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Clinical significance of molecular methods in the diagnosis of imported malaria in returning travelers in Serbia

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SUMMARY

Objectives: The goal of this study was to assess the clinical significance of conventional and PCR-based molecular diagnosis in patients with imported malaria in Serbia.
Methods: Giemsa microscopy, the rapid diagnostic test, and quantitative real-time PCR (qPCR) were used to detect *Plasmodium* species in 109 whole-blood samples from patients after their return from malaria endemic areas, including those clinically suspected for malaria ($n = 97$) and healthy travelers ($n = 12$) examined as part of epidemiological surveillance.
Results: A total of 45 patients were diagnosed with malaria: 42 (93.3%) by microscopy and three (6.7%) additional ones by qPCR. The agreement between the results of species-specific qPCR and microscopy was 73.3%; it was as high as 90.6% for *Plasmodium falciparum* infections. Follow-up analysis demonstrated persistence of *Plasmodium sp* DNA for a mean 6 days after the disappearance of parasitemia on microscopy.
Conclusions: Due to its sensitivity and specificity, qPCR is a helpful method complementary to microscopy, particularly in cases of low parasitemia. In addition, it is superior to microscopy for species identification.

1. Introduction

Malaria is the most important parasitic disease globally, affecting the populations of 97 countries. In 2012, 207 million cases and 627 000 deaths occurred in malaria endemic regions, concentrated in the tropical and subtropical areas.[1] Five different *Plasmodium* species infect humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, and *Plasmodium knowlesi*.

A prompt diagnosis with accurate identification of the species is crucial for appropriate treatment. Conventional microscopic diagnosis, although still the gold standard, is highly subjective and depends on the skill of the microscopist. This has been overcome by molecular methods, which are constantly being improved for increased sensitivity and specificity.[2–5]

The World Health Organization (WHO) officially declared Serbia (then within Yugoslavia) malaria-free in 1974; only imported cases have occurred ever since. From 1975 to 1988, 24 to 57 cases of imported malaria were reported per year. In the 1990s, amidst the political and economic turmoil surrounding the dissolution of the former Yugoslavia, there was a sharp decline in the number of malaria cases due to greatly reduced travel,[6] but since 2000, travel of Serbian citizens to tropical areas has been increasing steadily.

Most patients with suspected malaria in Serbia are referred to the Clinical Center of Serbia (CCS) for diagnosis and treatment. We recently analyzed a series of 2981 travelers examined for malaria between 2001 and 2009, of whom 102 were diagnosed with malaria.[7] Plasmodium was not detected microscopically in 10.8% of patients, indicating inadequate sensitivity of conventional diagnostic methods. This, along with the need to monitor asymptomatic travelers returning from malaria endemic areas as a measure of prevention of autochthonous transmission of malaria, locally re-established in the region of Southeast Europe,[8] prompted us to introduce molecular methods into the diagnosis of imported malaria in Serbia.

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In this study, we analyze the adequacy of quantitative real-time PCR (qPCR) for the diagnosis of imported malaria in diagnostically uncertain cases, and for the determination of the *Plasmodium* species.

2. Materials and methods

2.1. Study design

Microscopy and qPCR for the diagnosis of malaria were comparatively assessed in blood samples collected consecutively during 3 years from patients with suspected imported malaria at one reference center in Serbia.

Blood samples were initially examined by microscopy and the rapid diagnostic test (RDT), and the diagnosis of malaria was based on the detection of Plasmodium in blood smears. In a few patients, the diagnosis was based on a favorable effect of antimalarials administered because of a clinical and epidemiological suspicion of malaria, despite repeatedly negative blood smears (ex juvantibus).

Later, the stored blood samples were tested for the presence of the parasite genus-specific 18S rRNA gene by qPCR (screening qPCR); all samples positive on screening qPCR and/or RDT were subsequently analyzed with species-specific qPCR for the detection of four *Plasmodium* species including *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*.

The durations of microscopic parasitemia and Plasmodium DNA persistence were analyzed by testing subsequent samples from the malaria patients.

