Natural *Plasmodium inui* Infections in Humans and *Anopheles cracens* Mosquito, Malaysia

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We detected 2 natural, asymptomatic *Plasmodium inui* monoinfections in humans in Malaysia by using nested PCR on concentrated high-volume blood samples. We found a *P. inui*–positive *Anopheles cracens* mosquito in the same site as the human infections. Investigators should use ultrasensitive detection methods to identify simian malaria parasite transmission in humans.

Zoonotic transmission of simian malaria parasites to humans have been occurring in Southeast Asia and South America. Among the 3 simian malaria parasites in Southeast Asia experimentally shown to infect humans (*1*), *Plasmodium knowlesi*, *P. cynomolgi*, and *P. inui*, only *P. knowlesi* and *P. cynomolgi* have been reported in cases of natural infection (*2*). We report 2 natural, asymptomatic *P. inui* human infections detected by using nested PCR (nPCR) on concentrated high-volume blood.

The Study

We conducted an epidemiologic and entomological study at a campsite in Kem Sri Gading, Pahang, Malaysia (3°45′46.24″N, 102°34′20.32″E), because of frequent reports of human *P. knowlesi* infections acquired from this area. Kem Sri Gading is a receptive area, a location in which the ecosystem permits malaria transmission because vector and reservoir host populations both inhabit it.

On March 2, 2020, we obtained ≤3 mL of venous blood from 71 persons at the camp who provided consent. Participants had undergone training in the forest at Kem Sri Gading during January 27–28, 2020. The Medical Research and Ethics Committee, Ministry of Health Malaysia, approved this study (approval no. NMRR-15-672-23975 for the human study and approval no. NMRR-19-962-47606 for the mosquito study).

The 2 case-patients we report, PMAR0041, a 20-year-old woman, and PMAR0052, a 19-year-old woman, had no previous history of malaria. Before our study, PMAR0041 was in a nonreceptive city in Selangor 1–2 weeks before training at the camp; PMAR0052 regularly entered forested areas ≥2 times per month. During January 29–March 2, 2020, neither case-patient visited any potentially receptive areas. Both persons reported they were healthy before, during, and after blood collection.

Using the amount of DNA equivalent to 500 µL of whole blood (*3*), we detected *Plasmodium* in the 2 cases in separate nPCR assays (Appendix, https://wwwnc.cdc.gov/EID/article/27/10/21-0412-App1.pdf). We used primers targeting both the asexual and sexual 18S rRNA genes of *Plasmodium* (*4*). Sequence analysis of the cloned genus PCR products confirmed *P. inui* (Table). We performed species-specific nPCR assays to detect 5 known human malaria parasites, including *P. knowlesi*, and to detect *P. cynomolgi* and *P. inui*, by using previously published primers (*4*–*6*). However, the species-specific PCR amplification demonstrated spurious results; we were unable to produce consistent results over repeated tests. Thus, *P. inui* was detected only in case-patient PMAR0041 (Figure 1) because the protocol produced insufficient DNA, which hampered further analyses. However, we found likely trophozoites in thick blood smears of each case during 2 hours of observation (Figure 2).

On October 9, 2020, we obtained a second blood sample from case-patient PMAR0041; case-patient PMAR0052 did not consent to a second blood collection. Between the first and follow-up blood collections, PMAR0041 did not travel to any receptive areas. We did not detect any *Plasmodium* DNA in the second blood sample from PMAR0041 after repeated tests.

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Table. Nucleotide BLAST results of the PCR products sequenced in a study of natural transmission of *Plasmodium inui* in 2 humans and in *Anopheles cracens* mosquitoes, Malaysia*

