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Indigenous *Plasmodium malariae* Infection in an Endemic Population at the Thai–Myanmar Border

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Abstract. *Plasmodium malariae* is a neglected malaria parasite. It has wide geographic distribution and, although often associated with mild malaria, is linked to a high burden of anemia and nephrotic syndromes. Here, we report a cohort study conducted in the Kanchanaburi Province of Thailand during May 2013–June 2014 in which *P. malariae* infection was detected. Of the 812 study participants, two were found to be infected with *P. malariae*. One had an infection that led to acute malaria, but the other was positive for *P. malariae* at multiple visits during the study and apparently had chronic asymptomatic infection. Such persistent infection may explain how *P. malariae* has been able to thrive at very low prevalence and represents a challenge for malaria elimination.

INTRODUCTION

Plasmodium malariae is one of the five *Plasmodium* species causing human malaria. It has broad geographic distribution, being present in many parts of Asia, the western Pacific, South America, and Africa. Although commonly believed to cause mild malaria, *P. malariae* infection has been linked to a high burden of anemia and a chronic nephrotic syndrome.[1,2] *Plasmodium malariae* infection can last a very long time, sometimes several decades.[3–5]

Compared with *Plasmodium falciparum* and *Plasmodium vivax*, the two major malaria parasite species, *P. malariae* has received much less attention from the research community. Much of the current knowledge about the parasitology and clinical illness caused by this parasite was obtained through records of malaria therapy, a treatment of neurosyphilis by induced malaria in the first half of the 20th century.[6,7] There is only a dearth of information about the parasite in its natural settings. In Africa, *P. malariae* is mostly found as mixed infection with *P. falciparum*.[8–11] In Papua New Guinea (PNG), *P. malariae* contributed to approximately 4% of malaria infection.[12] In Brazil, asymptomatic infection of *P. malariae* was detected by nested polymerase chain reaction (PCR) in a few individuals at multiple time points during a 21-month follow-up, consistent with long-lasting low-density infection.[13] The recent use of sensitive molecular diagnosis suggested that the prevalence of *P. malariae* in many early studies may have been significantly underestimated because of the limited sensitivity of light microscopy (LM).[14–16] By quantitative PCR (qPCR), 15% *P. malariae* prevalence was observed in 5- to 9-year-old children from PNG.[17]

In this study, we report *P. malariae* infection in a hypoendemic area of Thailand near the Thailand–Myanmar border. Two unrelated individuals were found to be infected with the parasite during a cohort study of 812 participants between May 2013 and June 2014.

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METHODS

Characteristics of the study area. The study was carried out in Ban Bong Ti Bon village of Bong Ti subdistrict, Sai Yok district, Kanchanaburi Province. This village had a population of approximately 800, which was composed mostly of Thai and Karen ethnic origin. Agriculture was the main occupation. Malaria is seasonal with the peak season during May–August. According to a report from the Thai Ministry of Public Health, the number of malaria patients in the country has significantly declined during the years 2012–2016. The annual malaria case numbers were 16,196 (*P. malariae*: 48) in 2012, 14,740 (*P. malariae*: 80) in 2013, 11,352 (no *P. malariae* infection reported) in 2014, 12,637 (*P. malariae*: 26) in 2015, and 15,451 (*P. malariae*: 26) in 2016.[18] In Bong Ti subdistrict, *P. vivax* and *P. falciparum* were the predominant species contributing to more than 99% of all infections. Four clinical cases of *P. malariae* detected by LM and none for *Plasmodium ovale* and *Plasmodium knowlesi* were reported by the ministry during these 4 years.[18]

Sample collection. Blood samples were collected from each of 812 individuals every 4 weeks during May 2013–June 2014. In total, 14 active case detection (ACD) visits per individual were made during the study. In addition, samples were obtained by passive case detection (PCD) when participants presented to the local malaria clinic. Demographic and behavioral factors often associated with malaria infection were collected by interview based on questionnaire. Finger-prick blood samples (250 μL) were collected into BD microtainers™ with K_2 ethylene diamine tetraacetic acid (EDTA) as anticoagulant, 50 μL of which was transferred immediately to another tube with 250 μL of RNAprotect cell reagent (Qiagen, Hilden, Germany) for RNA preservation. Both the original blood and blood in RNAprotect were stored on dry ice until arrival at the laboratory in Bangkok where they were transferred to −20°C until DNA/RNA extraction. *Plasmodium* detection was completed approximately 1 month after each visit. At the time of the study (2013–2014), the Thai national guideline for malaria treatment required confirmation by positive blood smears within 7 days. As a result, no antimalarial treatment was given to PCR-positive individuals of our study.

