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Contribution of *Plasmodium knowlesi* to multi-species human malaria infections in North Sumatera, Indonesia

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Running Title: Multi-species malaria in North Sumatera

40-word summary:

Indonesia is aiming for malaria elimination by 2030. A parasitological survey of 3,731 individuals in North Sumatera province identified 1,169 parasitaemic individuals by PCR. Highly specific amplification of *sicavar* genes was used to identify *Plasmodium knowlesi* infection in 377 people.

Footnote page.

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TEXT: 3471 words

ABSTRACT

Background

As Indonesia works towards the goal of malaria elimination, information is lacking on malaria epidemiology from some western provinces. As a basis for studies of antimalarial efficacy, we set out to survey parasite carriage in three communities in North Sumatera Province.

Methods

A combination of active and passive detection of infection was carried out among communities in Batubara, Langkat and South Nias regencies. Finger-prick blood samples from consenting individuals of all ages provided blood films for microscopic examination and blood spots on filter paper. Plasmodium species were identified by nested PCR of rRNA genes, and a novel assay which amplifies a conserved sequence specific for the *sicavar* gene family of *P. knowlesi*.

Results

614 of 3,731 participants (16.5%) were positive for malaria parasites by microscopy. PCR detected parasite DNA in samples from 1,169 individuals (31.3%). In total, 377 participants (11.8%) harboured *P. knowlesi*. Also present were *P. vivax* (14.3%), *P. falciparum* (10.5%) and *P. malariae* (3.4%).

Conclusions

Amplification of *sicavar* is a specific and sensitive test for the presence of *P. knowlesi* DNA in humans. Subpatent and asymptomatic multi-species parasitaemia is relatively common in North Sumatera, and so PCR-based surveillance is required to support control and elimination activities.

INTRODUCTION

Malaria remains widespread across the South East Asian region. In Indonesia, 2 million cases of malaria are reported each year. *Plasmodium falciparum* and *P. vivax* are reported to be the two major causes of malaria [1]. Among the other species contributing to human infections, *P. malariae* malaria may require hospitalisation in the eastern province of Papua [2], but is not frequently encountered in western Indonesia. *P. knowlesi* is a parasite of long-tailed and pig-tailed macaques, also known to infect humans. The morphological features in the blood stage are similar to *P. falciparum* and *P. malariae*, which in routine practice has led to frequent misdiagnosis [3-5]. High *P. knowlesi* parasitaemia occurs in some individuals, and has been reported to cause fatal disease [6]. Despite this, a proportion of *P. knowlesi* infections are asymptomatic and submicroscopic across all age groups [7]. A small number of human cases of *P. knowlesi* have been documented in the province of Kalimantan, Indonesian Borneo [8] and in Aceh province [5] but this species has not yet emerged as a major cause of human malaria and is not considered in Indonesian Government guidelines.

The Ministry of Health of Indonesia has implemented malaria control, aiming for elimination by 2030. Malaria surveillance relies on passive case detection by microscopic examination and rapid diagnostic tests (RDT) at primary health care centres [9]. These tests are sufficient to detect clinical malaria infection caused by the two major species in Indonesia, *P. falciparum* and *P. vivax* [10]. However, identification of less common species, particularly at low density parasitaemia, is more difficult and can lead to under-diagnosis [11]. Modelling of data from low endemicity areas predicts that submicroscopic parasites may contribute 70 to 80% of all malaria infections [12], and *in vivo* studies demonstrate that these contribute to ongoing malaria transmission [13]. Hence, the use of routine microscopy and RDT in malaria surveillance fails to detect a substantial proportion of the human reservoir of infection and so may compromise malaria elimination strategies. One solution is to deploy molecular assays for parasite detection, as these can provide excellent sensitivity and specificity [14-17].

