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Current status of *Plasmodium knowlesi* vectors: a public health concern?

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SUMMARY

Plasmodium knowlesi a simian malaria parasite is currently affecting humans in Southeast Asia. Malaysia has reported the most number of cases and *P. knowlesi* is the predominant species occurring in humans. The vectors of *P. knowlesi* belong to the Leucosphyrus group of *Anopheles* mosquitoes. These are generally described as forest-dwelling mosquitoes. With deforestation and changes in land-use, some species have become predominant in farms and villages. However, knowledge on the distribution of these vectors in the country is sparse. From a public health point of view it is important to know the vectors, so that risk factors towards knowlesi malaria can be identified and control measures instituted where possible. Here, we review what is known about the knowlesi malaria vectors and ascertain the gaps in knowledge, so that future studies could concentrate on this paucity of data in-order to address this zoonotic problem.

Key words: malaria, vectors, forest, zoonosis, *Anopheles*, *Plasmodium knowlesi*.

INTRODUCTION

On a global scale there has been substantial progress in reducing malaria morbidity and mortality with both decreasing by 37 and 48%, respectively in the past decade (WHO, 2015). Overall countries in the Asia-Pacific region have reduced their malaria cases by 76% (Cotter *et al*. 2013). Thus, most countries in the Asia-Pacific region are now working towards elimination of malaria (Cotter *et al*. 2013). However, *Plasmodium knowlesi*, a simian malaria parasite has been found infecting humans in all countries in Southeast Asia with the exception of Lao PDR and Timor Leste (Jongwutiwes *et al*. 2004; Singh *et al*. 2004; Cox Singh *et al*. 2008; Luchavez *et al*. 2008; Ng *et al*. 2008; Vythilingam *et al*. 2008; Eede *et al*. 2009; Figtree *et al*. 2010; Jiang *et al*. 2010; Khim *et al*. 2011; William *et al*. 2013; Yusof *et al*. 2014). In Malaysia, *P. knowlesi* forms 38% of the reported malaria cases (MOH, 2012).

The success of the malaria control programme has been due to active case detection, better diagnosis and treatment coupled with vector control measures such as indoor residual spraying and insecticide-treated bed nets (Yukich *et al*. 2008). *Anopheles* mosquitoes are responsible for the spread of malaria and the species responsible have been well documented (Sinka *et al*. 2011). Knowlesi malaria, which is a zoonosis, is currently reported from countries where Leucosphyrus group of *Anopheles* mosquitoes are present along with the simian hosts the long-tailed and the pig-tailed macaques (Moyes *et al*. 2014).

Incrimination of the vectors of simian malaria is the first step in understanding their role and subsequently choosing appropriate control measures required to target their specific behaviour. However, although cases of knowlesi malaria have been reported throughout Malaysia and Southeast Asia, studies on the vectors of *P. knowlesi* are limited (Vythilingam, 2012; Vythilingam and Hii, 2013). Due to this paucity of data risk factors associated with *P. knowlesi* remain unknown.

VECTORIAL STATUS AND GEOGRAPHIC DISTRIBUTION OF LEUCOSPHYRUS GROUP OF *ANOPHELES* MOSQUITOES

Only *Anopheles* mosquitoes belonging to the Leucosphyrus group have been reported as vectors of simian malaria (Vythilingam and Hii, 2013). The Leucosphyrus group consists of three subgroups: Leucosphyrus, Hackeri and Riparis. According to (Sallum *et al*. 2005; Manguin *et al*. 2008), the Leucosphyrus subgroup consists of the Dirus and Leucosphyrus complexes, which includes eight and five sibling species, respectively. Species belonging to the Leucosphyrus complex are also important vectors of human malaria and lymphatic filariasis (Manguin *et al*. 2010) and are distributed in the South and Southeast Asia regions. The current vectorial status and geographical distribution of the Leucosphyrus group are listed in Table 1 which has been modified and updated from (Sallum *et al*. 2005; Vythilingam and Hii, 2013).

