Detection of remaining *Plasmodium* DNA and gametocytes during follow up after curative malaria treatment among returned travellers in Norway

Christel Gill Haanshuus1* and Kristine Mørch1,2

**Abstract**

**Background:** PCR can be positive weeks after effective malaria treatment, potentially leading to over diagnose of recrudescence and re-infections. The DNA detected by PCR post-treatment might stem from residuals of destroyed asexual parasites, or from live gametocytes. The objective of this clinical observational study was to describe the presence of positive PCR for *Plasmodium falciparum* and *Plasmodium vivax* in follow-up samples post-treatment from returned travellers, and the proportion of positive PCR due to gametocytes.

**Methods:** Whole blood was collected during hospitalization and outpatient routine follow-up from 13 patients with imported malaria. DNA was extracted applying QIAamp DNA Blood Mini Kit, while mRNA was collected and extracted applying PAXgene Blood RNA Tubes and Kit. All DNA samples (N = 25) were analysed with a genus-specific *cytb* real-time SYBR PCR, and *P. falciparum* DNA samples (N = 22) were also analysed with a falciparum-specific *varATS* real-time TaqMan PCR. All the mRNA samples (N = 18) were analysed with both a genus-specific 18S rRNA RT-PCR and a gametocyte-specific *Pfs25* (*P. falciparum*)/*Pvs25* (*P. vivax*) RT-PCR.

**Results:** Latest samples were collected at day 1 (n = 2) and from day 11–54 (n = 11) after treatment. Genus DNA *cytb* PCR was positive up to 49 days after effective treatment, and 18S rRNA transcripts from active *P. falciparum* parasites were detectable for at least 11 days. Gametocyte-specific mRNA was detected at latest only two days after treatment. Among six patients with late positive PCR for *P. falciparum*, four had high parasitaemia at admittance (6–30%), while two had parasitaemia < 2%. Late detection of *P. vivax* was not found by any of the PCR methods.

**Conclusions:** DNA-based PCR can be positive up to at least seven weeks after curative malaria treatment, potentially leading to over-diagnose of recrudescence and re-infections. Based on the observations in this study, it is unclear if the DNA origins from residuals of destroyed parasites or live gametocytes, warranting further investigations.

**Keywords:** Malaria, *Plasmodium falciparum*, *Plasmodium vivax*, Gametocyte, PCR, RT-PCR, mRNA, DNA, Travellers, Post-treatment

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re-infections [1]. Residuals of destroyed asexual parasites, and live or destroyed gametocytes, are possible sources of post-treatment *Plasmodium* DNA in patients cured of their malaria infection. Gametocytes are sexual forms of malaria parasites responsible for transmission between humans after completion of their cycle in mosquitoes. They cause no clinical symptoms in the human host. In *Plasmodium falciparum*, immature gametocytes seques-
ter in internal host organs, particularly in the bone mar-
row, and undergo five morphological development stages
in the course of 7–12 days, hence only mature gameto-
cytes circulate in the blood until they die of age [2, 3]. It
has been estimated from mathematical modelling that
*P. falciparum* gametocyte carriage may persist for up
to 55 days after treatment [4]. Primaquine (PQ) is the only
drug effective against mature gametocytes, and is added
to artemisinins in routine treatment of *P. falciparum* in
many malaria-endemic areas to prevent transmission [5,
6]. Artemisinin also has some effect on gametocyte carri-
age duration, through the effect on immature gameto-
cytes and rapid killing of asexual parasites [5]. Less is
known about *Plasmodium vivax* gametocytes. It seems
that immature *P. vivax* gametocytes seques ter mainly in
the bone marrow, similar to *P. falciparum* [7]. However
they mature much faster than *P. falciparum* gametocytes,
they are commonly present before symptoms and before
parasite detection by microscopy, and live only for up to
days [2, 8].

Several malaria PCR methods have been introduced
applying different amplification targets on the parasite
genome. The real-time PCR targeting *var* gene acidic ter-

minal sequence (varATS) on the chromosomal genome,
and cytochrome b (*cytb*) on the mitochondrial genome,
are among the most sensitive ones [9]. The varATS gene
exists in 59 copies for each parasite nucleus [10], while
the number of copies of the *cytb* gene varies depending
on parasite stage; ring-stage parasites have about 20 cop-
ies, and gametocytes about 160 copies [11, 12]. Game-
tocyte-specific mRNA transcripts can be detected by
reverse transcript (RT)-PCR methods [13, 14].

