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Short Report: A Modified Semi-Nested Multiplex Malaria PCR (SnM-PCR) for the Identification of the Five Human *Plasmodium* Species Occurring in Southeast Asia

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Abstract. We have modified an existing semi-nested multiplex polymerase chain reaction (PCR) by adding one *Plasmodium knowlesi*-specific nested PCR, and validated the latter against laboratory and clinical samples. This new method has the advantage of being relatively affordable in low resource settings while identifying the five human *Plasmodium* species with a three-step PCR.

Since the first Malaysian reports and the recognition of *Plasmodium knowlesi* as the fifth human *Plasmodium* species,[1–5] there have been continuous attempts to improve the molecular detection of this *Plasmodium* species of simian origin.[6–11] Initially, a nested 18S small subunit (ssu) ribosomal DNA (rDNA)-based polymerase chain reaction (PCR) identified the first large cohort of naturally acquired *P. knowlesi* infections in humans in Borneo.[1] However, the primers used to detect the *P. knowlesi* DNA (Pmk8-Pmkr9) can cross-react with the *Plasmodium vivax* ribosomal RNA (rRNA) gene, requiring systematic confirmation by either a secondary PCR (1) or sequencing.[6,12] Since then, more specific methods using nested[6] or non-nested PCR,[10,11] loop-mediated isothermal amplification,[8] or real time PCR[7,9] have been developed.

Plasmodium knowlesi infections in humans were reported in Vietnam[12] and this species needs to be accounted for in the newly defined national malaria elimination strategies.[13] Applying the multiplex semi-nested (SnM) PCR published by Rubio and others[14] for the detection of the four previously described human *Plasmodium* species, we recently showed that the malaria parasite reservoir in Central Vietnam was more complex than previously reported by standard microscopy.[15] We therefore added another secondary PCR, specific for *P. knowlesi*, into the existing SnM-PCR to enable the detection of the five *Plasmodium* species within a three-step PCR.

The existing SnM-PCR protocol was adapted from Rubio and others,[14] and consists of a first reaction *Plasmodium* genus specific (Figure 1A) followed by a semi-nested PCR including the *Plasmodium*-specific forward (PLF) primer used in the primary PCR, plus species-specific reverse primers for *Plasmodium falciparum*, *P. vivax, Plasmodium malaria*, and *Plasmodium ovale* (Figure 1B). We hereby describe the newly added semi-nested (Sn-) PCR for the identification of *P. knowlesi* (Figure 1C). The analysis of existing oligonucleotide primers for *P. knowlesi* showed that the reverse complement of the PkF1140 primer designed and validated by Imwong and others[6] was compatible with the PLF primer described by Rubio and others.[14] Therefore, the Sn-PCR of *P. knowlesi* includes the PLF forward primer, and a species-specific reverse primer (PKR4: 3′-GAT TCA TCT ATT AAA AAT TTG CT-5′), the latter being the reverse complement of the PkF1140 primer shortened by two base pairs (bp). The specificity of the PKR4 was subsequently assessed *in silico* by aligning sequences for the 18S ssu rRNA genes of different human and non-human *Plasmodium* species (*P. knowlesi*, *P. coatney*, *P. cynomolgi*, *P. inui*, *P. fragile*, *P. reichenowi*, *P. lophurae*, *P. gallinacae*) as well as 29 *P. knowlesi* sequences available on GenBank (http://www.ncbi.nlm.nih.gov/genbank/index.html).

The Sn-PCR was performed in 25 μL reaction mixture with final concentration for a 1× reaction of 1× Qiagen loading buffer (10× Qiagen buffer, Hilden, Germany), 100 μM of each dNTP (Eurogentec, Seraing, Belgium), 0.5 μM PLF, 0.2 μM PKR4, 1 Unit of Qiagen HotstarTaq plus polymerase, and 2 μL DNA (a 1/500 dilution of the primary PCR product was used as the template). Cycling conditions were as follows: a 5 min denaturation and activation of the Qiagen HotstarTaq plus polymerase step at 94°C, followed by 30 cycles of 20 sec at 51°C for annealing, followed by elongation at 72°C for 1 min, and a 30 sec denaturation at 94°C. The final cycle was followed by an extension time of 10 min at 72°C. The PCR products were detected by 2% agarose gel stained with ethidium bromide, and visualized under UV light.