Informed consent was obtained from each individual or a legal guardian before participation in the study. The study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University (Ethics Committee approval number TMEC 13-020).

DNA extraction and purification. Genomic DNA (gDNA) was extracted from 200 µL blood pellet samples with FavorPrep 96-well genomic DNA extraction kit (Favorgen, Ping-Tung, Taiwan) and stored in 100 µL elution buffer. Genomic DNA samples were kept at –20°C before analyses.

***Plasmodium* detection.** All DNA samples were screened by genus-specific qPCR assay (QMAL) targeting 18S rRNA genes as previously described.[19] Four microliters of purified DNA, corresponding to 8 µL whole blood, was used as the template for QMAL. Six hundred and nine samples were identified as *Plasmodium* positive. Analyses of *P. falciparum* and *P. vivax* infections are being performed for separate publication.

Detection of *P. malariae*, *P. ovale*, and *P. knowlesi*. Because of the limited quantity of available DNA, detection of *P. malariae*, *P. ovale*, and *P. knowlesi* was by quantitative reverse transcription PCR (qRT-PCR). Only QMAL-positive blood samples were examined. RNA was extracted from the 50-µL portion of blood stored in RNAprotect using the RNeasy Plus Mini Kit (Qiagen). Purified RNA was eluted in 50 µL water and stored at –80°C. Quantitative reverse transcription PCR was performed using 2 µL of RNA as the template, using the SuperScript III Platinum one-step qRT-PCR kit. Most primer and probe sequences have been previously published (Table 1).[19,20]

Nested PCR to confirm *Plasmodium* species in PCD samples. Nested PCR for malaria diagnosis was modified from the original method by Kimura et al.,[21] using purified gDNA as the template. The sequences of primers used in nested PCR are shown in Table 2 with modified oligonucleotides (MR_W2 and KR_W1). The first PCR uses primers P1F and P2R (genus-specific primers) to amplify *Plasmodium* 18S rRNA genes. The PCR product for this first reaction was then used as the template for species-specific semi-nested amplification with FR, MR_W2, OR, VR, or KR_W1 as the reverse primer and PF1 as the forward primer. The thermocycle of both the first and the nested reaction was as follows: 1) 94.0°C for 10:00 minutes; 2) 35 rounds of denaturation at 95.0°C for 0:30 minutes, annealing at 60.0°C for 1:30 minutes, and extension at 72.0°C for 1:00 minutes; and 3) 72.0°C for 5:00 minutes. The PCR product was then analyzed by agarose gel electrophoresis.

***Plasmodium malariae* merozoite surface protein-1 gene (*Pmmsp-1*) sequence analysis.** Nested PCR was used to amplify region 1, a highly polymorphic region, of *Pmmsp-1* as described previously.[22] The PCR amplicon was subjected to dye-terminator sequencing (Bioneer, Daejeon, Republic of Korea). DNA sequence chromatograms were examined visually to confirm good quality before sequence alignment.

TABLE 2

Primers and probes for nested PCR[21]

Primer name	Sequence
P1F	5′-ACG ATC AGA TAC CGT CGT AAT CTT-3′
P2R	5′-ACG ATC AGA TAC CGT CGT AAT CTT-3′
FR	5′-CAA TCT AAA AGT CAC CTC GAA AGA TG-3′
MR_W2	5′-AAG GAA GCT ATC TAA AAG AAA CAC TCA T-3′
OR	5′-ACT GAA GGA AGC AAT CTA AGA AAT TT-3′
VR	5′-CAA TCT AAG AAT AAA CTC CGA GAG GAA A-3′
KR_W1	5′-CTG AAG GAA GCA ATC TAA GAG TTC-3′

Primers used in nested PCR to identify the species of *Plasmodium* parasite.