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Table 1. Simian malaria parasites of Southeast Asia: their natural vectors of the Leucosphyrus group, hosts and geographical distribution (modified from Sallum *et al*. 2005; Vythilingam and Hii, 2013)

Subgroup	Complex	Vector species in nature	Species of *Plasmodium*	Vertebrate hosts in nature	Distribution
Leucosphyrus	Leucosphyrus	*An. leucosphyrus* Donitz (hv)	*Pf, Pv, Pm*	Human	Indonesia, Sumatra
		An. latens Sallum and Peyton (hv, sv, fv)	*Pf, Pv, Pm, Pk P. knowlesi, P. inui, P. coatneyi, P. fieldi, P. cynomolgi*	Human *M. fascicularis, M. nemestrina P. melalophos*	Indonesia, East and West Malaysia, Thailand
		An. introlatus Colless (sv)	*P. knowlesi, P. cynomolgi P. fieldi*	*M. fascicularis, M. nemestrina P. melalophos*	Indonesia, West Malaysia, Thailand
		An. balabacensis Colless (hv, sv, fv)	*Pf, Pv, Pm, Pk P. knowlesi, P. inui, P. coatneyi, P. cynomolgi*	Human *M. fascicularis*	Brunei, Indonesia, East Malaysia, Philippines
	Dirus	*An. dirus* Peyton and Harrison (hv, sv, fv)	*Pf, Pv, Pm, Pk P. knowlesi*	Human *M. fascicularis*	Cambodia, China, Vietnam, Laos, Thailand
		An. cracens Sallum and Peyton (hv, sv. fv)	Probable vector of human malaria *P. knowlesi, P. inui, P. cynomolgi*	*M. fascicularis*	Indonesia, West Malaysia, Thailand
		An. baimaii Sallum and Peyton (hv, fv)	*Pf, Pv, Pm*	Human	Bangladesh, India, Thailand, Myanmar, China
Hackeri	–	*An. mirans* Sallum and Peyton (sv)	*P. cynomolgi, P. inui shortii, P. fragile*		Sri Lanka, India
	–	*A. hackeri* Edwards (sv)	*P. cynomolgi, P. inui, P. fieldi, P. coatneyi, P. knowlesi*	*M. fascicularis*	East and West Malaysia, Philippines, Thailand
	–	*An. pujutensis* Colless (sv?)	Probable vector of simian malaria parasites	*M. fascicularis*	Indonesia, East and West Malaysia, Thailand
Riparis	–	*An. macarthuri* Colless (sv?)	Probable vector of simian malaria parasites	*M. fascicularis*	East and West Malaysia, Thailand

hv, sv and fv indicate human, simian and lymphatic filariasis vectors; sv? indicates vectorial status awaiting confirmation.

HISTORY OF NATURAL INFECTION IN MOSQUITOES

Simian malaria was detected in long tailed macaques in 1901 (Daniels, 1908), yet the vectors responsible for the transmission remained unknown until the 1960s. When the first case of *P. knowlesi* was reported in an American surveyor working in the jungles of Pahang by Chin *et al*. (1965); American scientists along with their counterparts from the Institute for Medical Research, Malaysia carried out entomological surveys in Pahang and other areas of peninsular Malaysia. Unfortunately they could not identify the vectors.

Extensive studies were carried out in Selangor and only *Anopheles hackeri* was incriminated as the vector from the mangrove swamp areas of Rantau Panjang (Wharton and Eyles, 1961). Besides, *An. hackeri* was also incriminated as the vector of all the other simian malaria parasites namely *Plasmodium cynomolgi*, *Plasmodium coatneyi*, *Plasmodium fieldi* and *Plasmodium inui* (Warren and Wharton, 1963). However, it was found that *An. hackeri* had a definite preference for monkey bait compared with human and preferred to feed at canopy compared with ground (Wharton *et al*. 1963). In those days, the sporozoites were identified by inoculating them into the rhesus monkeys. In earlier studies also in the same area *An. hackeri* (eight out of 399) and *Anopheles pujutensis* (two out of 83) were found with sporozoites, but the species were not determined (Reid and Weitz, 1961). In Hulu Gombak (Selangor) *Anopheles latens* (known at that time as *Anopheles leucosphyrus*) was found positive for *P. inui* (Wharton *et al*. 1964), and *An. introlatus* was positive for *P. cynomolgi* and *P. fieldi* (Eyles *et al*. 1963). Besides the Leucosphyrus group, *Anopheles maculatus* ss was positive for sporozoites but proved negative when inoculated into

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rhesus monkeys (Wharton *et al*. 1964). *Anopheles maculatus* is the predominant vector for *Plasmodium falciparum* and *Plasmodium vivax* in peninsular Malaysia (Reid, 1968).

