasite densities and positive and negative controls were extracted with the QIAamp column-based method and analyzed by cytb-qPCR with both one and old primers. The qPCR success rate was 100% (9/9; CI95% 66.4-100) for the new primers and 66.7% (6/9; CI95% 29.9-92.5) for the old primers (Figure 4). The cytb-qPCR reproducibility was relatively high and similar between the new and old primers (2-3 positives/triplicate).

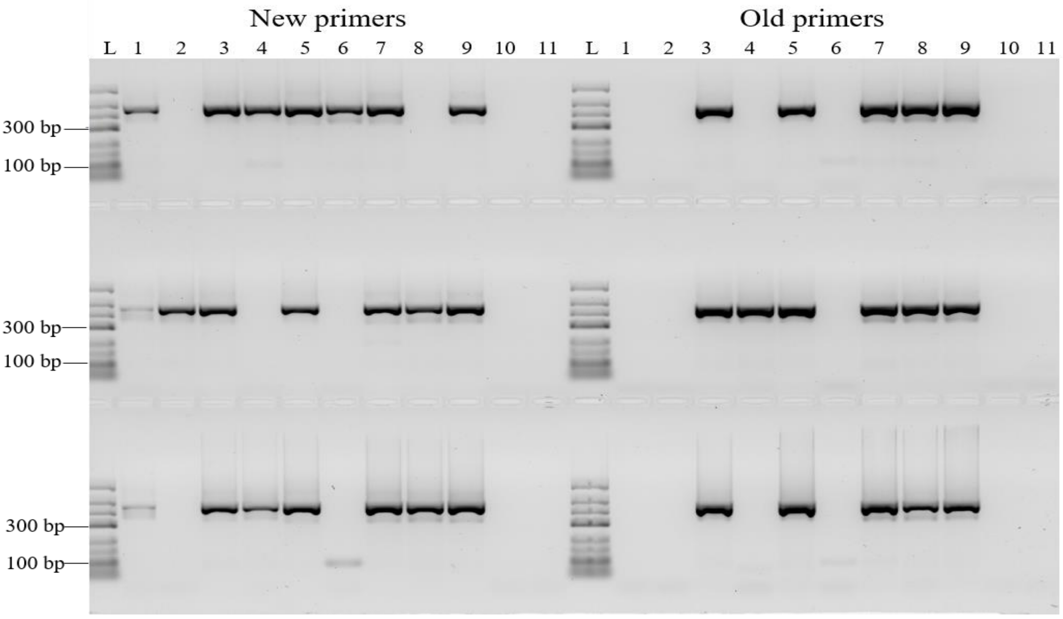


**Figure 5: Comparison of cytb-qPCR repeatability with new and old primers.** The cytb-qPCR method was tested using a new (left) and old set of primers (right). The cytb 430 bp amplicon was detected between the 500 and 400bp ladder bands. All samples run as triplicates. L= GeneRuler™ Low Range DNA Ladder; 1-3= *P. falciparum* field samples with parasite densities of 120 p/ µL, 104 p/ µL, 174 p/ µL, respectively; 4= *P. falciparum* control 1 p/ µL; 5= *P. falciparum* control 10 p/ µL; 6= *P. malariae* control 1 p/ µL; 7= *P. malariae* control 10 p/ µL; 8= *P. ovale* control 1 p/ µL; 9= *P. ovale* control 10 p/ µL; 10= parasite-negative blood; 11= NTC

### 4.5 Detection of human housekeeping gene and 18S rRNA gene

After continued low PCR success rate in the analysis of middle/low density field samples using QIAamp column-based method and new set of primers, qPCRs targeting the human beta-tubulin housekeeping gene and *Plasmodium* 18S rRNA gene were used as a control for ensuring successful DNA extraction from the filter papers. In total, 42 QIAamp extracted field samples and

7 controls were analysed. The PCR success rate was 100% (49/49; CI95% 92.7-100) for the human beta-tubulin and *Plasmodium* 18S rRNA (Figure 6). A Cq value around 30 was observed for the beta-tubulin. The reproducibility was high (2 positives/duplicate) for the two genes. In the case of 18S rRNA gene, signal in the parasite-negative blood samples, serving as negative controls, was obtained in three independent experiments indicating non-specific amplification or probe degradation (Figure 7). When the same negative controls were re-analysed with cytb-qPCR, no signal was obtained (Figure 8).

