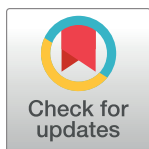


RESEARCH ARTICLE

Improved limit of detection for zoonotic *Plasmodium knowlesi* and *P. cynomolgi* surveillance using reverse transcription for total nucleic acid preserved samples or dried blood spots

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Abstract

Background

Zoonotic *P. knowlesi* and *P. cynomolgi* symptomatic and asymptomatic infections occur across endemic areas of Southeast Asia. Most infections are low-parasitemia, with an unknown proportion below routine microscopy detection thresholds. Molecular surveillance tools optimizing the limit of detection (LOD) would allow more accurate estimates of zoonotic malaria prevalence.

Methodology/Principal findings

An established ultra-sensitive *Plasmodium* genus quantitative-PCR (qPCR) assay targeting the 18S rRNA gene underwent LOD evaluation with and without reverse transcription (RT)

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for *P. knowlesi*, *P. cynomolgi* and *P. vivax* using total nucleic acid preserved (DNA/RNA Shield) isolates and archived dried blood spots (DBS). LODs for selected *P. knowlesi*-specific assays, and reference *P. vivax*- and *P. cynomolgi*-specific assays were determined with reverse transcription (RT). Assay specificities were assessed using clinical malaria samples and malaria-negative controls. The use of reverse transcription improved *Plasmodium* species detection by up to 10,000-fold (*Plasmodium* genus), 2759-fold (*P. knowlesi*) and 1000-fold (*P. vivax* and *P. cynomolgi*). The Kamau et al. *Plasmodium* genus RT-qPCR assay was highly sensitive for *P. knowlesi* detection with a median LOD of ≤ 0.0002 parasites/ μ L compared to 0.002 parasites/ μ L for *P. cynomolgi* and *P. vivax*. The LODs with RT for *P. knowlesi*-specific PCRs were enhanced for the Imwong et al. 18S rRNA (0.0007 parasites/ μ L) and Divis et al. real-time 18S rRNA (0.0002 parasites/ μ L) assays, but not for the Lubis et al. hemi-nested *SICAvar* (1.1 parasites/ μ L) and Lee et al. nested 18S rRNA (11 parasites/ μ L). The LOD for *P. vivax*- and *P. cynomolgi*-specific assays with RT were moderately improved at 0.02 and 0.002 parasites/ μ L, respectively (1000-fold change). For DBS *P. knowlesi* samples the use of RT also markedly improved the *Plasmodium* genus qPCR LOD from 19.89 to 0.08 parasites/ μ L (249-fold change); no LOD improvement was demonstrated in DBS archived beyond 6 years. The *Plasmodium* genus and *P. knowlesi*-assays were 100% specific for *Plasmodium* species and *P. knowlesi* detection, respectively, from 190 clinical infections and 48 healthy controls. Reference *P. vivax*-specific primers demonstrated known cross-reactivity with *P. cynomolgi*.

Conclusions/Significance

Our findings support the use of an 18S rRNA *Plasmodium* genus qPCR and species-specific nested PCR protocol with RT for highly-sensitive surveillance of zoonotic and human *Plasmodium* species infections.

Author summary

The monkey malaria parasite *Plasmodium knowlesi* has been found to increasingly infect humans across Southeast Asia via the bite of its anopheline mosquito vectors. Human infections with a similar monkey parasite, *Plasmodium cynomolgi*, have also been reported. The diagnostic tools commonly used to detect these malaria species are often unable to detect very low-level infections. We aimed to improve surveillance detection tools and blood sample collection methods to detect these zoonotic malaria species and understand the extent of transmission and the burden of disease. This study validated and compared the use of molecular laboratory assays targeting these species. We found that with the use of reverse transcription, large improvements in the limit of detection were possible, by up to 10,000-fold for initial malaria screening, and up to 2759-fold for specific *P. knowlesi* detection. Findings from this study support the use of ultrasensitive detection tools to improve surveillance approaches to emerging zoonotic malaria species.

