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

Manijeh Vafa Homann a,⁎, S. Noushin Emami b, Victor Ymana c, Christine Stenström d, Klara Sondén a,d, Hanna Ramström c,d,1, Mattias Karlsson c, Muhammad Asghar a,d,2, Anna Färnert a,d,2

A R T I C L E   I N F O

Article history:
Received 25 August 2017
Received in revised form 21 September 2017
Accepted 2 October 2017
Available online 4 October 2017

Keywords:
Malaria
Real-time PCR
msp2-PCR
Gametocyte
Traveler
Treatment

A B S T R A C T

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. Ct 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.

© 2017 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

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...
2. Methods

2.1. Study Population and Sample Collection

The study was performed on adults diagnosed with \textit{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 \textit{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, \textit{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 × 10⁶ 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). \textit{Plasmodium} species-specific qPCR was carried out following the 18S rRNA gene (Shokoples et al., 2009) on a QuantStudio™ 5 Real-Time PCR System (Applied Biosystems). A threshold cycle (C \textit{T}) value over 40 was considered negative. Parasite densities estimated by species-specific qPCR were calculated using the \(\Delta C \text{T} \quad \text{value} \).

\[
\Delta C \text{T} = 2^{\Delta C T} \times \text{Parasite Density}_{\text{positive control}}
\]

\(\Delta C \text{T} = C \text{T}_{\text{Control with known parasite density}} - C \text{T}_{\text{Sample with unknown parasite density}}\)

Genotyping of \textit{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

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

2.5. RNA Extraction and cDNA Synthetization

RNA was extracted from 500 μl of samples (blood and culture) collected in \textit{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 \textit{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 gametocytes, a detection limit of 1·5 and 0·5 gamocyte/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 \textit{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 QuantStudio™ 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 determined 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
proportion of positivity by PCR within 10 days intervals during follow-up, with patient study ID as a random effect in this model [fidelity function]. The correlation between the species-specific qPCR-CT value and microscopy-defined parasite densities (log) in microscopy positive samples was analyzed using linear regression model [lm], following a log-normal distribution.

Mixed effects linear model [lmer] was used to evaluate the effect of multiple covariates (sex, age, patient origin, number of years out of endemic area, and duration of fever before treatment) on days of PCR-positivity as the outcome.

The influence of parasite density, age, sex, country of origin, days of fever, previous treatment, recrudescence, and parasite clone number on presence of gametocytaemia was evaluated using logistic regression [GLMM: glm].

In all GLMM models, backward elimination was used for sequential removal of non-significant variables, to obtain the minimal statistically-significant model. Additional details of analyses are presented in Supplementary Material.

3. Results

3.1. Study Population Characteristics

Thirty-six patients with *P. falciparum* malaria were enrolled in the study. The study participants, aged 21–70 years (median 39·5), were either of European (non-endemic) (n = 10) or African (endemic) origin (n = 26) living in Europe for 2–46 (median 15) years. Majority of patients were infected during visits to Sub-Saharan Africa. Patient characteristics including clinical presentations and treatment are presented in Supplementary Table 1.

Four patients had late treatment failure after three weeks and are reported separately. In addition, one patient had some uncertainty regarding slow response to the drug as well as incomplete DNA/RNA series, and was excluded from further analysis.

In patients with successful treatment (n = 31), availability of samples (slides, DNA and RNA) for the respective study participants at different time points are presented in Supplementary Table 2.

3.2. Detection of Parasites by Microscopy

Microscopy-defined parasitemias were <0·1–17% (median 0·95) at admission, with eight patients having parasitemias higher than 2% (corresponding to 100,000 p/μl of blood). Parasitemia declined rapidly after treatment initiation (Fig. 1a). Asexual parasites were detected by microscopy in 11/20 (55%) slides on day 1, and in 4/17 (23·5%) slides on day 2 after treatment (Fig. 1b). Gametocytes were detected by microscopy only in one patient on day 2 (Fig. 1g). On day 3 and onward, all slides were negative by microscopy (Fig. 1b, g).