Studies conducted in Perlis, the Northern most state of peninsular Malaysia, incriminated *Anopheles cracens* (known at that time as *Anopheles balabacensis*) to be the vector of *P. inui* (Cheong *et al*. 1965). Most of the studies at that time were carried out in Malaya (peninsular Malaysia) which was covered with tropical rain forest and in the 1960s two-thirds of the country was virgin forest (Warren *et al*. 1970). The above studies have shown that it is possible for the mosquitoes to be infected with more than one species. Although sporozoite identification was a laborious task during those days, the work carried out was of great significance.

A similar study was also carried out in the monsoon forest region of Cambodia and it was found that *Anopheles dirus* sl (known at that time as *An. balabacensis*) was responsible for 50% of malaria prevalence in humans. It was interesting to note that *An. dirus* fed almost equally at ground level on humans and on monkeys in the forest canopy. Eyles and co-workers proved that *An. dirus* was responsible for human malaria but were not able to isolate simian malaria from infected mosquitoes (Eyles *et al*. 1964).

In studies conducted in the 1970s in Palawan Island in the Philippines, Tsukamoto and co-workers suspected *An. balabacensis* to be the vector of simian malaria. They found *An. balabacensis* was more attracted to a monkey-baited trap than traps baited with water buffalo or humans and individuals host seeking on macaques had oocyst and sporozoites (although malaria species unconfirmed) (Tsukamoto *et al*. 1978). Wild *An. balabacensis* (obtained from larvae in the field) fed on macaques were able to develop sporozoites; however, the species of simian malaria was not determined (Tsukamoto *et al*. 1978). It at least showed that *An. balabacensis* could develop the simian malaria parasites to the infective stage.

LABORATORY STUDIES ON MOSQUITO INFECTION

Most of the experimental studies on mosquito infection were carried out in the 1960s. Infection studies with *P. cynomolgi* showed that the following non Leucosphyrus group of mosquitoes were able to produce sporozoites and were successful in transmission: *Anopheles kochi, Anopheles lesteri, Anopheles letifer, An. maculatus, Anopheles sinensis, Anopheles subpictus, Anopheles sundaicus* and *Anopheles vagus* (Warren and Wharton, 1963). However, studies with *An. dirus* sl and *An. maculatus* sl showed that *An. dirus* was very susceptible to *P. knowlesi*, while only a few *An. maculatus* (three out of 57) were able to produce sporozoites (Collins *et al*. 1967). Further studies conducted by the same author confirmed *An. dirus* to be a successful vector in the transmission of *P. knowlesi* (Collins *et al*. 1971).

Although laboratory studies have shown that some of the *Anopheles* species besides Leucosphyrus group can transmit simian malaria, thus far no natural infection has been recorded from published studies. For example although in one study *An. kochi* comprised ~30% of the mosquitoes found in monkey-baited traps, none were infected (Jiram *et al*. 2012). Now that human cases of *P. knowlesi* malaria are on the increase it is timely for more studies to be carried out to identify the vectors involved in transmitting this parasite from its simian host to the humans.

IMPACT OF FOREST AND FARMS ON VECTORS OF KNOWLESI MALARIA TO-DATE IN MALAYSIA

Since the report of a large focus of *P. knowlesi* cases in Kapit, Sarawak, in 2004 (Singh *et al*. 2004), studies have been conducted in various parts of Malaysia (Fig. 1) especially in forest, farms and villages where people were suspected to have been infected. Only *Anopheles* mosquitoes belonging to the Leucosphyrus group have been incriminated as vectors: *An. latens* (Vythilingam *et al*. 2006) in Kapit; *An. cracens* (Vythilingam *et al*. 2008) in Kuala Lipis, *An. introlatus* (Vythilingam *et al*. 2014) in Hulu Selangor and *An. balabacensis* (Wong *et al*. 2015) in Kudat and Banggi Island. Of these *An. latens* and *An. balabacensis* are also vectors of human malaria (*Pf* and *Pv*) in Sarawak and Sabah, respectively (Hii *et al*. 1988; Chang *et al*. 1995). In peninsular Malaysia *An. maculatus* was the predominant mosquito species in malarious areas in Pahang and Leucosphyrus group of mosquitoes were seldom obtained during collections in those areas (Vythilingam *et al*. 1995). However, with occurrence of knowlesi malaria in humans, *An. cracens* has become the predominant species obtained in Pahang (Jiram *et al*. 2012). These mosquitoes were considered as forest mosquitoes and were rarely found in villages in the 1960s. Studies carried out in Sarawak and Sabah in the 1990s after environmental changes (forest converted to plantation) it was found that *Anopheles donaldi* was the predominant species which replaced *An. latens* and *An. balabacensis*, respectively (Chang *et al*. 1995; Vythilingam *et al*. 2005). However, deforestation and land use change have been associated with malaria vectors and incidence of malaria globally (Guerra *et al*. 2006). A recent study in Sabah showed that historical forest loss was more significantly associated with *P. knowlesi* incidence than forest loss occurring during the same year of reported incidence, suggesting that increased