Sequence source and length, bp (GenBank accession no.)	Description of sequence (GenBank accession no.)	% Identity	% Query cover
Patient PMAR0041, 234 (MW555281)	*Plasmodium inui* asexual type 18S rRNA, Celebes (AB287276)	99.57†	100
	P. inui asexual type, 18S rRNA, Thailand (EU400385)	99.57	100
	P. inui asexual type, 18S rRNA, Taiwan I (FN430724)	99.57	100
Patient PMAR0052, 243 (MW555282)	*P. inui* sexual type, 18S rRNA, Taiwan I (FN429982)	99.59†	100
	P. inui 18S rRNA, *Anopheles latens* mosquito, Sarawak (MN535358)	99.18‡	100
	P. inui sexual type, 18S rRNA from monkey (FJ619103)	99.18	100
	P. inui 18S rRNA, wild monkey, Thailand (EU400386)	99.18	100
An. cracens, 986 (MW555286)	*P. inui* asexual type, 18S rRNA, Celebes (AB287276)	99.90†	100
	P. inui 18S rRNA, *An. latens*, Sarawak (MN535320)	99.80‡	100
	P. inui asexual type 18S rRNA, wild monkey, Thailand (EU400385)	99.70	100
	P. inui asexual type 18S rRNA, South China (HM032051)	99.49	100
	P. inui asexual type 18S rRNA, Taiwan II (FN430725)	99.49	100

*BLAST, https://blast.ncbi.nlm.nih.gov. bp, base pair.
†This sequence had only a 1 single-nucleotide mismatch at the forward primer priming site.
‡This sequence had 2 single-nucleotide mismatches, 1 at the forward primer priming site.

We collected *Anopheles cracens*, *An. introlatus*, and *An. barbirostris* sensu lato mosquitoes at the camp by using human landing catches and Mosquito Magnet Independence Trap (Woodstream Corp., https://www.woodstream.com). *An. cracens* was the predominant mosquito species collected. Only 1 nonblood fed *An. cracens* mosquito, caught on August 24, 2020, was *Plasmodium*-positive in its head and thorax by nPCR (*4*). We found no oocysts upon dissection of the mosquito gut. We were unable to successfully dissect the salivary glands because the mosquito was dead. We used published primers (*7*) to amplify the *P. inui* 18S rRNA gene and confirmed *P. inui* by sequencing the PCR product (Table). We tested the entomological team by using the same PCR methods described for the case-patients but detected no *Plasmodium*.

Our analyses showed that the *P. inui* sequence obtained from case-patient PMAR0041 was identical to the corresponding region on the asexual type 18S rRNA sequence obtained from the *An. cracens* mosquito (Appendix Figure 2), but the *P. inui* sequence obtained from case-patient PMAR0052 was of the sexual type 18S rRNA (Table). The human *P. inui*–positive cases we detected originated from separate DNA extractions and PCR assays on different days by using dedicated benchtops for different procedures. The case-patients had the only *P. inui*–positive samples, but we identified a few *P. knowlesi*–positive samples among the 71 persons screened at the camp (Appendix Figure 1). The *An. cracens* mosquito was the only *Plasmodium*-positive mosquito we detected. We hypothesize that PMAR0041, PMAR0052, and the *Plasmodium*-positive mosquito were monoinfected