The study was approved by the University of Belgrade ethics committees at the Institute for Medical Research (EO 101/2012) and the Faculty of Medicine (EO 29/X-12).

2.2. Study population

The study group included all travelers returning from the tropics examined for malaria at the CCS Parasitological Laboratory between July 2010 and May 2013. These included patients with a clinical presentation suggestive of malaria, but also healthy travelers monitored as part of epidemiological surveillance, most because of a previous malaria episode. Healthy individuals who had not been exposed to malaria, patients diagnosed with toxoplasmosis and leishmaniasis, and AIDS patients with *Pneumocystis jirovecii* pneumonia (PCP) served as controls.

The medical records were reviewed for relevant epidemiological and clinical data.

2.3. Sampling

Blood for thick and thin blood smears and for the RDT was collected by finger prick. Samples for molecular diagnosis were collected by venipuncture using ethylenediaminetetraacetic acid (EDTA) vacutainer tubes. In patients with diagnosed malaria, blood sampling was repeated daily until the disappearance of parasitemia, followed by three times a week during their hospitalization and weekly after discharge from the hospital. The venous blood samples were stored at −70 °C until DNA extraction.

2.4. Microscopy

Five thick and five thin films were prepared from each blood sample. Three films each were stained with 10% Giemsa stain, and the remaining ones were stored in case staining needed to be repeated.

Thick and thin smears were examined under conventional light microscopy by expert microscopists. Before reporting a negative result, at least 500 oil immersion microscopic fields at a magnification of 1000× were examined. The level of parasitemia was expressed as the percentage of parasitized erythrocytes.

Microscopy results were available within 2 h of blood drawing; if the initial smear was negative, examination was repeated at least three times within the next 48 h.

2.5. RDT for the detection of P. falciparum histidine-rich protein 2

For the initial diagnosis of patients with suspected *P. falciparum* infection, the RDT VISITECT MALARIA (Omega Diagnostics Ltd, London, UK) was performed as per the manufacturer's instructions. The test is based on the detection of histidine-rich protein 2 (HRP-2) antigen of *P. falciparum*.

2.6. Quantitative real-time PCR

2.6.1. Extraction of DNA

Extraction of DNA was performed from 200 μl of collected blood using a commercial kit (GeneJET Genomic DNA Purification Kit; Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Extracted DNA was resuspended in 120 μl of nuclease-free water and stored at −70 °C until further analysis.

2.6.2. Screening of Plasmodium genus with qPCR

qPCR for detecting the 18S Plasmodium gene was performed according to the method of Rougemont et al. (2004).[9] Briefly, specific primers and probe were used to amplify a 157–165-bp segment of the 18S gene common to all four *Plasmodium* species. The qPCR reaction was performed in a final volume of 20 μl and contained Maxima Probe/Rox qPCR Master Mix (Fermentas, Thermo Fisher Scientific Inc., Waltham, MA, USA), uracil-DNA glycosylase (UNG), forward (Plasmo 1) and reverse (Plasmo 2) primers, probe (Plasprobe) (Metabion International AG, Germany), $MgCl_2$, nuclease-free water, and 3 μl of DNA template. Amplification was performed on a Mastercycler ep realplex4 (Eppendorf AG, Hamburg, Germany) using the following conditions: 2 min at 50 °C for UNG pre-treatment, 10 min at 95 °C initial denaturation, followed by 45 cycles of 15 s at 95 °C for denaturation and 1 min at 60 °C for annealing/extension. Each reaction was performed in duplicate and the cycle threshold number (Ct) was determined as their mean. A sample was considered positive if the fluorescent signal was detected in at least one replicate; conversely, if no signal was detected within 40 cycles, a reaction was considered negative. Negative controls consisted of nuclease-free water and DNA extracted from healthy, malaria-unexposed blood donors, while DNA extracted from the *P. falciparum* Dd2 strain maintained in vitro was used as a positive control.

