Multiplex real-time PCR for the diagnosis of malaria: correlation with microscopy

L. Dormond[1], K. Jaton-Ogay[1], S. de Vallière[2], B. Genton[2], J. Bille[1,3] and G. Greub[1,3]

1) *Institute of Microbiology, University Hospital Center and University of Lausanne, Lausanne;* 2) *Travel Clinic, Department of Ambulatory Care and Community Medicine, University Hospital, Lausanne and* 3) *Service of Infectious Diseases, University Hospital Center, Lausanne, Switzerland*

Abstract

Malaria is generally diagnosed by microscopy and rapid antigen testing. Molecular methods become more widely used. In the present study, the contribution of a quantitative multiplex malaria PCR was investigated. We assessed: (i) the agreement between PCR-based identification and microscopy and (ii) the correlation between the parasite load as determined by quantitative PCR and by microscopy. For 83 patients positive by microscopy for *Plasmodium* spp., the first EDTA-blood sample was tested by multiplex PCR to confirm smear-based species identification. Parasite load was assessed daily using both microscopy and PCR. Among the 83 patients tested, one was positive by microscopy only and 82 were positive by microscopy and PCR. Agreement between microscopy and PCR for the identification at the species level was 89% (73/82). Six of the nine discordant results corresponded to co-infections by two or three species and were attributed to inaccurate morphological identification of mixed cases. The parasite load generally decreased rapidly after treatment had been started, with similar decay curves being obtained using both microscopy and PCR. Our PCR proved especially useful for identifying mixed infections. The quantification obtained by PCR closely correlated with microscopy-based quantification and could be useful for monitoring treatment efficacy, at least in clinical trials.

Keywords: Diagnosis, disease severity, malaria, microscopy, *Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale*, *Plasmodium vivax*, real-time PCR

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Corresponding author: Gilbert Greub, Infectious Diseases Service and Institute of Microbiology, University Hospital Center and University of Lausanne, Switzerland

E-mail: gilbert.greub@chuv.ch

Introduction

In industrialized countries, the standard of malaria diagnosis in routine laboratories still comprises thin and thick blood film microscopy because of its rapidity and accessibility compared to molecular methods. Rapid diagnostic tests offer a good alternative, especially in emergency situations [1] and in the absence of danger signs.

Thick blood film microscopy has an acceptable sensitivity for clinical use, in the range of 50 000–500 000 parasites/mL [2–5]. It may thus detect relatively low parasitaemia that may be seen: (i) early in the course of the disease; (ii) among infected returning patients under chemoprophylaxis [6]; or (iii) among asymptomatic infected autochthonous subjects. However, thick blood film is not recommended for quantification and identification of *Plasmodium* species because it underestimates parasite density in the order of one log lower compared to PCR [4]. Thin blood film microscopy is superior for species identification and quantification is easier in such a monolayer preparation. Nevertheless, microscopy needs experienced microscopists, and even highly-trained technicians may fail to identify *Plasmodium* at the species level. Moreover, a two- to three-fold discrepancy in parasite quantification may be seen between technicians [7]. Quantification is important for estimating clinical prognosis and monitoring the treatment response, particularly in areas with parasites exhibiting drug resistance. Molecular methods such as real-time PCR tend to be more sensitive (down to 20 parasites/mL of blood) [8] and to be more specific than microscopy, especially in cases of mixed infections [9]. The high sensitivity of PCR was also recently demonstrated in endemic populations in low-transmission areas with low level of parasitaemia [10]. PCRs are used to evaluate other malaria rapid diagnostic tests [1], in malaria vaccine trials

[8,11] and to detect antigen polymorphism [12]. They can also be used to distinguish between recrudescence or reinfection in *in vivo* efficacy studies [13]. New molecular markers are being constantly developed [14].

Subsequent to January 2004, PCR has been used in our diagnostic laboratory to confirm species identification for every positive microscopy as a systematic internal quality control. The present study aimed to compare: (i) species identification obtained by microscopy and molecular approaches and (ii) parasitaemia diagnosed in thin smears with real-time PCR quantification.

Materials and Methods

Patients and samples

All samples received in our diagnostic laboratory from 1 January 2004 to 30 June 2008 and found to be positive for a *Plasmodium* infection by Giemsa-stained thin smear and PCR have been included in the present study. Inclusion was limited to samples taken from adults (>16 years old) hospitalized at the University Hospital, Lausanne, Switzerland, or seen at the Department of Ambulatory Care and Community Medicine. This project was approved by our local ethical committee.