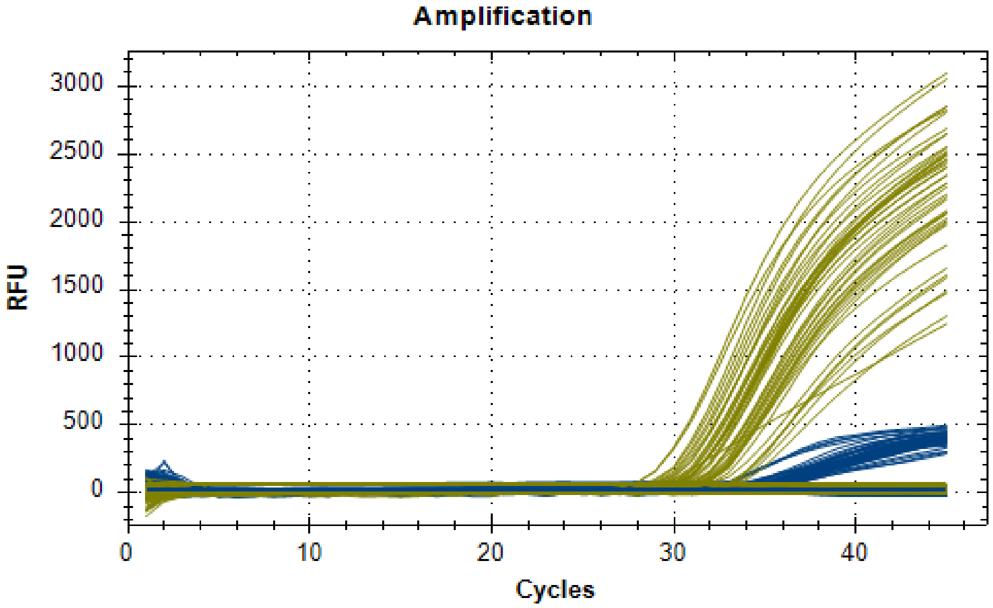


**Figure 6: Simultaneous detection of human beta-tubulin gene (light brown) and 18S rRNA gene of *Plasmodium* species (blue) in field samples.** The samples were run in duplicates.

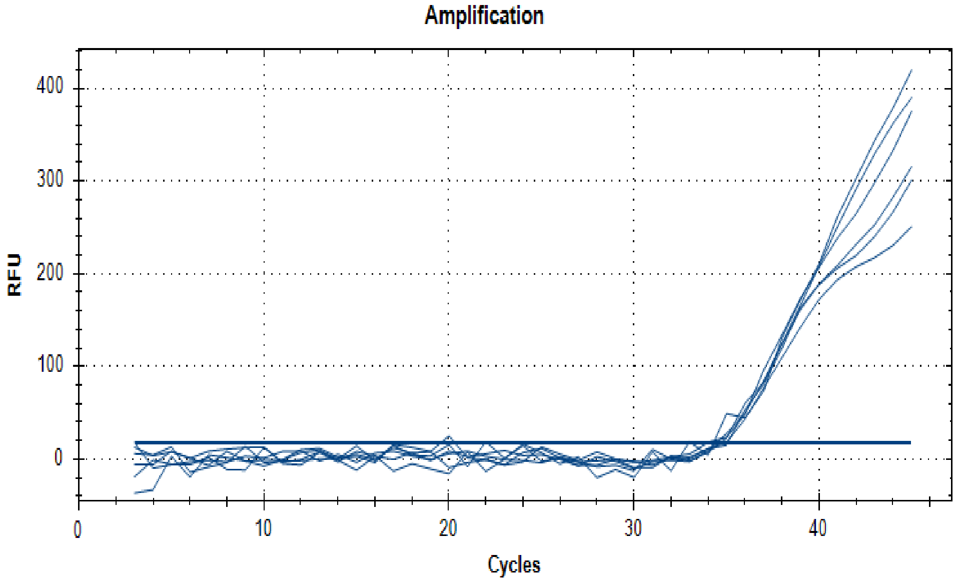


**Figure 7: Non-sigmoidal signal detection in the *Plasmodium* 18S rRNA qPCR in parasite-negative blood samples.** Three independent parasite-negative blood samples were used. The samples run in duplicates.