2.7. Species-specific qPCR

Plasmodium species were detected by targeting the 18S rRNA genes specific for *P. falciparum*, *P. vivax*, and *P. ovale* using primers and probes (Metabion International AG, Germany; Invitrogen, USA) as per the protocol of Perandin et al. (2004),[10] and for *P. malariae* according to the protocol of Rougemont et al. (2004) (Table 1).[9]

The species-specific qPCR reaction had a final volume of 25 μl and included Maxima Probe qPCR Master Mix (Fermentas), forward and reverse primers, probe, $MgCl_2$, nuclease-free water, and 3 μl extracted DNA. Species-specific primers and probes were separately mixed with the remaining solution and DNA samples were individually tested for the presence of DNA of each of the four *Plasmodium* species. Amplification conditions, interpretation of results, and negative controls were the same as for the screening qPCR. As positive controls, we used DNA extracted from the blood

Table 1
Primer and probe sequences for species-specific quantitative real-time PCR

Species	Primers and probes	Sequences
P. falciparum	FAL-F	5′-CTT TTG AGA GGT TTT GTT ACT TTG AGT AA-3′
	FAL-R	5′-TAT TCC ATG CTG TAG TAT TCA AAC ACA A-3′
	FAL probe	5′-Fam-TGT TCA TAA CAG ACG GGT AGT CAT GAT TGA GTT CA-TAMRA-3′
P. vivax	VIV-F	5′-ACG CTT CTA GCT TAA TCC ACA TAA CT-3′
	VIV-R	5′-ATT TAC TCA AAG TAA CAA GGA CTT CCA AGC-3′
	VIV probe	5′-Tet-TTC GTA TCG ACT TTG TGC GCA TTT TGC-Tamra-3′
P. ovale	OVA-F	5′-TTT TGA AGA ATA CAT TAG GAT ACA ATT AAT G-3′
	OVA-R	5′-CAT CGT TCC TCT AAG AAG CTT TAC AAT-3′
	OVA probe	5′-Yakima Yellow™-CCT TTT CCC TAT TCT ACT TAA TTC GCA ATT CAT G-Tamra-3′
P. malariae	Mal-F	5′-CCG ACT AGG TGT TGG ATG ATA GAG TAA A-3′
	Plasmo2	5′-AACCCAAAGACTTTGATTTCTCATAA-3′
	Malaprobe	5′-FAM-CTA TCT AAA AGA AAC ACT CAT-MGBNFQ

MGBNFQ, minor groove binding non-fluorescent quencher.

of infected patients; for *P. falciparum* and *P. malariae* it originated from our patients, while DNA of *P. vivax* and *P. ovale* was kindly provided by Dr Anna Färnert, Karolinska University Hospital, Stockholm, Sweden. The estimated time to obtaining the results was 3 h.

2.7.1. External quality control

External quality control was performed at the University Hospital, Reims, France, where the validity of the screening test was checked on 10 blinded patient samples, and at the Karolinska University Hospital where confirmatory analysis by nested PCR was performed on 11 inconclusive samples.

2.7.2. Analytical sensitivity and specificity of qPCR

The analytical sensitivity of the 18S rRNA screening qPCR method was estimated in serial 10-fold dilutions of DNA extracted from a patient infected with *P. falciparum* at a 3.3% parasitemia, and the sensitivity of the species-specific qPCR in serial 10-fold dilutions of DNA from *P. falciparum*, *P. ovale*, *P. vivax*, and *P. malariae*, with parasitemia levels of 2%, 0.001%, 0.05%, and 0.015%, respectively. The highest dilution with a positive PCR signal indicated the detection limit. The specificity of the assay was analyzed by testing DNA obtained from healthy, malaria-unexposed individuals and patients diagnosed with *Toxoplasma gondii*, *Leishmania sp*, and *P. jirovecii*.

2.8. Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics for Windows, Version 21.0 (IBM Corp., Armonk, NY, USA). The performance of the methods was analyzed by determining the classical measures including sensitivity, specificity, and positive and negative predictive values. The differences in the duration of parasitemia determined by microscopy and qPCR, and in the mean Ct between patient subgroups, were analyzed by *t*-test. The level of statistical significance was 5%.