Quantitative real-time PCR

All samples positive by direct microscopy were confirmed by *Plasmodium* quantitative real-time PCR adapted from Rougemont *et al.* [15]. To improve specificity, especially in case of mixed infections, we modified the forward primers used to amplify *Plasmodium ovale* and *Plasmodium malariae* as: forward primer for *P. ovale*: 5′-CCGACTAGGTTTTGGATGAAAG AT-3′ and forward primer for *P. malariae*: 5′-CGACTA GGTGTTGGATGATAGAGTA-3′. The specificity of these new primers was tested using DNA from humans, *Aspergillus*, *Toxoplasma*, *Pneumocystis*, *Neospora* and *Leishmania*. Cross-reactivity within other plasmodial species was also assessed. No cross-amplification was observed between the different *Plasmodium* species and between any of all other tested eucaryotes. The sensitivity of the new *P. ovale* and *P. malariae* PCR was assessed on ten-fold serial dilutions (from 10 000 to one copy) of positive plasmid controls, as described previously [15], and proved to be highly sensitive, repeatedly amplifying the ten copies positive control and amplifying only part of the one copy positive control, most likely as a result of the stochastic distribution of DNA in each PCR reaction tube. The PCR was performed on the EDTA blood sample already used for microscopy. This real-time Taqman PCR, which has also been recently implemented in other clinical diagnostic laboratories [9,16], targets the 18S rRNA encoding gene. It is a species-specific multiplex PCR that detects the four most common human *Plasmodium* species (*Plasmodium falciparum*, *P. ovale*, *Plasmodium vivax* and *P. malariae*) using four different specific Taqman probes. All positive results were confirmed by a subsequent monoplex PCR using the same primers and probes aiming to quantify the number of parasites per mL of blood.

Comparison of PCR results with microscopy

PCR identification of *Plasmodium* spp. was performed on all cases found to be positive by microscopy. All PCR results discordant with microscopy were tentatively solved by repeating microscopic examination (twice independently by two laboratory technicians) and by sequencing PCR products.

In addition, for samples positive for *P. falciparum* only, we compared quantification with microscopic examination with that obtained by real-time PCR on the first positive sample and on all subsequent samples. The latter analysis allowed us to compare the effect of treatment on the decay of the parasite load. The ratio of mean parasitaemia determined by microscopy and by PCR was also calculated.

For microscopy, parasitaemia was initially reported in percentages. The number of *P. falciparum* parasites per mL of blood was estimated assuming that 1 μL contains 5×10^6 red blood cells. Thus, 1% parasitaemia corresponded to 50 000 parasites/μL of blood and to 50 000 000 parasites/mL. PCR was expressed in DNA copies/mL.

Results

Patients and samples

Among 89 patients with *Plasmodium* infection, *P. falciparum* infection was more commonly observed than the three other species. Six patients with negative microscopy and a positive PCR on the first blood sample (five for *P. falciparum* and one for *P. vivax*) were excluded from all comparisons between microscopy and PCR. All six positive PCR results were true positive results because at least one subsequent sample was positive by microscopy. Two of these six had a positive rapid test, and PCR was performed for the other four patients based on a strong clinical suspicion of *Plasmodium* infection. These six patients had DNA loads in the range 67–480 000 DNA copies/mL.

Among the 83 remaining patients who had a first sample positive by microscopy for at least one *Plasmodium* species, PCR was negative in one single case, with only one trophozoite seen on the thin smear (parasitaemia of <500,000

parasites/mL; 0.01%); this patient had already received anti-malaria treatment (pyrimethamine/sulphadoxine) when in Rwanda, and was seen 2 weeks later in our outpatient travel clinic as a result of persistent fatigue.

Identification at the species level

Among the remaining 82 patients (positive by microscopy and by PCR), PCR and microscopy results were initially congruent at the species level for only 66 patients (80.5%). After a second independent blind assessment of the thin smears by two laboratory technicians who were specialized in parasitology, seven discordant results were resolved (i.e. seven single *P. falciparum* infections had been wrongly considered as *P. falciparum*/*P. malariae* co-infections by microscopy, most likely as a result of the over-interpretation of some old *P. falciparum* trophozoites as *P. malariae*).

