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# Cytochrome *b* Gene Quantitative PCR for Diagnosing *Plasmodium falciparum* Infection in Travelers[▽]

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**A cytochrome *b* (*cytb*) gene quantitative PCR (qPCR) assay was developed to diagnose malaria in travelers. First, manual and automated DNA extractions were compared and automated DNA extraction of 400 μl of blood was found to be more efficient. Sensitivity was estimated using the WHO international standard for *Plasmodium falciparum* DNA and compared to that of a previously published qPCR targeting the 18S rRNA coding gene (18S qPCR). The limit of detection of the *cytb* qPCR assay was 20 DNA copies (i.e., 1 parasite equivalent) per 400 μl of extracted whole blood and was comparable for the two qPCR assays. Both qPCR assays were used on blood samples from 265 consecutive patients seen for suspicion of malaria. There were no microscopy-positive and qPCR-negative samples. Positive *cytb* qPCR results were observed for 51 samples, and all but 1 were also 18S qPCR positive. Eight (16%) of these 51 samples were negative by microscopic examination. The 8 *cytb* qPCR-positive and microscopy-negative samples were from African patients, 3 of whom had received antimalarial drugs. Three non-*P. falciparum* infections were correctly identified using an additional qPCR assay. The absence of PCR inhibitors was tested for by the use of an internal control of mouse DNA to allow reliable quantification of circulating DNA. The high analytical sensitivity of both qPCR assays combined with automated DNA extraction supports its use as a laboratory tool for diagnosis and parasitemia determination in emergencies. Whether to treat qPCR-positive and microscopy-negative patients remains to be determined.**

In countries where malaria is not endemic, a significant rise in imported malaria cases has been observed in recent years due to the development of travel, tourism, and migration from areas in which malaria is endemic. Microscopic examination of stained blood films is still considered the gold standard for diagnosis. The main strengths of this method are that it can identify both the species and the stage of infection, as well as quantify parasite density. However, microscopy remains labor-intensive and time-consuming. Moreover, diversity in protocols and in the results obtained by different observers has been documented for both species identification and quantification (21). These problems are exacerbated in regions where malaria microscopy is performed infrequently to maintain expertise (14). Immunochromatographic tests (ICT) based on the detection of *Plasmodium* antigens in blood can be performed by nonskilled technicians within half an hour but are not more sensitive than microscopy, quantification of parasitemia is not possible, species other than *P. falciparum* species may not be detected, and negative results require microscopic confirmation (12, 20).

DNA amplification for malaria diagnosis began to attract attention as a possible alternative to microscopy as early as the early 1990s. Nested and other open-tube PCR methods are very prone to contamination with previously amplified products and require long turnaround times and are therefore not suitable for routine use (4). Moreover, these techniques do not allow parasitemia to be quantified. In contrast, real-time quantitative PCR (qPCR) technology has the potential to overcome these limitations and offers a simple, time-effective, and quantitative diagnostic option. With the use of specific fluorescently labeled probes in a closed system, amplicon formation can be detected, monitored, and quantified throughout the reaction with no risk of contamination of the environment with amplicons. Additionally, since the copurification of trace PCR inhibitors may reduce amplification efficiency, leading to erroneous quantification of the parasitic load or false-negative results, the use of an internal control (IC) is compulsory. This necessity is linked to the need for high-quality DNA extraction from blood samples by a rapid DNA extraction technique. Finally, the availability of results within 2 h allows a possible application in an emergency context to be envisaged (12).