The objective of this clinical observational study was to
describe the presence of positive PCR for *P. falciparum*
and *P. vivax* in follow-up samples post-treatment from
returned travellers, and the proportion of positive PCR
due to gametocytes, applying PCR methods detecting
*Plasmodium* DNA and gametocyte-specific mRNA.

**Methods**

**Patient material**

Blood samples from adult patients, diagnosed with malaria by microscopy and/or rapid diagnostic test at
Haukeland University Hospital, Bergen, were collected
in the period 2013–2015. Blood samples were collected
during hospitalization and at routine follow-up in the
outpatient clinic at 11–54 days after treatment. Clinical
information and results from routine microscopy was
collected retrospectively from patient records.

**PCR methods**

DNA was extracted from EDTA whole blood applying
QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Ger-
many), while mRNA was collected and extracted applying
PAXgene Blood RNA Tubes and Kit (PreAnalytiX,
Hombrechtikon, Switzerland), according to the manufac-
turer’s instructions.

All the DNA samples (N=25) were analysed with a
genus-specific *cytb* real-time SYBR PCR, and the *P. fal-
ciparum* DNA samples (N=22) were also analysed with
a *falciparum*-specific varATS real-time TaqMan PCR,
following previously published methods [9, 10]. All
the mRNA samples (N=18) were analysed both with a
genus-specific 18S rRNA RT-PCR (detecting mRNA
transcripts produced by all parasite stages), and a game-
tocyte-specific *Pfs25* (*P. falciparum*)/*Pvs25* (*P. vivax*)
RT-PCR detecting female gametocytes, following previ-
ously published methods [14]. In addition, quantitative
analysis was performed for the *cytb* real-time PCR and
*Pfs25/Pvs25* RT-PCR runs by applying tenfold dilution
series of customized plasmids designed with EcoRI lin-
erized q-PCR template and a pUCminusMCS vector
 backbone (OriGene Technologies, Rockville, MD, USA).
All samples were run in triplicates, and a positive result
was defined as at least two out of three detections.

**Results**

The PCR results applying DNA and mRNA template,
including mean C<sub>T</sub> values as well as quantitative
numbers, are presented in Table 1. Genus DNA *cytb* PCR
was positive up to 49 days after effective treatment, and
18S rRNA transcripts from active *P. falciparum* parasites
were detectable for at least 11 days. Microscopy and/or
PCR detected gametocytes in 67% (8/12) patients, but
gametocyte-specific mRNA was detected at latest only
two days after treatment. Late detection of *P. vivax*
was not found by any of the PCR methods.

All patients were asymptomatic and had negative
microscopy at outpatient follow-up visits. The association
between positive PCR, microscopy findings and clinical
characteristics is presented in Table 2. Six patients with *P.
falciparum* were treated with artesunate intravenously,
followed by a full oral course of artmether-lumefantrine (AL)
or atovaquone-proguanil (AP), while two received only AL
and one only AP. All *P. vivax* patients, and no *P. falciparum*
patients, received primaquine. Among the six patients with
late *P. falciparum* positive PCR (day 11–49), four had high
parasitaemia (6–30%) while two had parasitaemia <2% at
admittance, and three had gametocytes detected. A clear association between low/high *P. falciparum* parasitaemia and duration of positive PCR detections was not found. One patient with late positive PCR was of Norwegian origin, while five were from sub-Saharan Africa and had lived from 10–46 years in Norway. Two of the patients with late positive PCR were immunodeficient with HIV or sickle cell disease.

**Discussion**

In this observational study, DNA-based PCR was positive up to seven weeks after effective malaria treatment. No patients had clinical signs of recrudescence, and there was no risk of re-infection. This is in line with a previous report that found positive malaria PCR after six weeks in a group of returned travellers in Sweden [1]. Positive PCR for weeks after effective treatment of infections is a

