

CASE REPORT

Detection of *Plasmodium vivax* in a child returning from India by use of broad-range PCR and electrospray ionization mass spectrometry

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Plasmodium vivax is a common cause of imported malaria in the USA, second only to *P. falciparum*. We present a case of *P. vivax* malaria in a child returning from India. *P. vivax* was initially diagnosed by standard methodology and detected retrospectively by use of broad-range PCR and electrospray ionization mass spectrometry using a panel of primers designed to detect vector-borne pathogens. This is the first reported case of *P. vivax* detection using PCR and electrospray ionization mass spectrometry.

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CASE REPORT

A 12-year-old male presented on 13 August 2010 with a nine day history of fever. He had recently spent 6 weeks in India, where he did not take any malaria chemoprophylaxis or use a mosquito net. He developed a 105.7 °F fever on 5 August on the flight home, which recurred every morning between 6:00 and 11:00 a.m., ranged from 104 °F to 105 °F, and was associated with headaches. He had treated his fevers only with Tylenol. Additionally, he presented with a five day cough, and one episode of diarrhea and three episodes of emesis in the two days prior to presentation. The day after his flight home, the patient saw his primary care physician; a complete blood count and liver panel were ordered (Table 1). A malaria smear was also ordered and was negative for *Plasmodium* and *Babesia*. A second complete blood count and a liver panel were performed upon patient presentation on 13 August (Table 1). Urinalysis revealed small bilirubin, trace ketones and protein 30, but was otherwise negative. The patient was negative by Monospot test, as well as for influenza A and B, and had had a negative purified protein derivative test 3 years prior. Negative results were also obtained for antibodies (IgM and IgG) to Chickungunya and Dengue fever and for *Ehrlichia* PCR. The patient had evidence of mosquito bites. Blood films (Figure 1) demonstrated the diagnostic features of *Plasmodium vivax* infection: all parasite forms were present (trophozoite, schizont, and gametocyte); not all red blood cells were infected, unlike *P. falciparum* infections; and the schizonts were larger than uninfected red blood cells (Figure 1), and merozoites and Schuffner's dots were present, both characteristic of *P. vivax*.

Several months subsequent to treatment, remnant whole-blood and plasma specimens collected the day of presentation were de-identified

and sent to Ibis Biosciences for analysis with the Vector-Borne BPN assay using the PLEX-ID (Abbott Molecular, Des Plaines, IL, USA). This technology employs broad-range PCR and electrospray ionization mass spectrometry (PCR/ESI-MS) and has been used to detect a range of vector-borne organisms.^{1–4} Specifically, PCR/ESI-MS involves generation of short PCR amplicons, followed by electrospray mass spectrometry to determine the mass of the amplicon. Based on the amplicon mass, the numbers of each of the four nucleotides in the amplicon, the basecount, can be calculated and compared to a database of basecounts to identify the microorganism. This assay, which is not available for diagnostic use, is designed to detect species of *Anaplasma*, *Babesia*, *Bartonella*, *Borrelia*, *Ehrlichia*, *Francisella* and *Rickettsia*.⁵ Nucleic acids were extracted using a modified version of a previously published procedure.⁵ Modifications consisted of the following: homogenized lysates were incubated in lysis buffer for 1 h at 30 °C prior to loading on the Kingfisher Flex; isopropanol was used instead of ethanol in Wash I buffer; specimens were washed twice with Wash I buffer and twice with Wash II buffer; and the temperature of the initial binding step on the Kingfisher was changed to 30 °C for 22.5 min. Ten microliters of the resulting 200 µL extracts was used in each well of the Vector-Borne BPN assay plate. Negative controls included water extractions; all controls were negative. A novel base count, A28 G24 C16 T18, was detected in both specimen extracts (Figure 2) with primer pair INV4855, designed to target the *Babesia* β -tubulin gene. To identify the detection, initially thought to be a species of *Babesia*, the specimen extracts were amplified with 4 sets of primers targeting the 18S rDNA, ITS1, ITS2 and *hsp70* genes of *Babesia* spp.⁶ using the Platinum Taq High Fidelity kit (Invitrogen, Carlsbad, CA,

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Table 1 Laboratory test results

Test (units)	Days after symptom development	
	1 (8 August)	8 (13 August)
White blood cells (numbers/mm ³)	3600	2700
Hemoglobin (%)	12.7	11.1
Hematocrit (%)	37.6	32.1
Platelet (numbers/mm ³)	136 000	66 000
Alanine transaminase (IU/L)	33.0	18.0
Alkaline phosphatase (IU/L)	239.0	150.0
Albumin (g/dL)	4.2	3.5
Aspartate aminotransferase (IU/L)	29.0	28.0
Total bilirubin (mg/dL)	0.9	2.3
Conjugated bilirubin (mg/dL)	0.0	0.0
Serum total protein (g/dL)	6.8	6.8