We have therefore developed a strategy including (i) a commercial and automated DNA extraction protocol, (ii) a heterologous IC incorporated into each sample to monitor the yield of DNA amplification and to allow quantification, (iii) a positive diagnosis based on a qPCR assay, and (iv) differentiation between *Plasmodium falciparum* and non-*P. falciparum* species based not on melting curve analysis but on an additional qPCR assay. We developed a qPCR assay targeting the mitochondrial cytochrome *b* gene and compared our results to those of an already published qPCR method targeting the 18S rRNA-encoding gene (17). The 18S rRNA gene is one of the most often reported targets in qPCR (1, 3). However, there are some reports suggesting that mitochondrial targets could be more

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TABLE 1. Primers and probes used in this study

| Primer purpose and name | Sequence[b] | Modification(s)[a] | Amplicon length (bp) |
|---|---|---|---|
| *Plasmodium* sp. diagnosis by real-time qPCR | | | |
| MACH60 | 5′-ACATGGCTATGACGGGTAACG-3′ | None | 84 |
| MACH61 | 5′-TGCCTTCCTTAGATGTGGTAGCTA-3′ | None | |
| MACH62 | 5′-TCAGGCTCCCTCTCCGGAATCGA-3′ | 5′ FAM, 3′ TAMRA | |
| cyt *b* 5 | 5′-TGGWTATGTGGAGGATATACTGT-3′ | None | 203 |
| cyt *b* 2 | 5′-CCTTTAACATCAAGACTTAATAGATTTGGA-3′ | None | |
| cyt *b* Probe | 5′-G+TGC+TAC+CAT+GTA+AAT+GTAA-3′ | 5′ FAM-3′, TAMRA | |
| *P. falciparum* identification | | | |
| cyt *b falciparum* | 5′-TACTAACTTGTTATCCTCTATTCCAGTAGC-3′ | None | 240 |
| cyt *b* 2 | 5′-CCTTTAACATCAAGACTTAATAGATTTGGA-3′ | None | |
| cyt *b* Probe | 5′-G+TGC+TAC+CAT+GTA+AAT+GTAA-3′ | 5′ FAM, 3′ TAMRA | |
| IC | | | |
| IC 1 | 5′-GCGCTTCCCGAGGTACACTAT-3′ | None | 135 |
| IC 2 | 5′-ATGTCACATCTGCCCGAACTCC-3′ | None | |
| IC 3 | 5′-TGGTGATCCTGCCGTTTCCTTGTCTT-3′ | 5′ LCRed670, 3′ Ph | |
| IC 4 | 5′-GCCCTGATGTGGTCACAGTCAAGCA-3′ | 3′ FITC | |

[a] FITC, fluorescein isothiocyanate; Ph, phosphate; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.
[b] +, locked nucleic acid base.

sensitive than ribosomal ones (11, 28). Finally, we applied this qPCR strategy to a collection of 294 EDTA blood samples from 265 patients for which microscopy, quantification, and antigen detection had been performed.

## MATERIALS AND METHODS

**Validation of the *cytb* qPCR assay using the *Plasmodium* standard.** To calibrate and compare our results, the WHO international standard for *P. falciparum* DNA nucleic acid amplification technology assays was obtained from the National Institute for Biological Standards and Control (NIBSC; Hertfordshire, United Kingdom). This standard consists of a freeze-dried preparation of whole blood collected from a patient infected with *P. falciparum* by exchange transfusion. According to NIBSC recommendations, this lyophilized material was suspended in 0.5 ml of sterile, nuclease-free water. The concentration of this standard is $10^9$ IU/ml, corresponding to a parasitemia of 9.79 parasites/100 red blood cells (David Padley, NIBSC, personal communication).

For the cytochrome *b* gene (*cytb*) qPCR assay, the primers and probe (Table 1) were designed with the aid of Oligo v6.71 software (National Biosciences, Inc., Plymouth, MN) after the alignment of available GenBank sequences for the cytochrome *b* gene (*P. falciparum*, accession number AY910014; *P. vivax*, accession number AY598140; *P. ovale*, accession number AB182496; *P. malariae*, accession number AF69624). Since mutations responsible for *P. falciparum* atovaquone resistance had been described in the cytochrome *b* gene, primers and probes were chosen to avoid these polymorphic loci (16). We compared the present *cytb* qPCR assay to a previously published qPCR aimed to diagnose the main four *Plasmodium* species and targeting the 18S rRNA-encoding gene, hereafter 18S qPCR (17). All oligonucleotides were synthesized by Sigma-Aldrich (Lyon, France). All the samples, including the serial dilutions for establishing standard curves and the clinical samples, were tested with both qPCR assays.