Introduction

Plasmodium knowlesi is a unicellular protozoan malaria parasite present across Southeast Asia within the geographical range of its natural monkey hosts and vector mosquitoes [1,2]. *P.*

knowlesi is the most common cause of human malaria in Malaysia; capable of causing severe disease comparable to *P. falciparum* [3–5]. Human infections with other genetically similar zoonotic species, such as *P. cynomolgi* which share the same natural macaque hosts, have also been reported [6]. Accurate detection of emerging zoonotic species such as *P. knowlesi* and *P. cynomolgi* in co-endemic areas with other human *Plasmodium* species infections is necessary to understand the geographical extent of zoonotic malaria transmission and to improve regional estimates of the disease burden [7]. Improving national malaria control program detection and reporting of low-level *P. knowlesi* infections is also vital to demonstrate World Health Organization (WHO)-certified elimination for other non-zoonotic malaria species in Southeast Asia [8].

Conventional malaria diagnostic methods such as microscopy lack sensitivity and specificity for active surveillance of *P. knowlesi* due to common low-level sub-microscopic infections and an inability to accurately distinguish other morphologically similar *Plasmodium* species [7,9]; notably *P. malariae* and the early ring stages of *P. falciparum* [10,11]. Similarly, *P. cynomolgi* microscopically resembles *P. vivax* in human infections [12,13]. Current malaria rapid diagnostic tests which detect circulating *Plasmodium* species antigens also remain insufficiently sensitive for *P. knowlesi* passive case detection at the low parasite counts able to produce symptomatic infections [14–16].

Multiple molecular methods to detect *P. knowlesi* have been developed, including both quantitative and conventional qualitative PCR assays of different gene targets [7,17]. However, surveillance strategies for rare or low-level infections such as *P. knowlesi* require maximising initial screening limits of detection, with systematic comparisons of the lower limit of detection (LOD) of these assays currently lacking [7]. The degree to which the LOD is enhanced with a prior reverse transcription (RT) step is not well characterised despite potential benefits in improving the detection of very low-level parasitemia symptomatic or asymptomatic zoonotic *Plasmodium* species infections [18]. The *Plasmodium* genus real-time assay selected for evaluation in this study [19] aims to maximise the detection threshold for zoonotic malaria screening by targeting the known high multicopy number (5 to 10 copies per genome) of the 18S rRNA gene [20], in addition to amplification of both the A- and S- type genes and their RNA transcripts. Many of the most commonly used non-zoonotic [21] and zoonotic [22] *Plasmodium* species-specific assays also target the 18S rRNA gene and would fit within a clear integrated subsequent laboratory workflow, although the sensitivity of these assays that include a reverse transcription step has not been evaluated. Specificity for *P. knowlesi* detection also ideally needs to be validated against other macaque zoonotic *Plasmodium* species, including *P. cynomolgi* [7].

To support an improved molecular surveillance workflow for detection of low-level zoonotic *Plasmodium* species infections [7,23], we evaluated an established *Plasmodium* genus and species-specific PCR assays with the inclusion of a reverse transcription step to enhance the LOD in either total nucleic acid preserved media or more logistically feasible dried blood spot (DBS) collected samples.

Methodology

Ethics approval and consent to participate

Sample collection and diagnostic evaluation were approved as part of prospective malaria studies by the Medical Research and Ethics Committee (MREC), Ministry of Health Malaysia (NMRR-10-754-6684, NMRR-19-4109-52172 and NMRR-19-3229-49967), Universitas Sumatera Utara, Indonesia (#723/KEP/USU/2021) and by the Menzies School of Health Research, Australia (HREC-2010-1431 and HREC-2022-4417) in accordance with all applicable Federal and other regulations governing the protection of human subject research. Written informed

consent was obtained from all participants, including parents/guardians of those less than 18 years of age.

Clinical specimen collection and storage

Clinical venous whole blood samples and dried blood spots were collected prior to antimalarial treatment from individuals diagnosed with malaria by routine hospital microscopy, as part of an ongoing prospective malaria study in Sabah, Malaysia between April 2013 and May 2023. Adult healthy individuals were recruited as malaria-negative controls. Additional *P. vivax* clinical cases were enrolled from a prospective malaria study in western Indonesia between January 2022 and August 2023. Whole blood samples were collected in ethylenediaminetetraacetic acid (EDTA) and a small subset in DNA/RNA Shield (Zymo Research, Irvine, CA, USA) before being frozen at -80°C at the time of enrolment. DBS were concurrently made using 20µL whole blood spotted on Whatman 3MM cellulose chromatography filter paper and then stored in cardboard covers in individual sealed bags with desiccant at room temperature unexposed to direct light. A single donated *P. cynomolgi*-infected sample [24] obtained from a macaque host was frozen in glycerolyte and stored in liquid nitrogen prior to thawing, counting and immediately placing in DNA/RNA Shield.