In preparation for a study of antimalarial drug efficacy *in vivo*, we performed intensive malaria screening in three Regencies of the Province of North Sumatera, western Indonesia. In addition to microscopy, established PCR assays were deployed [18]. However, these tests have limitations for the identification of *P. knowlesi* infection, as the target region of the 18S ribosomal RNA can cross-react with *P. vivax* [19]. Therefore, we developed a sensitive and highly *P. knowlesi*-specific nested-PCR assay to ensure reliable determination of all *Plasmodium* species, including submicroscopic infection, in our study areas.

METHODS AND MATERIALS

Study sites

A parasitological survey was conducted between January and June 2015 among people attending out-patient clinics temporarily established in more than 80 localities across three selected regencies in North Sumatera province, Indonesia: Batubara regency, Langkat regency and South Nias regency (Figure 1). North Sumatera has a total area of 71,680.68 km^2 with a population of 13,215,401. The province is subject to stable low malaria transmission, and is currently planning for elimination by 2020. The three regencies were selected based on published malaria endemicity data [20]. Batubara Regency, situated on the east coast facing peninsular Malaysia, comprises semi-forested and plantation areas. Langkat Regency is a forested highland area (altitude 105-530m above sea level), and South Nias Regency is a cluster of islands in the Indian Ocean. Each regency is served by a district hospital and peripheral health clinics, however some rural villages in the study areas have very limited access to these services.

Ethics approval

The study was approved by the ethics committee of the University of Sumatera Utara, Indonesia (ID# 401/KOMET/FK USU/2014), and the ethics committee of the London School of Hygiene and Tropical Medicine, United Kingdom (ID# 8504‑01).

Sampling

Sampling strategies differed among the three sites, due to contrasting geography and inconsistencies in access to health facilities. In Batubara, most communities had good access to a health clinic, which was open to patients from 08:00 to 12:00, 6 days per week. We established a 24-hour, 7-day clinic for an 8-week period of screening, following intensive health promotion and education on malaria, and sensitisation as to our study objectives. This sensitisation was facilitated by local leaders and carried out at the level of the whole community, but clinic attendance was entirely voluntary. Thus sampling was not designed to reach the whole community. Local health clinics were also asked to refer patients diagnosed with malaria to our team. Langkat is a forested area with isolated villages that have poor access to health facilities; we therefore adopted a village-by-village approach in which a sensitisation meeting was followed by 2-3 days of screening, before the team moved to another village. The 31 communities we sampled in South Nias were spread across several islands, and sea journeys were required to move our team and the samples between each village and our study clinic / diagnostic lab which was temporarily set up in a central location on Tello Island.

Malaria testing was done on three groups of people. Firstly, patients with fever (axillary temperature $\geq$ 37.5 ^{o}C) or history of fever in the preceding 48 hours who presented at the health clinics were tested for malaria. Tests were also offered to healthy individuals who, following our community sensitisation activity, came for a voluntary malaria check. This second group includes children who volunteered during school sensitisation visits (Batubara and South Nias only). Thirdly, household members of any individuals slide-positive for malaria parasites were subsequently visited and also offered malaria testing. Finger-prick blood samples were taken from all participants for thick and thin blood films for microscopy examination (single read), and also spotted onto filter papers (3MM Whatman) for molecular analysis. Participants confirmed as malaria-positive by microscopy were clinically assessed and treated according to national guidelines, regardless of symptoms.

Laboratory Procedures

DNA extraction

Parasite DNA was extracted from filter papers using the Chelex method [21, 22].

Parasite species identification by rRNA gene amplification

A conventional nested PCR assay targeting the genes encoding the *Plasmodium* 18S ribosomal RNA was performed on all samples for species determination and detection of submicroscopic infections [3, 18]. Positive controls for *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* spp. and *P. knowlesi* were included in all nested-PCR assays.