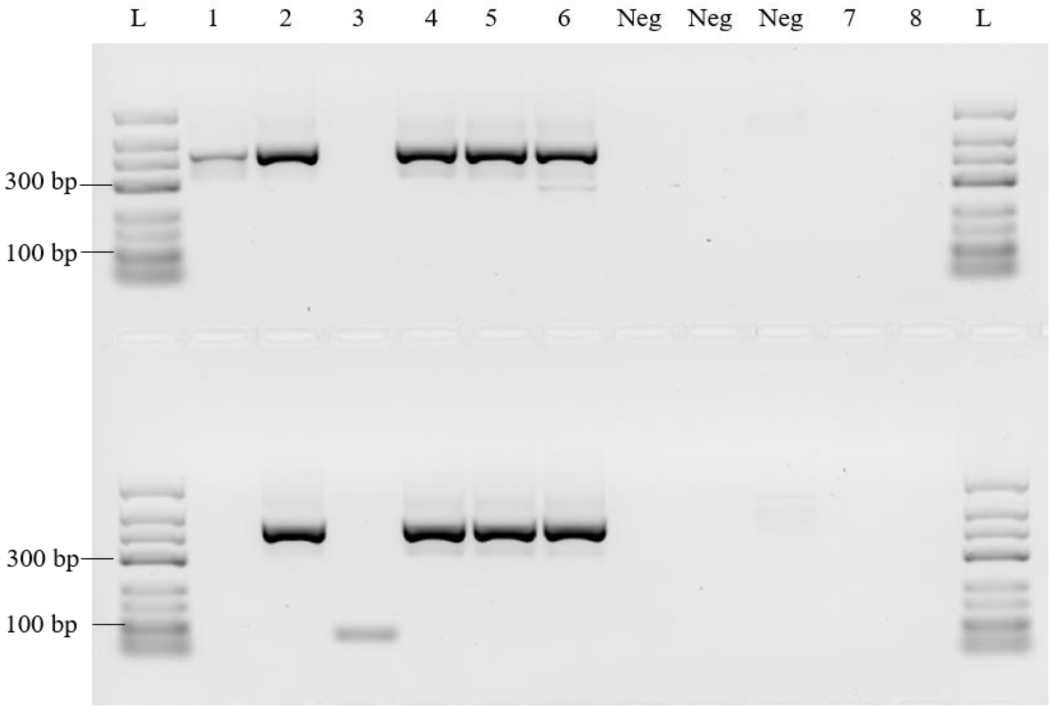


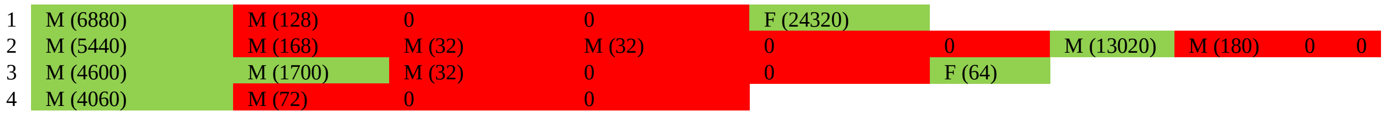
**Figure 8: Cytb-qPCR RFLP analysis of parasite-negative blood and positive controls.** The same parasite-negative blood controls as for the detection of 18S rRNA gene were used. The samples run in duplicates. L= GeneRuler™ Low Range DNA Ladder; 1= *P. falciparum* control 1 p/ µL; 2= *P. falciparum* control 10 p/ µL; 3= *P. malariae* control 1 p/ µL; 4= *P. malariae* control 10 p/ µL; 5= *P. ovale* control 1 p/ µL; 6= *P. ovale* control 10 p/ µL; Neg= parasite-negative blood; 7-8= NTC.

## 4.6 Measurement of DNA concentration

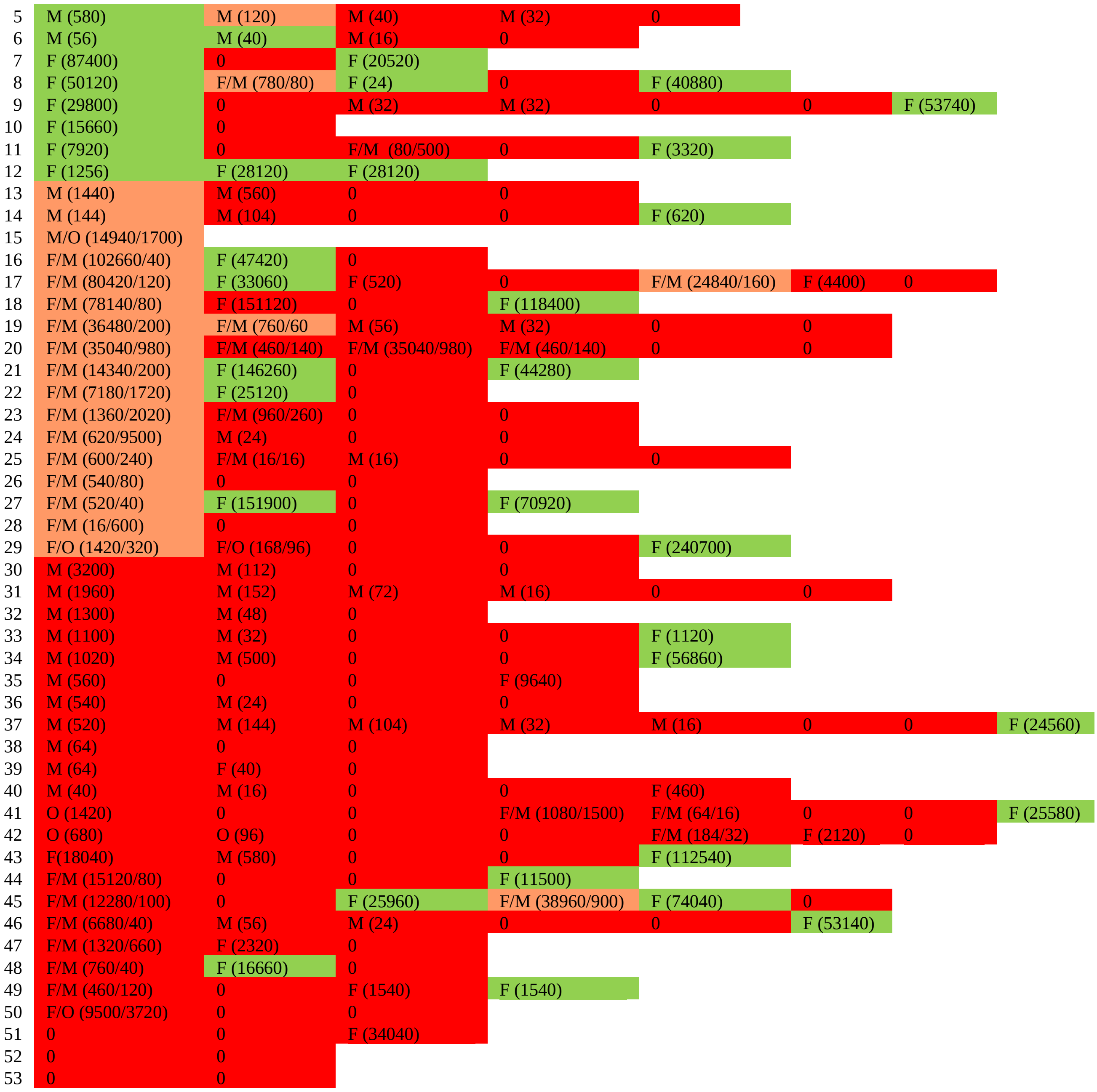
Having verified the successful QIAamp column-based DNA extraction of the field samples by detection of the human beta-tubulin gene, the DNA concentration and indicator of DNA purity was measured using a spectrophotometer. In total 30 QIAamp extracted samples were tested. From those, 10/30 samples had a DNA concentration higher or equivalent to 2 ng/ µL, i.e. the limit of detection of the spectrophotometer, with the A260/280 ratio fluctuating between 1.14 and 2.40 and 20/30 samples had DNA concentration below 2 ng/ µL. The acquired curves lacked the expected characteristic peaks at A260 and A280 wavelengths (data not shown).