3. Results

3.1. Characteristics of the study group

The study group comprised a total of 109 returning travelers, including 97 patients clinically suspected of malaria (83 patients with fever and 14 with other clinical manifestations) and 12 healthy travelers (10 with recent malaria and two who acknowledged mosquito bites). Controls included two healthy individuals who had not been exposed to malaria and four patients diagnosed with toxoplasmosis ($n = 2$), leishmaniasis ($n = 1$), and PCP ($n = 1$).

Malaria was diagnosed in 45 patients, all of whom were clinically suspected of malaria. The diagnosis was based on microscopy in 42 patients (93.3%); three patients (6.7%) had submicroscopic malaria (SMM) (diagnosis based on the favorable effect of antimalarials administered in clinically suspected patients but without microscopic confirmation).

3.2. Analytical sensitivity and specificity and external quality control of qPCR

The analytical sensitivity of screening qPCR was 0.04 parasites/μl and specificity was 100%. No positive reactions occurred in the samples of healthy individuals who had not been exposed to malaria or in the samples of individuals infected with *T. gondii*, *Leishmania sp*, and *P. jirovecii*. External quality control of the 10 blinded patient samples for the validity of the screening test showed 100% agreement.

The analytical sensitivity of species-specific qPCR was 6, 0.3, 0.13, and 0.09 parasites/μl for *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*, respectively. The results for the 11 samples examined in parallel by species-specific qPCR in Serbia and by nested PCR at Karolinska showed agreement for eight (72.7%). In the three discrepant samples, species-specific qPCR was negative, whereas nested PCR identified *P. ovale* in two cases (which was observed microscopically in both), but also *P. falciparum* in one recently treated patient.

3.3. Plasmodium detection according to method

A comparison of the results of all of the diagnostic methods is presented in Table 2.

By microscopy, asexual-stage Plasmodium parasites were found in 42 (38.5%), whereas in the others, neither asexual nor sexual stages of Plasmodium were found. Parasitemia was generally low (<2% in 82%). A single case of parasitemia >5% (9.5%) was observed in a patient who presented at the hospital 10 days after symptom onset.

The RDT, performed for 97 travelers, was positive for 35, including all 32 diagnosed with *P. falciparum* malaria (29 patients with microscopic confirmation and three SMM), as well as in three patients who had recently been treated with antimalarials (9, 13, and 30 days before examination, respectively). Conversely, the RDT was negative in all 13 non-falciparum malaria patients as well as in one microscopically identified as a mixed infection (*P. falciparum* + *P. vivax*).

Screening qPCR was positive in 51 (46.8%) patients, including 44 with malaria (all 42 cases with microscopic malaria and two with SMM), and seven without malaria (of whom six had recently been treated for malaria). The mean Ct, however, was significantly

Table 2
Comparative evaluation of the performance of methods applied in the diagnosis of malaria in returning travelers in Serbia

	Microscopy[a] (*n* = 109)		RDT[b] (*n* = 97)		Screening qPCR (*n* = 109)		Species-specific qPCR (*n* = 53)	
	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
Malaria patients (*n* = 45)	42	3	32	0	44	1	42	3
Non-malaria patients (*n* = 64)	0	64	3	62	7	57	1	7
Sensitivity	93.3		100.0		97.8		93.3	
Specificity	100.0		95.4		89.1		87.5	
Positive predictive value	100.0		91.4		86.3		97.7	
Negative predictive value	95.5		100.0		98.3		70.0	

RDT, rapid diagnostic test; qPCR, quantitative real-time PCR.
[a] For genus detection.
[b] For *Plasmodium falciparum*.

lower ($p < 0.03$) in the malaria patients (25.64 ± 5.9, range 16.97–39.47) than in the seven patients without current malaria (38.40 ± 1.7, range 35.36–39.86).

3.4. Species identification by species-specific qPCR vs. microscopy

The *Plasmodium* species identified according to the method of identification are shown in Figure 1. *P. falciparum* predominated, with a share of 71.1% (32/45).