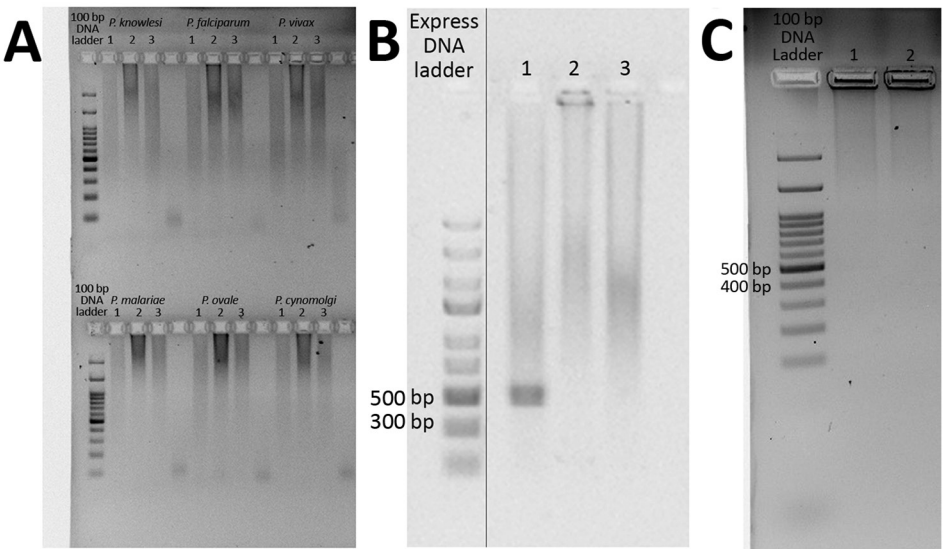

Figure 1. Species-specific nested PCR amplification products for a study of *Plasmodium inui* infections among humans, Malaysia. Samples were subjected to electrophoresis on a 1.5% agarose gel. A) Results for detection of *P. knowlesi*, *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. cynomolgi*. Lane 1, human case-patient PMAR0041; lane 2, human case-patient PMAR0052; lane 3, no-template control. B) Results for the detection of *P. inui* in human case-patient PMAR0041. Lane 1, case-patient PMAR0041; lane 2, negative control; lane 3, no-template control. The solid vertical line indicates these are separate parts of the same image. C) Results for the detection of *P. inui* in human case-patient PMAR0052. Lane 1, case-patient PMAR0052; lane 2, no-template control.

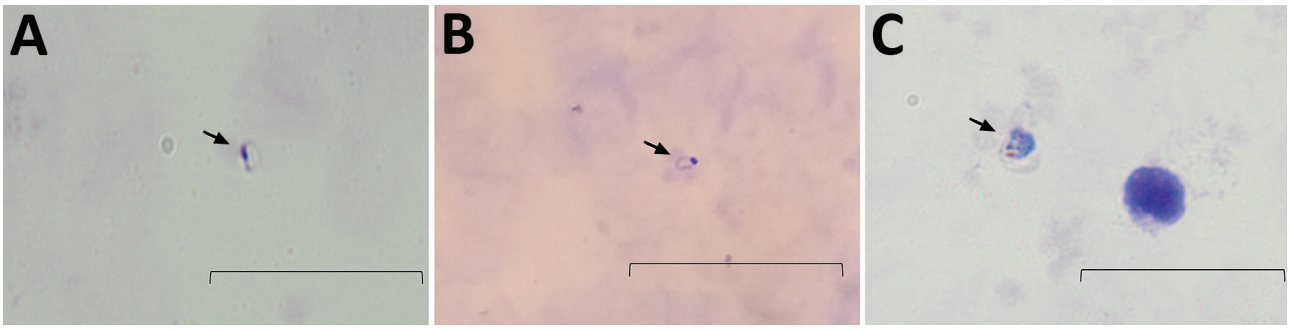

Figure 2. Micrographs of the thick blood smears showing *Plasmodium* trophozoites in 2 human cases of *Plamodium inui* infection, Malaysia. A) Smear from case-patient PMAR0041, taken by using an Olympus BX51 microscope (Olympus Corporation, https://www.olympus-lifescience.com). B) Smear from case-patient PMAR0041, taken by using Redmi Note 4 (Xiaomi Corporation, https://www.mi.com) smartphone camera. C) Smear from case-patient PMAR0052, taken by using an Olympus BX51 microscope. Arrows indicate *P. inui* trophozoites in each image. Scale bars indicate 20 μm.

with *P. inui* because we found no other *Plasmodium* species in any of them.

Conclusions

In experimentally infected humans, patent *P. inui* infections appeared ≥31 days after infectious mosquito bites (*8*). Similarly, both cases we report show a patent infection ≈30 days after alleged exposure. *P. inui* undergoes a 72-hour erythrocytic cycle, causing quartan fever (*8*). Infection by the quartan *P. inui* could be self-limiting in humans because the parasite was not detected in case-patient PMAR0041 ≈8 months after exposure. Indeed, *P. inui* infections in monkeys are usually low-grade and chronic and can be self-limiting (*9*,*10*). In addition, the *P. inui* OS strain parasite count in experimentally infected humans was <2,520/μL blood. Symptoms were mild, and parasitemia could be submicroscopic or undetectable for certain periods. Antimalarial intervention was deemed unnecessary in these experimental infections (*8*).

Natural human *P. inui* infection seems possible, but because of the very low number of parasites and sharp fluctuations between negative and moderate parasitemia by microscopy (*8*,*10*), previous studies that used less sensitive methods, including standard PCR, were not able to detect it (*11*). We show that nPCR on concentrated, high-volume blood was more sensitive at detecting low-grade infection than standard PCR (*12*), which highlights the need for ultrasensitive detection tools.