## 4.7 Plasmodium species detection and determination in field samples by cytb-qPCR RFLP analysis

In total 236 field samples from 53 patients were analysed by QIAamp DNA extraction followed by cytb-qPCR with the new set of primers and RFLP for species determination. The cytb-qPCR success rate was 48.2% (69/143 microscopy positive samples; CI95% 39.8-56.7) (Figure 9). Overall, 10 *P. malariae* monoinfections, 2 *P. ovale* monoinfection and 57 *P. falciparum* monoinfections were identified. No coinfections and no other *Plasmodium* species were detected by RFLP analysis. A relatively low cytb-qPCR reproducibility was observed (1-2 positives/triplicate or 1 positive/5 or 6 repeats).



| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | M (6880) | M (128) | 0 | 0 | F (24320) | | | | | |
| 2 | M (5440) | M (168) | M (32) | M (32) | 0 | 0 | M (13020) | M (180) | 0 | 0 |
| 3 | M (4600) | M (1700) | M (32) | 0 | 0 | F (64) | | | | |
| 4 | M (4060) | M (72) | 0 | 0 | | | | | | |



| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 5 | 🟩 M (580) | 🟧 M (120) | 🟥 M (40) | 🟥 M (32) | 🟥 0 | | | |
| 6 | 🟩 M (56) | 🟩 M (40) | 🟥 M (16) | 🟥 0 | | | | |
| 7 | 🟩 F (87400) | 🟥 0 | 🟩 F (20520) | | | | | |
| 8 | 🟩 F (50120) | 🟧 F/M (780/80) | 🟩 F (24) | 🟥 0 | 🟩 F (40880) | | | |
| 9 | 🟩 F (29800) | 🟥 0 | 🟥 M (32) | 🟥 M (32) | 🟥 0 | 🟥 0 | 🟩 F (53740) | |
| 10 | 🟩 F (15660) | 🟥 0 | | | | | | |
| 11 | 🟩 F (7920) | 🟥 0 | 🟥 F/M (80/500) | 🟥 0 | 🟩 F (3320) | | | |
| 12 | 🟩 F (1256) | 🟩 F (28120) | 🟩 F (28120) | | | | | |
| 13 | 🟧 M (1440) | 🟥 M (560) | 🟥 0 | 🟥 0 | | | | |
| 14 | 🟧 M (144) | 🟥 M (104) | 🟥 0 | 🟥 0 | 🟩 F (620) | | | |
| 15 | 🟧 M/O (14940/1700) | | | | | | | |
| 16 | 🟧 F/M (102660/40) | 🟩 F (47420) | 🟥 0 | | | | | |
| 17 | 🟧 F/M (80420/120) | 🟩 F (33060) | 🟥 F (520) | 🟥 0 | 🟧 F/M (24840/160) | 🟥 F (4400) | 🟥 0 | |
| 18 | 🟧 F/M (78140/80) | 🟥 F (151120) | 🟥 0 | 🟩 F (118400) | | | | |
| 19 | 🟧 F/M (36480/200) | 🟧 F/M (760/60 | 🟥 M (56) | 🟥 M (32) | 🟥 0 | 🟥 0 | | |
| 20 | 🟧 F/M (35040/980) | 🟥 F/M (460/140) | 🟥 F/M (35040/980) | 🟥 F/M (460/140) | 🟥 0 | 🟥 0 | | |
| 21 | 🟧 F/M (14340/200) | 🟩 F (146260) | 🟥 0 | 🟩 F (44280) | | | | |
| 22 | 🟧 F/M (7180/1720) | 🟩 F (25120) | 🟥 0 | | | | | |
| 23 | 🟧 F/M (1360/2020) | 🟥 F/M (960/260) | 🟥 0 | 🟥 0 | | | | |
| 24 | 🟧 F/M (620/9500) | 🟥 M (24) | 🟥 0 | 🟥 0 | | | | |
| 25 | 🟧 F/M (600/240) | 🟥 F/M (16/16) | 🟥 M (16) | 🟥 0 | 🟥 0 | | | |
| 26 | 🟧 F/M (540/80) | 🟥 0 | 🟥 0 | | | | | |
| 27 | 🟧 F/M (520/40) | 🟩 F (151900) | 🟥 0 | 🟩 F (70920) | | | | |
| 28 | 🟧 F/M (16/600) | 🟥 0 | 🟥 0 | | | | | |
| 29 | 🟧 F/O (1420/320) | 🟥 F/O (168/96) | 🟥 0 | 🟥 0 | 🟩 F (240700) | | | |
| 30 | 🟥 M (3200) | 🟥 M (112) | 🟥 0 | 🟥 0 | | | | |
| 31 | 🟥 M (1960) | 🟥 M (152) | 🟥 M (72) | 🟥 M (16) | 🟥 0 | 🟥 0 | | |
| 32 | 🟥 M (1300) | 🟥 M (48) | 🟥 0 | | | | | |
| 33 | 🟥 M (1100) | 🟥 M (32) | 🟥 0 | 🟥 0 | 🟩 F (1120) | | | |
| 34 | 🟥 M (1020) | 🟥 M (500) | 🟥 0 | 🟥 0 | 🟩 F (56860) | | | |
| 35 | 🟥 M (560) | 🟥 0 | 🟥 0 | 🟥 F (9640) | | | | |
| 36 | 🟥 M (540) | 🟥 M (24) | 🟥 0 | 🟥 0 | | | | |
| 37 | 🟥 M (520) | 🟥 M (144) | 🟥 M (104) | 🟥 M (32) | 🟥 M (16) | 🟥 0 | 🟥 0 | 🟩 F (24560) |
| 38 | 🟥 M (64) | 🟥 0 | 🟥 0 | | | | | |
| 39 | 🟥 M (64) | 🟥 F (40) | 🟥 0 | | | | | |
| 40 | 🟥 M (40) | 🟥 M (16) | 🟥 0 | 🟥 0 | 🟥 F (460) | | | |
| 41 | 🟥 O (1420) | 🟥 0 | 🟥 0 | 🟥 F/M (1080/1500) | 🟥 F/M (64/16) | 🟥 0 | 🟥 0 | 🟩 F (25580) |
| 42 | 🟥 O (680) | 🟥 O (96) | 🟥 0 | 🟥 0 | 🟥 F/M (184/32) | 🟥 F (2120) | 🟥 0 | |
| 43 | 🟥 F(18040) | 🟥 M (580) | 🟥 0 | 🟥 0 | 🟩 F (112540) | | | |
| 44 | 🟥 F/M (15120/80) | 🟥 0 | 🟥 0 | 🟩 F (11500) | | | | |
| 45 | 🟥 F/M (12280/100) | 🟥 0 | 🟩 F (25960) | 🟧 F/M (38960/900) | 🟩 F (74040) | 🟥 0 | | |
| 46 | 🟥 F/M (6680/40) | 🟥 M (56) | 🟥 M (24) | 🟥 0 | 🟥 0 | 🟩 F (53140) | | |
| 47 | 🟥 F/M (1320/660) | 🟥 F (2320) | 🟥 0 | | | | | |
| 48 | 🟥 F/M (760/40) | 🟩 F (16660) | 🟥 0 | | | | | |
| 49 | 🟥 F/M (460/120) | 🟥 0 | 🟥 F (1540) | 🟩 F (1540) | | | | |
| 50 | 🟥 F/O (9500/3720) | 🟥 0 | 🟥 0 | | | | | |
| 51 | 🟥 0 | 🟥 0 | 🟥 F (34040) | | | | | |
| 52 | 🟥 0 | 🟥 0 | | | | | | |
| 53 | 🟥 0 | 🟥 0 | | | | | | |