Amplification, detection, and quantification of *Plasmodium* sp. DNA were carried out using a LightCycler 480 instrument (Roche Molecular Biochemicals, Mannheim, Germany). PCR was set up in a final volume of 20 μl with the Probe Master 2X (Roche Molecular Biochemicals); primers and probes were used at concentrations of 0.5 μM and 0.25 μM, respectively, with 0.25 μl of uracil-DNA-glycosylase (New England BioLabs, Ozyme, Saint Quentin en Yvelines, France) and 5 μl of extracted DNA. The reaction mixture was initially subjected to incubation for 2 min at 50°C; this was followed by a 10-min step at 95°C. For both qPCR assays, the 50 cycles of amplification consisted of a step of denaturation (95°C for 15 s; ramping rate, 4.4°C/s) followed by a single step of annealing and extension (60°C for 1 min; ramping rate, 4.4°C/s). A single fluorescence reading for each sample was taken (annealing step for both qPCRs). Each sample was tested in duplicate. Each experiment included sterile water as a negative control and a positive control with a known parasitemia of $9.79. \times 10^{-4}$ parasites/100 red blood cells, corresponding to a concentration of $10^5$ IU/ml, obtained from the *Plasmodium* standard. The result was considered positive when a significant fluorescent signal above the baseline was detected as determined by the second-derivative algorithm method and was expressed as the quantification cycle (Cq) as recently proposed (5).

The linear range, the efficiency, and the limit of detection of the *cytb* qPCR were determined by analyzing 10-fold serial dilutions of the NIBSC standard in fresh, parasite-free blood from a healthy donor ($6 \times 10^3$ white blood cells/μl; $5 \times 10^6$ red blood cells/μl; 13.1 g hemoglobin/dl) with parasite loads ranging from 10 parasites/100 red blood cells to $1 \times 10^{-9}$ parasites/100 red blood cells. Each dilution was tested in duplicate in five independent runs. Accuracy was measured as the percentage difference between the observed and expected parasitemia for a known quantity dilution of the NIBSC standard. One sample's titer (parasitemia of $9.79 \times 10^{-4}$ parasites/100 red blood cells) was determined by qPCR 10 times in the same run to assess repeatability expressed as the standard deviation of the Cq variance. One sample's titer was determined by qPCR 15 times in 15 independent runs to assess reproducibility expressed as the coefficient of variation of parasitemia.

To estimate the analytical specificity of the two PCR assays, DNA was obtained from various reference species of *Trypanosoma* (*T. brucei brucei*, *T. cruzi* Brener, *T. cruzi* Y, *T. musculi*) and *Leishmania* (*L. infantum*, *L. chagasi*, *L. donovani*, *L. amazonensis*, *L. peruviana*, *L. pifanoi*, *L. guyanensis*, *L. aethiopica*, *L. lainsoni*, *L. braziliensis*, *L. tropica*, *L. major*, *L. garnhami*, *L. mexicana*, *L. panamensis*) and from *Toxoplasma gondii* (RH strain) and processed. Additionally, 10-fold serial dilutions of the NIBSC standard in water were tested to look for any impact of human DNA.

**DNA extraction.** In order to choose the best DNA extraction protocol, we first compared a manual (QIAamp DNA Blood Mini Kits; Qiagen, Courtaboeuf, France) and an automated (MagNA Pure compact instrument; Roche Diagnostics, Meylan, France) DNA extraction method using 5 positive samples with different parasitemia levels with 400 μl of whole blood eluted in 100 μl for both methods. We also compared the automated extraction kits using 400 μl of blood (MagNA Pure Compact Nucleic Acid Isolation Kit I; Roche Diagnostics) with a final elution volume of 100 μl versus the one using 1 ml (MagNA Pure Compact Nucleic Acid Isolation Kit I Large volume; Roche Diagnostics) with a final elution volume of 200 μl, according to the manufacturer's recommendations. The impact of freezing and thawing was studied by processing the extraction of 5 samples (the same ones as above) before and after storage at −20°C. After extraction, the DNA concentration and the ratio of absorbance at 260 nm/280 nm was determined by a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE).

