



## Rapid Communication

*Anopheles dirus* co-infection with human and monkey malaria parasites in VietnamShusuke Nakazawa<sup>a,\*</sup>, Ron P. Marchand<sup>b</sup>, Nguyen Tuyen Quang<sup>b</sup>, Richard Culleton<sup>a</sup>, Nguyen Duc Manh<sup>c</sup>, Yoshimasa Maeno<sup>d</sup><sup>a</sup> Department of Protozoology, Institute of Tropical Medicine and the Global Center of Excellence Program, Nagasaki University, Japan<sup>b</sup> Khanh Phu Malaria Research Center, Medical Committee Netherlands-Viet Nam, Viet Nam<sup>c</sup> Molecular Biology Department, National Institute of Malariology, Parasitology and Entomology, Viet Nam<sup>d</sup> Department of Virology and Parasitology, Fujita Health University School of Medicine, Japan

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## ABSTRACT

The feasibility of identifying parasite DNA and specific mRNAs from wild-caught *Anopheles dirus* mosquitoes was assessed using dried mosquito salivary glands preserved on filter paper. We were able to detect *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium knowlesi* DNA by conventional PCR and, furthermore, detected *P. falciparum* gametocyte-specific genes, *pfg377* and *pfs16* mRNA, *P. knowlesi* circumsporozoite protein (CSP) and sporozoite surface protein 2 (SSP2) mRNA by reverse transcription-PCR. Using this technique, we were able to confirm the presence of *P. vivax*, *P. falciparum* and *P. knowlesi* in one particular wild-caught mosquito. These results indicate that *P. knowlesi* may be transmitted by the primary human malaria vector in forested areas in Vietnam. This study also shows that the preservation of mosquito salivary glands on filter paper, and the down-stream extraction of parasite DNA and RNA from those, offers a powerful resource for molecular epidemiological studies on malaria.

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Malaria transmission occurs in the forested areas in the southern and central provinces of Vietnam (Erhart et al., 2004) and constitutes a considerable public health problem in these areas. In order to fully understand the complexities of malaria transmission, it is important to identify and characterize malaria parasites in mosquito vectors. Evidence for the generation of genetic diversity during sexual proliferation in mosquito vectors has previously been accumulated using laboratory isolates of genetically diverse parasites, as well as with samples from field settings (Babiker et al., 1994; Menegon et al., 2000). However, there have been relatively few reports focusing on the molecular characterization of parasites isolated from vectors in a natural setting. This is mainly due to the laborious nature of work involving the collection of large numbers of infected mosquitoes, and the difficulties involved in isolating parasite genetic material from those. Here, we report the results of attempts to isolate parasite DNA and mRNA from stocked dried mosquito salivary glands collected in Vietnam and the down-stream analysis of this material to identify parasite species, stage and genotype. Recently, the monkey malaria parasite *Plasmodium knowlesi* has been reported infecting humans in south-east Asian countries, Malaysia in particular. As Vietnam is known to harbor both the natural hosts and vectors of *P. knowlesi*

(Cox-Singh and Singh 2008), we included an assay for the detection of this parasite as well as the human malaria parasites *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale* in our attempts to isolate parasite DNA and mRNA from mosquito salivary glands.

Mosquitoes were collected by outdoor human baited landing catches in the forest and forest fringe area around Khan Phu commune, Khanh Vinh district, Khanh Hoa province, Vietnam, and brought to the field station for mosquito species identification and parasite rate determination. Female anopheline mosquitoes were dissected and salivary glands and midguts examined by microscopy for the presence of sporozoites and oocysts. Sporozoite-infected glands were applied to filter paper and dried in an ambient atmosphere, before storage in closed vials at 4–6 °C. Previously, in 2007 and 2008, 4,609 human-biting *Anopheles dirus* were caught and dissected in the Khanh Vinh district (average human-biting density 3.75 per person per night) of which 77 were positive for sporozoites (1.67%). Midguts were examined for oocysts in 2,791 of these and 28 were positive (1.00%). Fourteen sporozoite samples were analyzed at the Biomedical Primate Research Center in the Netherlands in 2007. The presence of *P. falciparum* and *P. vivax* was confirmed, but *P. knowlesi* was not detected in these samples. Of a total of 22 sporozoite-infected glands found in 2008, eight were preserved (as described above) on separate filter papers. In the present study, we analyzed three (randomly selected) of these dried filter samples (numbered 28–30) which were collected between April and August 2008. All three samples