Microscopic parasite count quantification

Microscopic diagnosis of *Plasmodium* species was undertaken by experienced research microscopists using thick and thin Giemsa-stained blood films. Microscopic quantification of parasitemia (parasites per microlitre) was performed using thick blood smears calculated from the number of parasites per 200 white blood cells, multiplied by the individual patient's total white cell count obtained from routine hospital laboratory flow cytometry [25].

Total nucleic acid extraction and PCR amplification

Total nucleic acids were directly extracted from 200 µL of whole blood using a QIAamp DNA Blood Mini Kit (QIAGEN, Cat. No. 51106), with DNA/RNA Shield samples eluted in 50µl AE buffer to account for the preservative dilution factor. DNA and RNA extraction from DBS were carried out using an in-house method, established by Zainabadi *et al*, 2017 [26]. Briefly, DBS equivalent to 40µl whole blood was incubated with 900µl lysis buffer at 65°C, with shaking at 250rpm for 90 minutes. Lysate was transferred to QIAamp DNA Blood Mini Kit (QIAGEN, Cat. No. 51106) columns, washed with modified buffers, dried at 65°C for 10 minutes and eluted in 40µl buffer AE. The primers, annealing temperatures and/or probe sequences for each PCR assay are described in Table 1. Real-time PCR amplifications were performed on a QuantStudio 6 Flex (Applied Biosystems). Conventional PCR was performed on a DNA thermal cycler (Bio-Rad T100™ thermal cycler). The amplified nested PCR products were separated by electrophoresis using 2% agarose gels, stained by SYBR Safe™ (Invitrogen), and visualised on a UV transilluminator (Gel Doc XR+ imaging system, Bio-Rad). Each PCR amplification included a *Plasmodium* species positive and negative control and molecular weight standards (Applied Biosystems). Positive (clinical malaria cases) and negative (healthy afebrile study participant) control samples were derived from venous whole blood collected in EDTA from Sabah participants with malaria status confirmed using validated reference PCR [21,22,27].

Plasmodium species confirmation using reference PCR

A validated reference PCR targeting the *Plasmodium* 18S rRNA genes was used to confirm the *Plasmodium* species using EDTA-whole blood clinical samples, consisting of an initial

Table 1. Primer sequences and annealing temperatures of *Plasmodium* genus and species-specific PCR assays.

Assay ID	PCR assays	Primer / Probe	Sequence (5'– 3')	Annealing temp. (°C)	PCR method	<i>Plasmodium</i> gene target
<i>Plasmodium</i> spp. screening	Kamau et al, 2011 [19]	KamGF	GCTCTTCTTGATTCTTGATG	60	RT-qPCR	18S rRNA
	(rRNA template)	KamGR	AGCAGGTTAAGATCTCGTTCG			
		KamGP probe	^{FAM} -ATGGCCGTTTTAGTTCGTG- ^{BHQ1}			
Test A (Pk)	Divis et al, 2010 [28]	Plasmo 1	GTTAAGGGAGTGAAGACGATCAGA	60	qPCR	18S rRNA
		Plasmo 2	AACCCAAAGACTTTGATTTCTCATAA			
		Pk probe	^{FAM} -CTCTCCGGAGATTAGAACTCTTAGATTGCT- ^{BHQ1}			
Test B (Pk)	Lubis et al, 2017 [29]	SICAf1	GGTCCTCTTGTTAAAGGAGG	55	Hemi-nested	SICAv
		SICAr1	CCCTTTTGGACATTCGTCC			
		SICAf2	CTTGGTAAAGGAGGACCACG			
		SICAr1	CCCTTTTGGACATTCGTCC			
Test C (Pk)	Lee et al, 2011 [22]	rPLU 1	TCAAAGATTAAGCCATGCAAGTGA			
		rPLU 5	CCTGTTGTTGCCTTAACTTC			
		Kn1f	CTCAACACGGGAAACTCACTAGTTTA	66	Nested	18S rRNA
		Kn3r	GTATTATTAGGTACAAGGTAGCAGTATGC			
Test D (Pcyn)	Lee et al, 2011 [22]	CY2F	GATTTGCTAAATTGCGGTCG	66		
		CY4R	CGGTATGATAAGCCAGGGAAGT			
Pk reference	Imwong et al, 2009 [27]	PkF1160	GATGCCTCCGCGTATCGAC	55	Hemi-nested	18S rRNA
		PkF1150	GAGTTCTAATCTCCGAGAGAAAAGA			
		PkF1140	GATTCATCTATTAAAAATTTGCTTC	50		
		PkR1150	GAGTTCTAATCTCCGAGAGAAAAGA			
Human-species reference	Snounou et al. (1993) [21]	rPLU6	TAAAAATTGTTGCAGTTAAACG	58	Nested	18S rRNA
		rPLU5	CCTGTTGTTGCCTTAACTTC			
		rFAL 1	TTAAACTGGTTTGGGAAAACCAATATATT			
		rFAL 2	ACACAATGAACTCAATCATGACTACCCGTC			
		rVIV 1	CGCTTCTAGCTTAATCCACATAACTGATAC			
		rVIV 2	ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA			
		rMAL 1	ATAACATAGTTGTACGTTAAGAATAACCGC			
		rMAL 2	AAAATTCCCATGCATAAAAAATTATACAAA			
		rOVA 1	ATCTCTTTTGCTATTTTCTAGTATTGGAGA			
		rOVA 2	GGAAAAGGACACATTAATTGTATCCTAGTG			