RESULTS

***Plasmodium malariae* infection.** Parasite detection workflow is summarized in Figure 1. Finger-prick blood samples were collected from each of the 812 participants at monthly intervals. In total, 14 ACD visits were made, resulting in 10,198 blood samples. These samples were examined for *Plasmodium* infection by a genus-specific qPCR assay QMAL,[19] 609 of which were positive for *Plasmodium* sp. Quantitative reverse transcription PCR was performed to identify infection of *P. ovale*, *P. knowlesi*, and *P. malariae*. Of the 609 *Plasmodium*-positive samples, 12 were positive for *P. malariae* by qRT-PCR and none for *P. ovale* and *P. knowlesi*. In addition to these 12 *P. malariae*–positive samples from ACD, two additional *P. malariae*–positive samples were identified by nested PCR; these two samples were obtained from PCD visits when the participants presented to the local malaria clinic. These 14 *P. malariae*–positive samples were derived from only two study participants, 11 from the first and three from the second.

The first infected participant was a 41-year-old man. Despite repeated detections of *P. malariae* infection, he had no malaria-like symptoms at all ACD visits. The participant presented once to the Bong Ti malaria clinic on November 29, 2013 (week 27), with fever, but his blood sample was negative by LM, and he did not receive antimalaria treatment. Parasite densities of these *P. malariae*–positive samples were always very low. Their 18S rDNA copy numbers from QMAL were at least 100-fold lower than that of the second participant who suffered a clinical episode of malaria (Figure 2). Samples were not collected at weeks 12 and 16 because the participant temporarily left the study site. The samples from weeks 20 and 45 were negative by QMAL, suggesting that the parasite was either absent or present below to the limit of detection.

TABLE 1

Primers and probes for quantitative reverse transcription PCR (qRT-PCR)

Species	Primer	Sequence (5′ > 3′)
Plasmodium malariae[20]	Mal_fw	TGC CGA CTA GGT GTT GGA TGA T
	Mal_rev	CTA GTG AGT TTC CCC GTG TTG AGT
	Mal_probe	HEX – TGT TTC TTT TAG ATA GCT TCC TTC AG – BFQ
Plasmodium ovale[20]	Ova_fw	CCA GCT CCA ATA GCG TAT ATT AAA
	Ova_rev*	ACA CAT TTT GSA TAA GGA ATG CAA AG
	Ova_probe*	HEX – TAT AAG ATG CTT AGR CAA TAC AAC GTA TCT G – BFQ
Plasmodium knowlesi	Kno_fw	GTT AGC GAG AGC CAC AAA AAA GCG AAT
	Kno_rev*	ACT CAA AGT AAC AAA ATC TTC CAT A
	Kno_probe*	HEX – TGC TTT ATG TGC GCA TCC TCT ACC TA – BFQ

Primers and probes validated for qRT-PCR targeting 18S rRNA.
* Oligo includes a wobble: S = G/C; R = A/G.