3.3. Detection of Parasite DNA by Species-specific qPCR

Species identification by microscopy and species-specific qPCR agreed 100% and confirmed mono-infections by *P. falciparum* in all patients. Once treatment was initiated, CT values increased rapidly until day 5 and thereafter raised gradually until the cycle 40 (threshold limit) (Fig. 1c). The species-specific qPCR was positive in all samples available between day 0 and 9 after treatment (Fig. 1d). Thereafter, PCR remained positive in 17/22 (77%) of patients with samples available during days 10–19, in 1/5 (20%) patients during days 20–29, in 4/10 (40%) patients during days 30–39, and in 1/5 (20%) patients during days 40–49. All samples thereafter until one year after treatment were negative by species-specific qPCR (Fig. 1d). The species-specific qPCR results were verified by re-analyzing subsets of samples (n = 20) in different settings (See Methods), confirming positive and negative PCR results in all samples by another investigator in the same laboratory, and only one discordant result of a 30 day sample with Ct value of 37·6 that was negative by the reference laboratory (data not shown).

3.4. msp2-PCR positivity and number of clones over time

The msp2-genotyping PCR was positive in all (n = 23) available samples collected on day 0, with 1–3 (median 2) *P. falciparum* clones per individual. The number of clones decreased over time after treatment within the respective individuals (Fig. 1e). The msp2-PCR assay was positive in all individuals on day 1 and remained positive in 4/5 (80%) on day 2, in 10/12 (83%) on days 3–9, in 12/22 (54·5%) on days 10–19, in 1/5 (20%) on days 20–29, and the longest in one individual until day 31 after treatment (Fig. 1f). All follow-up samples thereafter, up to twelve months were negative by msp2-PCR.

3.5. Detection of Gametocytes by Sex-specific qPCR

One hundred and eighty-five slides from 31 patients, at different time-points after treatment were screened by microscopy and gametocytes were seen without asexual parasites in one patient on day 2. RNA tubes were introduced later in the study (see Methods) and thus 55 RNA samples were available from 12 patients in total. Three of these patients are amongst treatment failure cases. Thirty-seven RNA samples were available from nine patients with successful treatment (Supplementary Table 2). Gametocytes were detected by PCR in all available samples collected at the time of admission (5/5) (Fig. 1g). No gametocyte was found at later time points (days 2–389) in any patient (32 RNA samples from nine participants).

3.6. Modelling of Kinetics of Species-specific qPCR-Generated Ct Values over Time

Mean Ct value of available samples at different time points is presented in Fig. 2. Owing to long intervals between time points and incomplete sample series, it was not possible to determine the exact duration of PCR-positivity (i.e. the day that Ct value reaches 40) in individual patient. Therefore, modelling of available data was performed to estimate the length of species-specific qPCR-positivity after treatment, which predicted 48 days for Ct values to reach 40 [univariate Cox-PHZ with patient as random covariate, [HR = 0·75 (CI 95%: 0·69–0·79); p = 0·001]. In addition, a logistic regression model [glm] for *P. falciparum* positivity with time modelled in 10 day strata, estimated that 56% (±0·07% SE) of samples are positive by species-specific qPCR between days 20 and 26% (±0·05% SE) remain positive during 30–39 days. Proportion of patients with positive microscopy and qPCR over time was also analyzed using Cox-PHZ, which showed that number of days for post-treatment qPCR-positivity are significantly higher than those estimated for microscopy [HR = 0·60 (CI 95%: 0·45–0·76), p < 0·001]. By microscopy, all samples (100%) are predicted to be negative by day 3 (Fig. 3).

3.7. Duration of PCR Positivity and Ct Kinetics in Relation to Microscopy-defined Parasite Densities

Parasite densities defined by microscopy before treatment were not correlated to days of positivity by microscopy [F = 2·7; (df = 1·5); p = 0·10]; msp2-PCR[F = 0·94; (df = 1·11); p = 0·35], and species-specific qPCR[F = 0·58; (df = 1·12); p = 0·45]. Although, the qPCR applied here is not absolute-, rather relative quantitative method, a significant correlation was noted between Ct values and microscopy-defined parasite densities before and/or on the day treatment started [linear regression, lm, F = 6·28; (df = 20); R² = 0·24; p = 0·02]. This association was lost for microscopy positive samples collected one day after treatment and onwards [F = 1·80; (df = 10); R² = 0·15; p = 0·20] (Supplementary Fig. 1). In PCR positive samples that were negative by
microscopy (n = 39), Ct values ranged between 23·1–39·8, which according to our serial dilution of positive samples with known parasitemia estimates parasite densities of 0·12 p/μl (Ct 39·8) and 25,500 p/μl (Ct 23·1).

