Use of qPCR and Genomic Sequencing to Diagnose *Plasmodium ovale wallikeri* Malaria in a Returned Soldier in the Setting of a Negative Rapid Diagnostic Assay

Robert Cohen, Karla Feghali, Saba Alemayehu, Jack Komisar, Jun Hang, Peter J. Weina, Patricia Coggeshall, Edwin Kamau, and Michael Zapor*

Infectious Disease Service, Walter Reed National Military Medical Center, Bethesda, Maryland; Parasitology Section, Department of Pathology, Walter Reed National Military Medical Center, Bethesda, Maryland; Malaria Vaccine Branch, Walter Reed Army Institute of Research, Silver Spring, Maryland; Viral Diseases Branch, Walter Reed Army Institute of Research, Silver Spring, Maryland; Headquarters, Walter Reed Army Institute of Research, Silver Spring, Maryland

Abstract. *Plasmodium ovale* is one of several clinically relevant malaria species known to cause disease in humans. However, in contrast to *Plasmodium falciparum* and *Plasmodium vivax*, which are responsible for most cases of human malaria, *P. ovale* has a wide distribution but low prevalence in tropical regions. Here, we report the case of a soldier returning from Liberia with *P. ovale wallikeri* malaria. This case highlights the limitations of both microscopy and the malaria rapid diagnostic test for diagnosing infection with *P. ovale* and for distinguishing *P. ovale wallikeri* from *P. ovale curtisi*. To our knowledge, this is the first case report in which quantitative real-time polymerase chain reaction amplification using the *Cytochrome B* gene, coupled with genomic sequencing of the *potra* locus, was used for definitive diagnosis of *P. ovale wallikeri* malaria.

INTRODUCTION

The diagnosis of malaria is made by identifying parasites of the genus *Plasmodium* in the peripheral blood of symptomatic individuals. In clinical laboratories, this is usually done by microscopy and/or use of the malaria rapid diagnostic tests (mRDT). Unlike microscopy, mRDT do not require specialized equipment, and specially or highly trained personnel. However, they are limited in their ability to distinguish between species and vary widely with respect to sensitivity and specificity. Although the World Health Organization (WHO) launched a program to provide comparative data on the performance of mRDT,¹ their reliability is still questionable and currently only one, BinaxNOW (Alere, Waltham, MA), is cleared by the Food and Drug Administration (FDA) and the only one used by the U.S. Department of Defense medical activities. Microscopy, long considered the gold standard for malaria diagnosis, is limited by the skill of the microscopist and its sensitivity may be diminished when the parasites have atypical morphologies.

Plasmodium ovale is one of several clinically relevant malaria species known to cause disease in humans. However, in contrast to *Plasmodium falciparum* and *Plasmodium vivax*, which are responsible for most human malaria, *P. ovale* has a low reported prevalence despite its wide distribution across tropical regions.^{2,3} It is speculated that the paucity of confirmed *P. ovale* infections in endemic areas may be the result of acquisition of species-specific immunity,⁴ misdiagnosis caused by morphological similarity between *P. ovale* and *P. vivax*, co-infection with *P. falciparum* or *P. vivax*, and the relative insensitivity of mRDT in identifying non-falciparum species. Moreover, it has been proposed that *P. ovale* actually comprises two co-circulating non-recombining sympatric species: *P. ovale curtisi* and *P. ovale wallikeri*.^{5,6}

Here, we report a case of a soldier returning from Liberia with a clinical presentation consistent with malaria, but non-diagnostic mRDT and inconclusive speciation by microscopy. Conclusive diagnosis was made by use of molecular

methods. To our knowledge, this is the first case report in which quantitative real-time PCR (qPCR) amplification using the *Cytochrome B* gene, coupled with genomic sequencing of the *potra* gene was used for definitive diagnosis of *P. ovale wallikeri* malaria.