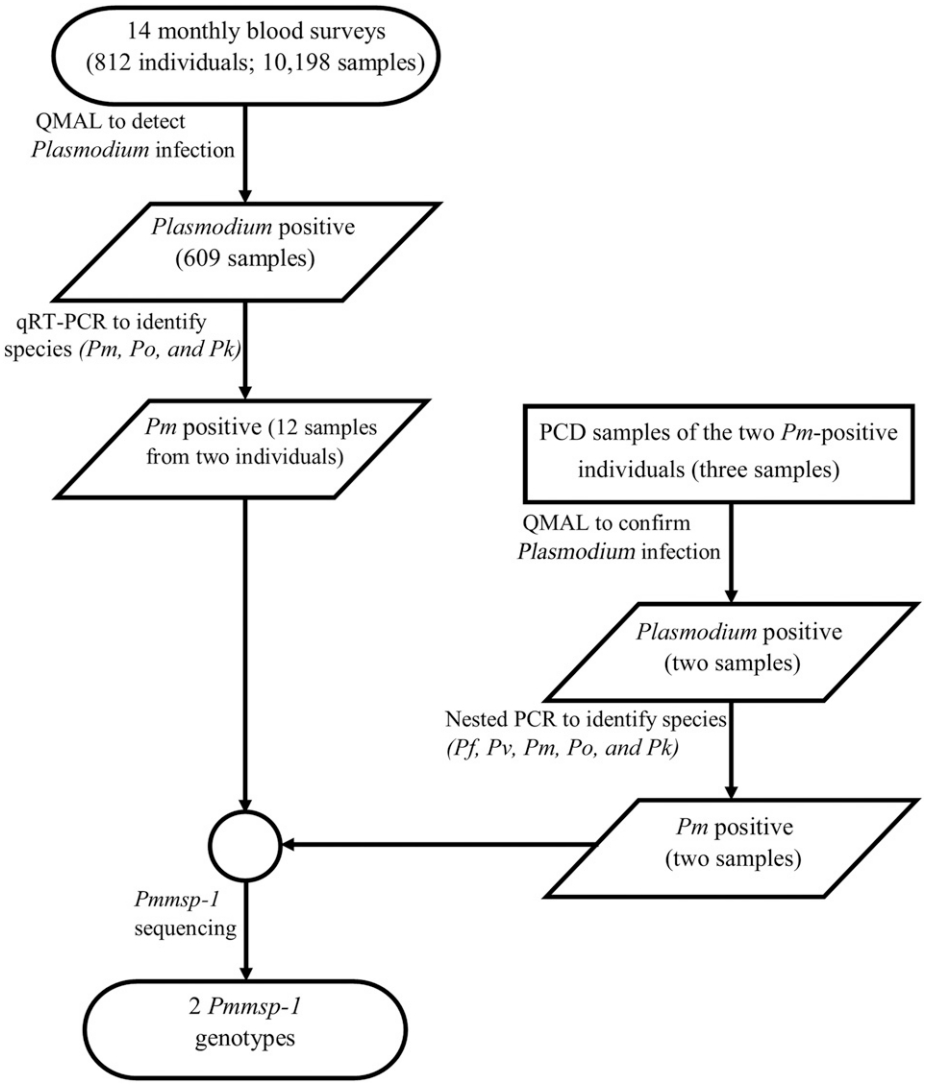

FIGURE 1. Study outline. Pf = *Plasmodium falciparum*; Pk = *Plasmodium knowlesi*; Pm = *Plasmodium malariae*; *Pmmsp-1* = *Plasmodium malariae* merozoite surface protein-1 gene; Po = *Plasmodium ovale*; Pv = *Plasmodium vivax*; PCD = passive case detection; QMAL = genus-specific quantitative PCR assay; qRT-PCR = quantitative reverse transcription PCR.

The second infected participant contributed three infected blood samples. This participant was a 54-year-old woman who lived in the same village as the first participant. She reported never to have had malaria before the study. The first two samples were from two consecutive ACD visits on July 19, 2013 (week 9), and August 13, 2013 (week 12). At both times, this individual appeared healthy and had none of the usual malaria symptoms. The third sample was obtained from PCD on August 20, 2013 (week 13), when the participant presented to the Bong Ti malaria clinic with fever. This time, the participant's blood was positive for malaria parasites by LM with 0.014% parasitemia. The parasite showed characteristics of *P. malariae* trophozoites, including lack of erythrocyte enlargement and lack of Schüffner's dots (Figure 3A). Nested PCR of this infection confirmed that the infection was due to *P. malariae* (Figure 3B). The parasite was apparently cleared by the *P. vivax* radical cure prescribed by the malaria clinic staff. None of the following nine blood samples collected from this individual over the next months was PCR positive for any *Plasmodium* species. Taken the results together, it appears that a new infection which was first detected on July 19, 2013 (week 9), developed into acute illness on August 20, 2013 (week 13).