USA). The reactions were performed on an MJ Thermocycler with the following PCR cycle: 95 °C for 2 min, followed by eight cycles of 95 °C for 15 s, 50 °C for 45 s and 68 °C for 90 s with the 50 °C annealing temperature increasing 0.6 °C each cycle. The PCR was then continued for 37 additional cycles of 95 °C for 15 s, 60 °C for 15 s and 68 °C for 60 s. The cycle ended with a final extension of 4 min at 72 °C and a 4 °C hold. PCR products were cloned with the ZeroBlunt TOPO PCR kit (Invitrogen) according to the manufacturer's instructions, and then sequenced with SP6 and T7 promoter primers by SeqWright

(Houston, TX, USA). Sequences were obtained only for the ITS2 region. Six of the ITS2 clones tested had sequences bearing 97.3% (397/408) identity with *Plasmodium vivax* (GenBank accession number AF316892.1).

Based upon the initial clinical findings in August 2010, the patient was diagnosed with vivax malaria and treated with Malarone (atovaquone-proguanil 250/100 mg) for 3 days, and then received 14 days of primaquine (15 mg base per day). The patient had a complete recovery.

COMMENTS

Malaria is an important cause of illness in travelers returning to the USA from endemic countries. *P. vivax* is the second most common etiological agent of malaria after *P. falciparum* and, although vivax malaria is not as severe as that caused by *P. falciparum*, *P. vivax* is more geographically widespread, particularly in temperate climates.⁷ 2010 saw a significant upswing in the number of imported malaria cases in the USA, with the highest reporting levels seen since 1980.⁸ Of the 1479 cases of imported malaria from all infecting species, 216 (15%) were acquired in India, a 57% increase in the number of imported cases acquired in India over 2009.⁸ Among the 1366 cases of imported malaria in the USA for which the etiological agent and the region of acquisition were known, infections caused by *P. vivax* comprised 87% of the infections acquired in Asia.⁸