CASE REPORT

A 35-year-old Army sergeant was transferred to Walter Reed National Military Medical Center (WRNMMC) from another military hospital where he had presented with 3 days of intermittent fever, myalgia, headache, nausea with emesis, and diarrhea. He had been in Liberia for 2 weeks in November 2011 and again in February 2012 and became ill in March 2012, shortly after his return to the United States. He stated that he had taken doxycycline while overseas for malaria prophylaxis but did not take primaquine on his return for terminal prophylaxis. On presentation, the patient was febrile at 107°F and his physical exam was significant for scattered petechiae. Notable laboratory results included a platelet count of < 10,000/μL and a thick and thin peripheral blood smear with features suggestive of malaria (Figure 1). However, a BinaxNOW mRDT, run twice on two different specimens was negative for malaria. A whole blood sample along with thick and thin blood smears prepared at WRNMMC were sent to the Walter Reed Army Institute of Research (WRAIR), where DNA extracted from the blood sample was analyzed by qPCR using a previously described method⁷ for *Plasmodium* genus specific and *P. falciparum* targets. The genus-specific qPCR assay was also used for relative quantification of the sample and the parasite burden was estimated to be 597 parasites/μL (0.011% parasitemia). *Plasmodium ovale* and *P. malariae* species-specific qPCR assays were performed as described in the Materials and Methods, and the results were consistent with *P. ovale*. The identity of *P. ovale* was confirmed by sequencing the *potra* locus using the Roche GS FLX Titanium System. The assembled sequence was 764 bp and had nucleotide homology with *P. ovale potra* gene partial sequences HM594180-594183 of 92.9%, 92.7%, 87.2%, and 85.1%, respectively. The high sequence homology and alignment suggested that the *potra* gene of the sample was homologous

* Address correspondence to Michael Zapor, 8901 Wisconsin Avenue, Bethesda, MD 20889. E-mail: Michael.J.Zapor.mil@health.mil

FIGURE 1. Thick peripheral blood smears showing intra-erythrocytic structures consistent with malaria (1,000 $\times$).

to the *P. ovale wallikeri* type 1 *potra* gene (HM594180) and the *P. ovale wallikeri* type 2 *potra* gene (HM594181, Figure 2).

Initial microscopic observations suggested either *P. vivax* or *P. ovale*. Although the patient's recent travel to Liberia made *P. ovale* more likely than *P. vivax*, microscopy was non-diagnostic. For the most part, infected cells were slightly larger than uninfected cells and stippling, consistent with either *P. vivax* or *P. ovale*, was present. However, neither oval-shaped or fimbriated cells, suggestive of *P. ovale*, nor ameboid-shaped cells, suggestive of *P. vivax*, were evident. Thin smears prepared on three successive days shortly after the patient was admitted were used for subsequent diagnosis. On one slide, very few infected cells were elongated, enlarged, and fimbriated, features that are characteristic of *P. ovale* (Figure 3). One parasite had a somewhat ameboid appearance, suggestive of *P. vivax* (although *P. ovale* parasites can also occasionally have an ameboid shape), and some infected cells were smaller than uninfected cells, an attribute

characteristic of *P. malariae* infection and contrary to the usual appearance of *P. ovale*-infected cells (Figure 4).⁸

Two thick smears were examined to quantify parasite density. The first slide had 25 parasites per 210 white blood cells and the second slide had 16 parasites per 204 white blood cells, corresponding to an average parasite density of 792 parasites/ μ L (0.015% parasitemia). These results were consistent with the estimated parasite density as determined by qPCR.

MATERIALS AND METHODS

Rapid diagnostic test. Malaria rapid diagnostic testing was performed using the BinaxNOW rapid diagnostic test (Alere) following the manufacturer's recommendations. This mRDT targets aldolase, a pan-malarial antigen common to *Plasmodium* species capable of infecting humans and the histidine-rich protein II (HRPII) antigen specific to *Plasmodium falciparum*.