**IC.** The copurification of PCR inhibitors was tested for by using an IC as previously described (8). Briefly, mouse DNA (Sigma-Aldrich, Lyon, France)

was added at 0.31 pg/μl in a reaction volume of 20 μl containing 5 μl of extracted DNA. Each PCR was tested in duplicate.

**Species identification.** To distinguish between *P. falciparum* and non-*P. falciparum* species, we designed a *P. falciparum*-specific primer in the cytochrome *b* gene (Table 1) that we used instead of the forward primer in the *cytb* qPCR assay. Amplification was achieved as for the *cytb* qPCR and gave negative results for non-*P. falciparum* species. Species identification was carried out by nucleotide sequencing of the amplified cytochrome *b* fragment with a Big Dye terminator sequencing kit on an ABI Prism 3130 capillary sequencer (Applied Biosystems, Courtaboeuf, France) according to the manufacturer's instructions. Alignment of the sequences was performed using the single-stranded mitochondrial cytochrome *b* sequences deposited in the GenBank database (see above).

**Patients and sample collection.** We studied 294 whole-blood samples collected in EDTA tubes from 265 travelers or migrants with clinical signs suggestive of malaria consecutively admitted to the emergency ward from 1 January 2007 to 31 December 2008; the samples were kept at −40°C until DNA analysis. Giemsa-stained thick and thin blood films were examined by different experienced microbiologists and were considered negative if no parasite was seen in at least 100 fields of the slide (20-min reading). When blood films were positive, species identification was made and parasitemia was calculated by determining the number of parasitized red blood cells seen per 10,000 red blood cells and expressing the number of parasitized cells as a percentage (19). All blood films were retrospectively reexamined by a single experienced microbiologist to confirm the results. The immunochromatographic BinaxNOW malaria test (Inverness Medical International, Bedford, United Kingdom) was performed to detect circulating *Plasmodium* antigens according to the manufacturer's instructions. In parallel, an aliquot of 1 ml of EDTA blood was stored at −20°C until use for PCR diagnosis. All clinical and epidemiological data were collected for patients with negative microscopy results and positive PCR results.

**Statistical analysis.** Statistical significance was tested using the Wilcoxon matched-pair signed-rank test. The level of significance was a $P$ value of $<0.05$. Linear regression and a Bland-Altman plot were used to determine agreement between parasitemia measured by qPCR and parasitemia determined by blood film (XLSTAT software).

## RESULTS

**Validation of qPCR assays using the international standard.** Linear regression analysis indicated that the *cytb* qPCR assay has a reproducible linearity over a $10^6$-fold range with a coefficient of determination ($R^2$) of 0.998. The results of the *cytb* qPCR assays using serial dilutions of the *P. falciparum* NIBSC standard in fresh, parasite-free blood and in water are shown in Fig. 1. Based on the slope, the efficiency of amplification was calculated to be 94.5%. The accuracy of the *cytb* qPCR assay was 2.5%, the repeatability was 0.10, and the reproducibility was 6.8% for a concentration of $9.79. \times 10^{-4}$ parasites/100 red blood cells. No positive test results were observed for any of the non-*Plasmodium* protozoan species (*Leishmania* spp., *Trypanosoma* spp., and *Toxoplasma gondii*), and no amplification products were visible in negative controls (no DNA template).