***Plasmodium malariae* infections in the two participants were genetically distinct.** Because the two participants infected with *P. malariae* lived only 1.2 km from each other, it was possible that the infection was transmitted from one to the other through local *Anopheline* mosquitoes. To determine whether the parasites carried by the participants shared the same genotype, we sequenced segments of the highly polymorphic gene *Pmmsp-1* from all *P. malariae* samples. All samples from the first participant had the same *Pmmsp-1* sequence, but this sequence was different from that of the second participant (Table 3). Thus, the parasites from the two participants were clearly distinct.

DISCUSSION

Only a handful of studies have described *P. malariae* infections in the malaria-endemic areas of mainland Southeast Asia.[23–26] *Plasmodium malariae* malaria is sporadically reported and caused only 0.2% of total malaria cases in Thailand

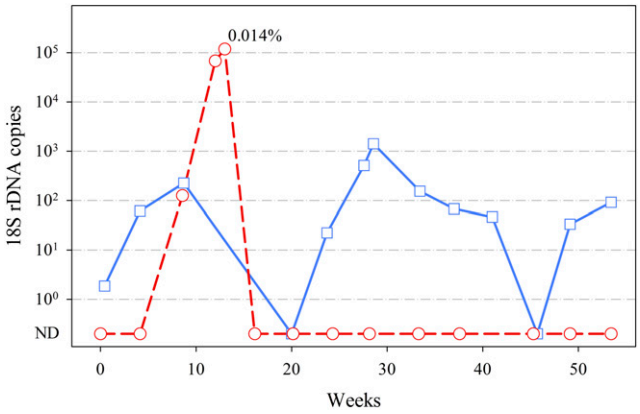

FIGURE 2. Time course of *Plasmodium malariae* infections. Shown are the copy numbers of *Plasmodium* 18S rDNA detected in each QMAL reaction (equivalent to 8 μL of blood). Blue solid line with squares, 1st participant; red dashed line with circles, 2nd participant. 18S rDNA copy below 10^0 considered as QMAL-negative samples in which parasite was ND; 0.014% indicates the parasitemia of participant 2 at the clinical passive case detection visit on August 20, 2013, as determined by light microscopy. ND = not detected; QMAL = genus-specific quantitative PCR assay. This figure appears in color at www.ajtmh.org.

during 2012–2016.[18] Our study, conducted between May 2013 and June 2014, identified two individuals out of 812 residents infected with *P. malariae*. The finding was consistent with the recent report by the Thai Ministry of Public Health, in which *P. malariae* was present in the study area.[16]

Our first infected participant was a 41-year-old male agriculturist. Throughout the study, this participant had no symptoms at all ACD visits. However, he was positive for *P. malariae* at multiple visits. Because this participant reported to have had malaria several times in the past, it is likely that he had acquired protective immunity from previous exposure to the parasite. DNA sequencing revealed a single *Pmmsp-1* genotype, suggesting persistent infection of a single parasite line. The absence of parasites at ACD visit 6 (week 20) and visit 12 (week 46) was likely due to fluctuation of parasitemia to a level lower than the detection limit of qPCR. The participant presented to the local malaria clinic with fever once on November 29, 2013 (week 27), but was diagnosed negative for malaria by LM. He was not treated with antimalarial drug. Given that the parasitemia at this time was low (on the order of 0.0001%) and that he did not return to the clinic again during the study period, the fever was likely due to another cause. The repeated detections of a clonal line of the parasite at multiple time points from this participant provide direct evidence for chronic low-density asymptomatic infection of *P. malariae* in its natural setting.

In contrast to the first participant, the second participant developed clinical malaria after two consecutive positive detections by qPCR. The timing of the first detection suggests that the parasite could be detected as early as 5 weeks before the onset of symptoms. This incubation time sits well within the range of the reported pre-patent period (time from mosquito transmission to first appearance of parasite on thick film) of 16–59 days.[11] Although Giemsa smear showed features of