| Patient | Species | No days since treatment | Microscopy (parasite %) | Genus DNA Cytb PCR (\(C_t\)) | No copies/rxn | Genus 18S mRNA PCR (\(C_t\)) | Genus 18S mRNA PCR (\(C_t\)) | No. copies/rxn |
|---------|---------|-------------------------|-------------------------|-------------------------------|---------------|-------------------------------|-------------------------------|---------------|
| No. 1   | Pf+Pm   | 0                       | Pos (2)                 | 1.1 \(\times\) 10^5          | Pos (26)      |                                |                                |               |
|         |         | 1                       | Pos (1)                 | 4.3 \(\times\) 10^2          | Pos (28)      | Pos (17)                       | Pos (29)                       | 3.2 \(\times\) 10^3 |
| No. 2   | Pf      | 0                       | Pos (4)                 | 5.7 \(\times\) 10^5          | Pos (19)      |                                |                                |               |
|         |         | 1                       | Pos (4)                 | 7.0 \(\times\) 10^5          | Pos (12)      | Pos (34)                       |                                | 95            |
| No. 3   | Pf      | 0                       | Pos (12)                |                                |               |                                |                                |               |
|         |         | 1                       | Neg                     | Pos (22)                      | 3.7 \(\times\) 10^4 | Pos (26) |                           |               |
|         |         | 2                       | Neg                     | Pos (23)                      | 1.5 \(\times\) 10^6 | Pos (29) |                           |               |
|         |         | 11                      | Neg                     | Pos (30)                      | 200           | Pos (35)                       | Pos (25)                       | Neg            |
| No. 4   | Pf      | 0                       | Pos (< 1)*              |                                |               |                                |                                |               |
|         |         | 31                      | Neg                     | Neg                           |               |                                |                                | Neg            |
| No. 5   | Pf      | 0                       | Pos (< 1)               |                                |               |                                |                                |               |
|         |         | 32                      | Neg                     | Pos (37)                      | 2             | Neg                           | Neg                           |               |
| No. 6   | Pf      | 0                       | Pos (2)                 | 3.9 \(\times\) 10^3          | Pos (20)      |                                |                                |               |
|         |         | 1                       | Pos (< 0.5)             | Pos (24)                      | 7.7 \(\times\) 10^5 | Pos (27) | Pos (17)                       | Pos (31)                       | 420           |
|         |         | 37                      | Neg                     | Pos (39)                      | 1             | Neg                           | Neg                           |               |
| No. 7   | Pf      | 0                       | Pos (10)                | 4.0 \(\times\) 10^6          | Pos (16)      |                                |                                |               |
|         |         | 4                       | Neg                     | Pos (25)                      | 6.3 \(\times\) 10^5 | Pos (29) | Pos (23)                       | Neg                           |               |
|         |         | 11                      | Neg                     | Pos (29)                      | 400           | Pos (35)                       | Pos (24)                       | Neg            |
|         |         | 39                      | Neg                     | Neg                           | Neg           | Neg                           | Neg                           | Neg            |
| No. 8   | Pf      | 0                       | Pos (30)                | 1.8 \(\times\) 10^6          | Pos (18)      |                                |                                |               |
|         |         | 1                       | Pos (6)                 | 3.0 \(\times\) 10^5          | Pos (17)      | Pos (7)                        | Pos (29)                       | 2.2 \(\times\) 10^3 |
|         |         | 45                      | Neg                     | Pos (34)                      | 16            | Neg                           | Neg                           | Neg            |
| No. 9   | Pf      | 0                       | Pos (6)*                |                                |               |                                |                                |               |
|         |         | 49                      | Neg                     | Pos (34)                      | 11            | Pos (36)                       | Neg                           | Neg            |
| No. 10  | Pv      | 0                       | Pos (0.5)               |                                |               |                                |                                |               |
|         |         | 2                       | Pos*                    |                                |               |                                |                                | Neg            |
| No. 11  | Pv      | 0                       | Pos (0.5)*              |                                |               |                                |                                | Neg            |
|         |         | 6                       | Neg                     | Pos (39)                      | 1             | Neg                           | Neg                           |               |
| No. 12  | Pv      | 0                       | Pos (22)                | 4.8 \(\times\) 10^4          | Pos           | Neg                           | Neg                           | Neg            |
| No. 13  | Pv      | 0                       | Pos                     |                                |               |                                |                                |               |
|         |         | 54                      | Neg                     | Neg                           | Neg           | Neg                           | Neg                           | Neg            |

*rxn reaction

* Gametocytes seen by microscopy
common phenomenon; a study investigating detectable DNA after treatment of *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and *Trichomonas vaginalis* infections, reported that PCR could be positive up to three weeks post-treatment [15].