Development and validation of a novel highly-specific P. knowlesi *PCR assay*

To overcome the cross-reactivity between *P. knowlesi* and *P. vivax* which occurs in the rRNA gene PCR assays, we developed a hemi-nested PCR assay based on a conserved region of the amino-terminal exon of the 50-70 members of the gene family encoding *P. knowlesi*-specific variant antigen family SICAvar [23]. The primers used were as follows: SICAf1, 5'-GGTCCTCTTGGTAAAGGAGG-3'and SICAr1, 5'- CCCTTTTTGACATTCGTCC-3' for the first amplification and SICAf2, 5'-CTTGGTAAAGGAGGACCACG-3' and SICAr1 for second amplification, generating a final amplicon of 228 – 249bp, encoding 76-83 amino acids. This sequence occurs in both Type 1 and Type 2 *sicavar* genes (Supplementary Table 1 in [23]). For the first round of amplification, 5 μL of DNA template was used in a total of 25 μL volume, and 0.2 μL of this product was template for the 25 μL reaction mixture of the hemi-nested amplification round. Amplifications were performed under the following

conditions: 94 °C for 3 min, then 30 cycles of 94 °C for 30 secs, 55 °C for 30 secs, and 65 °C for 1 min, with extension at 65 °C for 5 min. This assay was tested against control DNA from all human malaria parasites (*P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale curtisi* and *P. ovale wallikeri*), simian malaria parasites (*P. knowlesi*, *P. inui*, *P. cynomolgi*, *P. coatneyi* and *P. fieldi*), clinical *P. knowlesi* isolates obtained from Kapit of Malaysian Borneo and human DNA from malaria-free individuals to assess specificity. To determine the limit of detection of the assay, *P. knowlesi* culture of known parasitaemia (kindly provided by F. Mohring) was serially diluted in whole human blood, spotted on filter paper and DNA extracted by in a QIASymphony automated DNA extraction system. All field isolates were tested for *P. knowlesi* infection using this novel PCR assay. *Sicavar* amplicons from a subset of samples were verified by direct sequencing using BigDye Terminator v3.1 cycle sequencing kits and analysis on an ABI 3730 Sequencer (Applied Biosystems). Results were aligned and compared to *P. knowlesi* strain H reference genome using Geneious v.8.0.5 and BLAST.

RESULTS

Validation of SICAvar assay for *P. knowlesi*-specific identification

The novel SICAvar gene assay was validated against DNA from a range of *Plasmodium* species DNA, and from *in vitro* cultured *P. knowlesi*. The test was found to be specific, as the primers did not generate bands from the DNA of any of the *Plasmodium* species tested other than *P. knowlesi* (Figure 2). The *sicavar*-targeted primers detected 377 *P. knowlesi* infections, suggesting significantly higher sensitivity than the rRNA assay, which identified only 76 *P. knowlesi*–infected individuals (Table 1). Further, comparison of the results showed that only 10 cases (2.3%) demonstrated a positive test result in both assays. The finding that 66 individuals were seen as *P. knowlesi*-positive by rRNA nested PCR alone suggests the test cross-reacts with *P. vivax* DNA in the absence of *P. knowlesi* under the conditions used in this study. As we did not deploy a "tie-breaker" test for *P. vivax*, we cannot say for certain whether the *P. vivax* rRNA gene primers also cross-react with *P. knowlesi* DNA. We did find that 18 individuals were positive for both *P. vivax* and *P. knowlesi* rRNA amplicons in the nested PCR. Given this ambiguity, and the fact we were not able to repeat test the sample set, we proceeded with analysis taking the *sicavar* assay result as definitive for *P. knowlesi*. Nearly half of all *sicavar*-positive *P. knowlesi* infections were also rRNA amplicon-positive for at least one other species, *P. vivax* being the most common co-infection (Table 1). The limit of detection of the assay, as performed on dried filter paper blood samples, was estimated as 0.1 parasites per μL of whole blood (data not shown).

As further confirmation of our results, seven *P. knowlesi* isolates detected by SICAvar were PCR-amplified and the products directly sequenced. The sequences exhibited high variability, as expected for variant antigens, even in this most conserved exon (Figure 3). Interestingly, two of the seven sequences obtained harboured an insert encoding an additional 7 amino acids. These two forms were used to probe the current *P. knowlesi* reference genome (http://www.sanger.ac.uk/resources/downloads/protozoa/plasmodium-knowlesi.html) and both queries identified a number of distinct sequences in the reference genome (Suppl. Table 1).