The last consistently positive dilution corresponded to 1 parasite equivalent/PCR, or 20 parasites in the initial 400 μl of whole blood. The limit of detection of the *cytb* qPCR assay was around 20 copies of the cytochrome *b* gene, assuming that there are 20 copies in one *P. falciparum* parasite (23, 27) with low variability (16, 27). The limit of detection of the *cytb* qPCR assay determined on positive clinical specimens with known parasitemia was 0.67, 0.35, 0.53, and 0.3 parasites/PCR for *P. falciparum*, *P. ovale*, *P. vivax*, and *P. malariae*, respectively. Similar results were obtained with the 18S qPCR assay.

**Comparison of the extraction methods.** The impact of one freezing-thawing cycle was tested with 40 samples (5 positive samples with 8 different DNA extractions). Fresh samples provided a lower Cq than thawed samples (median, −0.15; range, −0.79 to 0.68; $P = 0.02$).

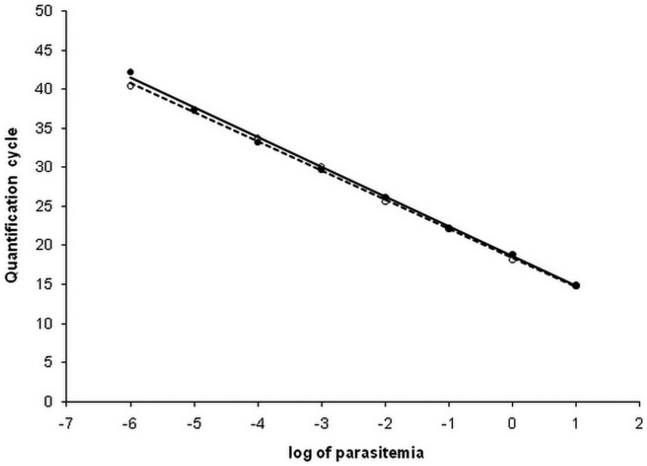


FIG. 1. Sensitivity of *cytb* qPCR assay calculated from serial dilutions of a *P. falciparum* standard (NIBSC) at 9.79 parasites/100 red blood cells in blood (plain line) and in water (dashed line). Cq values are plotted against the quantity of parasites expressed as the logarithm of the parasitemia level. The plot of the mean Cq values against quantity fits a linear function ($R^2$, >0.998).

Using 20 positive samples with different parasitemia levels, there was no statistically significant difference either between the Cq obtained by manual or automated DNA extraction ($P = 0.28$) or the Cq obtained with the two volumes tested with automated DNA extraction ($P = 0.23$). This last result was obtained despite the fact that the 1-ml protocol yielded significantly more DNA than the 400-μl one (median, 143 ng/μl; range, 66 to 302 ng/μl; $P = 0.005$). There was no significant difference between the 260-nm/280-nm absorbance ratios of the two automated protocols ($P = 0.11$).

**Analysis of clinical samples.** We obtained DNA using the 400-μl blood volume automated DNA extraction for the clinical samples from 265 patients with a suspected diagnosis of imported malaria. A microscopic diagnosis was established for 43 of them (16.2%), 36 on thin films and 7 on thick blood films only. All of them were positive in both qPCR assays. All of them were also ICT positive but one, and that one showed a *P. malariae* infection. Eight patients were *cytb* qPCR positive (Cq range, 29.5 to 40.5) and microscopy negative, and two of them were also ICT positive. Only one of these 8 *cytb* qPCR-positive patients was 18S qPCR negative.

Among the 43 microscopy-positive patients, there were 40 *P. falciparum* infections and 3 non-*P. falciparum* infections (1 *P. vivax* infection [parasitemia, 0.53/100 red cells]; 1 *P. ovale* infection [parasitemia, 0.35/100 red cells]; and 1 *P. malariae* infection [parasitemia, 0.03/100 red cells]). No mixed infection was observed. The complementary qPCR for species identification correctly identified these 3 non-*P. falciparum* cases. Sequencing correctly identified the three species.