**Figure 9: Plasmodium species detection and determination in field samples by cytb-qPCR RFLP analysis.** Each line represents a patient (1-53) and contains samples of different time points from the same patient. The *Plasmodium* species (F= *P. falciparum*; M= *P. malariae*; O= *P. ovale*) as well as the parasite densities (as p/ µL; mentioned in brackets) as defined by the microscopic examination are mentioned in each sample. Three different colours were used for the direct comparison of the microscopy and cytb-qPCR results: green= the gene amplification with cytb-qPCR was successful and the identified *Plasmodium* species was in agreement with the microscopy data; red= no gene amplification was achieved with cytb-qPCR regardless the microscopy data; orange= the gene amplification with cytb-qPCR was successful but the identified *Plasmodium* species was different than in microscopy data.

## 4.8 Cytb-qPCR detection capability in the field samples

Due to the low PCR success rate, the field samples were further classified and screened in subgroups from higher to lower parasite densities. The cytb-qPCR success rate was 73.2% (60/82; CI95% 62.2-82.4) in field samples with parasite densities higher or equivalent to 600 p/ µL and 14.7% (9/61; CI95% 6.9-26.2) in field samples with parasite densities below 600 p/ µL (Table 4).

**Table 4: Cytb-qPCR positivity in field samples.**

| Parasite density | Number of microscopically confirmed samples | Number of cytb-qPCR RFLP confirmed samples n (% of microscopy positive samples detected) |
|---|---|---|
| >2000 p/ µL | 60 | 46 (76.7%) |
| 1001-2000 p/ µL | 14 | 8 (57.1%) |
| 600-1000 p/ µL | 8 | 6 (75.0%) |
| 200-599 p/ µL | 15 | 3 (20.0%) |
| 1-199 p/ µL | 46 | 6 (13.0%) |
| **Total Positive** | 143 | 69 (48.2%) |