Following this successful validation of the SICAvar PCR assay, we were able to deduce robust estimates of the contribution of each species to malaria infections in each of our three sites. Either or both *P. falciparum* and *P. vivax* were the most abundant in both Langkat and South Nias, as expected. However in Batubara regency, *P. knowlesi* (39.7%) was more abundant than *P. vivax* (35.1%) among our tested participants (Figure 4). Of patients reporting fever symptoms in the previous 72 hours, 22 were carrying *P. knowlesi* mono-infection by PCR, with a further 47 having *P. knowlesi* double infections with *P. vivax* (n=20), *P. falciparum* (n=19) or *P. malariae* (n=2), or various combinations of triple species infection.

Parasite carriage

A total of 3731 individuals from Batubara (n = 1270), Langkat (n = 544) and South Nias (n = 1917) were included in the malaria screening (Supplementary Table 2). At each regency, 117 (9.2%), 98 (18.0%) and 397 (21.3%) individuals were positive for *Plasmodium* infections by microscopy. Three species (*P. falciparum*, *P. vivax* and *P. malariae)* were identified. A considerable number of participants with malaria-positive slides were negative by PCR subsequently performed on stored blood spots. Thus the total number of patent infections confirmed by both microscopy and PCR decreased to 93 (8.1%), 74 (13.6%) and 169 (9.1%), respectively, indicating poor specificity of microscopy in South Nias in particular. Conversely, a substantial number of submicroscopic infections were identified by PCR alone, with a PCR-confirmed parasite carriage in 25.2%, 33.5% and 34.8% of tested individuals, respectively, in the three sites (Table 2). All *Plasmodium* species with the exception of *P. ovale* spp. were detected among our samples by a combination of the rRNA gene and SICAvar PCR assays.

Carriage of submicroscopic infections

PCR analysis revealed that the majority of the 1169 infected individuals (71.3%) harboured submicroscopic parasites (Table 2). Among these submicroscopic infections, 77.7% (647/833) were single-species infections predominated by *P. vivax* (227) and *P. knowlesi* (220). Submicroscopic infections of any species were more often found in older individuals, the mean age in this group being 23.0 years, (95% CI 21.7 - 24.3 years). The mean age of individuals with patent infections was significantly lower at 18.0 years (95% CI 16.2 - 19.7; P < 0.001, 2-sided t-test) (Table 3). Submicroscopic carriage was more often observed in Batubara regency (OR 1.67, 95% CI: 1.14 – 2.45) and South Nias regency (OR 2.02, 95% CI: 1.43-2.85) compared to Langkat regency. However, individuals with multi-species infections were not significantly older than those infected with a single species (P = 0.66, 2-sided t-test).

DISCUSSION

In this study, a total of 3,731 febrile and non-febrile residents of three regencies in North Sumatera province were screened for malaria infection by both microscopy and PCR detection of parasite DNA. Microscopy identified 612 infected participants, whereas PCR identified 1,169 individuals harbouring at least one of the four *Plasmodium* species identified: *P. falciparum*, *P. knowlesi*, *P. malariae* and *P. vivax*. Using a novel assay developed for this study which detects a conserved motif in the multi-copy *sicavar* gene family, we found that *P. knowlesi* was present in 377 individuals (10.1%), including both patent and sub-patent infections. *P. vivax* and *P. falciparum* were both frequently detected, occurring in 11.3% and 8.2% of all individuals tested.