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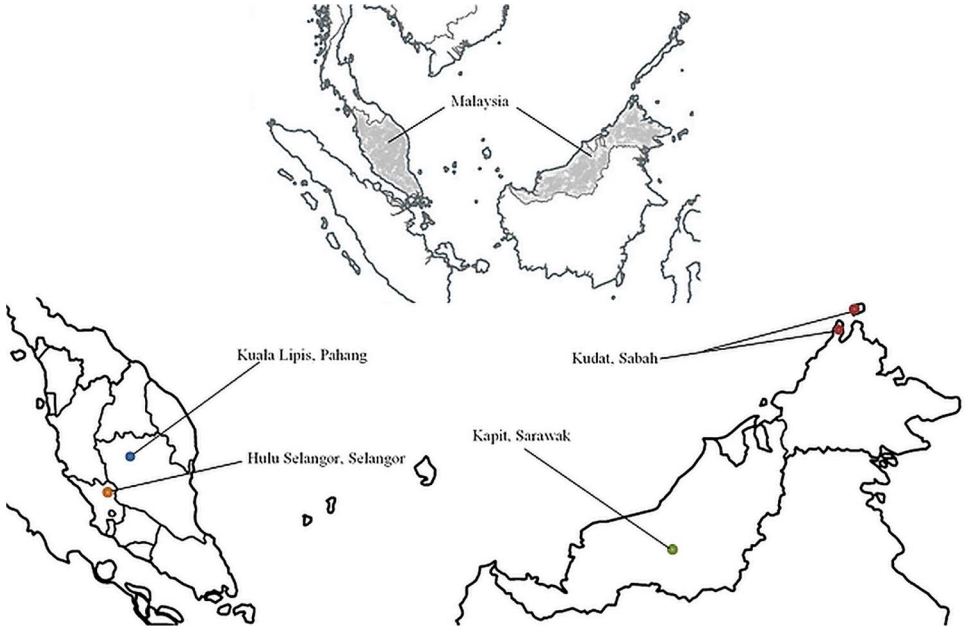


Fig. 1. Map showing study sites of knowlesi malaria vectors in Malaysia.

transmission is related to long-term changes in vector, host or human populations involved (Fornace *et al*. 2016).

In Malaysia deforestation has caused the movement of the long-tailed macaques from forested areas to farms and semi-urban areas. The mosquitoes may have followed their host and adapted to forest edge and farms. Figure 2 below shows consolidated data that has been published and it is obvious that most of these vector species are now predominant in farms followed by forest and village. However, *An*. *balabacensis* was still predominant in villages in the studied areas (Wong *et al*. 2015).

Biting habits of vectors

Current studies have shown that the biting times of the vectors have changed. As seen in Fig. 3 all species were biting in the early part of the night (in Sabah and Sarawak it gets dark early around 6 pm compared with peninsular Malaysia where it gets dark around 7 pm) where most people would be either outside their houses or returning home after working in the farms. The fact that there have been no reports of epidemics of *P*. *knowlesi* in any area coupled with low or zero sporozoite rates in mosquitoes from the villages suggest that people were getting infected in forest or farming areas.

Biological characteristics of vectors

Table 2 shows the biological characteristics of the vectors from published studies. The sporozoite rate for all vectors has been highest in the forest areas. However, human biting rate for *An*. *latens* and *An*. *cracens* has been highest in farms while for *An*. *balabacensis* it appears to bite humans most often in the villages. The parous rate of all the vectors was more than 50% in all sites and thus the daily survival rate was more than 0·8. On the whole more than 20% of the vectors were able to live the 10 days necessary for *P*. *knowlesi* to develop into transmission stage sporozoite, with the exception of *An*. *latens* in the forest (13%) and *An*. *balabacensis* in the village (16%). Those surviving the 10 days had a further life expectancy ranging from 4·7 to 8·6 days. The vectorial capacity was highest in *An*. *balabacensis* (3·85) from the forest area.