Reference blood samples from *P. knowlesi*-infected patients (N = 13) were kindly provided by the IMR, Kuala Lumpur. Furthermore, 80 blood samples (filter paper) from malaria-infected patients collected during a previous survey carried out in Vietnam[15] were used to further validate the specificity of the PLF-PKR4 primers. The DNA extraction was done using the QIAamp DNA Micro Kit (Qiagen, Hilden Germany).

Primary and nested PCR products were cloned into plasmid vectors (pCR4 -TOPO vector) and transformed into Mach1 $T1^B$ *Escherichia coli* cells using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Thirty cut out bacterial colonies, 10 from two distinct *P. knowlesi* samples harboring the amplified primary PCR fragment, and 10 harboring the secondary PCR fragment (five colonies for each of the two *P. knowlesi* samples) were purified and sequenced. In addition, DNA extracts from the 13 *P. knowlesi* reference samples were subjected to the new Sn-PCR and the amplified PCR fragments of expected size (actual size amplicon = 498 bp), were of the agarose gel, purified, cloned, and sequenced. The sequences obtained were analyzed with the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and compared with available sequences in GenBank. All sequences obtained

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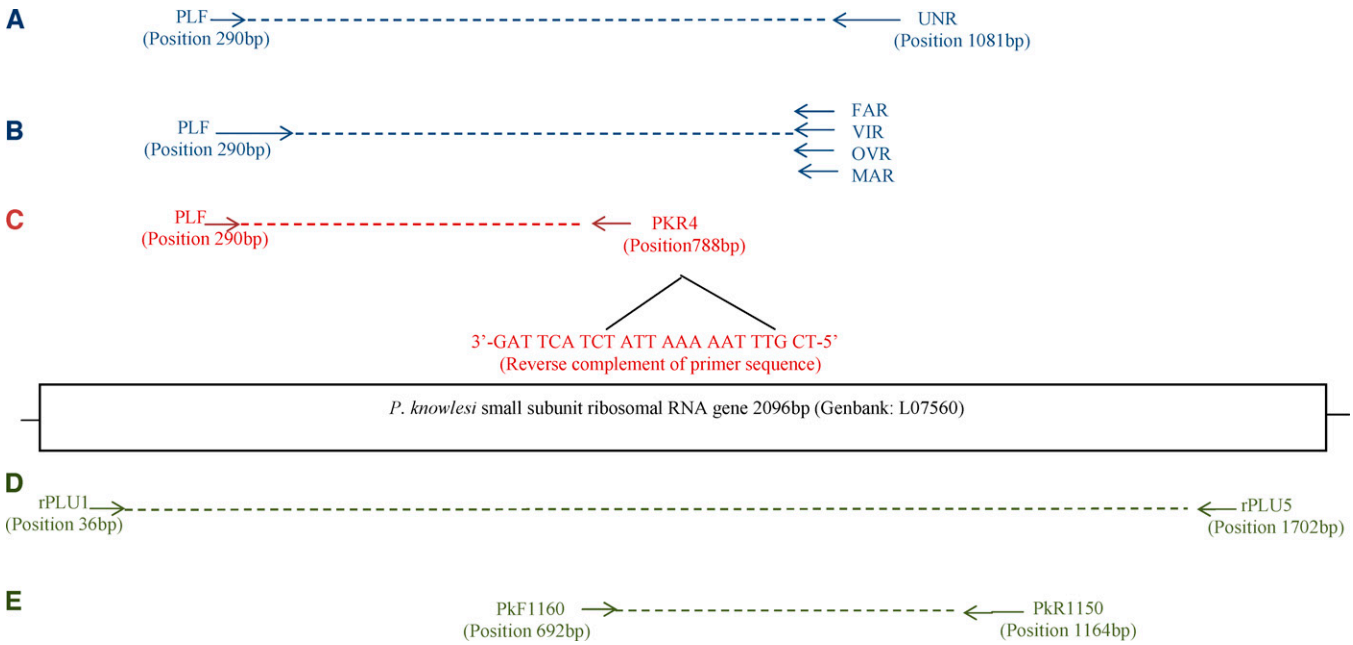

FIGURE 1. Primers position for the modified SnM-PCR amplification are shown for the single primary reaction (**A**), followed by two secondary PCRs: one for the four usual human *Plasmodium* species (**B**), following protocol by Rubio and others,[15] and the new secondary PCR with the PkR4 primer (**C**). The relative positions of the different primers used for the nested PCR amplification to detect *P. knowlesi* by Imwong and others[6] are indicated below the gene: primary PCR (**D**) and nested PCR (**E**).