After blind re-assessment of all discordant results, 67 patients were determined to be infected by *P. falciparum* alone, five by *P. vivax* only, one by *P. malariae* only and none by *P. ovale* only (Table 1). The final identification at the species level diverged between PCR and microscopy for the nine remaining patients (11%), even after blind retrospective assessment of the thin smears. Thus, microscopy and PCR were congruent at the species level for 73 patients (89%). In three of the nine discordant cases, a mixed *P. falciparum*/*P. malariae* infection was diagnosed based on initial microscopy. Although blind reassessment was again in favour of such co-infection, no *P. malariae* parasites were detected using our sensitive real-time PCR and, even after two independent re-assessments, no gametocytes could be observed; this strongly suggests that the PCR result was correct. In four of the nine divergent cases, *P. falciparum* only was detected by microscopy, whereas a mixed infection with *P. ovale* and/or *P. malariae* was detected by PCR with relatively low DNA copy numbers in the range 90–9300 copies/mL (Fig. 1). The final divergent cases were wrongly identified as *P. falciparum*/*P. vivax* co-infection and *P. vivax* infection whereas, based on PCR, they were both *P. falciparum*/*P. ovale* co-infections. The latter case is the only one where *P. falciparum* was missed by microscopy. Moreover, both patients acquired malaria in Cameroon, a country endemic for *P. ovale* and where *P. vivax* is extremely rare [17]. To further investigate the nine discordant results, we sequenced the PCR products and sequences were congruent with real-time PCR identification.

TABLE 1. Correlation between thin smear microscopy and real-time PCR at the level of *Plasmodium* species identification for 89 patients with malaria in a 4-year-period

PCR	Results of thin smear microscopy
Plasmodium falciparum (n = 75)	*Plasmodium falciparum* and *P. malariae* (n = 3)
	Plasmodium falciparum (n = 67)
	Negative microscopy (n = 5)
Plasmodium vivax (n = 6)	*Plasmodium vivax* (n = 5)
	Negative microscopy (n = 1)
Plasmodium malariae (n = 1)	*Plasmodium malariae* (n = 1)
Plasmodium falciparum and *Plasmodium ovale* (n = 3)	*Plasmodium falciparum* (n = 1)
	Plasmodium vivax (n = 1)
	Plasmodium falciparum and *P. vivax* (n = 1)
Plasmodium falciparum and *Plasmodium malariae* (n = 2)	*Plasmodium falciparum* (n = 2)
Plasmodium falciparum, *Plasmodium ovale* and *Plasmodium malariae* (n = 1)	*Plasmodium falciparum* (n = 1)
PCR negative	*Plasmodium falciparum* (n = 1)

PCR-based quantification at day 0

For day 0 samples exhibiting a parasitaemia ≤ 500 000 parasites/mL (≤0.01%) by microscopy (which corresponds to approximately 5.69 log), logarithms of DNA copies ranged between 2.58 (376 copies/mL) and 6.53 (3 300 000 copies/mL) (Fig. 2). Among day 0 samples with a parasitaemia >500 000 parasites/mL (>0.01%), we observed a clear correlation between microscopy and PCR quantification (Fig. 2).

Mean ± SD parasitaemia determined by microscopy was 7.69 ± 0.62 log copies/mL, and that determined by PCR was 7.01 ± 0.84 log copies/mL for samples with parasitaemia

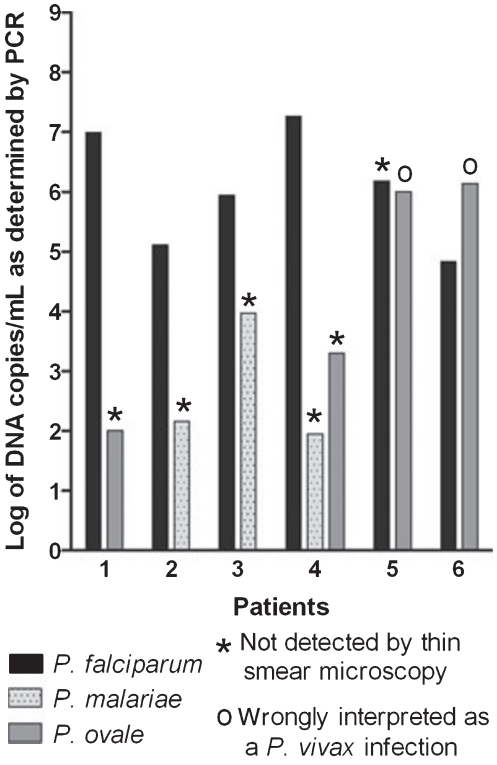

FIG. 1. Cases of discordant identification at the species level when comparing microscopy and PCR.