DNA extraction and genetic analysis. The DNA was extracted from whole blood collected in EDTA tubes using the EZ1 DNA blood kit on the EZ1 Advanced XL automated sample purification system (Qiagen, Valencia, CA), as recommended by the manufacturer. Species-specific primers were designed to amplify either 18S rRNA genes as previously published⁷ or the *Cytochrome B* gene. The design and testing of the *Cytochrome B* gene primers will be reported elsewhere. The qPCR analysis was performed in the background of QuantiFast SYBR green Master Mix (Qiagen). Amplification and real-time measurements were performed in the Applied Biosystems (Foster City, CA) 7500 analytical PCR system with the following thermal profile for qPCR: 5 min at 95 $^{\circ}$ C, 45 cycles of 10 s at 95 $^{\circ}$ C, 15 s at 60 $^{\circ}$ C, and 30 s at 72 $^{\circ}$ C. For the reaction, 1 μ L of template was added to 9 μ L of reaction master mix containing QuantiFast SYBR green Master and 0.5 μ M of each primer.

Sequencing. A region encoding the *P. ovale* sp. tryptophan-rich antigen (*potra*) gene was amplified using primers previously described⁶ and sequenced by next-generation high throughput pyrosequencing. The PCR amplification product was purified and ligated to rapid library multiplex identifier (RL MID) adaptors using the Roche Rapid Library Preparation kit (Roche 454 Life Sciences, Branford, CT). The barcoded

FIGURE 2. Alignment of *Plasmodium ovale* *tra* gene sequences. Nucleotide sequences were aligned and compared using ClustalW alignment program in Geneious Pro v5.6.4. Five partial *tra* sequences were used in the analysis (from top): *P. ovale wallikeri* type 1 *tra* gene (HM594180), *P. ovale wallikeri* type 2 *tra* gene (HM594181), *P. ovale curtisi* type 1 *tra* gene (HM594182), *P. ovale curtisi* type 2 *tra* gene (HM594183), and the *Potra* gene sequenced in this study. The numbers for nucleotide positions are shown above the alignment graph. Each vertical line indicates a nucleotide that is different from the other sequences. Sequence comparison for the variable region is shown in the box. Nucleotides different from the other sequences, i.e., corresponding to the vertical lines in the alignment graph are shown in red.

FIGURE 3. Thin smear showing structures suggestive of *Plasmodium ovale* malaria. (A) Enlarged “comet” cell (an elongated cell with fimbriae resembling a comet); (B) oval cell with fimbriae; (C) enlarged oval cell with fimbriae; (D) enlarged oval cell with rough edge and James’ dots.

FIGURE 4. Thin smear showing structures atypical for *Plasmodium ovale* malaria. (A–E) Infected cells that are smaller than uninfected cells, suggestive of *P. malariae*; (F) ameboid cell and cytoplasm suggestive of *P. vivax*.

amplicon was sequenced using the Roche GS FLX Titanium System and assembled *de novo* using Roche GSAssembler software (Newbler) version 2.5.3.

The putative protein-coding sequence was identified by alignment of the consensus sequence to GenBank non-redundant nucleotide (nr/nt) and protein (nr) databases using NCBI BLAST tools.⁹ Probable open reading frame shifts caused by pyrosequencing homopolymer miscalling were corrected by use of sequence analysis software Geneious Pro v5.6.4 (Biomatters Ltd., Auckland, New Zealand).¹⁰

Microscopic analysis. Thin smears were prepared by placing 10 μ L of blood collected in EDTA tubes on the slides close to the label area and spreading it rapidly toward the end of the slide to obtain a feathered edge. Smears were air dried for 5 min and then fixed in methanol and allowed to dry. They were then stained with a 20% solution of Giemsa stain (Sigma Life Science, St. Louis, MO) in water for 30 min and then rinsed. Thick smears were prepared by spreading 10 μ L of blood on a slide into a circle slightly more than 1 cm in diameter, air dried for 30 min at room temperature, stained with 20% Giemsa stain for 30 min, rinsed in water, and then air dried. Slides were then examined under oil immersion at 1,000 $\times$ magnification.