Although *P. knowlesi* infection has been widely recorded in Southeast Asia, only a handful of confirmed cases have been from Indonesia, from Kalimantan, eastern Borneo [8, 24], and more recently 20 cases from Aceh province, on the north-west extremity of Sumatera [5]. This latter study of 1532 individuals used a combination of passive and reactive case detection to identify a total of 20 *P. knowlesi* infections, 15 *P. vivax* and 8 *P. falciparum* infections, almost all of which were symptomatic. This contrasts with our findings of more frequent parasite carriage, and a significant proportion of subpatent and asymptomatic infections. Malaria transmission intensity is much lower in Aceh than in North Sumatera, and this neighbouring province is closer to elimination of the disease. North Sumatera's natural forests have been affected by deforestation in the last few years. In rural districts, people may live in close proximity to semi-forested, forested or plantation areas, with a high likelihood of forest exposure, but may not have adequate access to health facilities and antimalarial medication. In the Aceh study, the more frequent occurrence of symptoms in people with knowlesi malaria reflects observations in Malaysian Borneo where, as in Aceh, other *Plasmodium* species have decreased in prevalence over the past decade [4, 25]. In North Sumatera, as our data show, *P. falciparum* and *P. vivax* remain common and it may be that acquired immunity to these human parasites protects individuals subsequently infected with *P. knowlesi*, although asymptomatic infections have also been reported in Sabah [7].

Our novel PCR assay identified an unexpectedly large number of *P. knowlesi* infections, and so we made some effort to validate its sensitivity and specificity (Figure 2). Schizont-infected cell agglutination (SICA) variant genes encode an antigen family unique to *P. knowlesi*, estimated to number over a hundred members, including both multi-exon and truncated forms randomly distributed across all 14 chromosomes [23]. SICAvar proteins undergo antigenic variation in the course of a single infection [26, 27], and are likely to play a key role in maintaining chronic parasitaemia in semi-immune hosts. Sequencing of a handful of *sicavar* amplicons from our samples confirmed nucleotide diversity in our short target sequence, double peaking indicative of multiple

loci being amplified in some cases, and distinguished a variant form with a 7 amino acid insert (Figure 3). Probing the *P. knowlesi* reference genome with these two forms generated many hits with both length variants, including both Type 1 and Type 2 loci (Suppl. Table 1). These findings suggest our assay is performing as hoped and is a useful tool for identifying *P. knowlesi* infections among complex mixtures of *Plasmodium* species.

The national malaria control programme focuses on case management through passive surveillance at primary health centres, deploying microscopy or RDT to detect malaria cases to be treated [9]. In our study, microscopy identified many *Plasmodium* spp. infections, but lacked accuracy in distinguishing among the four species present, as previously reported in Malaysia [28]. Although personnel differed between sites, identification of the less common species was problematic in all sites. *P. malariae* was only detected in 1 case and *P. knowlesi* was left unrecognised. In South Nias, microscopy results showed poor specificity (Supplementary Table 2), leading to a number of false positives, whereas specificity was good at the other two sites. RDT have limitations for detection of *P. knowlesi* because only *P. falciparum* and *P. vivax* parasite LDH monoclonal antibodies are included [11]. Most importantly, almost half of the infections detected by PCR were not identified by either of the conventional tests. Therefore, although RDT and microscopy remain satisfactory for diagnosis of symptomatic falciparum and vivax malaria requiring treatment, these are not adequate tools for malaria elimination and control activities, as sub-microscopic *Plasmodium* carriage is associated with subsequent transmission to mosquitoes [13].

On the other hand, molecular tests are highly sensitive and specific, provide the capacity to detect low density infections missed by microscopy or RDT, and are well established for detection of human malaria infections [11, 29]. To overcome cross-amplification of *P. vivax* isolates with ribosomal gene PCR assays [3, 19], we have recently described a PCR-sequencing approach to detect *P. knowlesi cytb* DNA, which also provided satisfactory sensitivity [30]. However, our *sicavar* target assay provides greater sensitivity for *P. knowlesi* identification in our hands, and was effective in detecting submicroscopic parasites. This is a species-specific test, as the *sicavar* gene family is unique to *P. knowlesi* (Fig. 2). As a result of this improved specificity and sensitivity, we can confidently report moderate numbers of *P. knowlesi* co-infections with *P. vivax* in the present study.