3.8. Duration of PCR-positivity in Relation to Patient Characteristics

The number of post-treatment PCR-positive days was evaluated in relation to patient characteristics using linear mixed effects model...
Individuals receiving initial artesunate became qPCR negative in a significantly longer time as compared to those receiving only AL [$\chi^2_1 = 4.79; p = 0.005$], however, these patients also had high initial parasitemias. The potential interaction could not be tested due to sample size. Patients of malaria-endemic origin ($n = 24$) became qPCR negative significantly faster than those born in Europe ($n = 7$) [$\chi^2_1 = 6.29; p < 0.001$]. Nonetheless, 2/4 (50%) of patients with long qPCR-positivity (days 30–39) were of African origin. Furthermore, the number of years living out of endemic area did not significantly affect the clearance time of parasite DNA (defined by PCR positivity) after treatment [$\chi^2_1 = 1.09; p = 0.38$]. The other analyzed characteristics of patients (sex, age, and duration of fever before treatment) had no significant effect on days of PCR-positivity and were not included as covariates in the final model ($p > 0.05$).

3.9. Dynamics of PCR-positivity in Patients With Treatment Failure

In the four patients with late treatment failure, asexual parasites were seen by microscopy latest two days after treatment initiation of their first episode, while gametocytes were seen without asexual parasites in one individual until day 6. One of the patients was enrolled in the study at the time of recrudescence and thus had no DNA and RNA samples during the first infection. Gametocytes were detected by PCR, in all other three individuals, until day 3 post-treatment and remained detectable in one patient until day 13. The very last samples collected before recrudescence (day 13 in one and day 14 in two patients) were positive by both species-specific qPCR and $msp2$-PCR. Patients sought care again due to fever between 19 and 29 days after the first treatment. After recrudescence and second treatment initiation, asexual parasites and gametocytes were seen by microscopy latest on day 4 as well. Species-specific qPCR and $msp2$-PCR were positive until days 33 and 12, respectively. Additional details about these treatment failures including clinical presentation and management, parasite genotypes, drug resistance markers, and plasma drug concentrations are presented elsewhere (Sonden et al., 2017).

3.10. Gametocytaemia in Relation to Patient Characteristics and Recrudescence

Despite the low number of individuals with RNA samples, owing to the importance of gametocytes in all aspects of malaria, possible correlation between gametocytaemia and patient characteristics was tested. These analyses were performed on pooling data from 14 patients including all successfully treated individuals with available RNA samples ($n = 9$), patients with recrudescence ($n = 4$), and patient with microscopy detected gametocyte but missing RNA sample ($n = 1$). A significant association between the presence of gametocytes and recrudescence was noted ($\chi^2_1 = 3.79; p = 0.049$). No other significant associations were found between gametocytaemia and country of origin, age, days of fever, and number of parasite clone, or parasite density at enrolment [all $p > 0.08$]. The presence of gametocytes, at any time point, had no effect on the duration of PCR positivity after treatment ($n = 15; \chi^2_1 = 1.68; p = 0.19$).

4. Discussion

Many studies have compared methods to detect and quantify malaria parasites and have established advantages of PCR, especially for low-density infections and species identification (Hanscheid and Grobusch, 2002; Lima et al., 2011; Okell et al., 2012). In addition, PCR based-genotyping of $P. falciparum$ parasites is universally used to distinguish new infections from recrudescence in order to monitor the effect of antimalarial drugs in clinical trials (WHO, 2015b). Here we demonstrate that in a malaria-free area with no risk of re-infection, PCR can remain positive up to six weeks after curative treatment. Moreover, using RNA-based analysis in a subset of individuals, we show that the DNA detection by PCR.