### 4.9 Comparison of microscopy and molecular analysis data

A direct comparison between the microscopy data and the data acquired from the molecular analysis of the field samples with cytb-qPCR RFLP method was done. According to microscopy data, 143/236 samples were parasite-positive and 93/236 were parasite negative (Table 5, and Figure 10). The cytb-qPCR success rate was 48.2% (69/143; CI95% 39.8-56.7), with 70.8% (167/236) of samples identified as parasite-negative. The cytb-qPCR RFLP method identified only 17.5% (10/57) and 33.3% (1/3) of the microscopically defined *P. malariae* and *P. ovale* monoinfections, respectively. Kappa analyses showed low levels of agreement between the microscopy data and cytb-qPCR RFLP method for *Plasmodium* species detection and identification ($\kappa=0.42$ and $\kappa=0.38$, $p<0.0005$, respectively). Two microscopically defined *P. malariae* monoinfections were identified as *P. ovale* and *P. falciparum* monoinfections by cytb-qPCR RFLP. No microscopy negative sample defined as positive by cytb-qPCR. Overall, *P. falciparum* infections accounted for 82.6% (57/69), *P. malariae* for 14.5% (10/69) and *P. ovale* for 2.9% (2/69) of samples detected by cytb-qPCR RFLP.

**Table 5: Summary of the molecular analyses with cytb-qPCR RFLP method and comparison with the microscopy data**

| ***Plasmodium* species** | **Microscopy data** | **Cytb-qPCR RFLP data** |
|---|---|---|
| • *P. falciparum* monoinfections | 47 | 57 |
| • *P. malariae* monoinfections | 57 | 10 |
| • *P. ovale* monoinfections | 3 | 2 |
| • *P. falciparum* + *P. malariae* coinfections | 32 | 0 |
| • *P. falciparum* + *P. ovale* coinfections | 3 | 0 |
| • *P. malariae* + *P. ovale* coinfections | 1 | 0 |

| | | |
|---|---|---|
| **Parasite densities** | | |
| • ≥ 600 parasite/ µL | 82 | 60 |
| • < 600 parasite/ µL | 61 | 9 |
| **Total samples** | | |
| • Parasite-positive | 143 | 69 |
| • Parasite negative | 93 | 167 |

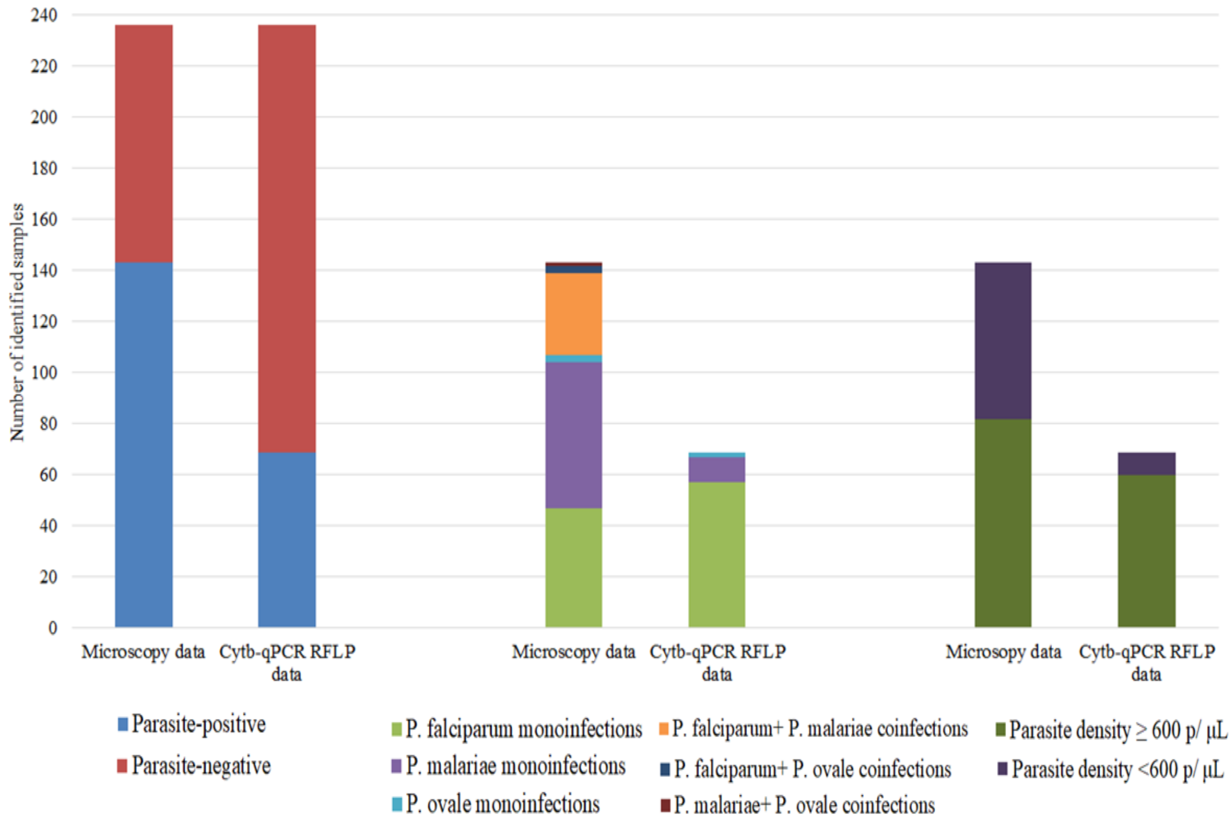


**Figure 10: Molecular analysis of all samples using cytb-qPCR RFLP method in comparison with the microscopy results.** Each colour represents a different variant