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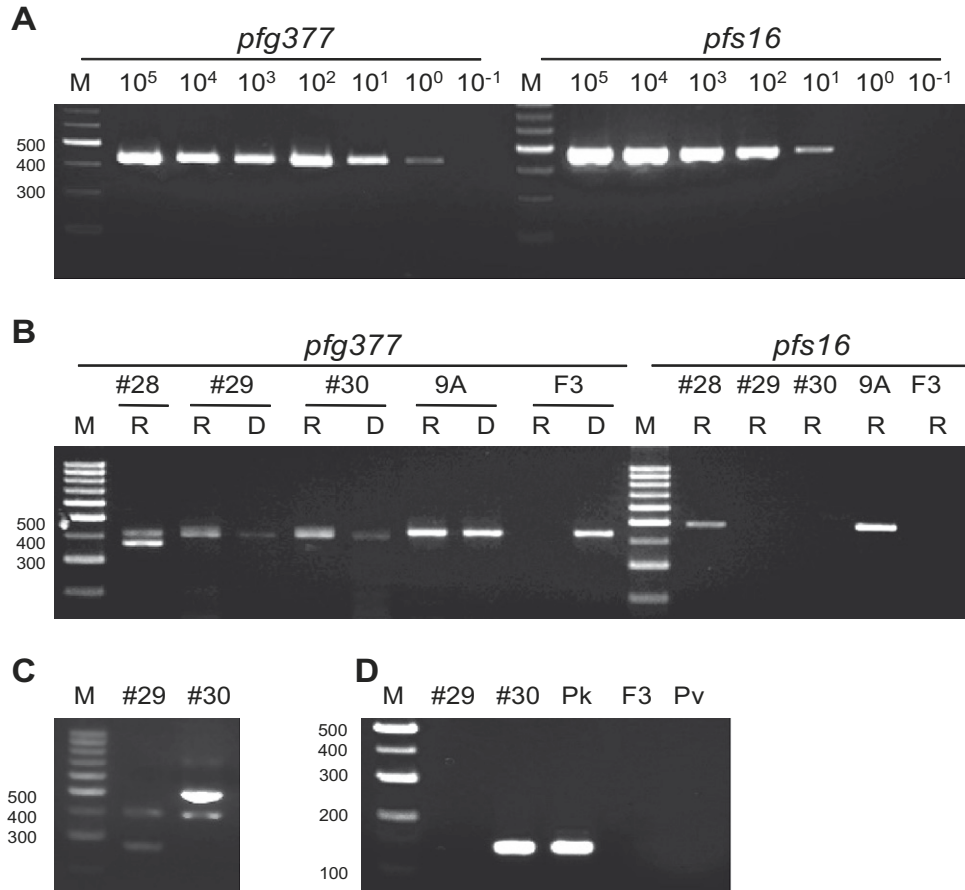
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were oocyst negative by microscopic examination. The remaining five filter paper preserved samples have not yet been analysed. Twenty-three sporozoite-negative salivary glands collected in May, 2009 were used as negative controls. Two of the mosquitoes analysed here were partially blood fed (Nos. 28 and 30), and one was fully engorged (No. 29). The sporozoite-negative mosquitoes varied from non-engorged to fully engorged. In order to assay the sensitivity of the parasite DNA and mRNA extraction process and down-stream molecular analyses, *P. falciparum* clone 3D7-9A was used as a gametocyte-producing positive control and clone FCR3-F3 was used as a negative control (Maeno et al., 2008) for PCR and reverse transcription (RT)-PCR experiments. Positive control DNA for *P. vivax* and *P. malariae* was obtained from Giemsa stained blood films. *Plasmodium knowlesi* H stain (ATCC No. 30158) donated by Dr. Kawai, Dokkyo University, Japan, was used as a positive control. *Plasmodium knowlesi*-infected blood was taken at a parasitemia of 28.3% from a 4 year-old male *Macaca fuscata* inoculated with the frozen isolate of *P. knowlesi*, treated with 0.15% saponin after removing white blood cells, washed with PBS and frozen. Twenty-five microliter aliquots of parasitized culture blood, 3D7-9A, FCR3-F3 and the frozen isolate of *P. knowlesi*, were spotted onto chromatography-grade filter papers (ET31CHR; Whatman, Maidstone, UK). Each blood-spotted filter paper was immediately air-dried and stored in a sealed plastic bag at room temperature until RT-PCR or PCR analysis was performed. To examine the detection limit for mosquito salivary gland-extracted parasite mRNA, salivary glands from laboratory reared *Anopheles stephensi* were prepared in the same way as were the wild-caught *A. dirus*. Salivary glands were dissected from laboratory colony reared *A. stephensi* more than 1 week after blood feeding on either mouse or human blood and dried on E31CHR filter paper at room temperature. Gametocyte suspensions were obtained from cultured 3D7-9A by pyrimethamine treatment at  $10^{-6}$  M from day 7 after thawing until harvest on day 12. Ten times serially diluted cultured gametocyte suspension was added to the spot on the filter paper where the salivary glands were attached. Extraction of RNA and reverse transcription was carried out as previously described (Maeno et al., 2003). Briefly, dried spotted filter paper was cut into small pieces and total RNA and genomic DNA (gDNA) was extracted with ISO-GEN (Nippon gene, Tokyo, Japan) according to the manufacturer's instructions. The extracted total RNA was transcribed to synthesize cDNA which was subsequently subjected to PCR using specific oligonucleotide primers. For PCR analysis of *P. falciparum* gametocyte-specific protein genes, *pfg377*, *pfs16* and RT-PCR analysis of *pfg377*, *pfs16*, circumsporozoite protein (CSP) and sporozoite surface protein 2 (SSP2) mRNA, reaction mixtures (25  $\mu$ l) comprised 1  $\mu$ l of cDNA or gDNA as a template, 0.5 mM each primer, 200 mM dNTP, 0.625 units of Blend Taq DNA polymerase (Toyobo, Osaka, Japan), and  $1 \times$  PCR buffer (containing 2 mM  $MgCl_2$ ). Target cDNA or gDNA was amplified using a PCR protocol consisting of a denaturation step (94 °C, 2 min) followed by 35 amplification cycles (94 °C, 1 min; 55 °C, 30 s; and 72 °C, 1 min), and a final extension step (72 °C, 5 min). Detection and identification of ssrRNA-DNA of human *Plasmodium* spp. in mosquitoes were carried out as previously described by a semi-nested multiplex PCR (Lardeux et al., 2008). *Plasmodium knowlesi* ssrRNA-DNA in mosquitoes was separately detected by two methods (Singh et al., 2004; Putaporntip et al., 2009). Detection of the CSP gene of *P. knowlesi* was carried out as previously described (Vythilingam et al., 2008). Primer sequences for region 3 of *pfg377* mRNA and gDNA (Menegon et al., 2000), *pfs16* mRNA (Niederwieser et al., 2000), ssrRNA-DNA of human *Plasmodium* spp. (Lardeux et al., 2008), and ssrRNA-DNA of *P. knowlesi* (Singh et al., 2004) and the CSP gene of *P. knowlesi* (Vythilingam et al., 2008) were as previously described. Alternative primer sequences for the ssrRNA-DNA of *P. knowlesi* were obtained from Putaporntip et al. (2009). Primer se-