We found 2 forms of *Plasmodium* 18S rRNA genes: the asexual type, which is expressed during the parasite's asexual life cycle in the vertebrate host; and the sexual type, which is expressed during its sexual life cycle in the mosquito vector. The *Plasmodium*-genus PCR primers we used amplify asexual and sexual 18S rRNA, but the *P. inui*–specific primers only amplify the asexual type, which explains the negative results from the species-specific nPCR despite the positive amplifications in the *Plasmodium*-genus PCR. Nonetheless, successful PCR amplification is compounded by low levels of parasites and the subsequent chance effect that can lead to occasional spurious results, as we experienced.

P. inui sporozoites have been found naturally occurring in *An. cracens* mosquitoes (*2*). Other mosquito species from the Leucosphyrus group can transmit *P. inui* naturally (*2*). In addition, laboratory experiments showed *P. inui* adapted to co-indigenous *Anopheles* mosquito species (*13*).

P. inui has a wide geographic range in Asia, including southern India, Southeast Asia, and Taiwan (*13*). A surveillance study reported that the prevalence of *P. inui* among wild macaques in Pahang was 66.7% (26/39 macaques sampled); 76.9% of these infections were co-infections with other *Plasmodium* species (*14*). Given the high prevalence of *P. inui* among macaques and natural *Anopheles* mosquito vectors (*2*), humans could be exposed to *P. inui* via vectorborne transmission from infected macaques, particularly at a location where humans, macaque hosts, and mosquito vectors co-exist. Furthermore, studies report that *P. inui* often occurs in co-infections with *P. knowlesi* and *P. cynomolgi* in monkeys and mosquitoes (*2*), and that humans frequently can be exposed to a mix of non-human primate malaria sporozoites (*15*). Because human *P. inui* infections can be asymptomatic, *P. inui* could evolve to efficiently infect humans (*2*), especially considering patent human infection can be established by just a few parasites (*8*). Strains from different geographic locations might even exhibit different infection patterns. Investigators should use ultrasensitive methods for epidemiologic and entomological studies of simian malaria transmissions in Malaysia and other countries in malaria elimination efforts.