DISCUSSION

Prevalence of *P. ovale*. *Plasmodium ovale* accounts for 5–10% of malaria in much of sub-Saharan Africa, including Liberia, but *P. vivax* is rare. *Plasmodium ovale* is also found in New Guinea, the Indonesian portion of Timor, the Philippine island of Palawan, and a few other Western Pacific locations.¹¹

Current malaria diagnostic practices. Malaria is commonly diagnosed by microscopy, antigen detection, and molecular techniques. Currently, microscopy is the gold standard for the detection of *Plasmodium* sp. in the blood. However, the sensitivity of microscopy depends upon the skill of the microscopist and proficiency requires continued training and practice.¹² Because of their convenience and simplicity, mRDT are increasingly popular, especially in low resource settings and in laboratories with less experience with malaria. However, as with microscopy, low levels of parasitemia may reduce the sensitivity of these tests. Although more labor and resource intensive, molecular techniques such as qPCR permit detection of parasitemia $\sim$ 1 log unit lower than can be detected with microscopy or rapid antigen assays.^{13,14}

Challenges associated with detection and identification of malaria using microscopy. The blood stages of *P. ovale* can be reliably distinguished from those of *P. falciparum* by microscopy. However, *P. malariae* shares some morphological features with *P. ovale*, such as compact, regular trophozoites with little vacuolation,^{15,16} and it may be difficult to distinguish between the two. Discriminating *P. ovale* from *P. vivax* by microscopy may be even more difficult, and the identification of *P. ovale* as a distinct species was controversial for many years.¹⁷ Features that may be useful for distinguishing *P. ovale* from *P. vivax* include the oval distortion of the *P. ovale*-infected red cell, heavy and early (James') dots, a cycle of 50 rather than 48 hours, large nuclei, absence of much ameoboidicity, $\sim$ 8–10 merozoites per schizont, darker pigment and smaller size, and the fimbriated appearance of cells infected with *P. ovale*.^{18,19} Both the oval shape and fimbriation of *P. ovale*-infected cells are artifactual but useful for

diagnosis nonetheless. Levels of parasitemia are usually low with *P. ovale* (as well as with *P. malariae* and to a lesser extent, *P. vivax*), therefore a prolonged search may be necessary to find the parasites on a thin film, and diagnosis often depends on the examination of a thick film. This can lead to misinterpretation because some of the distinguishing features of *P. ovale*, such as stippling and the oval shape of infected erythrocytes are not reliably seen on a thick smear. For this reason, some authorities consider the identification of *P. ovale* using only a thick smear to be unreliable.²⁰

Challenges associated with detection and identification of malaria using mRDT. A number of mRDT are available or are under development.^{21–23} The BinaxNOW mRDT relies on identification of certain *Plasmodium* antigens that are conserved among species within the genus. These include a pan-malaria antigen (aldolase) and histidine-rich protein II, which is specific for *P. falciparum*. Although the BinaxNOW mRDT meets the World Health Organization (WHO) recommended performance criterion of detection at > 200 parasites/ μ L for *P. falciparum*, it does not meet this criterion for all non-falciparum species²⁴; in one study, the sensitivity of the BinaxNOW mRDT for detecting *P. vivax* at parasitemia $> 1,000$ parasites/ μ L was 94.1%. However, sensitivity fell to 55% and 50% for parasitemia of 101–1,000 parasites/ μ L and 1–100 parasites/ μ L, respectively²⁵; although *P. ovale* and *P. vivax* are closely related and the protein detected by the BinaxNOW mRDT occurs in both species, one might expect that the sensitivity of the test would be similar for both. However, a number of studies suggest that the currently available rapid immunochromatographic tests, including the BinaxNOW mRDT, are generally unreliable in diagnosing *P. ovale* malaria. Proposed explanations for this include low parasite density, low production of aldolase, and regional variations in the genetic determinants of immunochromatographic test panmalarial antigens.²⁶