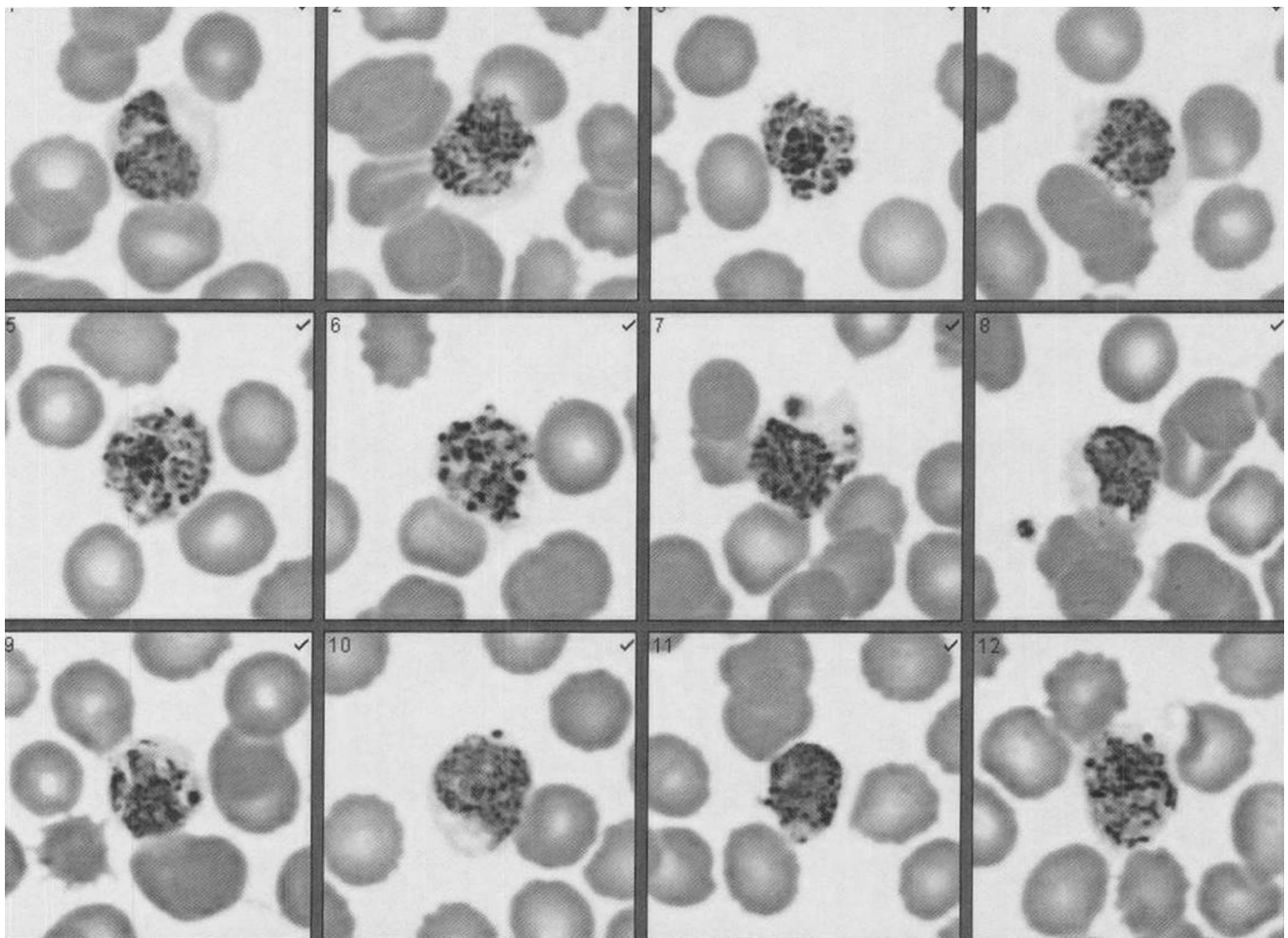


Figure 1 Blood film slides. Slides are taken from a blood film performed on 14 August and are similar to slides prepared on 13 and 16 August. Schizonts are larger than uninfected red blood cells and Schuffner's dots are present, both characteristic of *P. vivax*.

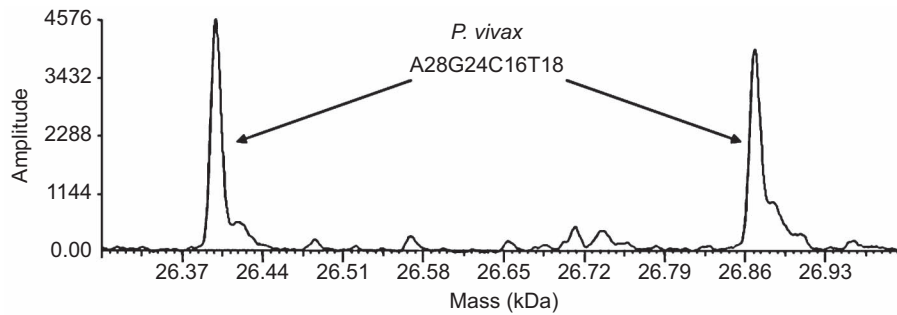


Figure 2 Mass spectra for *Plasmodium vivax*. Mass spectra of the forward and reverse strands of the amplicon produced by primer pair INV4855, which was designed to target the β -tubulin gene of *Babesia* spp. The base count A28 G24 C16 T18 represents the number of adenines, guanines, cytosines, and thymines found in the forward strand of the amplicon.

P. vivax infection also displays a seasonal transmission cycle;⁷ in 2010, the incidence of imported *P. vivax* cases peaked in August, with a secondary peak in November.⁸

This case marks the first report of malaria detection using the PCR/ESI-MS technique with the PLEX-ID. This technique has previously been used to rapidly detect and identify nucleic acids from pathogens in specimens collected from patients diagnosed with various infectious diseases.^{2,9–11} The particular assay used in this report, the Vector-Borne BPN assay, was designed to detect and identify common vector-borne pathogens from the USA and Europe,⁵ including *Babesia*. While not specifically designed to target *Plasmodium*, the broad-range primer, INV4855, designed to target the related *Babesia* β -tubulin gene, amplified *P. vivax* and produced a unique basecount signature allowing for the identification of the organism. Bioinformatic analysis has since determined that primer pair INV4855 can also detect and differentiate *P. falciparum* and *P. knowlesi*. That there is conservation in β -tubulin gene between *Babesia* and *Plasmodium* species is not surprising, as both genera are members of the class Aconoidasida.

There are several advantages of using PCR/ESI-MS over traditional means of malaria detection. The technique offers a rapid molecular method for the identification of pathogen DNA from human specimens,¹² which does not require the screening of multiple blood films to identify infected red blood cells and provides a molecular basis for species differentiation rather than one based on visual inspection of parasite forms. Additionally, the Vector-Borne BPN assay was able to detect *P. vivax* DNA in a plasma specimen, indicating that sample types other than whole blood can be used to detect active malaria infection. Finally, the use of the Vector-Borne BPN assay in particular offers the advantage of being able to screen for multiple vector-borne pathogens simultaneously, which proved useful in this case for differentiating an imported *P. vivax* infection from infection with domestic vector-borne pathogens associated with high fevers, such as *Babesia microti* and *Ehrlichia chaffeensis*.

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