The overall agreement between the results of species determination by qPCR and microscopy was 73.3% (33/45). Importantly, for *P. falciparum* it was as high as 90.6% (29/32), and the three discrepant findings were cases of SMM not identified on microscopy (Table 3). The agreement between the methods was lower for *P. vivax*, *P. ovale*, and *P. malariae* (1/4, 1/6, and 2/3), respectively, whose identification accounted for all nine remaining discrepancies (Table 3). Three of these were missed by qPCR, one *P. vivax* case that could not be detected as venous blood was not available until the fourth day after microscopic diagnosis (and immediate initiation of treatment) and two cases of *P. ovale*. The six (13.3%) cases of misidentification on microscopy included both microscopic mixed infections that qPCR revealed as mono-infections (one species was correctly determined by microscopy), and four cases of incorrect morphological identification; for all of these, repeated microscopy revealed the correct species.

3.5. Duration of parasitemia

Figure 2 depicts the duration of parasitemia estimated by both microscopy and screening qPCR. The overall mean duration of microscopic parasitemia was 2.2 ± 1.2 (range 0–5) days and of parasite DNA was 7.9 ± 6.5 (range 0–28) days ($p < 0.000$). Importantly, the persistence of DNA was at least that long, since in 55% of patients PCR was still positive for the last available sample. The difference was even more pronounced ($p < 0.000$) for *P. falciparum* infections, where the mean duration of parasitemia was 1.9 ± 1.0 (range 0–5) days and of DNA at least 9.2 ± 7.2 (range 0–28) days. For *P. vivax*, *P. ovale*, and *P. malariae*, parasitemia was observed during 2.3, 3, and 4 days, respectively, whereas the respective DNA was detected for 4, 3.8, and 8.3 days, respectively; DNA apparently persisted longer, but the groups were too small for statistical analysis.

3.6. Characteristics of malaria patients

Patients were predominantly male (95.6%). The mean age was 50.0 ± 11.8 (range 23–70) years. Malaria was imported predominantly from Africa (95.6%), by Serbian workers (88.9%). The vast majority (93.4%) had not taken any prophylaxis. At admission, all patients with malaria had fever (Table 4).

Overall, symptoms of malaria manifested within 35.8 ± 72.7 (range 0–365) days after entering Serbia, but this period differed greatly among the *Plasmodium* species. For *P. falciparum*, the mean time between arrival in Serbia and onset of fever was 7.8 ± 6.9 (range 0–30) days, for *P. vivax* 61.0 ± 99.7 (range 0–210) days, for *P. ovale* 165.0 ± 113.1 (range 72–365) days, and for *P. malariae* 42.7 ± 67.0 (range 2–120) days. The mean time from symptom onset to presentation at the hospital was 4.8 ± 4.4 (range 0–20) days. Thirteen (28.8%) patients had already started antimalarial treatment before presentation.

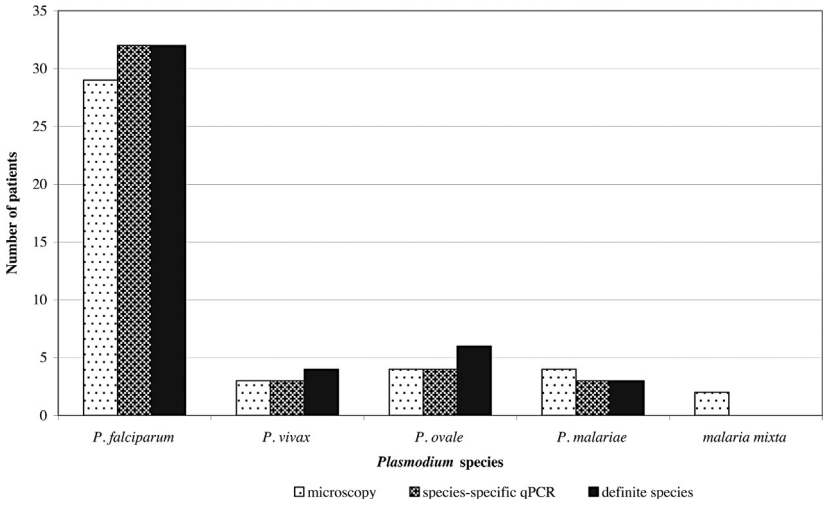

Figure 1. *Plasmodium* species by method and final species identification in patients with malaria (*n* = 45).