quences for *P. knowlesi* CSP and SSP2 mRNA were 5' GCG TGT TAC ATC AGT TGG AAC AA 3', 5' CGG CCA AGA GAA TGA AGT TCT T3'; 5'ACA CCA GTA TGT CGT CTG CTC TAT CA3' and 5'GCT GGT TAA TGC CTT GCT CAA TTC3', respectively. These were designed based on GenBank sequences M19749 and XM\_002259951 in *P. knowlesi* strain H SSP2 (PKH\_121770) mRNA (Pain et al., 2008). Human G3PDH primers were obtained from Toyobo (Osaka, Japan). The GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT, USA) was used for all PCRs. PCR products were separated by electrophoresis on 1.5% agarose gels and stained with ethidium bromide. Detection and size analysis of PCR products was confirmed with Lane & Spot Analyzer software (Atto, Tokyo, Japan). Specific PCR products of mRNA and gDNA for *pfg377* and gDNA for the CSP gene were purified using the Wizard SV Gel and PCR Clean-up System (Promega, Tokyo, Japan) according to the manufacturer's instructions (260/280, 1.8), and were then sequenced at a concentration of 50 ng/ $\mu$ l with a DYEnamic ET Terminator Cycle Sequencing Premix Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The reaction products for sequencing were separated with an ABI Prism 310 Genetic Analyzer (Perkin Elmer) and the resulting nucleotide sequences were compiled using Vector NTI Advance (Invitrogen, Carlsbad, CA, USA).

