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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