Table 3
Analysis of discordant results ($n = 12$) between microscopy and species-specific qPCR in patients with malaria

	Species-specific qPCR						
	P. falciparum	*P. vivax*	*P. ovale*	*P. malariae*	Mixed infection	Negative	Total
Microscopy							
P. falciparum	0	0	0	0	0	0	0
P. vivax	0	0	1	0	0	1	2
P. ovale	0	1	0	0	0	2	3
P. malariae	0	0	2	0	0	0	2
Mixed infection	0	1[a]	0	1[b]	0	0	2
Negative	3	0	0	0	0	0	3
Total	3	2	3	1	0	3	12

qPCR, quantitative real-time PCR.
[a] Microscopically *P. falciparum* + *P. vivax*.
[b] Microscopically *P. malariae* + *P. vivax*.

Of the 45 patients, four were cases of recrudescence of falciparum malaria and three were cases of relapse of ovale malaria. In all four patients with recrudescence of *P. falciparum* malaria, the infection was treated immediately prior to entering Serbia and recrudescence had occurred 10–15 days later. Of these, three were infected in Equatorial Guinea; two patients were treated with artesunate, while data for one were not available. The fourth patient was treated with Co-Arinate in Gabon. One relapse of *P. ovale* was due to incorrect microscopic identification of the species, initially reported to be *P. malariae*.

All patients with malaria were hospitalized, for a mean period of 12.0 ± 11.2 (range 1–85) days. Six patients had severe disease, including two cases of cerebral malaria. One fatality occurred, in a patient with cerebral falciparum malaria and a parasitemia of 2% at admission.

4. Discussion

In this study of returning travelers in Serbia, malaria was diagnosed in 45/109 (41.3%) travelers, all of them with fever.[11] Patients with malaria were predominantly working-age males. Malaria was predominantly imported from Africa, as in other European countries.[4,5,11–13] However, in contrast to these previous reports,[11,13] for 86.7% of patients in the present study, this was not the first attack, in comparison to, for example, only 17.8% in Slovenia, and a vast majority of patients did not take any prophylaxis. Moreover, nearly a third of the patients had started antimalarial treatment before presentation. These differences reflect the differences in the patient populations. The dominant mode of importing malaria in Serbia has always been by Serbian workers hired at construction sites in malaria endemic areas,[6,7] where many stay for more than 6 months; in contrast, in Western Europe malaria mostly occurs in immigrants and tourists.[5,12]

SMM was registered in 6.7% of patients, all of whom had started treatment before presentation at the hospital. A study in Madrid showed 35.5% cases of SMM, which was attributed to immunity in a large proportion of immigrants from endemic regions.[5]

The dominant species in this study was *P. falciparum*. Although this species commonly causes higher parasitemia, parasitemia was generally low in our series on account of the high proportion of patients who had already started treatment before diagnosis. On the other hand, this is likely the reason for the high sensitivity (100%) of the RDT for the detection of HRP-2 antigen of *P. falciparum* in the present study. With 95.4% specificity, this test proved to be of great help for the confirmation of the most virulent *Plasmodium* species, particularly for SMM. However, long