The detection limit of parasite mRNA by RT-PCR was one gametocyte per salivary gland for *pfg377* mRNA and 10 gametocytes per gland for *pfs16* mRNA (Fig. 1A). We extracted both parasite gDNA and mRNA from the salivary glands of wild-caught mosquitoes. Region 3 of *pfg377* mRNA was detected in all of the three dried salivary gland samples and both the mRNA and gDNA of *pfg377* were detected in two samples (Fig. 1B). The PCR products showed the same molecular size as the RT-PCR products by electrophoresis. No PCR product was observed by RT-PCR for *pfg377* mRNA and *pfs16* mRNA using RNase-treated RNA as a template, while DNase treatment of the RNA preparation did not abolish the positive RT-PCR amplification (data not shown). Similarly, no PCR product was observed using DNase-treated gDNA as a template. These results indicate that the RT-PCR product was not derived from gDNA contaminants. The types of *pfg377* allele detected in the three samples were A and C according to our previously reported categorization (Maeno et al., 2008) and were confirmed by sequencing of the *pfg377* PCR products (Table 1). We then carried out conventional PCR for further malaria parasite species typing of *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. ssrRNA-DNA of *P. falciparum* and *P. malariae* was detected in sample No. 29 (Fig. 1C). ssrRNA-DNA of *P. falciparum*, *P. vivax* and *P. knowlesi* were detected from sample No. 30 (Fig. 1C and D). The detection of *P. knowlesi* ssrRNA-DNA in sample No. 30 was confirmed by PCR with alternative primers targeting different DNA sequences of the ssrRNA-DNA. Twenty-three sporozoite-negative samples were negative for both ssrRNA-DNA and CSP DNA of *P. knowlesi* (Table 1). The presence of *P. knowlesi* parasites in sample No.30 was confirmed by PCR for the CSP gene and by sequencing of the specific PCR product. However, the molecular size of the PCR product was not identical to that of the *P. knowlesi* positive control (Fig. 2A). The CSP gene DNA sequence showed a homology of more than 97.9% with the reported sequences of the *P. knowlesi* CSP gene (Fig. 2B). To confirm that *P. knowlesi* produced sporozoites in *A. dirus*, CSP and SSP2 mRNA were detected by RT-PCR. Both of these mRNAs were not detected in the *P. falciparum*, *P. vivax* or *P. knowlesi* gDNA controls but were detected in sample No. 30 (Fig. 2C). To exclude the possibility that the mosquito salivary glands were contaminated with human blood containing parasites, the presence of human G3PDH mRNA was examined and was positive only in sample No. 29.

We were able to successfully extract malaria parasite RNA and DNA from mosquito salivary glands dried on filter paper prepared from laboratory mosquitoes (*A. stephensi*) and wild-caught mos-



**Fig. 1.** Detection of malaria parasite-specific DNA and mRNA from dried mosquito salivary glands. (A) Reverse transcription (RT)-PCR products for *Plasmodium falciparum* gametocyte-specific genes, *pfg377* and *pfs16* mRNA in dried salivary glands of uninfected mosquitoes with serial dilution of cultured gametocytes added. M, 100 bp DNA ladder marker;  $10^{-1}$ ,  $10^0$ ,  $10^1$ ,  $10^2$ ,  $10^3$ , number of gametocytes per gland. (B) RT-PCR and PCR products for *pfg377* and RT-PCR products for *pfs16* mRNA in dried sporozoite-positive salivary glands and cultured parasites. #28–30, salivary gland samples; 9A and F3, 3D7-9A clone and FCR3-F3 clone; M, 100 bp DNA ladder marker; R, mRNA; D, genomic DNA. (C) Conventional PCR products for *ssrRNA*-DNA of *P. falciparum*, *Plasmodium vivax*, *Plasmodium malariae*. M, 100 bp ladder marker; #29 and #30, salivary gland samples; Product size: 499 bp, *P. vivax*; 395 bp, *P. falciparum*; 269 bp, *P. malariae*. (D) PCR products for *ssrRNA*-DNA of *Plasmodium knowlesi*. #29–30, salivary gland samples; P.k, *P. knowlesi* positive control; F3, FCR3-F3 clone; Pv, *P. vivax* positive control.