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References

1. Coatney GR, Collins WE, Warren M, Contacos PG. The primate malarias. Washington DC: National Institutes of Health; 1971.
2. Jeyaprakasam NK, Liew JWK, Low VL, Wan-Sulaiman W-Y, Vythilingam I. *Plasmodium knowlesi* infecting humans in Southeast Asia: what's next? PLoS Negl Trop Dis. 2020;14:e0008900. https://doi.org/10.1371/journal.pntd.0008900
3. Imwong M, Hanchana S, Malleret B, Rénia L, Day NPJ, Dondorp A, et al. High-throughput ultrasensitive molecular techniques for quantifying low-density malaria parasitemias. J Clin Microbiol. 2014;52:3303–9. https://doi.org/10.1128/JCM.01057-14
4. Singh B, Bobogare A, Cox-Singh J, Snounou G, Abdullah MS, Rahman HA. A genus- and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. Am J Trop Med Hyg. 1999;60:687–92. https://doi.org/10.4269/ajtmh.1999.60.687
5. Imwong M, Tanomsing N, Pukrittayakamee S, Day NP, White NJ, Snounou G. Spurious amplification of a *Plasmodium vivax* small-subunit RNA gene by use of primers currently used to detect *P. knowlesi*. J Clin Microbiol. 2009;47:4173–5. https://doi.org/10.1128/JCM.00811-09
6. Lee KS, Divis PCS, Zakaria SK, Matusop A, Julin RA, Conway DJ, et al. *Plasmodium knowlesi*: reservoir hosts and tracking the emergence in humans and macaques. PLoS Pathog. 2011;7:e1002015. https://doi.org/10.1371/journal.ppat.1002015
7. Chua TH, Manin BO, Daim S, Vythilingam I, Drakeley C. Phylogenetic analysis of simian Plasmodium spp. infecting Anopheles balabacensis Baisas in Sabah, Malaysia. PLoS Negl Trop Dis. 2017;11:e0005991. https://doi.org/10.1371/journal.pntd.0005991
8. Coatney GR, Chin W, Contacos PG, King HK. *Plasmodium inui*, a quartan-type malaria parasite of Old World monkeys transmissible to man. J Parasitol. 1966;52:660–3. https://doi.org/10.2307/3276423
9. Garnham PCC. The mosquito transmission of *Plasmodium inui* Halberstaedter and Prowazek, and its pre-erythrocytic development in the liver of the rhesus monkey. Trans R Soc Trop Med Hyg. 1951;45:45–52. https://doi.org/10.1016/S0035-9203(51)90524-X
10. Schmidt LH, Fradkin R, Harrison J, Rossan RN, Squires W. The course of untreated *Plasmodium inui* infections in the rhesus monkey (*Macaca mulatta*). Am J Trop Med Hyg. 1980;29:158–69. https://doi.org/10.4269/ajtmh.1980.29.158
11. Siner A, Liew ST, Kadir KA, Mohamad DSA, Thomas FK, Zulkarnaen M, et al. Absence of *Plasmodium inui* and *Plasmodium cynomolgi*, but detection of *Plasmodium knowlesi* and *Plasmodium vivax* infections in asymptomatic humans in the Betong division of Sarawak, Malaysian Borneo. Malar J. 2017;16:417. https://doi.org/10.1186/s12936-017-2064-9
12. Hofmann NE, Gruenberg M, Nate E, Ura A, Rodriguez-Rodriguez D, Salib M, et al. Assessment of ultra-sensitive malaria diagnosis versus standard molecular diagnostics for malaria elimination: an in-depth molecular community cross-sectional study. Lancet Infect Dis. 2018;18:1108–16. https://doi.org/10.1016/S1473-3099(18)30411-0
13. Collins WE, Sullivan JS, Galland GG, Nace D, Williams A, Williams T, et al. Isolates of *Plasmodium inui* adapted to *Macaca mulatta* monkeys and laboratory-reared anopheline mosquitoes for experimental study. J Parasitol. 2007;93:1061–9. https://doi.org/10.1645/GE-1035R.1
14. Amir A, Shahari S, Liew JWK, de Silva JR, Khan MB, Lai MY, et al. Natural *Plasmodium* infection in wild macaques of three states in peninsular Malaysia. Acta Trop. 2020;211:105596. https://doi.org/10.1016/j.actatropica.2020.105596
15. Maeno Y, Quang NT, Culleton R, Kawai S, Masuda G, Nakazawa S, et al. Humans frequently exposed to a range of non-human primate malaria parasite species through the bites of *Anopheles dirus* mosquitoes in south-central Vietnam. Parasit Vectors. 2015;8:376. https://doi.org/10.1186/s13071-015-0995-y

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Appendix

Materials and Methods

High-volume DNA Purification

We used Gentra Puregene Blood Kit (QIAGEN, https://www.qiagen.com) to perform DNA purification by using the following methods. We collected ≤3 mL of blood from each study participant into EDTA blood tubes. We centrifuged blood at 3,000 rpm for 10 min to separate it into distinct layers of plasma, buffy coat, and packed red blood cells. We noted the volume of the whole blood and packed red blood cells. We discarded the plasma, then thoroughly removed the buffy coat. Then, we dispensed 1.5 mL of red blood cell lysis solution into a 2-mL microcentrifuge tube. We added packed red blood cells per 500 µL of whole blood to the tube and mixed by inverting 10 times. We left the mixture to incubate at room temperature for 1 min, with ≥1 inversion during the incubation period. We centrifuged the mixture for 30 s at 13,000 × g. We discarded the supernatant, leaving ≈10 µL of the residual liquid and the DNA pellet. Then, we vigorously vortexed the tube to resuspend the pellet in the residual liquid, after which we added 500 µL of Cell Lysis Solution (QIAGEN) to the tube, and mixed vigorously for 10 s. Next, we pipetted 167 µL of Protein Precipitation Solution (QIAGEN) into the tube, then mixed vigorously for 20 s at high speed. We centrifuged the mixture for 1 min at 13,000 × g before transferring the supernatant into a 1.5 mL tube containing 500 µL of isopropanol. We gently inverted the mixture 50 times until the DNA was visible as threads or a clump. Then, we centrifuged the mixture for 1 min at 13,000 × g. We discarded the supernatant before adding 500 µL of 70% ethanol to wash the DNA pellet. We centrifuged the tube for 1 min at 13,000 × g then left the DNA pellet to air dry for 5 min. Then, we added 50 µL of DNA Hydration Solution (QIAGEN) to the tube and vortexed for 5 s at medium speed to mix. We incubated the mixture at

65°C for 5 min to dissolve the DNA pellet before incubating at room temperature overnight with gentle shaking by using a rotator. Finally, we lyophilized or concentrated the DNA solution to only 5 µL. Using this protocol, we concentrated DNA from 500 µL of whole blood into 5 µL of solution.