![Fig. 2. Local regression fitting of $C_T$ value over time. a) Empty circles represent individual samples at a certain time point and solid red line shows the overall uncertainty which is measured as how well the estimated curve fits the $C_T$ value. b) The solid blue line displays local weighted polynomial regression of $C_T$ value and shaded area shows the 95% confidence interval (CI) using lm model, followed by loess function in ggplot2.](image)

![Fig. 3. Predicted proportion of $P. falciparum$ parasite positive samples by microscopy and species-specific qPCR, over time. The shaded area shows 95% confidence interval (CI).](image)
detected weeks after treatment does not seem to be originated from circulating sub-microscopic gametocytes.

We found that according to microscopy, parasites were rapidly cleared by AL treatment (in some cases AL + artesunate), with a significant decline to 55% already one day after treatment and dropped further down to 23-5% on day 2. On day 3 after treatment, all samples were negative by microscopy for presence of both asexual parasites and gametocytes. These results are in line with another study on travelers where according to microscopy, complete clearance of asexual parasites and gametocytes were achieved by day three and seven, respectively, after a six-dose AL regimen (Hatz et al., 2008).

Using species-specific qPCR, we detected *P. falciparum* DNA up to day 42 after treatment was started. Since the sampling intervals did not allow determining the exact duration of PCR-positivity, a statistical model was carried out which predicted; that positive PCR could last in 26% of the samples at 40 days; and that 48 days was needed for $C_{1}'$ values to reach 40 (negative threshold).

We additionally performed a PCR targeting *msp2*-gene, which is widely used as a genetic marker to characterise *P. falciparum* populations and to distinguish reinfection from recrudescence in drug efficacy studies. The *msp2*-PCR also remained positive in a subset of samples between days 20–39 after treatment, with a decreasing number of concurrent genotypes over time suggesting gradual clearance of the different infecting parasite clones. Detection of parasites with the same genotype longer than 28 days after treatment in drug trials following the 28-days treatment reaches the LoD of microscopy performed by microscopists with different levels of experience. A Limit of Detection (LoD) of 5

Duration of PCR positivity was correlated neither with the initial (sexual) parasite densities nor with the initial presence of gametocytes (defined by microscopy and PCR). Contrary to expectations, patients receiving initial intravenous artesunate, remained qPCR-positive significantly longer as compared to those treated with AL only. This effect could be owing to the sudden release of sequestered parasites killed by artesunate, into circulation, which may require longer time to clean up.

Patients of African origin cleared the infection faster as compared to those born and grown up in Europe, suggesting that previous exposure and partial immunity may speed up removal of parasite debris from circulation. However, number of years living out of endemic areas did not significantly affect the length of PCR-positivity. In addition, individuals with long PCR positivity (days 30–39) were also of endemic origins. Regardless of differences in earlier exposure between non- and semi-immune patients, inter-individual variation in PCR-positivity duration might partly result from diversity in the proportion of antibody classes/sub-classes, which in turn may contribute to evading the ingestion by host immune cells as well as stabilisation of parasite DNA in the form of immune complexes and thus delaying their clearance from circulation (Schiølner and Taylor, 1989).

A recent review on post-treatment gametocytaemia has reported that treatment with AL does not potentially promote the development of gametocytes and rather rapidly clears the pre-existing gametocytaemias (Group, 2016). In line with this, we observed that gametocytes were no longer detected by microscopy or PCR later than day 2 post AL treatment. It has also been shown that regardless of anti-malarial medicine, individuals experiencing treatment failure by day 28 were significantly more probable to be gametocytaemic at any time point during follow up (Group, 2016). In concordance with this, a significant correlation between gametocytaemia and recrudescence was observed in our study. However, in this small subset of patients, we did not find that gametocytaemia correlated with various host- and parasite-related factors such as age, country of origin, fever, as well as initial parasite density, observed in other studies (Group, 2016). This discrepancy could partly be due to the smaller size of our study population as well as variation in previous exposure and consequently different levels of immunity in one-time infected travelers and immigrants living years in malaria-free areas as compared to individuals living in endemic areas.