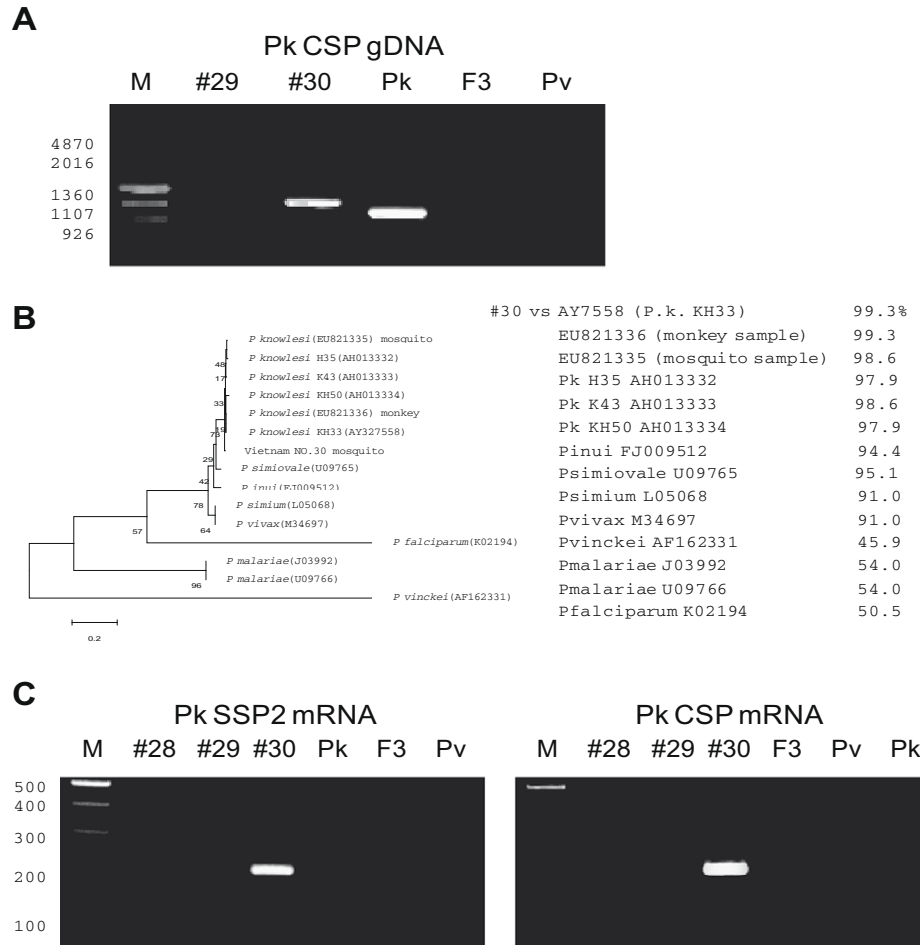
**Table 1**  
Detection of transcripts of *Plasmodium falciparum* gametocyte-specific genes, *pfg377* and *pfs16* and *Plasmodium* spp. in mosquito salivary glands.

Sample	RT-PCR		PCR						
	<i>pfg377</i>	<i>pfs16</i>	<i>pfg377</i>	<i>ssrRNA</i>					CSP
				Pf	Pv	Pm	Po	Pk	Pk
No. 28	A & C	(+)	nd	nd	nd	nd	nd	nd	nd
No. 29	A	(-)	A	(+)	(-)	(+)	(-)	(-)	(-)
No. 30	A	(-)	A	(+)	(+)	(-)	(-)	(+)	(+)
23 spz (-) samples	nd	nd	nd	nd	nd	nd	nd	(-)	(-)

RT-PCR, reverse transcription-PCR; CSP, circum sporozoite protein; Pf, *Plasmodium falciparum*; Pv, *Plasmodium vivax*; Pm, *Plasmodium malariae*; Po, *Plasmodium ovale*; Pk, *Plasmodium knowlesi*; spz (-), sporozoite-negative. A, C, allele types of region three variable region; A, (D-Q/H-Q/H-D/N-H-H-I)<sub>11</sub>, C, (D-Q/H-Q/H-D/N-H-H-I)<sub>8</sub>; nd, not done.