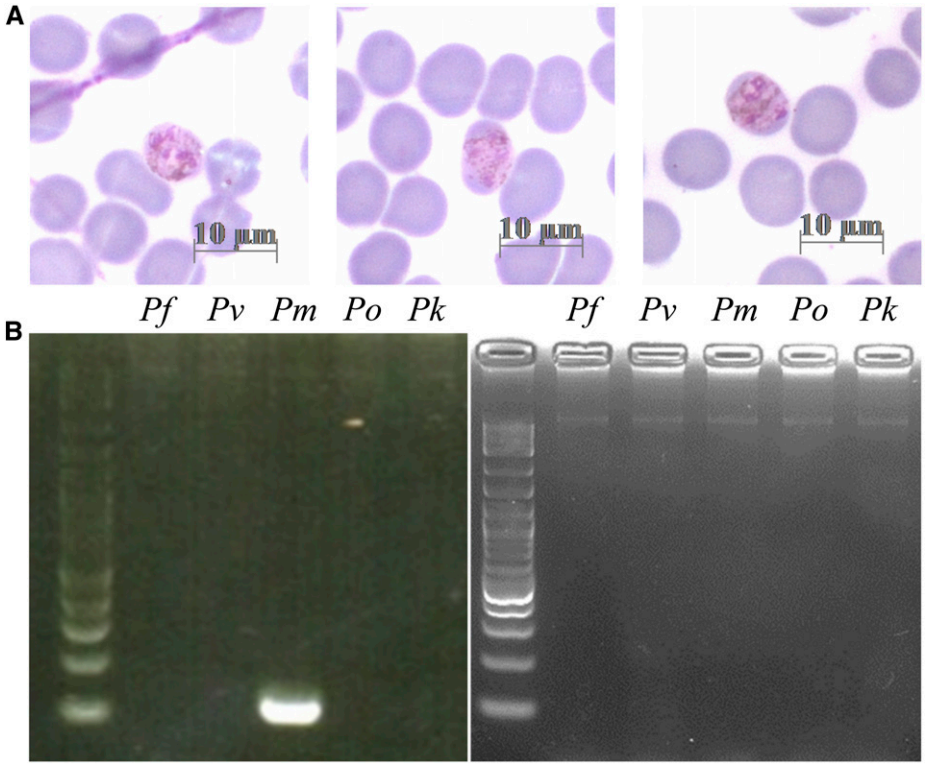

FIGURE 3. Blood sample diagnosis of clinical *Plasmodium malariae*. (**A**) A thin smear of clinical *P. malariae* infection from the second participant on August 20, 2013. (**B**) Nested PCR to identify *Plasmodium* species. Left panel: gel electrophoresis of PCR products using blood DNA of the second participant from August 20, 2013, as the template. Right panel: negative controls of PCR without DNA template. Left lane of each panel: 100-bp molecular weight marker. Pf = *Plasmodium falciparum*; Pk = *Plasmodium knowlesi*; Pm = *Plasmodium malariae*; Po = *Plasmodium ovale*; Pv = *Plasmodium vivax*. This figure appears in color at www.ajtmh.org.

TABLE 3

Nucleotide sequences of the polymorphic regions (base 140–146, 175–220, and 240–265) of *Plasmodium malariae* merozoite surface protein-1 gene (*Pmmsp-1*) from 14 *P. malariae*–positive blood samples