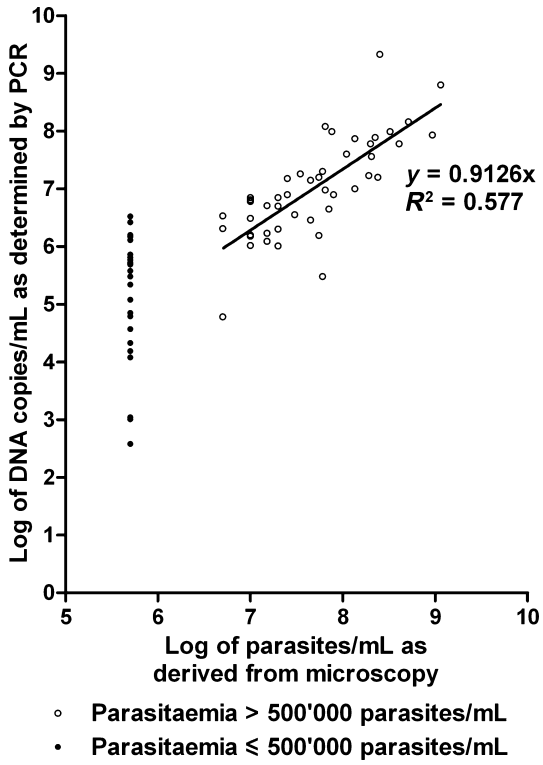

FIG. 2. Comparison of quantification assessed by thin smear microscopy versus PCR. Only the first sample positive for single *Plasmodium falciparum* (70 patients) has been analyzed. Black dots represent samples with a parasitaemia determined by microscopy of ≤0.01% (i.e. the limit of positivity below which accurate quantification was not possible by microscopy).

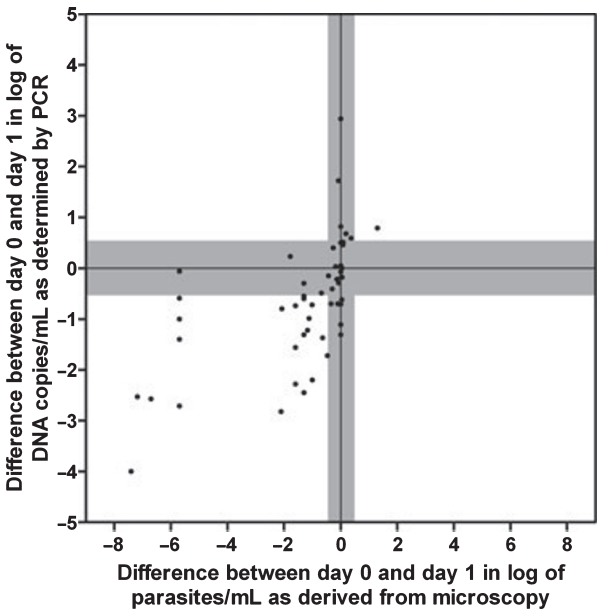

FIG. 3. Log decrease of DNA copies/mL as determined by PCR in correlation with log decrease as derived from thin smear microscopy between day 0 and day 1 of treatment among 56 patients presenting a single *Plasmodium falciparum* infection. The shaded area represents a decrease/increase of <0.5 log, which approximatively corresponds to the variability of PCR quantification and therefore is not considered as a significant variation.

>0.01%, whereas the ratio of mean parasitaemia by PCR and by microscopy was 0.92. This shows that microscopy does not underestimate parasite density.

Evolution of PCR results under treatment

Among the 70 patients with single *P. falciparum* infection according to PCR identification, 52 had PCR quantification at day 0 and day 1 with the first positive microscopy (Fig. 3). No discrepant results were observed.

For six patients, a follow-up was available for six to ten samples during a 4–12-day-period (Fig. 4). There is a close correlation between PCR and microscopy quantification, as long as the parasitaemia by microscopy remains above 500 000 parasites/mL (0.01%; estimated log of 5.8).