Despite the aim to achieve malaria elimination by 2020 in Sumatera, our study demonstrated that a substantial number of individuals in our study areas carried parasites. *P. falciparum* contributed one-fifth of infections, while *P. vivax* was slightly more often seen. Interestingly, our findings also demonstrate that *P. knowlesi* carriage is not uncommon. Many *P. knowlesi*-infected individuals harboured additional *Plasmodium* species (Table 1), in contrast to areas in Malaysian Borneo where *P. vivax* and *P. falciparum* are now very scarce [4, 7]. Multiple-

species infections in our study were equally distributed across all age groups with both females and males exposed to a similar risk of infection (data not shown), whereas sub-microscopic infections were more common in older individuals (Table 2), suggesting a role for acquired immunity [31]. The observation of asymptomatic *P. knowlesi* infections in our study is consistent with recent findings in Malaysia [7], but does not necessarily support the occurrence of human-mosquito transmission of *P. knowlesi.* Macaques were present in all sites, and the communities shared established risk factors for malaria transmission by forest-dwelling *Anopheles* thought to be the vector of *P. knowlesi*. These data may suggest that acquired immunity permits sustainable chronic infections with this simian parasite. Cross-protection among the four human *Plasmodium* species may maintain overall parasite density at low levels [31], and this could also plausibly apply to *P. knowlesi,* particularly in settings where the closely related *P. vivax* is present. One weakness of our study is the lack of a systematic sampling procedure which may have introduced bias; as we had not previously worked in these three regions, methodology was adapted for each site to reflect the facilities available and particular local challenges. For South Nias, these included frequent sea journeys in small boats. Future studies could deploy a more systematic approach and collect sufficient data to better understand and characterise asymptomatic infections, now that community contacts have been established and baseline information is available.

Our study demonstrated the importance of submicroscopic infections of four *Plasmodium* species, including *P. knowlesi*, to malaria transmission in North Sumatera. There is an urgent need for the national malaria programme to include in malaria guidelines the recommendation that microscopists are trained to identify *P. knowlesi* infection in Indonesian clinics. Molecular detection of infection is also needed, to strengthen control and elimination programmes by accurately defining the true extent of the malaria reservoir, so as to achieve the current goal of elimination [32].

Acknowledgements

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Figure Legends

Figure 1.   **Map of North Sumatera province, Indonesia.**

The three studied regencies (Batubara regency, Langkat regency, South Nias regency) are indicated.
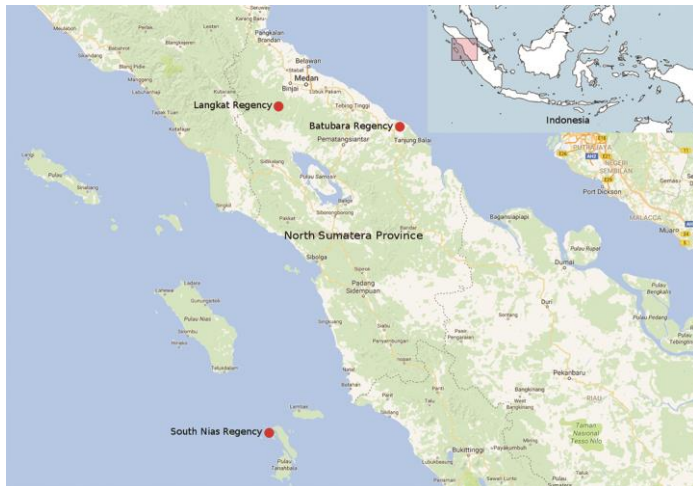
Figure 2.   **Validation of *P. knowlesi* primers targeting *sicavar* against human and simian malaria parasites reported from SE Asia.**

Control DNA of human malaria species were from imported cases in the UK (courtesy of the PHE Malaria Reference Laboratory), 2 different isolates each for *P. falciparum, P. vivax* and *P. malariae* are shown.