We tested 29 additional samples from 20 patients. For 7 patients, despite a negative first microscopy result, a second was required 1 to 30 days after the first one. Microscopy and qPCR results were again negative. For one patient, 3 additional samples were tested because of persistent ICT positivity

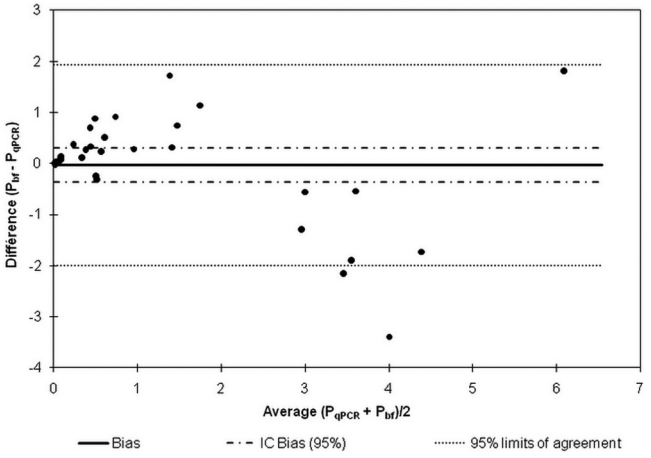


FIG. 2. Bland-Altman plot for comparison of the use of thin blood film (bf) and that of the *cytb* qPCR assay (qPCR) for the quantification of parasitemia (P).

and were shown to be both microscopy and qPCR negative. The result was interpreted as a false-positive ICT result. For 12 patients, 19 further samples were obtained as part of the normal follow-up of microscopy-positive patients, which recommends retesting at 3 and 7 days. The increase in Cq values was around 9, i.e., a decrease of 1 $\log_{10}$ in 3 days after therapy. Gametocytes were observed for three samples, which were also positive by both qPCRs.

**Quantification of parasitemia.** For *cytb* qPCR, a Cq value of $<25$ correlated with $>0.01\%$ parasitemia, between 25 and 29, the Cq value corresponded to negative thin and positive thick blood smears. When the Cq value was $>29$, the microscopy was negative and corresponded to $<10^{-3}$ parasites/100 red blood cells when using the NIBSC standard.

The IC was amplified for all the samples tested, showing the absence of PCR inhibitors after the extraction procedure (mean IC Cq value, 37.16 ± 0.60). Therefore, we calculated parasitemia using the *cytb* qPCR results and the calibration curve with the *P. falciparum* standard for the clinical specimens with positive thin blood films. Linear regression between parasitemia checked by a single microbiologist and the *cytb* qPCR parasitemia showed an $R^2$ value of 0.88. The Bland-Altman plot exhibited a bias of 0.032 (95% confidence interval, −0.336 to 0.302) (Fig. 2). The trend was to observe the highest divergences with high parasitemia, with microscopy usually giving a parasitemia level higher than that obtained by *cytb* qPCR. When the linear regression was performed with parasitemia obtained by different microbiologists on a routine basis, the coefficient of determination was lower ($R^2 = 0.76$) and the Bland-Altman plot exhibited a higher bias of −0.315 (95% confidence interval, −0.787 to 0.158).

## DISCUSSION

Our results show that our *cytb* qPCR assay combined with automatic DNA extraction is a reliable tool for the diagnosis of malaria infection. The sensitivity of microscopy, about 5 to 100 parasites/μl of blood (12, 19), is surpassed by that of the *cytb* PCR assay, with a limit of detection of around 20 parasites/400 μl. Many qPCR assays have been described; most of them target the 18S rRNA-encoding gene of *Plasmodium* spp. (3, 9, 13, 17, 22, 25, 26, 29). The mitochondrial genes have been less frequently studied (3, 11, 26). We show here that our *cytb* qPCR and the 18S qPCR published by Lee et al. (17) have similar sensitivities. Rather than choose between two DNA targets, it could be useful to combine them to overcome possible sequence variation in oligomer targets (2).