Molecular studies have shown that most individuals with asexual parasites also have sub-microscopic gametocyte carriage [16]. In general, the level of circulating gametocytes is low, about 5% compared to other parasite-stages. Gametocytes were detected by microscopy and/or RT-PCR in 67% (8/12) of the patients in the present study. However, gametocyte-specific mRNA was not detected in the late follow-up samples, similar to that reported by Vafa Homann et al. in Sweden [1], which might indicate that the detection of *Plasmodium* DNA origin from residuals of destroyed parasites. The phagocytic system has the potential to remove up to 40–80% of malaria infected red blood cells (RBCs) in a few days, but due to sequestration of infected RBCs in organs, the time of complete clearance of parasite residuals is unknown.

| Patient | Species | No. days since treatment | Microscopy (parasite%) | PCR | Fever duration before treatment (days) | Treatment | Origin/years out of endemic area | Comorbidity |
|---------|---------|--------------------------|------------------------|-----|---------------------------------------|-----------|--------------------------------|-------------|
| No. 1   | *P. f* + *P. m* | 0 | Pos (2) | Pos | 35 | AL | SSA/1 | HIV |
|         |         | 1 | Pos (1) | Pos |     |     |     |     |
|         |         | 2 | Neg | Pos |     |     |     |     |
|         |         | 11 | Neg | Pos |     |     |     |     |
| No. 2   | *P. f* | 0 | Pos (4) | Pos | 2 | A, AL | SSA/8 | HIV |
|         |         | 1 | Pos (4) | Pos |     |     |     |     |
| No. 3   | *P. f* | 0 | Pos (12) | Pos | 3 | A, AP | Norway |     |
|         |         | 1 | Neg | Pos |     |     |     |     |
|         |         | 2 | Neg | Pos |     |     |     |     |
|         |         | 11 | Neg | Pos |     |     |     |     |
| No. 4   | *P. f* | 0 | Pos (< 1) | Pos | 38 | AP | SSA/10 |     |
|         |         | 31 | Neg | Neg |     |     |     |     |
| No. 5   | *P. f* | 0 | Pos (< 1) | Neg | 7 | AL | SSA/46 |     |
|         |         | 32 | Neg | Pos |     |     |     |     |
| No. 6   | *P. f* | 0 | Pos (2) | Pos | 9 | Q, A, AL | SSA/21 |     |
|         |         | 1 | Pos (< 0.5) | Pos |     |     |     |     |
|         |         | 37 | Neg | Pos |     |     |     |     |
| No. 7   | *P. f* | 0 | Pos (10) | Pos | 21 | A, AL | SSA/10 | HIV |
|         |         | 4 | Neg | Pos |     |     |     |     |
|         |         | 11 | Neg | Pos |     |     |     |     |
|         |         | 39 | Neg | Neg |     |     |     |     |
| No. 8   | *P. f* | 0 | Pos (30) | Pos | 3 | A, | Norway |     |
|         |         | 1 | Pos (6) | Pos |     |     |     |     |
|         |         | 45 | Neg | Pos |     |     |     |     |
| No. 9   | *P. f* | 0 | Pos (6) | Pos | 13 | A, AP, AL | SSA/17 | Sickle cell disease |
|         |         | 49 | Neg | Pos |     |     |     |     |
| No.10   | *P. v* | 0 | Pos (0.5) | Pos | 10 | AP, P | SEA/30 |     |
|         |         | 2 | Pos | Pos |     |     |     |     |
| No. 11  | *P. v* | 0 | Pos (0.5) | Neg | Not known | AP, P | SSA |     |
|         |         | 6 | Neg | Pos |     |     |     |     |
| No. 12  | *P. v* | 0 | Pos | Pos | 2 | A, AP, P | Norway |     |
|         |         | 23 | Neg | Neg |     |     |     |     |
| No. 13  | *P. v* | 0 | Pos | Neg | 6 | C, P | Norway |     |
|         |         | 54 | Neg | Neg |     |     |     |     |

AL: artesunate-lumefantrine, ART: artesunate IV (intravenous), PA: atovaquone-proguanil, Q: quinine IV, C: chloroquine, P: primaquine, HIV: human immunodeficiency virus, SSA: sub-Saharan Africa, SEA: Southeast Asia

* Gametocytes seen by microscopy
* Detection of 18S mRNA transcripts (Produced by all parasite stages)
* Detection of gametocyte-specific mRNA
Drug treatment may also have contributed to clear gametocytes. All the P. falciparum patients were treated with artemisinin, which has some gametocidal effect, and all P. vivax patients were treated with PQ, which has a strong gametocidal effect. In a study from Kenya and Tanzania estimating gamocyte carriage following treatment with non-artemisinin drugs, artemisinin, and artemisinin in combination with PQ, duration of gametocytaemia was 55, 13 and 6 days, respectively [4].