Discussion

In the present study, we demonstrated that our in-house real-time quadriplex PCR is useful for detecting mixed *Plasmodium* infections and that identified discrepancies between microscopy and PCR were mainly the result of the inherent difficulties of accurate identification using phenotypic criteria. PCR should thus be performed systematically on positive samples to confirm phenotypic identification, at least in cases of suspected mixed infection. We also showed that our PCR allows precise assessment of parasite load during follow-up under treatment.

Discordances at the species level identification

Identification at the species level between microscopy and PCR was initially congruent for 80.5% of the cases. A second blind retrospective reading allowed us to improve the congruence of the *Plasmodium* identification to 89%. Discrepancies between microscopy and PCR were of three kinds: (i) the misinterpretation of some old *P. falciparum* trophozoites as *P. malariae* trophozoites; (ii) the misinterpretation of *P. ovale* infections as *P. vivax* infections; and (iii) the failure to detect mixed infections when the co-infecting species was in very low numbers.

Microscopical misinterpretation of some old *P. falciparum* trophozoites as *P. malariae* trophozoites occurred in ten of 82 cases. Most of them (7/10) were probably a result of the lack

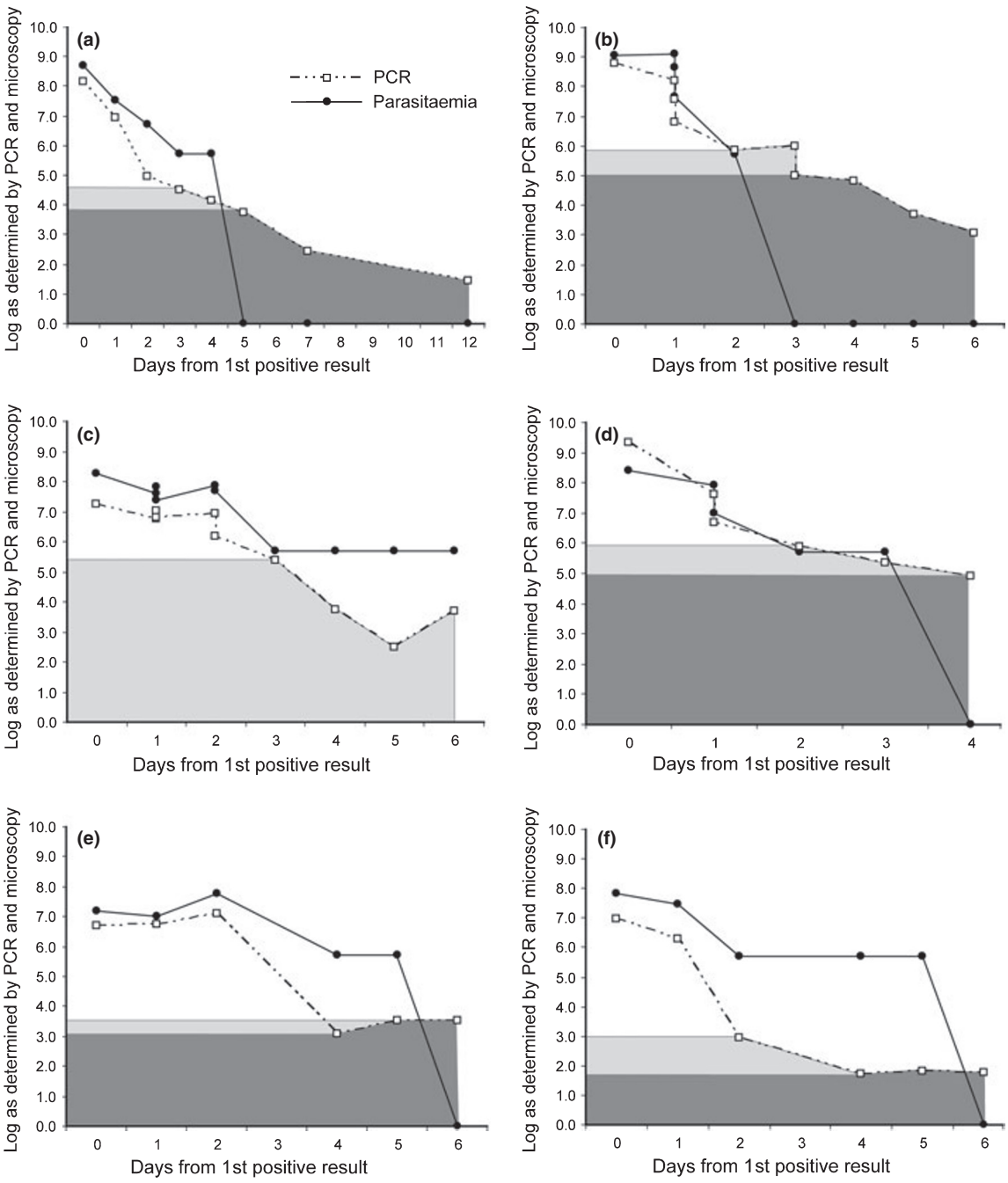

FIG. 4. Follow-up of parasitaemia as determined by thin smear microscopy and PCR for six different patients (a to f) with single *Plasmodium falciparum* infection for whom at least six successive samples were available. Shaded areas indicate the quantity of DNA copies present in the blood at the time of the first microscopy ≤0.01% (light grey) and the first negative microscopy (dark grey).

of experience of the first microscopy reader and were correctly considered as single *P. falciparum* when retrospectively re-interpreted blindly by two different laboratory technicians. One important diagnostic hint was the subnormal size (microcytes) of infected erythrocytes. The presence of anisocytosis could have mislead diagnosis. Misinterpretation may also happen in cases of superposition of platelets on red blood cells [18]. Finally, thin blood films were automatically stained by the haematology ward at a pH of 6.8; this relatively low pH may have impaired the detection of *Plasmodium* by microscopy because granulations are almost absent at pH 6.8 [18]. Staining should optimally be performed at a pH of 7.2 when staining

properties of red blood cells become apparent (i.e. as Maurer's dot of *P. falciparum*).

Misinterpretation of *P. ovale* infections as *P. vivax* infections occurred only twice. Both patients were coinfected with *P. falciparum*. They presented with amoeboid trophozoites. The fimbriated ends, which are typical of *P. ovale* infection and may be present in up to 25% of *P. ovale* infected cells [19], were not found.