From the above published results, the relatively high survival and sporozoite rates in these vectors coupled with the potentially increased contact of human–vector–macaques have likely made major contributions to the increase in *P*. *knowlesi* cases in these areas. It is obvious that current vector control measures have little impact on these vectors.

Anopheles dirus *and* P. knowlesi *transmission in Vietnam*

Besides Malaysia, Vietnam has also made efforts to identify the vectors of *P*. *knowlesi*. *Anopheles dirus* has been incriminated as the vector of *P*. *knowlesi* (Nakazawa *et al*. 2009), and it is also the vector of human malaria in countries in the Mekong region (Trung *et al*. 2004). However, only in Vietnam both human malarias (*Pf* and *Pv*) and *P*. *knowlesi* were present in *An*. *dirus* (Marchand *et al*. 2011). The average annual entomological inoculation rate of *An*. *dirus* was 25·4 (all species) infective bites per person per year (Marchand *et al*. 2011). Of the 86 sporozoite-positive mosquitoes, 73 underwent PCR analysis for malaria parasite detection, of

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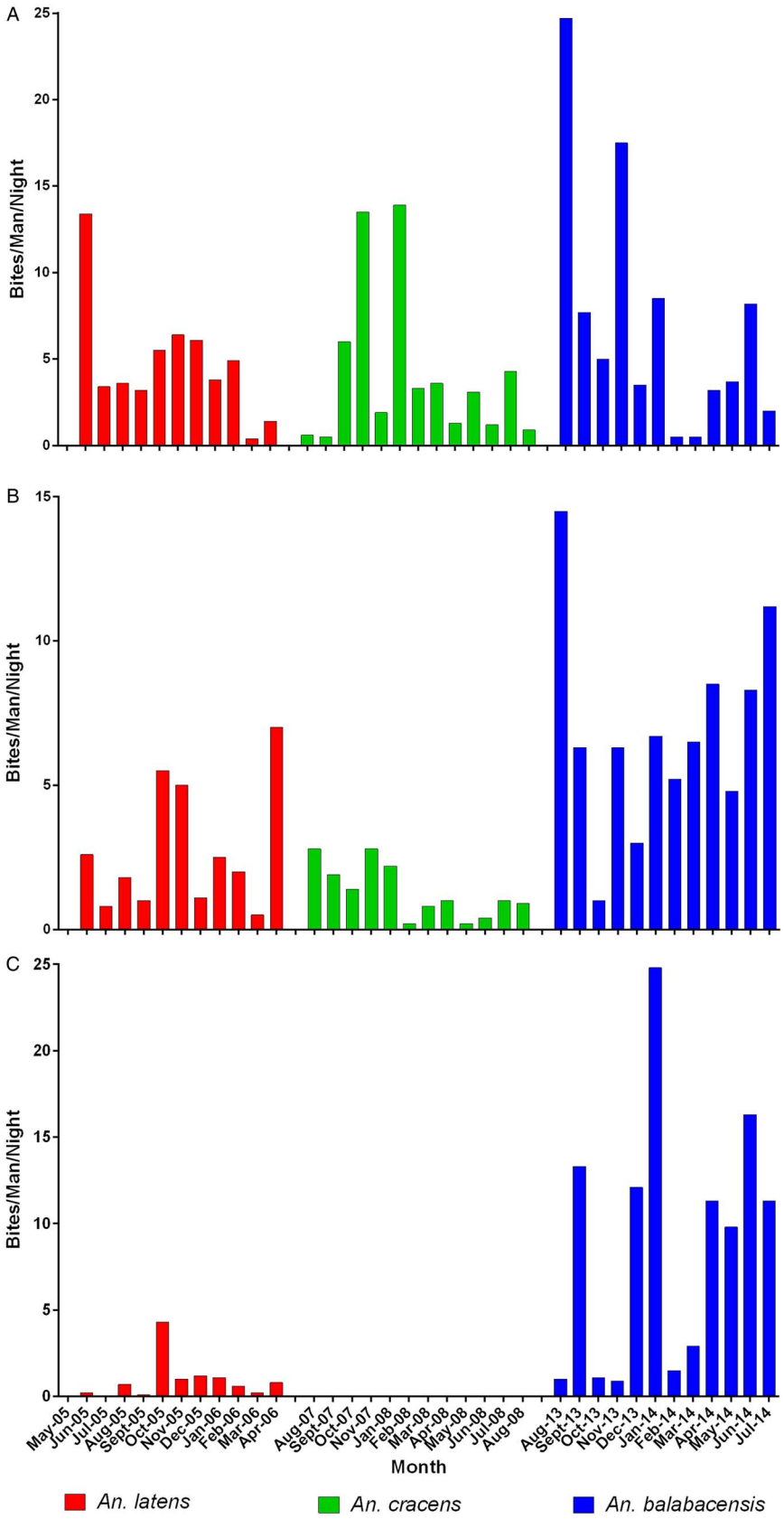