from plasmids with the primary and nested PCR inserts were confirmed as *P. knowlesi.*

The sensitivity and specificity of our Sn-PCR protocol was assessed using two previously published nested PCR protocols for the detection of *P. knowlesi*: one using Pmk8-Pmkr9 primers[1] (hereafter called PCR1) and the one using the PkF1150-PkR15560 primers[6] (hereafter called PCR2). The relative sensitivity was determined by a 10-fold serial dilution of the *P. knowlesi* H strain (*Plasmodium knowlesi* Genomic DNA-*P. knowlesi* H strain, MRA-456G obtained at the MR4 [http://www.mr4.org/]) using DNA extract from human blood collected from healthy donors. The analytic sensitivity was determined using a 10-fold serial dilution of the Primary PCR product of PCR 1 and 2 (using primers rPLU1 and 5). This PCR product was first cloned, extracted, and sequenced. The extracted plasmid DNA concentration was determined using three measurements of the Nanodrop (Thermo Scientific, Landsmeer, The Netherlands). A 10-fold serial dilution of the plasmid DNA containing the *P. knowlesi* fragment was made to determine the sensitivity of the different PCRs. The PCR1 showed the highest in both experiments with an analytical sensitivity at dilutions as low as 1 fg/µL compared with the 100 fg/µL for PCR2 and our Sn-PCR. In both panels the *P. knowlesi* MR4 H strain as well as the plasmid dilution series, our Sn-PCR and the PCR2 showed the same sensitivity.

The specificity of the three protocols was tested using different genomic DNA controls from *Plasmodium* species originating from monkeys (1 *P. cynomolgy*, 1 *P. simium*, 1 *P. fragile*; reference strains from NIMPE, Hanoi), and humans (4 *P. vivax*, 2 *P. falciparum*, 1 *P. ovale*, and 1 *P. malariae*; reference strains from ITM, Antwerp), 1 *P. knowlesi* (IMR, Kuala Lumpur) as well as genomic DNA (gDNA) of non-*Plasmodium* origin (1 *Leishmania donovani*, 1 *Schistosoma mansoni*, 1 *Trypanosoma cruzi*, 1 HIV provirus, 1 *Mycobacterium tuberculosis*, and 1 *Mycobacterium ulcerans*). Our Sn-PCR and PCR2 showed no cross-reaction, whereas PCR1 showed a false positive *P. knowlesi* with a 153 bp band for one of the four *P. vivax* controls.

The specificity was further tested with the 80 clinical samples, previously analyzed by SnM-PCR for the identification of the four human *Plasmodium* species.[15] Filter paper dried blood spots were then tested for the presence of *P. knowlesi* using the three nested PCR protocols (Table 1). Results showed that PCR1 identified six *P. knowlesi* infections, whereas our Sn-PCR and PCR 2 did not detect any. Sequencing confirmed the absence of *P. knowlesi* in all six samples, and the cross-reactivity of PCR1 primers with human DNA (97% homology to region 6853624 bp to 6853772 bp on the Human

TABLE 1
Specificity of the three nested protocols using 80 clinical samples from Vietnam

Mono- and mixed malaria infections*	Total tested n	Positive result for *P. knowlesi* by protocol: Nested PCR1† (Pmk8-Pmkr9)	Nested PCR2‡ (PkF1160-PkR1150)	Sn-PCR (PLF-PKR4)
P. falciparum (*P.f.*)	38	–	–	–
P. vivax (*P.v.*)	14	–	–	–
P. malariae (*P.m.*)	5	–	–	–
P. ovale (*P.o.*)	1	–	–	–
P.f. +*P.v.*	7	2	–	–
P.f. +*P.m.*	3	–	–	–
P.m. +*P.o.*	1	–	–	–
P.f. + *P.v.* + *P.m.*	5	2	–	–
P.f. + *P.m.* + *P.o.*	2	1	–	–
P.v. + *P.m.* +*P.o.*	3	1	–	–
P.f. + *P.v.* +*P.m.* + *P.o.*	1	–	–	–
Total (*N*)	**80**	**6**	**0**	**0**

*Previously confirmed by SnM-PCR.[15]
†Following protocol by Singh and others.[1]
‡Following protocol by Imwong and others.[6]

chromosome 16 genomic contig GenBank accession number: NW 001838290.1). No evidence of contamination of the PCR products was found.