If a qPCR assay is to be recommended as a routine test, the preanalytical step is important. DNA extraction should be convenient and rapid. In our experience, automated DNA extraction is as efficient as manual extraction. The time required for DNA extraction is around 40 min for both methods. Therefore, automation provides reduced hands-on time and better safety and applicability in routine clinical diagnosis. We also found that a freeze-thaw cycle reduces the quantification of the parasite, which suggests that qPCR assays would be more sensitive on a routine basis when dealing with fresh samples. Additionally, no gain in the detection of *P. falciparum* DNA is achieved by increasing the blood volume, probably because the increase in the DNA concentration is attributable mainly to human DNA.

Quantification of the parasites in blood is an important element of the prognosis of *Plasmodium* infection and therapeutic management. Quantification is usually achieved by microscopy, with variation depending on the examiner (21). For qPCR, an IC of the amplification is mandatory to avoid erroneous quantification of the parasitic load. In the absence of qPCR inhibitors, we obtained an $R^2$ value of 0.88 for the correlation between the parasitemia calculated by one single microbiologist and the *cytb* qPCR quantification. However, divergences appear when dealing with high parasitemias. As expected, the correlation was worse when using the routine values obtained by different microbiologists. With regular quality controls using international standards, quantification of parasitemia by qPCR should be more reproducible than that by visual reading.

Since qPCR is more sensitive than microscopy, this raises the issue of the clinical relevance of positive qPCR and negative microscopy results. We observed negative microscopy and positive qPCR results after treatment with a dramatic decrease in the parasite load evaluated by the Cq values, as already reported (10, 25). However, we also observed microscopy-negative and qPCR-positive African patients who had recently traveled to their native country, as already described (18). This finding can be misleading if fever is due to other infectious diseases. To our knowledge, these patients were not treated and did not return to our emergency ward. Whether this finding warrants any medication should be addressed by clinical studies. In countries where malaria is endemic, epidemiological studies suggest that these patients can increase malaria transmission (21, 28).

For *Plasmodium* identification, we opted for a second amplification to exclude non-*P. falciparum* species. This step adds about 40 min to the time required to reach a final diagnosis. In an emergency, this second qPCR should not delay a therapeutic decision targeting the most virulent *Plasmodium* species. Once the diagnosis of non-*P. falciparum* species has been made, there is time to confirm the identification by microscopy or by sequencing. With the present qPCR, no discordance

between microscopy and molecular identification was observed, in contrast to other studies, especially for non-*P. falciparum* species (3). However, we observed only three non-*P. falciparum* species (one isolate each of *P. ovale*, *P. malariae*, and *P. vivax*). In mixed infections, molecular identification could be impossible, but we have not observed such cases. We have not encountered any *P. knowlesi* infection either, but the homologies between the *P. knowlesi* sequences available in GenBank and the primers and probes used in this study suggest that such an infection would not have been missed.

Other strategies for *Plasmodium* identification are possible, such as the use of multiplex PCR (7, 25, 29) and/or the analyses of melting curves (3, 9, 26). The use of several primer sets and probes could potentially lead to competitive amplifications with variable yields. This could hamper the efficiency of the most important analysis, that is, *P. falciparum* detection in mixed infections (2). It could also lead to unreliable quantification since it is difficult to monitor the amplification yield of simultaneous qPCR assays. Analyzing melting curves to differentiate between species is another possibility, but this needs special technician skill and raises the question of the transferability of results between different laboratories.

The use of qPCR does not eliminate the need for confirmatory diagnosis using microscopy to prevent false-negative results due to molecular variants (6, 15) or newly described species (24) and to identify the parasitic stage. However, in the face of the predictable shortage of skilled people for emergency diagnosis, the present study strongly supports the use of qPCR for the diagnosis of *Plasmodium* infection in travelers, since it can be performed within a clinically relevant time frame. Additionally, qPCR can address the issue of quantification, which cannot be done with antigen detection, and for which qPCR should be more reproducible than microscopic determination. From a financial point of view, the cost of equipment and reagents should be compared with the cost of a 24/24 7/7 specialized microbiologist.

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