High-Volume Nested PCR to Detect *Plasmodium* 18S rRNA Gene

By using the DNA extracted in the previous step, we performed *Plasmodium* genus-specific PCR using primers rPLU1 and rPLU5 for the first PCR reaction, then primers rPLU3 and rPLU4 for the nested PCR (nPCR) reaction, as previously described (*1*). We used 2 µL of DNA in a 25 µL PCR reaction containing 1 U of GoTaq G2 Flexi DNA polymerase (Promega, https://www.promega.com), 4 mmol $MgCl_2$, 0.2 mmol of each dNTP, and 0.25 µmol of primers. We used the following cycling parameter for the first PCR reaction: initial denaturation at 94°C for 4 min, then 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 8 min. Next, we used 3 µL of the PCR product from the first PCR reaction in the nPCR reaction with the same PCR mixture as mentioned above. We used the following cycling parameter for the second nPCR reaction: initial denaturation at 94°C for 4 min, then 30 cycles of 94°C for 30 s, 58°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. We incorporated negative controls in the assays by using DNA from blood of a healthy donor, purified by using the same protocol, as a template in the PCR reaction. We used no-template controls in all PCR reactions by using ddH_2O in place of DNA templates in the PCR reactions.

We conducted similar species-specific nPCR to detect *P. falciparum*, *P. vivax*, *P. malariae* (*1*), *P. knowlesi* (*2*), *P. ovale* (*3*), and *P. cynomolgi* (*4*), except we used 35 cycles for the nest 2 PCRs specific for *P. inui* (*4*) at an annealing temperature of 55°C. We cloned positive PCR products into pGEM-T vector (Promega) and sequenced positive clones.

DNA Extraction and nPCR to *Plasmodium* Species in *Anopheles* Mosquito

We extracted genomic DNA from the salivary gland or head and thorax of mosquitoes by using the DNeasy tissue kit (QIAGEN) according to the manufacturer's protocol. We performed nPCR assay targeting the *Plasmodium* small subunit ribosomal RNA (18S rRNA) gene to identify human malaria parasites (*P. falciparum*, *P. malariae*, *P. ovale*, and *P. vivax*) and simian malaria parasites (*P. coatneyi*, *P. cynomolgi*, *P. fieldi*, *P. inui* and *P. knowlesi*) by using genus-

specific primers rPLU1 and rPLU5 for the nest 1 amplification (*1*), then species-specific primers in the nest 2 amplification (*1–4*).

We performed the first PCR assay in a final volume of 50 µL containing 5 µL of DNA template, 1× GoTaq Green reaction buffer (Promega), 3 mmol $MgCl_2$ (Promega), 0.2 µmol of dNTP mixture (Promega), 0.25 µmole each of forward (rPLU1) and reverse (rPLU5) primers and 1.25 U of GoTaq G2 Flexi DNA polymerase (Promega). We used the following cycling parameter for the first PCR: initial denaturation at 94°C for 4 min, then 35 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. Then for each 20 µL of nest 2 PCR amplification, we used 3 µL of PCR product from the first PCR reaction as DNA template. We used identical concentrations of reagents in the nest 2 amplifications to those used in the nest 1 reactions, except the final concentration of the GoTaq G2 Flexi DNA polymerase (Promega) was 1 U. We also used identical PCR conditions as in the first PCR amplification, except for the annealing temperatures; *P. knowlesi* and *P. inui* were annealed at 58°C; *P. coatneyi* and *P. cynomolgi* were annealed at 60°C; and *P. fieldi* was annealed at 63°C. We also used 4 µL of PCR product from the first PCR reaction as a DNA template to detect human-specific malaria parasites *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* by using primers and protocols described previously (*1*). We analyzed the amplification products by using 1.5% agarose gel electrophoresis.