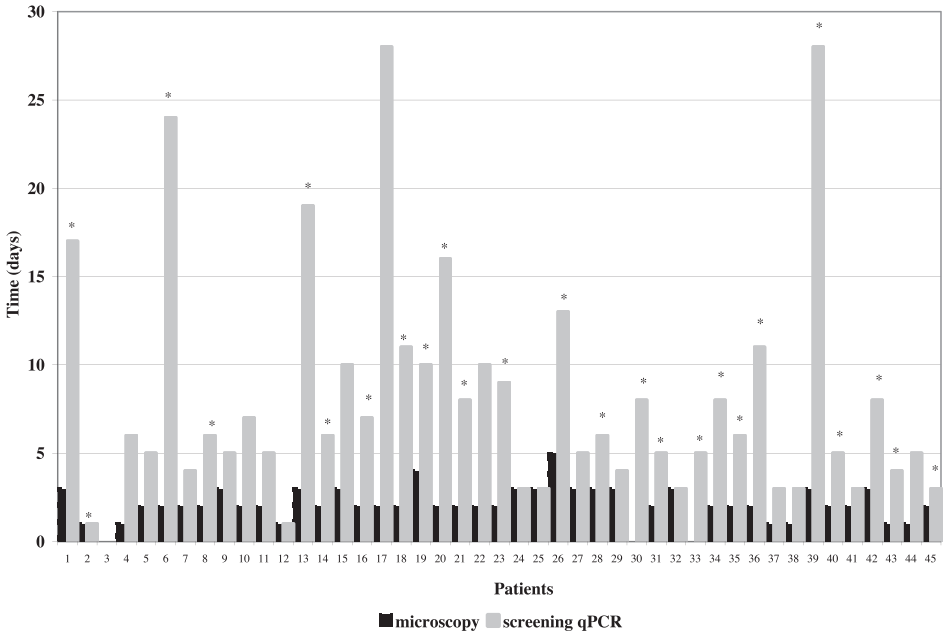

Figure 2. Duration of parasitemia and parasite DNA in patients with malaria ($n = 45$). *Positive in last available sample. Patients 3, 30, and 33 were cases of submicroscopic malaria.

Table 4
Demographic, epidemiological, and clinical characteristics of the 45 malaria patients

Characteristic	
Male, *n* (%)	43 (95.6)
Age, years, mean ± SD (range)	50.0 ± 11.8 (23–70)
Nationality, *n* (%)	
Serbian	40 (88.9)
Non-Serbian	5 (11.1)
Reason for travel, *n* (%)	
Work/business	43 (95.6)
Tourism	2 (4.4)
Duration of stay, *n* (%)	
<3 weeks	1 (2.2)
3 weeks to 6 months	7 (15.6)
>6 months	37 (82.2)
Prophylaxis, *n* (%)	
Yes, not regularly	2 (4.4)
Alternative prophylaxis	1 (2.2)
No prophylaxis	42 (93.4)
Previous malarial attacks, *n* (%)	39 (86.7)
Origin of imported malaria, *n* (%)	
Africa (*n* = 43; 95.6%)	
Angola	4 (8.9)
Equatorial Guinea	21 (46.7)
Ethiopia	2 (4.4)
Gabon	3 (6.7)
Ghana	2 (4.4)
Democratic Republic of the Congo	1(2.2)
Nigeria	9 (20.0)
Sierra Leone	1 (2.2)
Asia[a] (*n* = 2; 4.4%)	2 (4.4)
Clinical signs and symptoms, *n* (%)	
Fever	45 (100.0)
Shaking, chills, and sweats	42 (93.3)
Headache	21 (46.7)
General malaise	28 (62.2)
Myalgia	32 (71.1)
Arthralgia	25 (55.6)
Dry cough	17 (37.8)
Nausea/vomiting	13 (28.9)
Diarrhea	10 (22.2)
Enlarged spleen	25 (55.6)
Enlargement of the liver	27 (60.0)
Enlargement of the liver and spleen	20 (44.4)
Jaundice	9 (20.0)
Dark urine	16 (35.6)

SD, standard deviation.

antigenemia[14] may account for false-positive results, which we obtained for three recently treated patients.

Various PCR-based methods have been used in the diagnosis of malaria, including different qPCR protocols.[3,9,10,15,16] Sensitivity depends on the PCR method, DNA extraction method, and the volume of blood used for extraction.[17] For example, limits of detection of 0.002 and of 0.01 parasites/μl have been reported for a quantitative reverse-transcriptase PCR[18] and for a semi-nested PCR,[4] respectively. In our study, the analytical sensitivity of the screening qPCR was 0.04 parasites/μl, allowing the detection of low parasitemia. A single finding was missed, in a case of SMM. This may have been due to the poor quality of the long stored blood samples and DNA degradation, or the inhibition of PCR amplification.[3,19] However, as this sample was positive by species-specific qPCR and RDT for *P. falciparum*, a technical error is more likely. The false-positive results obtained for a few patients in samples collected after antimalarial treatment may be attributed to the persistence of gametocytes (although sexual stages of Plasmodium were not detected independently of asexual parasites), circulating DNA from therapy-damaged parasites,[20] or to cross-reactivity with homologous DNA of other eukaryotic species.[9]