Genus-specific primers for the cytochrome B gene (*cytb*) were used to confirm presence of detectable *Plasmodium* DNA in each sample, indicated by + symbol.
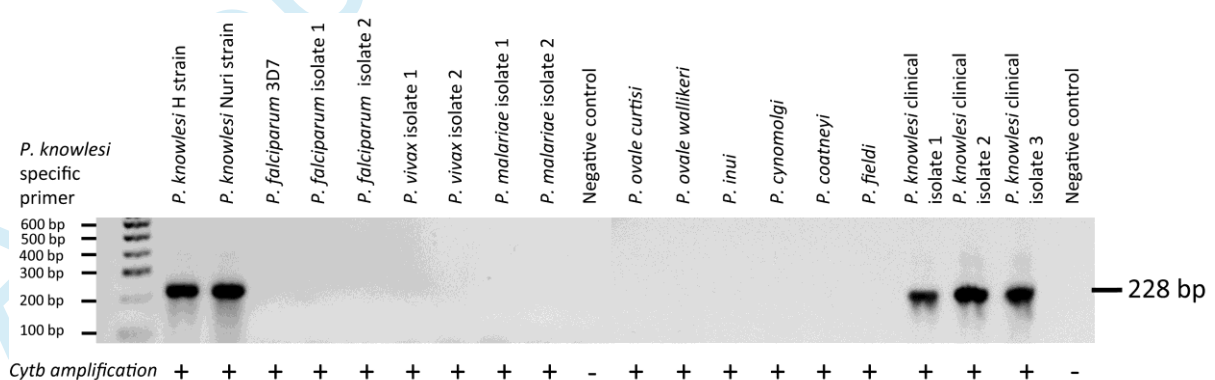
Figure 3.   **Sequence alignment of representative 120 and 141 nucleotide sequences of *SICAvar* amplicons from the peripheral blood DNA of four participants from Batubara (BB), and two each from Langkat (LK) and South Nias (NS).**

Amplicons were produced by hemi-nested PCR as described in Materials and Methods. Sample order is determined by the alignment. Representative amplification products were chosen for this sample, and sequenced directly using amplification primers to prime forward and reverse sequencing reactions. Sequences shown were confirmed in both directions. Two loci from the *P. knowlesi* strain H reference genome (PKNH_0932000, Type I SICAvar, chrom 9, centrally located; PKNH_0118500, Type II SICAvar, chrom 1, sub-telomeric) are shown for comparison. Double-peaking was seen in some samples – only the peak with highest amplitude was read for this analysis.

Figure 4. **Proportion of *Plasmodium* species and multiplicity of infections in each regency.** Denominators for each site (total number of individuals tested) are given under each regency name. The number of parasite-positive individuals is shown at top-left of each bar graph. Coloured bars denote species, grey bars denote proportion of mixed-species infections identified in each site. Pf = *P. falciparum*, Pv = *P. vivax*, Pm = *P. malariae*, Pk = *P. knowlesi.*
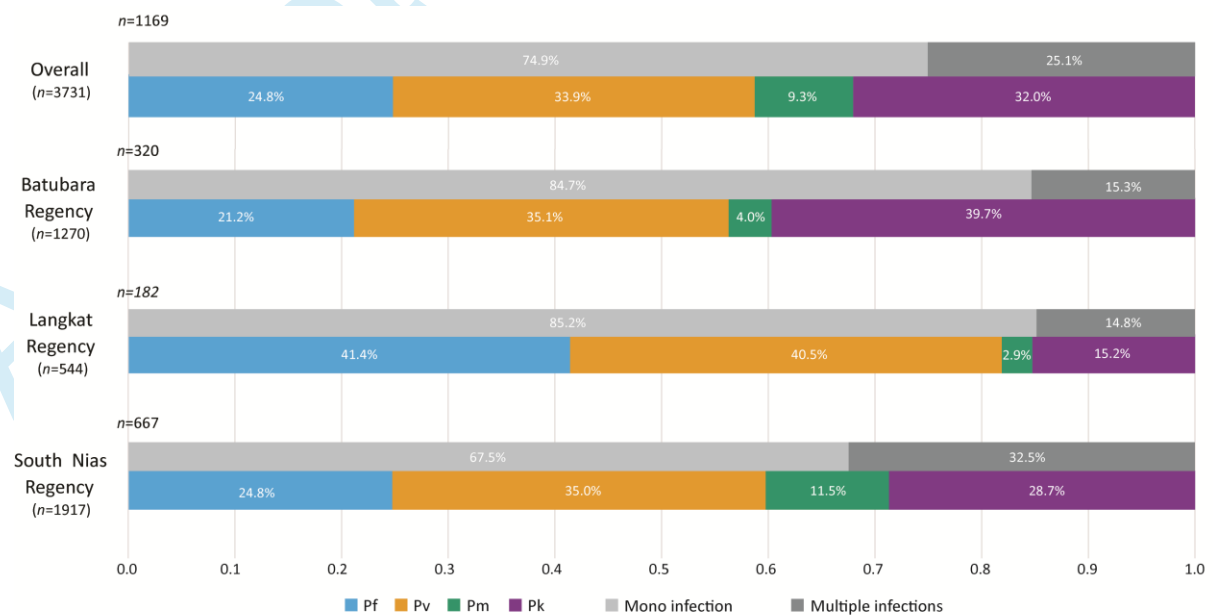
Table 1. Comparison of two PCR assays for *P. knowlesi* cases detection