18S rRNA Sequencing of *P. inui* from *Anopheles cracens* Mosquito

We subjected the *P. inui*–positive mosquito sample to another nPCR targeting a larger fragment of the 18S rRNA gene. We performed PCR amplification reaction for the first PCR assay in a final volume of 50 µL containing 5 µL of DNA template, 1× GoTaq Long PCR master mix (Promega), and 0.25 µmol each of forward (rPLU1) and reverse (rPLU5) primers. We used the following cycling parameter for the first PCR reaction: initial denaturation at 94°C for 4 min, followed by 40 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1.5 min, and a final extension at 72°C for 10 min. We performed PCR amplification reaction for the nest 2 assay by using a universal forward primer UMSF (*5*) combined with species-specific primer INAR3 (*4*). We performed the nest 2 PCR assay in a final volume of 25 µL containing 3 µL of DNA template from nest 1, 1× GoTaq Green Reaction Buffer (Promega), 2 mmol $MgCl_2$ (Promega), 0.2 µmol of dNTP mixture (Promega), 0.4 µmol each of the forward and reverse primer, and 1.5 U of GoTaq G2 Flexi DNA Polymerase (Promega). We used a similar cycling parameter for nest 2 to

the first PCR reaction, except the duration for the first denaturation was 5 min, then 35 cycles of 1 min each of denaturation, annealing, and extension. We analyzed the amplified products by using 1.5% agarose gel electrophoresis. We observed the correct band in the gel which we excised and sent for sequencing.

References

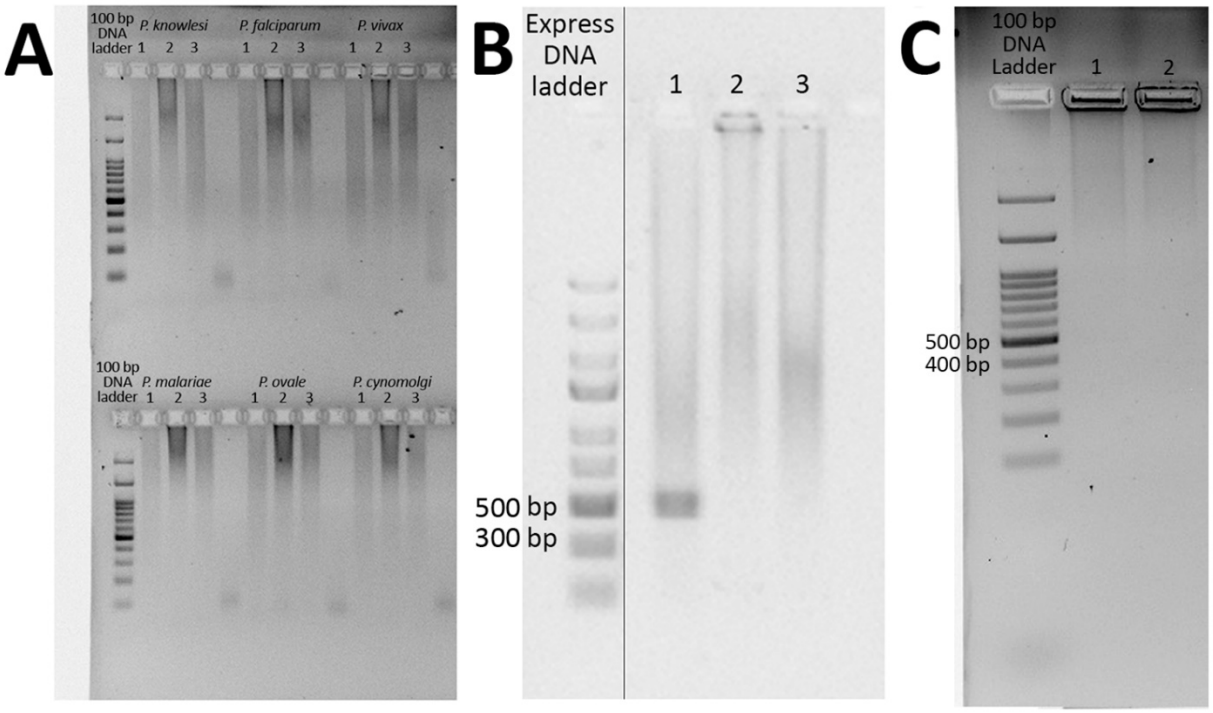
1. Singh B, Bobogare A, Cox-Singh J, Snounou G, Abdullah MS, Rahman HA. A genus- and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. Am J Trop Med Hyg. 1999;60:687–92. PubMed https://doi.org/10.4269/ajtmh.1999.60.687