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Plasmodium genus (hereafter abbreviated to *P. genus*) nest 1, followed by species-specific nest 2 for *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* spp. [21]. The *P. knowlesi*- [27] and *P. cynomolgi*-specific [22] reference assays used also target 18S rRNA genes. The *P. knowlesi* reference assay has a previously reported high sensitivity (LOD less than 10 parasite genomes) when validated without reverse transcription (RT) against four *P. knowlesi* strains (Malayan, H, Philippine, and Hackeri); specificity was assessed against the major human *Plasmodium* species in addition to other simian *Plasmodium* species present in Southeast Asia including *P. cynomolgi*, *P. inui*, and *P. simiovale* [27].

Selection of PCR assays for LOD evaluation

A reverse transcriptase real-time hydrolysis probe (RT-qPCR) assay designed to detect the *P. lasmodium* genus was evaluated for its utility in enhancing LOD (Fig 1A) [19]. Three published *P. knowlesi* species-specific assays, which have not previously had their LODs evaluated using reverse transcription, were compared to the reference assay (Fig 1B). Test A is a qPCR assay [28] in current use in the State Public Health Laboratory (Makmal Kesihatan Awam; MKA) Sabah, Malaysia, for routine *P. knowlesi* malaria detection [30]. Test B is a hemi-nested PCR targeting the multi-copy *SICAvar* genes, previously validated for *P. knowlesi* detection against *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* spp., in addition to clinical *P. knowlesi* isolates from Sarawak, Malaysia [29]. Test C is a nested conventional PCR assay targeting 18S rRNA genes specific for *P. knowlesi* that has been robustly validated against *P. vivax* and other zoonotic *Plasmodium* species [22].

Limit of detection evaluation for PCR assays with and without RT

The initial microscopy-quantified *Plasmodium* species infected samples collected in DNA/RNA Shield were diluted with malaria-negative whole blood (also at the same manufacturer recommended DNA/RNA Shield ratio) to prepare individual parasite count dilutions ranging between 20 to 0.0002 parasites/μL. Total nucleic acids from samples at each serial parasite count dilution were then extracted and duplicate aliquots prepared. High-capacity cDNA reverse transcription (Applied Biosystems, Thermo Fisher Scientific, MA, USA) was then