The oval shape of *P. ovale*, which may be present in 30–85% of parasitized erythrocytes [20], was rarely observed in the two cases infected with *P. ovale* and misdiagnosed as *P. vivax*. The pH of 6.8 may also have impaired *Plasmodium* identification. Indeed, Schüffner's dots of *P. ovale* and *P. vivax* can be seen much more easily at a pH of 7.2 [19,21]. The size and number of Schüffner's dots may also differ with species, with *P. ovale* exhibiting larger but less numerous dots than *P. vivax*. Both patients that were infected with *P. ovale* and wrongly interpreted as *P. vivax* had come from Cameroon, where *P. ovale* is endemic and *P. vivax* extremely rare, supporting the suspicion that the PCR result was correct. These examples demonstrate the importance of correlating clinical information, such as country of exposure as well as country of origin, to correctly interpret microscopy.

It is particularly difficult to identify mixed infections when one or other of the co-infecting species is present in low numbers [9]. Thus, in the present study, four of the six discordant results not solved by blind reassessment were the result of a very low parasite load of the co-infecting species (Fig. 1). Mixed infections have been misdiagnosed by microscopy in approximately 71% of cases [2,22]. The systematic use of PCR may therefore be especially useful for detecting co-infection with a second species present in low quantity. This may be important for *P. ovale* and *P. vivax* infections because they establish hypnozoites in the liver that are responsible for relapses, and which might be prevented using schizonticides [23].

PCR-based quantification

There was correlation between the number of *P. falciparum* copies/mL as determined using our real-time PCR and parasitaemia determined by microscopy among samples with parasitaemia >0.01% (Fig. 2). Most patients with quantification at treatment day 0 and day 1 had a decrease of parasite load detected by both methods and no significant discordances were observed. PCR-based quantification might especially be useful in clinical trials to precisely compare the decrease of parasite load upon treatment because it allows the precise quantification of parasitaemia ≤0.01% (based on microscopy) on a five-log scale. Another advantage of our real-time quantitative PCR over microscopy is that it can be performed in large batches with 384-well plates. PCR also gives objective results that are not dependant on the experience of the laboratory technicians.

Conclusion

In conclusion, our real-time quadriplex PCR proved to be very useful for correcting microscopy-based misidentifications and for detecting mixed infections, especially when one of the species was present in low quantities. This real-time PCR also proved to be more sensitive than microscopy and might be performed systematically in addition to rapid tests and microscopy when the clinical suspicion of malaria is high.

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Transparency Declaration

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