quitoes (*A. dirus*), to experimentally detect as few as one parasite in a salivary gland using *pfg377* mRNA, and determine parasite species in the salivary glands of three wild-caught mosquitoes. It is possible that the positive results we achieved for the amplification of parasite RNA and DNA from mosquito salivary glands may have been due to contamination of the salivary glands by infected human blood (from the mosquito's most recent blood meal), and not due to the presence of sporozoites in the glands. However,

we consider this unlikely, as in samples Nos. 28 and 30, sporozoites were identified in salivary glands by microscopic observation and human G3PDH mRNA was not detected. We have shown, therefore, that filter paper stored dried salivary glands of mosquitoes can be used in molecular epidemiological studies of malaria transmission. In this study we found that one mosquito carried *P. falciparum*, *P. vivax* and *P. knowlesi* parasites. We initially identified the presence of *P. knowlesi* in this sample by PCR for *ssrRNA*-DNA and by PCR and sequencing of the CSP gene. Furthermore, we identified the presence of sporozoite stage-specific mRNA in this sample by RT-PCR of CSP and SSP2, and excluded the possibility of infected human blood contamination of the glands by the failure to detect human G3PDH mRNA. *Plasmodium knowlesi*, the non-human primate malaria parasite (Coatney et al., 1971), is currently in the process of being accepted as the fifth human malaria parasite (Nishimoto et al., 2008). Based on a study by Chin et al. (1968), we can offer at least two scenarios for the presence of both *P. knowlesi* and the human malaria parasites in this mosquito; firstly, the mosquito was infected with all three species either at once or sequentially from humans only; second, the mosquito was infected with *P. falciparum* and *P. vivax* from feeding on a human, and independently acquired *P. knowlesi* from feeding on a monkey. This mosquito was caught in the forest fringe zone, about 800 m from Nga Hai village, which is situated in a clearing surrounded by forest and a banana plantation. Local people report regular sightings of monkeys in this



**Fig. 2.** Confirmation of sporozoite production by *Plasmodium knowlesi* in *Anopheles dirus* by PCR (A), sequencing (B) and RT-PCR (C). (A) PCR products for *P. knowlesi* circumsporozoite protein (CSP) gene. M, 100 bp ladder marker; #29 and #30, salivary gland samples; Pk, *P. knowlesi* positive control; F3, FCR3-F3 clone; Pv, *Plasmodium vivax* positive control genomic DNA (gDNA). (B) Phylogenetic tree based on CSP gene nucleotide sequences of *Plasmodium* spp. and nucleotide sequences. Percentage of homology between #30 and malaria parasites. *Plasmodium knowlesi* CSP gene: Ay327558, EU8231336-1, EU821335-1, Vietnam #30 Mos, Pk H35 AH013332, Pk K43@AH013333, Pk KH50 AH013334; other primate malaria parasite CSP gene: Pini FJ009512, Psiovale U09765, Psimum L05068; human malaria parasite CSP gene: Pvivax M34697, Pmalariae J03992, Pmalariae U09766, Pfallciparum K02194; rodent malaria parasite CSP gene: Pvincke AF162331; nucleotide sequences of parasite CSP genes were obtained from GenBank. Phylogenetic tree was produced by Neighbour Joining method using MEGA software version 4. Scale bar indicates the number of base substitutions per site (0.2). (C) Reverse transcription-PCR (RT-PCR) products for CSP mRNA and sporozoite surface protein 2 (SSP2) mRNA in the mosquito and parasite samples. M, 100 bp ladder marker; #28–30, salivary gland samples; Pk, *P. knowlesi* positive control; F3, FCR3-F3 clone; Pv, *P. vivax* positive control.

area, which are presumably attracted by the banana plantation. One mosquito was infected with *P. knowlesi* among 17 sporozoite-positive mosquitoes from this area on which malaria parasite species typing was performed. Many more mosquito samples will require analysis in order to assess the prevalence of *P. knowlesi* in this mosquito population. To our knowledge this is the first report of the detection of *P. knowlesi* together with *P. falciparum* and *P. vivax* in an individual mosquito from a population that acts as the principal human malaria vector in a given area. Studies on *P. knowlesi* transmission in Malaysia (Vythilingam et al., 2006, 2008) identified a vector species that, although belonging to the *Anopheles leucosphyrus* group, is at present not implicated in the transmission of human malaria parasites. Our report shows that *P. knowlesi* transmission occurs in the Khanh Phu area of Vietnam, and has the very real potential to infect the human population in this region.

Our mosquito sample preservation and parasite DNA and RNA extraction processes were performed without the harsh conditions associated with methods such as Chelex 100 extraction. Furthermore, salivary glands were considered to be free of the PCR inhibitors associated with other mosquito tissues (Schriefer et al., 1991; Snounou et al., 1993; Arez et al., 2000). We consider that these fac-

tors contributed to our success in identifying mixed species infections in wild-caught mosquitoes.

The analysis of malaria parasites from dried mosquito salivary glands provides a powerful molecular epidemiological technique which will be of great benefit in elucidating the relationships between human and monkey malaria parasites in regions where the transmission of both types of parasite occurs.

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