2. Imwong M, Tanomsing N, Pukrittayakamee S, Day NP, White NJ, Snounou G. Spurious amplification of a *Plasmodium vivax* small-subunit RNA gene by use of primers currently used to detect *P. knowlesi.* J Clin Microbiol. 2009;47:4173–5. PubMed https://doi.org/10.1128/JCM.00811-09

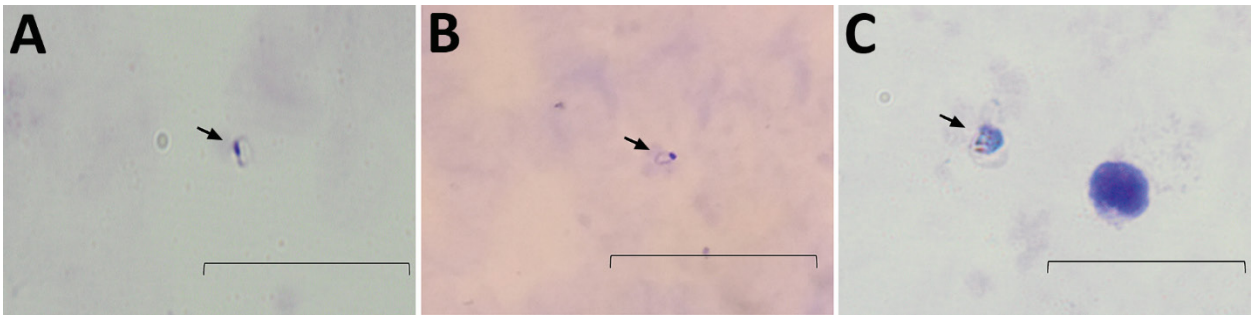
3. Fuehrer HP, Stadler MT, Buczolich K, Bloeschl I, Noedl H. Two techniques for simultaneous identification of *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri* by use of the small-subunit rRNA gene. J Clin Microbiol. 2012;50:4100–2. PubMed https://doi.org/10.1128/JCM.02180-12

4. Lee KS, Divis PC, Zakaria SK, Matusop A, Julin RA, Conway DJ, et al. *Plasmodium knowlesi*: reservoir hosts and tracking the emergence in humans and macaques. PLoS Pathog. 2011;7:e1002015. PubMed https://doi.org/10.1371/journal.ppat.1002015

5. Chua TH, Manin BO, Daim S, Vythilingam I, Drakeley C. Phylogenetic analysis of simian *Plasmodium* spp. infecting *Anopheles balabacensis* Baisas in Sabah, Malaysia. PLoS Negl Trop Dis. 2017;11:e0005991. PubMed https://doi.org/10.1371/journal.pntd.0005991



Appendix Figure 1. *Plasmodium* genus-specific nested PCR results of 2 cases of human *Plasmodium inui* infection, Malaysia. Samples were subjected to electrophoresis on a 1.5% agarose gel. A) Results for case PMAR0041. Lane 1, 100 bp DNA ladder; lane 2, case PMAR0041; lanes 3 and 4, negative control; lane 5, *P. knowlesi*–positive control; lane 6, no-template control. B) Results for case PMAR0052. Lane 1, 100 bp DNA ladder; lane 2, case PMAR0052; lane 3, negative control; lane 4, no-template control. The unlabeled lanes represent *P. inui*-negative samples.



Appendix Figure 2. CLUSTAL OMEGA alignment of *Plasmodium inui* 18S rRNA sequences from human cases PMAR0041 and PMAR0052 and part of the *P. inui* sequence obtained from an *Anopheles cracens* mosquito collected in the same area as the 2 human cases. GenBank accession numbers are in parentheses. The asterisks indicate identical nucleotides in all the 3 sequences at that position.