Samples	Nucleotide position of *Pmmsp-1*		
	140–146	175–220	240–265
MSP1-MM1A*	TAATAT	ACATCGCTGATGAGAATAAAAAATTAGAGGCTCCTAGTGAATCAGG	AAGAATTGTAATGAAAAACAGAAAAT
1st participant			
Week 0	TAA**A**AT	ACATC**G**CTACTAA**G**AATAAA**G**AATTAGA**G**GC**TCA**TA**G**TG**G**AT**C**A**G**G	AA**G**AATTGTG**C**TAAAAAACAG**G**AAAT
Week 4	TAA**A**AT	ACATC**G**CTACTAA**G**AATAAA**G**AATTAGA**G**GC**TCA**TA**G**TG**G**AT**C**A**G**G	AA**G**AATTGTG**C**TAAAAAACAG**G**AAAT
Week 9	TAA**A**AT	ACATC**G**CTACTAA**G**AATAAA**G**AATTAGA**G**GC**TCA**TA**G**TG**G**AT**C**A**G**G	AA**G**AATTGTG**C**TAAAAAACAG**G**AAAT
Week 24	TAA**A**AT	ACATC**G**CTACTAA**G**AATAAA**G**AATTAGA**G**GC**TCA**TA**G**TG**G**AT**C**A**G**G	AA**G**AATTGTG**C**TAAAAAACAG**G**AAAT
Week 27†	TAA**A**AT	ACATC**G**CTACTAA**G**AATAAA**G**AATTAGA**G**GC**TCA**TA**G**TG**G**AT**C**A**G**G	AA**G**AATTGTG**C**TAAAAAACAG**G**AAAT
Week 28	TAA**A**AT	ACATC**G**CTACTAA**G**AATAAA**G**AATTAGA**G**GC**TCA**TA**G**TG**G**AT**C**A**G**G	AA**G**AATTGTG**C**TAAAAAACAG**G**AAAT
Week 33	TAA**A**AT	ACATC**G**CTACTAA**G**AATAAA**G**AATTAGA**G**GC**TCA**TA**G**TG**G**AT**C**A**G**G	AA**G**AATTGTG**C**TAAAAAACAG**G**AAAT
Week 37	TAA**A**AT	ACATC**G**CTACTAA**G**AATAAA**G**AATTAGA**G**GC**TCA**TA**G**TG**G**AT**C**A**G**G	AA**G**AATTGTG**C**TAAAAAACAG**G**AAAT
Week 41	TAA**A**AT	ACATC**G**CTACTAA**G**AATAAA**G**AATTAGA**G**GC**TCA**TA**G**TG**G**AT**C**A**G**G	AA**G**AATTGTG**C**TAAAAAACAG**G**AAAT
Week 49	TAA**A**AT	ACATC**G**CTACTAA**G**AATAAA**G**AATTAGA**G**GC**TCA**TA**G**TG**G**AT**C**A**G**G	AA**G**AATTGTG**C**TAAAAAACAG**G**AAAT
Week 53	TAA**A**AT	ACATC**G**CTACTAA**G**AATAAA**G**AATTAGA**G**GC**TCA**TA**G**TG**G**AT**C**A**G**G	AA**G**AATTGTG**C**TAAAAAACAG**G**AAAT
2nd participant			
Week 9	TAA**T**AT	ACATC**A**CTACTA**C**GAATAA**C**GAATTAGT**G**AC**GTC**TAATG**T**A**CC**AA**G**	AC**G**AATTGTG**A**TAAAAAACAG**A**AAAT
Week 12	TAA**T**AT	ACATC**A**CTACTA**C**GAATAA**C**GAATTAGT**G**AC**GTC**TAATG**T**A**CC**AA**G**	AC**G**AATTGTG**A**TAAAAAACAG**A**AAAT
Week 13†	TAA**T**AT	ACATC**A**CTACTA**C**GAATAA**C**GAATTAGT**G**AC**GTC**TAATG**T**A**CC**AA**G**	AC**G**AATTGTG**A**TAAAAAACAG**A**AAAT

Bases with bold characters represent single-nucleotide polymorphism found. Only highly polymorphic segments of the gene are shown for clarity. All other sequenced gene segments display the same clustering within each individual.

* Reference sequence (GenBank: FJ824669.1).

† Samples obtained through passive case detection.

P. malariae, the parasite was initially misidentified as *P. vivax* by the malaria clinic personnel, and the patient was treated with *P. vivax* radical cure according to Thailand's malaria treatment guideline, that is, 3 days of chloroquine (25 mg/kg total dose) + 14 days of primaquine (15 mg/day). Although the treatment was successful, this misdiagnosis exemplifies the frequent incorrect species identification of *P. malariae*[27,28] and microscopist bias toward the more prevalent *P. vivax*.

In summary, two cases of *P. malariae* infection were identified in a cohort of 812 participants living in an endemic site of Thailand. The characteristics of infection suggest that one infection was chronic and asymptomatic, whereas the other culminated in acute illness. The observed persistence of infection without symptoms may provide a source of transmission that helps sustain *P. malariae* at very low prevalence. To accelerate malaria elimination in Thailand and other endemic countries, intervention such as mass screening and treatment aiming to remove such a residual source of transmission may be necessary.

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