# 5. Discussion

The overall aim of this study was to confirm the microscopy findings of the WANECAM longitudinal study [28] regarding the efficacy of the ACTs against low density *Plasmodium malariae* and *Plasmodium ovale* infections in West Africa using a more sensitive molecular tool i.e. qPCR. Our initial interest was the low effectiveness of the artemether-lumefantrine treatment against *Plasmodium malariae* and *Plasmodium ovale.* However, 241 samples, which had previously been collected from the clinical trial site in Mali, from patients treated with dihydroartemisinin-piperaquine were immediately available for molecular analysis whereas the samples from the artemether-lumefantrine treatment arm were planned to be collected during the spring had it not been for the global Covid-19 pandemic. Nevertheless, the cytb-qPCR success rate was only 48.2% (69/143) among microscopy positive samples, with most of the analyzed samples (167/241) being identified as parasite-negative. Although the cytb-qPCR was capable to detect 1 parasite/ µL and consistently positive at 10 parasites /µL in the parasite-positive controls, a noteworthy lower detection capacity was observed in field samples, especially for parasite densities below 600 parasites/ µL. In total, 10 *Plasmodium malariae* monoinfections, 2 *Plasmodium ovale* monoinfection, 57 *Plasmodium falciparum* monoinfections, two misclassifications and no coinfections were identified by cytb-qPCR RFLP.

### The choice of DNA extraction method is important for cytb-qPCR success rates

We initially set out to optimize the molecular method for parasite detection in the field samples by comparing Chelex®-100 and QIAamp column based extraction methods. Chelex®-100 boiling method is a widely used inexpensive, simple extraction method with documented higher sensitivity of DNA extraction from samples with low parasite densities in comparison to other extraction methods [40–42]. Contrary to expectations, we found that the QIAamp column based extraction method performed better than the Chelex®-100 extraction method in the analysis of low parasite density samples as indicated by the cytb-qPCR success rate. This discrepancy could be due to the possible presence of PCR inhibitors in the Chelex-extracted DNA that set the extracted nucleic acid inappropriate for downstream analysis with highly sensitive molecular methods. Other previously published studies [39,43,44] have shown that the QIAamp column based extraction method is actually better suited for older samples, which is in accordance with our findings since the samples utilized in this study have been stored for more than 3 years in tropical conditions.

### Efforts to improve the cytb-qPCR reproducibility

The unexpected low cytb-qPCR success rate led us to check various parameters in attempt to optimize the method. A change in the final elution volume (from 150 to 100 µL) followed by a slightly increased final incubation time with the elution buffer (from 1 to 5 minutes) was added to the extraction protocol in order to increase the yield and concentrate the extracted DNA. The amplification capability of the primers, by comparison of a new and old set of primers, as well as testing different volumes of the DNA template and the final reaction volume (data not shown), were also tested as part of our effort to improve the cytb-qPCR reproducibility. Finally, the detection of a human housekeeping gene and the measurement of the DNA concentration in the extracted field samples, as an indicator of the successful DNA extraction method and the DNA purity, respectively, were both examined during the overall evaluation of the molecular method. Still, we did not manage to improve the cytb-qPCR success rate and reproducibility in the field samples to the same levels achieved in the positive controls.

### High gene copy number important for PCR success rate

Historically, the *Plasmodium* 18S ribosomal RNA gene has been widely used as target for the detection and identification of *Plasmodium* species [37,45,46]. The 18S rRNA exists in 5-8 copies in different *Plasmodium* species, and in some species such as *Plasmodium malariae* and *Plasmodium ovale*, the gene polymorphism results in the presence of at least two variants. This genetic polymorphism in the non-falciparum species requires the specific design of primers based on the circulating variant, a fact that sets the simultaneous detection of the *Plasmodium* species extremely tough [47–50]. Various studies have evaluated and verified the conserved cytochrome b gene as a suitable target for the detection and identification of *Plasmodium* species, especially in low density infections. The cytb gene in the mitochondrial DNA has one identical copy per mitochondrion and the total gene copy numbers fluctuate between 30-100 based on the number of mitochondria per parasite [36,51,52]. Although the cytb gene sequence and copy number differs among the *Plasmodium* species and the copy number also differs during the lifecycle of the parasite, the probability of detecting and amplifying 30-100 gene copies is much higher than the 5 copies of the 18S rRNA gene per parasite. Taking into consideration that the efficacy and the sensitivity of a molecular method, i.e. qPCR, is highly dependent on the target gene copy number, we believe that the cytb gene was the most suitable target for this project.

### Impact of DNA quantity/quality on the molecular analyses

The human beta-tubulin gene in a fraction of extracted field samples was successfully detected. This observation acted as a clear indicator that the DNA had been correctly extracted from the selected samples and that the QIAamp column based method was efficient for the extraction. Based on our results, the cytb-qPCR had lower success rate and detection capacity in the analysis of the field samples compared to the parasite-positive controls, whose analysis defined the detection limit of the method as 1 parasite/ μL. Xu *et al.* developed the cytb-qPCR RFLP method especially for the analysis of low density *Plasmodium* infections [36]. On the contrary, we observed low cytb-qPCR applicability in both the medium-low density infections and the relatively higher density infections. In details, 78.3% (54/69) of samples with medium-low parasite densities (≤1000 p/μL) and 27% (20/74) of the samples with relatively high parasite densities (>1000 p/ μL) did not yield results during the molecular analysis. These unexpected data in combination with the previous housekeeping gene analyses indicates a potential problem in the samples themselves and the biological material preserved on the filter papers rather than inadequate extraction method and low sensitivity of the cytb-qPCR method per se.