Interestingly, persistence of positive PCR long after curative treatment has also been reported in sleeping sickness (Deborghgraeve et al., 2011) and brucellosis (Marei et al., 2011), suggesting that slow clearance of pathogen debris is not a rare phenomenon. All together, these findings highlight the shortcoming of PCR to distinguish active infections from dead pathogens or their debris. Importantly, our results emphasize that real-time qPCR might underestimate drug efficiency in clinical management and trials, overestimate after-treatment parasite prevalence in epidemiological studies, and possibly misinterpret the results of other interventions. Nonetheless, our findings do not deny the advantage of real-time qPCR for diagnosis and quantification of initial parasite load before treatment, as in challenge studies of controlled human malaria infections (Kamau et al., 2014). It rather points out that the choice of detection method(s), e.g. microscopy or PCR, should be selected according to the purpose of analysis. Novel RNA-based methods, which allow for more accurate detection of viable parasites of both sexual and asexual forms (Tadesse et al., 2017), could be considered as the alternative approach in diagnosis of malaria parasite in clinical practice as well as in all area of malaria research. However, such advanced methods are less feasible and more costly to be routinely performed in all study settings.
Regardless of the underlying reason for vast inter-individual variation in clearance-time of parasite debris after treatment, this report underscores; an important diagnostic matter essentially in infectious diseases and particularly in malaria; the need for a detection tool as sensitive as PCR and as accurate as microscopy; and the necessity for further studies to uncover the actual cause of slow clearance of parasite debris.

**Contributors**

MVK was the main organizer of experiments and data analysis; carried out majority of experiments; compiled the data and drafted the manuscript. SNE cultured gametocytes; performed statistical analyses and modelling; contributed with result presentation and manuscript revision. VY was involved in organizing the follow-up and collection of clinical data. CS performed microscopy analysis. KS was involved in patient inclusion and follow-up. HR and MK repeated the DNA extraction and realtime PCR for a subset of samples. MA designed the gametocyte-specific PCR; contributed with experiments, result preparation and manuscript revision. AF was the principle supervisor of the study; responsible for clinical supervision and treatment of the patients; critically revised the manuscript. All authors reviewed and approved the final report.

**Declaration of Interests**

We declare no competing interests.

**Funding**

This study was funded by: The Swedish Research Council (521-2012-3311 and 348-2013-6573) and Stockholm County Council (20130207), grants to Anna Färnert; and by The Swedish Society for Medical Research (SSMF), Stiftelsen Sigurd och Elsa Goljes Minne, and Karolinska Institutet Research Foundation, grants to Muhammad Asghar. The funders had no role in study design, data analysis, manuscript preparation or the decision for publication.

**Acknowledgments**

We are grateful to the patients for their invaluable participation in the study. We also wish to thank the clinicians and laboratory staff for assistance with patient recruitment, Ingrid Andrén for sample collection as well as Nicholas Dufin and Ulf Hammar for valuable comments on the manuscript.

**Appendix A. Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2017.10.003.

**References**

Avaí, P.E., Kirchhageter, K., Brunialti, K.C., Oliveira, A.M., Siciliano, R.F., Di Santi, S.M., 2002. Evaluation of a rapid dipstick test, Malar-Check, for the diagnosis of Plasmodium falciparum malaria in Brazil. Rev. Inst. Med. Trop. Sao Paulo 44, 293–296.

Aydin-Snibd, B., Mubi, M., Norris, U., Petzold, M., Ngesala, B.E., Premji, Z., Bjorkman, A., Martensson, A., 2013. Usefulness of Plasmodium falciparum-specific rapid diagnostic tests for assessment of parasite clearance and detection of recurrent infections after artesiminin-based combination therapy. Malar. J. 12, 349.

Barker Jr., R.H., Banchongaksorn, T., Courval, J.M., Suwonkerd, W., Rimwungtragoon, K., Wirth, D.F., 1994. Plasmodium falciparum and P. vivax: factors affecting sensitivity and specificity of PCR-based diagnosis of malaria. Exp. Parasitol. 79, 41–49.