Detection and identification of malaria using molecular techniques. Molecular techniques are generally more sensitive in the detection of *Plasmodium* than are microscopy or mRDT and are likely to have an increasing role in the diagnosis of malaria. This is facilitated by the development of new platforms and more technically advanced assays (such as Cepheid's [Sunnyvale, CA] GeneXpert), which use a cartridge (laboratory-in-a-cartridge approach), require minimum training and experience (most assays are Clinical Laboratory Improvement Amendments [CLIA] waived), and are highly accurate and dependable. In addition, PCR assays in which the amplicon is analyzed using lateral flow detection, similar to mRDT, have been described²⁷; these assays will likely become increasingly common in both resources abundant and resource-constrained laboratories.

CONCLUSION

To our knowledge, this is the first case report in which qPCR coupled with genomic sequencing was used for definitive diagnosis of *P. ovale wallikeri* malaria. This case highlights the limitations of both microscopy and the BinaxNOW mRDT for diagnosing *P. ovale* malaria and for distinguishing *P. ovale wallikeri* from *P. ovale curtisi*. This is compounded by the fact that *P. ovale wallikeri* and *P. ovale curtisi* co-circulate in endemic areas (raising a number of intriguing questions to include potential differences between the two with respect to

clinical presentation and drug susceptibility). A limitation of this report is the fact that we only used the one mRDT available to us. It remains to be seen if other brands are similarly insensitive before concluding that current generation mRDT are generally unreliable for diagnosing infection with *P. ovale*. Until mRDT more sensitive for detecting non-falciparum species are developed and qPCR is more widely available, the proper and continuous training of microscopists capable of diagnosing *Plasmodium* infections is advisable.

Case Follow up. The patient presented here was treated for his malaria with 1 g atovaquone/400 mg proguanil hydrochloride (Malarone, GlaxoSmithKline; Research Triangle Park, NC) daily for 3 consecutive days, along with primaquine 30 mg base daily for 14 days, and he had a prompt clinical response. His exceptionally profound thrombocytopenia was attributed to immune-mediated thrombocytopenic purpura, which is a known complication of malaria (although more typically *P. falciparum* and, to a lesser extent, *P. vivax*²⁸), and his platelet count rapidly normalized after treatment with intravenous immune globulin.

Received December 3, 2012. Accepted for publication June 9, 2013.

Published online July 8, 2013.

Acknowledgments: We wish to acknowledge Timothy Burgess, MD, Chief, Infectious Diseases Service, Walter Reed National Military Medical Center, for his critical review of the manuscript. The American Committee on Clinical Tropical Medicine and Travelers' Health (ACCTMTH) assisted with publication expenses.

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Authors' addresses: Robert Cohen, Walter Reed National Military Medical Center - Infectious Disease Service, Bethesda, MD, E-mail: cohen.robby@gmail.com. Karla Feghali, Saba Alemayehu, and Jack Komisar, Walter Reed Army Institute of Research - Malaria Vaccine Branch, Silver Spring, MD, E-mails: Karla.C.Feghali.ctr@us.army.mil, Saba.Alemayehu.ctr@us.army.mil, and Jack.Komisar@us.army.mil. Jun Hang, Walter Reed Army Institute of Research - Viral Diseases Branch, Silver Spring, MD, E-mail: Jun.Hang.ctr@us.army.mil. Peter J. Weina, Walter Reed Army Institute of Research - Headquarters, Silver Spring, MD, E-mail: peter.weina@us.army.mil. Patricia Coggeshall, Walter Reed National Military Medical Center - Parasitology Section, Department of Pathology, Bethesda, MD, E-mail: Patricia.A.Coggeshall.civ@health.mil. Edwin Kamau, Walter Reed Army Institute of Research - Malaria Vaccine Development, Silver Spring, MD, E-mail: edwin.kamau@us.army.mil. Michael Zapor, Infectious Disease Service, Department of Medicine, Walter Reed National Military Medical Center, Bethesda, MD, E-mail: Michael.J.Zapor.mil@health.mil.

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