Fig. 2. Man biting rate of *Anopheles latens* (Kapit), *Anopheles cracens* (Kuala Lipis) and *Anopheles balabacensis* (Kudat and Banggi Island) collected from different study sites (A) farm, (B) forest and (C) village. The first author was involved in all the studies and the methods used were the same.

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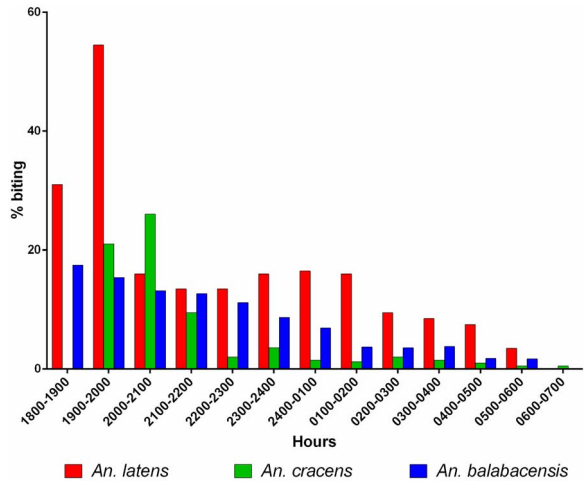


Fig. 3. Biting cycles of *Anopheles latens*, *Anopheles cracens* and *Anopheles balabacensis* in study sites. *Anopheles latens* collected from Kapit, Sarawak; *Anopheles cracens* collected from Kuala Lipis, Pahang; and *Anopheles balabacensis* collected from Kudat, Sabah. The first author was involved in all the studies and the methods of collection were the same.

which 72 were successfully assayed. Thirty-one (43%) of these 72 salivary glands were PCR positive for *P. knowlesi* csp and for *P. knowlesi* 18S rRNA.

CHALLENGES ASSOCIATED WITH VECTOR STUDIES

The way forward

One of the greatest challenges associated with vector studies is the method of collecting these vector mosquitoes. Unfortunately in Malaysia, the human landing catch method remains the best method for collecting these mosquitoes. Recent studies have shown that light trap is not as efficient as human landing catch method (Rohani *et al*. 2015). Both human landing catches and monkey-baited traps come with associated ethical problems yet for these particular vector species light traps do not provide a good alternative method. Indeed, for a successful knowlesi vector study, monkey traps ideally should be placed at different canopy heights. Work is therefore urgently needed to find sampling methods that can match the efficacy of both monkey traps and human bait.

It is still not clear if there are certain species of the vector mosquitoes biting only the monkeys. Studies in the 1960s did prove that *An. hackeri* was only attracted to monkeys (Wharton *et al*. 1964). However, in a longitudinal study carried out in Kuala Lipis the infection rate in *An. cracens* was very low but the infection in long-tailed macaques was very high (Vythilingam *et al*. 2008; Jiram *et al*. 2012). A previous laboratory study has shown one infective mosquito bite can infect human (Chin *et al*. 1968) and similarly a recent study showed it

