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Research Paper

Detection of Malaria Parasites After Treatment in Travelers: A 12-months Longitudinal Study and Statistical Modelling Analysis

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ABSTRACT

The rapid clearance of malaria parasite DNA from circulation has widely been accepted as a fact without being systemically investigated. We assessed the persistence of parasite DNA in travelers treated for *Plasmodium falciparum* malaria in a malaria-free area.

Venous blood was collected at the time of admission and prospectively up to one year. DNA and RNA were extracted and analyzed using species-specific and gametocyte-specific real-time PCR as well as merozoite surface protein 2 (*msp2*)-PCR.

In 31 successfully treated individuals, asexual parasites were seen by microscopy until two days after treatment, whereas parasite DNA was detected by *msp2*- and species-specific PCR up to days 31 and 42, respectively. Statistical modelling predicted 26% (±0·05 SE) species-specific PCR positivity until day 40 and estimated 48 days for all samples to become PCR negative. Gametocytes were detected by microscopy and PCR latest two days after treatment. C_T values correlated well with microscopy-defined parasite densities before but not after treatment started.

These results reveal that PCR positivity can persist several weeks after treatment without evidence of viable sexual or asexual parasites, indicating that PCR may overestimate parasite prevalence after treatment.

1. Introduction

Microscopy has been the gold standard method for detection of malaria parasites for more than a century. Despite being a rather simple technique, sensitivity of this method depends on the experience of the microscopist as well as the quality of the microscope (Roth et al., 2016). Moreover, microscopy may fail to distinguish between *Plasmodia* species with similar morphology and often misses mixed infections (Di Santi et al., 2004). During the past decades, rapid diagnostic tests (RDTs) (Avila et al., 2002), immunoassays (Huong et al., 2002), and molecular methods have been increasingly used to detect malaria parasites. Several studies have compared the sensitivity and specificity of different techniques and showed that polymerase chain reaction (PCR) is the most sensitive method enabling more accurate identification of the parasite species especially in mixed infections. It also detects infections with parasite densities below the detection threshold of microscopy (sub-microscopic infections) (Barker et al., 1994; Lima et al., 2011; Okell et al., 2012; Tusting et al., 2014). Furthermore, real-time quantitative PCR (qPCR) has the potential to quantify the parasite density, is fast, and can be automated (Perandin et al., 2004). Despite the increasing number of studies reporting the advantages of PCR, the likelihood of PCR positive results due to amplification of DNA from dead parasites killed by treatment and/or host immune response has not been examined in depth. Assessments of post-treatment PCR positivity performed in malaria endemic areas are affected by the risk of re-infection as well as partial immunity in study populations, which may influence parasite clearance.

In this longitudinal study, we examined the duration of PCR positivity as well as the presence of gametocytes in travelers treated for *Plasmodium falciparum* malaria and followed up to 12 months in a malaria-free setting, using microscopy, species-specific qPCR, merozoite

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surface protein 2 (*msp2*)-genotyping PCR, and gametocyte-specific qPCR.

2. Methods

2.1. Study Population and Sample Collection

The study was performed on adults diagnosed with *P. falciparum* malaria ($n = 36$), enrolled in a malaria immunology study, at Karolinska University Hospital in Stockholm, Sweden. Patients with known HIV infection, planned visits to malaria endemic areas during the follow-up, or any other planned absence interfering with the sampling schedule, were not included in the study. Patients were treated according to the national guidelines for *P. falciparum* malaria with a full regimen of six doses of artemether-lumefantrine (AL) (20 mg/120 mg Riamet®), four tablets per dose at 0, 8, 24, 36, 48, and 60 h administered together with fatty meal or drink by a ward nurse as long as patients were hospitalized. Eight patients received one to three initial dose(s) of intravenous artesunate (2·4 mg/kg per dose) before a full course of AL due to hyperparasitaemia, of which three patients had also other signs of severe malaria (Supplementary Table 1).

Venous blood was collected in EDTA tubes at the time of admission, on consecutive days until discharge, after 10 days, as well as at one, three, six, and twelve months after treatment, according to a predefined protocol of an ongoing malaria immunology study. As a result of preliminary data on PCR positivity, Tempus™ Blood RNA tubes (Applied Biosystems) were added at each sampling occasion for gametocyte analysis, thus available only for a subset of patients ($n = 12$). The study was approved by the Ethical Review Board in Stockholm and informed consent was given by all participants.

2.2. Microscopy

Conventional light microscopy of Field's stained thin and thick smears was performed to detect and enumerate the parasites as proportion of infected erythrocytes expressed as percentage and as number of parasites per microliter (p/μl) of blood, assuming 5×10^6 erythrocytes per microliter whole blood.