Further simplification of the protocol into a five-species SnM-PCR should be feasible as preliminary data have shown that the *P. knowlesi*-specific primer was compatible *in silico* with the other four human *Plasmodium*-specific primers. This requires additional optimization efforts, however if successful, will further enhance the value of the current protocol.

Our modified SnM-PCR, offers the advantage of being feasible and affordable for poor income settings where real time PCR is not available yet, while reliably detecting the five human *Plasmodium* species within a three-step assay.

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REFERENCES

1. Singh B, Kim Sung L, Matusop A, Radhakrishnan A, Shamsul SS, Cox-Singh J, Thomas A, Conway DJ, 2004. A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. *Lancet 363:* 1017–1024.
2. Cox-Singh J, Singh B, 2008. *Knowlesi* malaria: newly emergent and of public health importance? *Trends Parasitol 24:* 406–410.
3. Cox-Singh J, Davis TM, Lee KS, Shamsul SS, Matusop A, Ratnam S, Rahman HA, Conway DJ, Singh B, 2008. *Plasmodium knowlesi* malaria in humans is widely distributed and potentially life threatening. *Clin Infect Dis 46:* 165–171.
4. Vythilingam I, Noorazian YM, Huat TC, Jiram AI, Yusri YM, Azahari AH, Norparina I, Noorrain A, Lokmanhakim S, 2008. *Plasmodium knowlesi* in humans, macaques and mosquitoes in peninsular Malaysia. *Parasites & Vectors 1:* 26.
5. White NJ, 2008. *Plasmodium knowlesi*: the fifth human malaria parasite. *Clin Infect Dis 46:* 172–173.
6. Imwong M, Tanomsing N, Pukarittayakemee S, Day NP, White NJ, Snounou G, 2009. Spurious amplification of *P. vivax* small subunit RNA gene using the primers currently used to detect *P. knowlesi*. *J Clin Microbiol 47:* 4173–4175.
7. Babady NE, Sloan LM, Rosenblatt JE, Pritt BS, 2009. Detection of *Plasmodium knowlesi* by real-time polymerase chain reaction. *Am J Trop Med Hyg 81:* 516–518.
8. Iseki H, Kawai S, Takahashi N, Hirai M, Tanabe K, Yokoyama N, Igarashi I, 2010. Evaluation of a loop-mediated isothermal amplification (LAMP) method as a diagnostic tool of zoonotic simian malaria parasite *Plasmodium knowlesi* infection. *J Clin Microbiol 48:* 2509–2514.
9. Divis PCS, Shokoples SE, Singh B, Yanow SK, 2010. A TaqMan real-time PCR assay for the detection and quantitation of *Plasmodium knowlesi*. *Malar J 9:* 344.
10. Lucchi NW, Poorak M, Oberstaller J, DeBarry J, Srinivasamoorthy G, Goldman I, Xayavong M, da Silva AJ, Peterson DS, Barnwell JW, Kissinger J, Udhayakumar V, 2012. A new single-step PCR assay for the detection of the zoonotic malaria parasite *Plasmodium knowlesi*. *PLoS ONE 7:* e31848.
11. Chew CH, Lim YAL, Lee PC, Mahmud R, Chua KH, 2012. A hexaplex PCR detection system for the identification of five human *Plasmodium* species with internal control. *J Clin Microbiol 50:* 4012–4019.
12. Van den Eede P, Nguyen Van H, Van Overmeir C, Vythilingam I, Ngo Duc T, Xuan Hung L, Nguyen Manh H, Anne J, D'Alessandro U, Erhart A, 2009. Human *Plasmodium knowlesi* infections in southern Vietnam. *Malar J 8:* 249.
13. Ministry of Health, 2012. National malaria control and elimination programme. National Institute of Malariology, Parasitology, and Entomology. National strategy for malaria control and elimination for the period of 2012–2015. Hanoi, December 2011. Internal Report.
14. Rubio JM, Post RJ, van Leeuwen WM, Henry MC, Lindergard G, Hommel M, 2002. Alternative polymerase chain reaction method to identify *Plasmodium* species in human blood samples: the semi-nested multiplex malaria PCR (SnM-PCR). *Trans R Soc Trop Med Hyg 96:* S199–S204.
15. Hong Nguyen V, Van den Eede P, Van Overmeir C, Thang Ngo D, Hung Le X, D'Alessandro U, Erhart A, 2012. The distribution of human *Plasmodium* species in central Vietnam is complex with marked age-dependent prevalence of symptomatic and patent infections. *Am J Trop Med Hyg 87:* 989–995.