The agreement between the results of species determination by qPCR and microscopy was 73.3%. This is relatively similar to the 86% and 71% accuracy of species identification reported by Perandin et al. (2004) and Rougemont et al. (2004), respectively.[9,10] However, a recent study in Canada reported a misdiagnosis of the *Plasmodium* species by microscopy vs. qPCR in only 4%.[15] In the cases of disagreement in our series, the accuracy of PCR was 75% (9/12). In addition to the undetectable *P. vivax* case because of a sampling issue (late sampling of venous blood), two *P. ovale* infections were actually missed. The low performance of our PCR for the detection of *P. ovale* reflects the limitations of the primers and probe used in distinguishing between the two *P. ovale* subspecies, *P. ovale curtisi* and *P. ovale wallikeri*.[19,21,22] Since the primers we used for the detection of *P. ovale* detect only *P. ovale curtisi*, we indirectly confirmed that the four detected cases were *P. ovale curtisi* and the other two undetected ones were *P. ovale wallikeri*. Overall, the introduction of molecular diagnostics demonstrated a remarkably higher share (13.3%) of *P. ovale* among the *Plasmodium* species imported into Serbia than considered before the introduction of qPCR (1%).[7] Generally, *P. ovale* was underestimated before the PCR era in terms of both geographical distribution and severity.[19,21] Microscopic errors mostly occur between *P. ovale* and *P. vivax*, because of the highly similar morphology of these two species.[3,9] Fortunately, the misidentification of one of these species for the other does not have clinical ramifications since the therapeutic approach is the same for both. Conversely, misidentifying *P. ovale* as *P. malariae* results in the failure to administer anti-relapse therapy for *P. ovale* (primaquine); this occurred in two patients in our study (because of poor staining which did not allow for the visualization of Schüffner's granules), and one relapsed.

With a rate of 71.1% among all *Plasmodium* species in our series, *P. falciparum* was dominant, as elsewhere.[11–13] The highest agreement (90.6%) between the methods was, as expected, for *P. falciparum*,[10,15,23] since because of its frequency, microscopists are most familiar with this species. Also, higher parasitemia in falciparum malaria facilitates finding the typical morphology. However, the three infections missed by microscopy due to low parasitemia were *P. falciparum* cases, all after self-initiated antimalarial therapy.

No mixed infections were identified by species-specific qPCR. This may seem surprising, since the use of PCR has revealed an increased rate of mixed infections.[3,16] The failure of qPCR to detect mixed infections has been described in dual or multiplex qPCR due to primer competition,[9] and has been overcome by introducing species-specific forward primers in combination with a conserved reverse primer and species-specific probes.[16] However, the absence of mixed infections did not indicate a failure of our protocol, since primer competition was avoided by testing for each species individually, and moreover, no mixed infections were detected by nested PCR either.

As shown in previous studies,[14,24,25] parasite DNA was detected markedly longer than parasitemia, particularly in the case of *P. falciparum*. A positive qPCR result does not differentiate among the DNA of live parasites, residual DNA of destroyed asexual blood stage parasites, and circulating gametocytes, which limits the clinical significance of qPCR for monitoring the effect of treatment. In acute *P. falciparum* infection, gametocytes are formed only 7–15 days after the appearance of parasites in the blood and they are present longer than asexual parasitemia.[26]

In conclusion, this study showed that qPCR may be useful as a method complementary to microscopy, particularly in cases of low parasitemia, and for species determination, especially in non-*P. falciparum* cases where most instances of misdiagnosis occur. In low risk areas, qPCR may also be included in the epidemiological surveillance of returning travelers. Further work should focus on constant refinement of the techniques to overcome the current limitations. Inclusion of testing for *P. knowlesi* should be considered.

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