Collection, handling and analysis of mRNA is challenging [18, 19], and due to the instability of mRNA versus DNA, difference in sensitivity between the methods can be a factor that may underestimate late gametocytaemia in this study. A study investigating a similar gamocyte-specific Pf25 RT-PCR, reported that in dilution series down to 0.05 and 0.01 gametocytes/µl, the lowest density samples were often negative [20]. In the present study, the quantitative values detected by cytb DNA PCR in samples >30 days after treatment correspond to \( \leq 0.05 \) gametocytes/µl, so potentially these could have gametocytaemia below detection level of mRNA RT-PCR.

In the study from Kenya and Tanzania investigating gametocytaemia, a nucleic acid sequence based amplification (NASBA) method detecting Pf25 mRNA was applied, a method slightly more sensitive than Pf25 RT-PCR; gametocytes were found in 5–78% of the samples at day 14, and 12–48% at day 28 [4]. However, compared to returned travellers in the present study, patients in malaria-endemic areas may have a higher level of gamocyte carriage post-treatment due to reasons such as late admission and delayed treatment, use of less effective drugs, self-medication with sub-optimal regimes, re-infection, or gamocyte carriage that originate from previous undetected low-density infections.

Looking only at the two DNA-based PCR assays, the cytb PCR detected more late follow-up samples than varATS PCR. Hypothetically this could be explained by detection of gametocytes, since the cytb method detect mitochondrial DNA present in large amounts in gametocytes. In regard to copy number, the cytb PCR is about 2.5 times more sensitive in detecting gametocytes than varATS PCR, while the varATS PCR is about three times more sensitive than cytb PCR in detecting asexual parasites. In a study applying field samples from Tanzania, the sensitivity in detecting low level parasitaemia by different real-time malaria PCR methods where compared, and the varATS PCR was then found to be more sensitive than cytb PCR [9].

Two samples 11 days after treatment were positive by 18S rRNA RT-PCR (mRNA), but not by the gamocyte specific Pf25 RT-PCR, indicating that possible live asexual parasites circulated in the bloodstream in low densities at this point. Although, negative gamocyte

specific Pf25 RT-PCR due to lower sensitivity could also be possible. The quantitative levels by the DNA PCR after 11 days correspond to <5 parasites/µl. Post-treatment asexual parasites that survive until they are cleared by the immune system continue to produce gametocytes, which also could support the reported phenomenon of gamocyte carriage for several weeks after treatment [4].

For the P. vivax samples, a high level of mRNA from gametocytes was detected by the Pvs25 RT-PCR at day 2 after treatment, but both mRNA assays were negative for the late follow-up samples. The cytb DNA PCR was positive at day 6, though with parasitaemia as low as 0.025 parasites/µl. These results are consistent with the short duration of gametocytaemia in P. vivax [2]. The results for P. vivax also support that persistent positive P. falciparum PCR could be caused by gametocytes; if positive P. falciparum PCR was caused only by DNA residuals, it would be expected that also P. vivax was PCR positive days/weeks after treatment similar in P. falciparum infections. An association between high P. falciparum parasitaemia and persistent PCR would intuitively be expected. However, similar to that reported by Vafa Homann et al. among Swedish travellers [1], no clear association between low/high level of parasitaemia and persistent positive PCR was identified in the present study.

Conclusions
DNA-based PCR can be positive up to at least seven weeks after curative malaria treatment, potentially leading to over-diagnose of recrudescence and re-infections. Based on the observations in this study, it is unclear if the DNA origin from residuals of destroyed parasites or live gametocytes. Further studies of both P. vivax and P. falciparum are needed, since different gametocytaemia biology and treatment-regimes have the potential to give informative answers.

Abbreviations
PCR: Polymerase chain reaction; PQ: Primaquine (PQ); varATS: var Gene acidic terminal sequence; cytb: Cytochrome b; RT: Reverse transcript; AL: Artemether-lumefantrin; AP: Atovaquone-proguanil; RBCs: Red blood cells.

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Authors’ contributions
CGH performed the laboratory analyses, and wrote the first draft of the manuscript. KM recruited patients for the sample collection, recorded and analysed clinical data, and supervised the study. Both authors contributed to the planning of the study, revision of the manuscript. Both authors read and approved the final manuscript.

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Availability of data and material
The datasets used during the current study are available from the correspond-
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Ethics approval and consent to participate
The study was approved by the regional ethics committee in Norway
(2017/47/REK vest), and the patients gave written informed consents to par-
ticipate. Treatment and monitoring of patients was done according to clinical
routine practice.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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