### Effect of storage conditions in the DNA preservation on filter papers

Dried blood spots preparation and storage of the filter papers play crucial roles for the further analysis of the samples with highly sensitive molecular techniques such as PCR. Already in 1999, Färnert *et al.* revealed that a 30-day storage of the filter papers at 30 $^{o}$C in humid conditions (60% humidity) resulted in declined PCR sensitivity compared to samples stored at 4 $^{o}$C or room temperature (20 $^{o}$C) [53]. Furthermore, several studies demonstrated the DNA degradation and fragmentation in samples kept in wrong conditions for a long period of time (more than 2 years), supporting the negative correlation between the high temperature and humidity and the PCR sensitivity of DNA detection from dried blood spots, especially in low density infections [43,54–56]. Taking into consideration all these previous studies in combination with the long storage of our samples for at least 3 years (with maximum 8 years) in plastic bags, without desiccant and within paper boxes, prone to absorb humidity, in an environment with average temperature of 35$^{o}$C and average humidity of 60% (April-October) in Mali, we assume that the bad preservation of the genetic material and the DNA degradation on filter papers are the main causes of the discordance between the microscopy and the cytb-qPCR RFLP method as well as of the unsuccessful analysis of the samples especially those with parasite densities below 600 parasites/ μL. Consequently, the present study highlights the importance of the proper preparation and storage of the dried blood spots on filter papers by selection of the proper validated filter paper (Whatman 3MM-type paper), the adequate drying of the blood spots prior to storing, the usage of desiccant and its frequent replacement when it absorbs humidity and the right storing temperature (either, ideally, at -20 $^{o}$C or ~20 $^{o}$C) as the fundamental factors for maintaining the valuable genetic information of the dried blood spots on the filter papers for further analysis and epidemiological studies in malaria research and development.

### Alternative molecular methods to overcome the suspected DNA damage

There are various alternative molecular methods, which could be used to tackle the observed obstacles in this study and the major problem of the suspected DNA degradation. As the amplified product was relatively big for a qPCR protocol (430 bp), the designing of a new set of primers with a smaller amplicon, ideally 100-150 bp, would probably help to increase the cytb-qPCR efficiency if the sample degradation has resulted in DNA fragmentation. However, the highly AT-rich genome (average AT-content: 76%) as well as the presence of many introns and intergenic regions make the designing of primers for *Plasmodium* genes extremely challenging. Even though the detection of small-sized amplicons in gel electrophoresis can be problematic, a nested PCR targeting the cytb *Plasmodium* gene could replace the cytb-qPCR for species detection and determination. Digital droplet PCR (ddPCR) is a robust molecular method, which has become increasingly used during the last decade to overcome difficulties with extremely low biological material and/or rare targets. Few studies have already demonstrated its applicability in the malaria field [57–59]. Another alternative could be the enrichment of the *Plasmodium* genome extracted from the filter papers by selective whole genome amplification (sWGA) technique. SWGA will allow the enhancement of the parasite genome versus the human genome and will increase the probability of detection even in samples with limited preserved parasite DNA [60,61]. One of the requirements of sWGA is high quality extracted nucleic acid, which may not be an alternative in degraded samples.

### Conclusion

The efficacy of the artemisinin-based combination therapies (ACTs) against *Plasmodium malariae* and *Plasmodium ovale* is not as extensively documented as for the most common and dangerous species, *Plasmodium falciparum* and *Plasmodium vivax*. We aimed to evaluate the efficacy of the dihydroartemisinin-piperaquine treatment against *Plasmodium malariae* and *Plasmodium ovale* infections with low parasite densities in samples collected in Mali, but we did not achieve our initial goal as we faced difficulties during the molecular analyses with the highly sensitive cytb-qPCR RFLP method. We suspect that the inadequate storage of the filter papers in tropical conditions for more than 3 years had probably affected the quality of the preserved genetic material leading to noteworthy DNA degradation. Dried blood spots on filter papers are widely used in clinical trials as an easy, rapid and inexpensive way to store important genetic information thus their proper preparation and storage are vital. Our future goals are to develop an improved cytb-qPCR method by designing new primers for a smaller amplicon to increase the capacity of the technique as well as to evaluate the efficacy of the remaining ACTs used in the WANECAM study (artemether-lumefantrine, artesunate-pyronaridine, artesunate-amodiaquine) against *Plasmodium malariae* and *Plasmodium ovale* infections.

## Acknowledgements

Firstly, I would like to thank Professor Anders Björkman for giving me the opportunity to be member of his research team and conduct my master thesis project in the field of malaria. A great thank to my lovely supervisor Ulrika Morris, who was there for me, available and willing to discuss and solve the problems during my project and for her important help, guidance and feedback during the writing of my thesis. Thanks also to my co-supervisor Leyre Pernaute Lau for her important help and supervision during my first months in the lab, Berit Aydin-Schmidt for her positive energy and for her patience to teach me how to use the stepper pipette, José Pedro Gil for sharing his experience and useful tips and Sanna Luijcx for her nice company during the extractions.

Most of all, I would like to thank my family and all my friends (old and new) for their love and support. ‘Sweden enlightens you’ and these two years were wonderful but also very difficult, especially at the beginning. I wouldn’t have come to the end without your love and continuous support. Special thanks to you!!

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