Bouma, T., Okell, L., Shekalaghe, S., Grierson, J.T., Omar, S., Sawa, P., Sutherland, C., Sauweirin, R., Ghan, A.C., Drakeley, C., 2010. Revisiting the circulation time of Plasmodium falciparum gametocytes: molecular detection methods to estimate the duration of gametocyte carriage and the effect of gametocytocidal drugs. Malar. J. 9, 136.

Carter, C., 2014. Gametocyte carrier in uncomplicated Plasmodium falciparum malaria: screening treatment with an gametocytocidal drug: a systematic review and meta-analysis of individual patient data. BMC Med. 14, 9.

Deborgraeve, S., Lejon, V., Elangu, R.A., Mumba Njouj, D., Piti Pyana, P., Ilunga, M., Mulunda, J.P., Buscher, P., 2011. Diagnostic accuracy of PCR in gambianese sleeping sickness diagnosis, staging and post-treatment follow-up: a 2-year longitudinal study. PLoS Negl. Trop. Dis. 5, e792.

De Santi, S.M., Kirchhageter, K., Brunialti, K.C., Oliveira, A.M., Ferreira, S.R., Boussem, E., 2004. Molecular markers for sensitive discrimination of female and male Plasmodium falciparum gametocytes by reverse transcriptase quantitative PCR. Mol. Biochem. Parasitol. 199, 29–33.

Deborgraeve, S., Lejon, V., Elangu, R.A., Mumba Njouj, D., Piti Pyana, P., Ilunga, M., Mulunda, J.P., Buscher, P., 2011. Diagnostic accuracy of PCR in gambianese sleeping sickness diagnosis, staging and post-treatment follow-up: a 2-year longitudinal study. PLoS Negl. Trop. Dis. 5, e792.

Deborgraeve, S., Lejon, V., Elangu, R.A., Mumba Njouj, D., Piti Pyana, P., Ilunga, M., Mulunda, J.P., Buscher, P., 2011. Diagnostic accuracy of PCR in gambianese sleeping sickness diagnosis, staging and post-treatment follow-up: a 2-year longitudinal study. PLoS Negl. Trop. Dis. 5, e792.

Wirth, D.F., 1994. Plasmodium falciparum and P. vivax: factors affecting sensitivity and specificity of PCR-based diagnosis of malaria. Exp. Parasitol. 79, 41–49.

Bouma, T., Okell, L., Shekalaghe, S., Grierson, J.T., Omar, S., Sawa, P., Sutherland, C., Sauweirin, R., Ghan, A.C., Drakeley, C., 2010. Revisiting the circulation time of Plasmodium falciparum gametocytes: molecular detection methods to estimate the duration of gametocyte carriage and the effect of gametocytocidal drugs. Malar. J. 9, 136.

Carter, C., 2014. Gametocyte carrier in uncomplicated Plasmodium falciparum malaria: screening treatment with an gametocytocidal drug: a systematic review and meta-analysis of individual patient data. BMC Med. 14, 9.

Deborgraeve, S., Lejon, V., Elangu, R.A., Mumba Njouj, D., Piti Pyana, P., Ilunga, M., Mulunda, J.P., Buscher, P., 2011. Diagnostic accuracy of PCR in gambianese sleeping sickness diagnosis, staging and post-treatment follow-up: a 2-year longitudinal study. PLoS Negl. Trop. Dis. 5, e792.

Deborgraeve, S., Lejon, V., Elangu, R.A., Mumba Njouj, D., Piti Pyana, P., Ilunga, M., Mulunda, J.P., Buscher, P., 2011. Diagnostic accuracy of PCR in gambianese sleeping sickness diagnosis, staging and post-treatment follow-up: a 2-year longitudinal study. PLoS Negl. Trop. Dis. 5, e792.

Deborgraeve, S., Lejon, V., Elangu, R.A., Mumba Njouj, D., Piti Pyana, P., Ilunga, M., Mulunda, J.P., Buscher, P., 2011. Diagnostic accuracy of PCR in gambianese sleeping sickness diagnosis, staging and post-treatment follow-up: a 2-year longitudinal study. PLoS Negl. Trop. Dis. 5, e792.