Table 2. Biological characteristic of the known vectors of knowlesi malaria

	Anopheles latens (Tan *et al*. 2008)			*Anopheles cracens* (Jiram *et al*. 2012)		*Anopheles balabacensis* (Wong *et al*. 2015)		
	Farm	Forest	Longhouse (village)	Fruit Orchard	Forest	Farm	Forest	Village
Sporozoite rate (95% CI)	0·69 (0·18–2·18)	1·37 (0·56–3·11)	–	0·60 (0·52–0·68)	2·90 (2·11–3·30)	1·93 (0·85–4·12)	3·42 (1·91–5·93)	1·03 (0·45–2·20)
Oocysts rate (95% CI)	0·46 (0·08–1·84)	1·37 (0·56–3·11)	–	–	–	3·23 (1·76–5·74)	3·16 (1·72–5·60)	1·03 (0·45–2·21)
Man-biting rate (ma)	4·7[a]	2·8[a]	1·0[a]	4·15	1·24	6·80	7·00	8·80
Entomological inoculation rate (EIR)	11·98	14·10	–	0·08	0·08	0·13	0·24	0·09
Parous rate (95% CI)	65·80 (61·20–70·10)	53·70 (48·90–58·30)	65·80 (57·00–73·90)	65·70 (62·00–69·40)	71·50 (65·90–77·10)	63·78 (58·63–68·65)	65·00 (59·94–69·75)	57·53 (53·68–61·29)
Probability of daily survival-p	0·87	0·81	0·87	0·87	0·89	0·86	0·87	0·83
P^{10} (%)	25	13	25	25	31	22	24	16
Life expectancy	7·2	4·7	7·2	7·2	8·6	6·7	7·0	5·4
Vectorial capacity	2·86	0·60	0·85	2·46	1·09	3·36	3·85	2·50

[a] Man-biting rate was calculated based on Tan *et al*. (2008).
P^{10} Percentage of population expected to live long enough to become infective with an extrinsic cycle of 10 days based on *P. knowlesi* extrinsic incubation period.

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was sufficient to infect a monkey as well (Murphy *et al*. 2014). Perhaps there are other species biting macaques deeper in the forest. Thus, extensive studies need to be carried out at various distances into the forest and at various heights using monkey-baited traps. This would provide a better understanding of the behaviour of the vectors which in turn can be used for better control strategies to manage the vectors. Data on habitat preferences, movement patterns and social dynamics of macaques are important factors in *P. knowlesi* transmission (Brock *et al*. 2015).

It is also not known if vector mosquitoes can pick up the knowlesi parasites from humans and transmit to other humans or long-tailed macaques. Experimental studies should be conducted by feeding infected human blood through membrane feeders to mosquitoes. By this method at least it can be established if the mosquitoes will be able to develop the sporozoites. If sporozoites can develop within those infected mosquitoes, human-to-human transmission can occur. This will be a step closer to prove that human-to-human transmission can take place.

The utilization of modern analytical tools such as GIS is crucial in estimating hotspot areas for targeted control strategies. With the elimination of human malaria, cases of knowlesi malaria are on the increase as shown in Malaysia (Vythilingam *et al*. 2014; William *et al*. 2014; Yusof *et al*. 2014; Rajahram *et al*. 2016). Thus, there is a greater need for multidisciplinary research to combat simian malaria affecting humans. Besides, it is also important to use molecular-based species identification methods and to have a more precise geographical distribution maps coupled with descriptions of behaviour and ecology of specific species of vectors as carried out in Thailand for human malaria vectors (Tainchum *et al*. 2015).

Concluding remarks

Currently it has been shown that the Leucosphyrus group mosquitoes are able to transmit *P. knowlesi*. However, data on the distribution of the vectors throughout peninsular Malaysia remain scarce. In many areas, the prevalence of the parasites in the macaques are high (Ho *et al*. 2010; Akter *et al*. 2015), but the vectors remain unknown. Vector species may vary across the regions.

The limited studies that have been carried out seem to demonstrate that knowlesi malaria is a problem in the forest and farming areas. People seem to get infected while working in the forest or farms since infected mosquitoes have been obtained in these areas. The problem of *P. knowlesi* malaria is compounded by the hidden reservoirs of malaria parasites in the macaques which have not been fully addressed. This is further complicated by the unknown vectors and the changing environment.

It is also known that the above-mentioned vectors can also harbour the other simian malaria parasites; thus they could be the conduit for other zoonotic malaria spillovers to humans. As most countries in Southeast Asia are moving towards malaria elimination, it is timely to map the vector distribution, determine the home range of the macaques and carry out sero-epidemiological studies so as to prevent emergence of this epidemic zoonosis.

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