2.3. Species-specific qPCR and msp2-genotyping PCR

DNA was extracted from 400 μl blood using a magnetic bead separation method with Hamilton Chemagic Star Robot® (Bonadouz, Switzerland). *Plasmodium* species-specific qPCR was carried out targeting the 18S rRNA gene (Shokoples et al., 2009) on a QuantStudio™ 5 Real-Time PCR System (Applied Biosystems). A threshold cycle (C_T) value over 40 was considered negative. Parasite densities estimated by species-specific qPCR were calculated computing the $\Delta\Delta C_T$.

$$\Delta\Delta C_T = 2^{\Delta CT} \times \text{Parasite Density}_{\text{positive control}}$$

$$\Delta C_T = C_{T\ \text{Control with known parasite density}} - C_{T\ \text{Sample with unknown parasite density}}$$

Genotyping of *P. falciparum msp2* gene was carried out using nested PCR followed by capillary electrophoresis (Liljander et al., 2009). All PCR analyses were repeated twice and sample identity was blinded during experiments. The detection limit, defined by serial dilutions of positive controls, was 0·12 and 5 parasite/μl of whole blood for species-specific and *msp2*-PCR, respectively.

2.4. Gametocyte Culture and Preparation of Positive Control

P. falciparum gametocyte culture was set up according to standard procedure (Carter et al., 1993), and aliquots were collected in Tempus ™ Blood RNA tubes for further RNA extraction and cDNA synthetization.

2.5. RNA Extraction and cDNA Synthetization

RNA was extracted from 500 μl of samples (blood and culture) collected in Tempus Blood RNA tubes using Stabilized Blood-to-CT™ Nucleic Acid Preparation Kit for qPCR (ThermoFisher Scientific) following the manufacturer instructions. cDNA was synthesized by reverse transcriptase (RT)-PCR using Superscript® Vilo™ cDNA Synthesis Kit (Invitrogen). TaqMan® GAPDH Assay (Applied Biosystems) was carried out to verify the synthetization of cDNA.

2.6. Detection of Male and Female Gametocytes

Sex-specific real-time qPCR was performed on synthesized cDNA to detect both male and female *P. falciparum* gametocytes (modified from Schneider P et al.) (Schneider et al., 2015). Primer and probe sequences as well as PCR condition are presented in Table 1. Using positive controls with known gametocytaemia, a detection limit of 1·5 and 0·5 gametocyte/μl of blood was estimated for female and male gametocytes, respectively.

2.7. Validation of Real-time PCR Results

Although following a well-established protocol (Shokoples et al., 2009) for multiplex species-specific qPCR, a subset of samples were verified in singleplex reactions adopted to detect only *P. falciparum* in order to exclude probable inter-species cross-binding of primers. In addition, a randomly selected subset of blood samples ($n = 20$), blinded to previous results and sample identity, were re-analyzed (from DNA extraction to PCR) by a second researcher in our laboratory. In addition, the same subgroup of samples was analyzed by the Department of Clinical Microbiology at Karolinska University Hospital, serving as reference laboratory for malaria PCR in Sweden. DNA was extracted from whole blood using the Universal Pathogen protocol on MagNA Pure 96 system (Roche diagnostics). Real-time PCR was performed following a modified protocol of Shokoples et al. (2009) and Divis et al. (2010) on a QuanStudio™ 6 Flex Real-Time PCR System (Applied Biosystems).

2.8. Statistical Analyses

Statistical analyses were performed using R statistical software [v3.2.2; lme4, lmer, glmer, Cox proportional hazards model (Cox-PHZ), rms, simr, powersurvEpi].

The duration of microscopy PCR positivity over time [time-to-becoming negative] was analyzed using the Cox-PHZ; with time of follow-up treated as the main covariate for evaluating the proportion of positive samples (by microscopy and species-specific qPCR) as the outcome, with patient ID incorporated as a random effect [frailty function].

The correlations between parasite density defined by microscopy before treatment and the number of positive days by microscopy and PCRs were assessed using a linear regression model [lm]. Moreover, logistic regression [GLMM: glmer] was used to define the estimated

Table 1
P. falciparum gametocyte sex-specific primers and probes.

Name	Sequence	Sex
Pfs230p-FW	5′-CCCAACTAATCGAAGGGATGAA-3′	Male
Pfs230p-RV	5′-TTTGTTGTTCGATTCCAGTTGGT-3′	Male
Pfs230p-Probe	VIC-AAACGATCAAACCATCTCA-MGB	Male
Pfs25-FW	5′-TAAAATAGATGGAAATCCCGTTTC-3′	Female
Pfs25-RV	5′-TACCGTTACCACAAGTTACATTCTTAC-3′	Female
Pfs25-Probe	FAM-ATGTAATCTTGGATATGATATGG-MGB	Female

Real-time PCR was carried out separately for male and female gametocytes targeting sex-specific transcripts (Schneider et al., 2015). PCR reactions were run in 25 μl total volume containing cDNA, 1× TaqMan® Multiplex Master Mix (ThermoFisher Scientific), 200 nM of each primers, and 150 nM of probes (Life Technologies) using similar programme (an initial denaturation step at 95 °C for 20 s followed by 45 cycles of 95 °C for 1 s and 60 °C for 20 s) for both assays.

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