	18 ssu rRNA assay	SICAvar assay
Total *P. knowlesi* cases	76	377
P. knowlesi mono infection	42 (55.3%)	215 (57.0%)
P. knowlesi + *P. vivax*	16 (21.1%)	65 (17.2%)
P. knowlesi + other *Plasmodium* spp. infections	18 (23.7%)	97 (25.7%)
Cases positive by both assays	10	
Total *P. knowlesi* cases detected with any assay	443	
P. knowlesi mono infection	254/443 (57.34%)	

Relative frequencies (percentages) read vertically.

Table 2. Sub-microscopic infections in 1169 *Plasmodium* spp. PCR-positive participants

	All PCR Positive (%)	PCR positive microscopy negative
PCR-positive	1169 (31.33)	833 (71.26)
P. falciparum	247 (6.62)	165 (14.11)
P. vivax	335 (8.97)	227 (19.41)
P. malariae	40 (1.07)	35 (2.99)
P. knowlesi	254 (6.80)	220 (18.81)
Mixed infections	293 (7.85)	186 (15.91)
Species multiplicity by PCR		
1	876 (74.94)	647 (77.67)
2	256 (21.90)	163 (19.57)
3	35 (2.99)	21 (2.52)
4	2 (0.17)	2 (0.24)
PCR-positivity by regency		
Batubara	320/1270 (25.19)	227/320 (70.93)
Langkat	182/544 (33.45)	108/182 (59.34)
South Nias	667/1917 (34.79)	498/667 (74.66)

Frequencies are shown together with relative frequency expressed as a percentage (in brackets) of the total number of participants (N=3731), all PCR positives (n=1169) or all sub-microscopic infections (n=833).

Top row percentages read horizontally; the percentages for other indented categories read vertically within the appropriate sub-group, apart from the regency-specific data (denominators as shown).

Table 3. Age and gender of participants PCR-positive for *Plasmodium* spp.

	PCR positive (%)
Age	
P. falciparum	
<5 years	24 (9.72)
5-14 years	91 (36.84)
>15 years	132 (53.44)
P. vivax	
<5 years	41 (12.24)
5-14 years	143 (42.69)
>15 years	151 (45.07)
P. malariae	
<5 years	4 (10.00)
5-14 years	18 (45.00)
>15 years	18 (45.00)
P. knowlesi	
<5 years	28 (11.02)
5-14 years	96 (37.80)
>15 years	130 (51.18)
Parasite carriage among females	608 (52.01)
P. falciparum	137 (55.47)
P. vivax	171 (51.04)
P. malariae	15 (37.50)
P. knowlesi	129 (50.79)

Frequencies are shown together with relative frequency expressed as a percentage (in brackets) of the total number of participants.

Top row percentages read horizontally; the percentages for all other indented categories read vertically.

Age and sex data are presented for single species infection only.