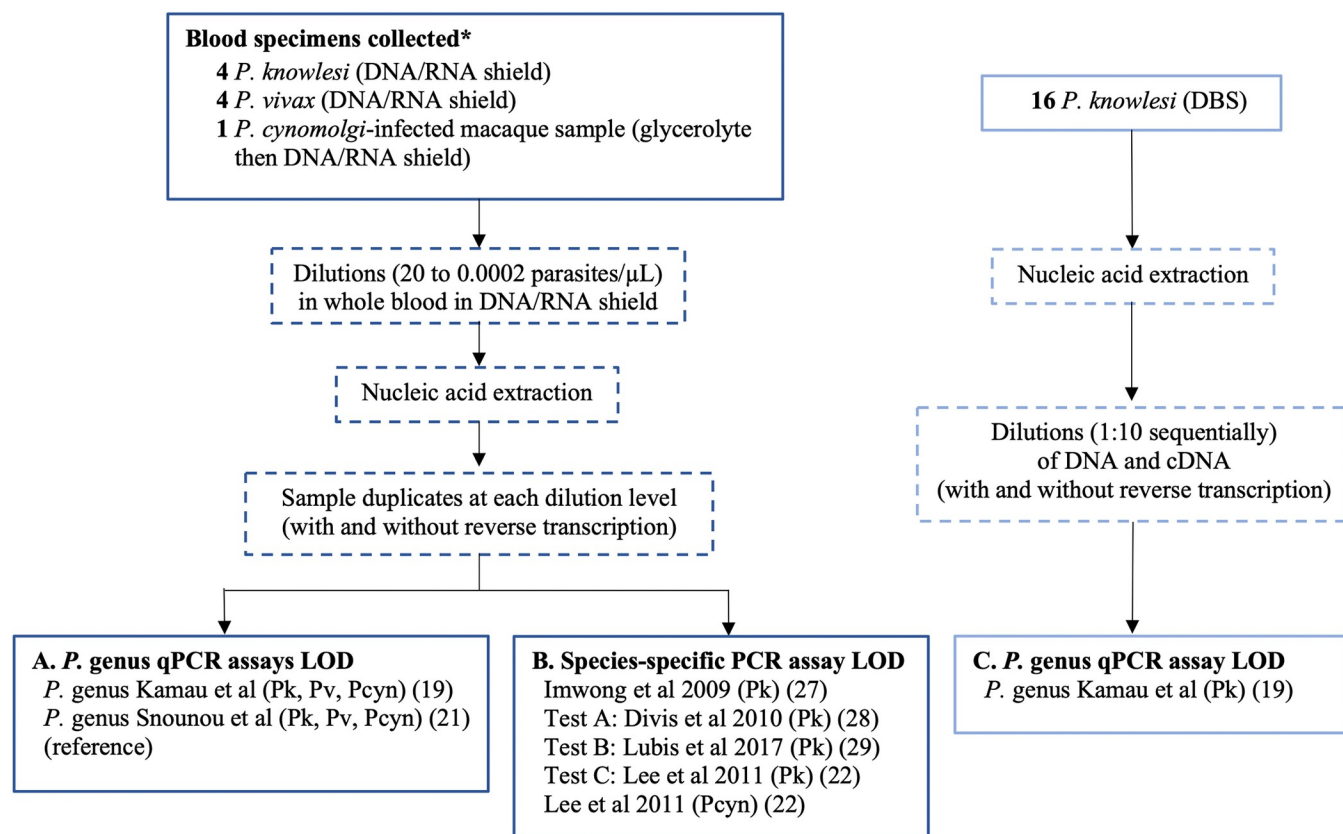


Fig 1. Limit of detection workflow for (A) *P. genus*, (B) *Plasmodium* species-specific, and (C) *P. genus* dried blood spot PCR assays. Abbreviations: DBS = dried blood spot; LOD = limit of detection; qPCR = real-time quantitative PCR; Pk = *P. knowlesi*; Pf = *P. falciparum*; Pv = *P. vivax*; Pcyn = *P. cynomolgi*; RT = reverse transcription.

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performed on one aliquot from each pair according to manufacturer's recommendations. PCR was performed on the paired aliquots to detect *P. genus* by the qPCR assay [19], followed by species-specific assays for: *P. knowlesi* by the reference hemi-nested assay [27] and test assays A, B, C [22,28,29]; *P. vivax* [21] and *P. cynomolgi* [22] by reference assays. The LOD was expressed as the lowest parasite count per microlitre of whole blood detected by an individual PCR assay in both amplification replicates (Fig 1C). All *P. genus* qPCR assays were run in duplicate for individual samples. The nested species-specific assays were run in singlicate for each sample.

Limit of detection of *P. genus* qPCR assay for dried blood spots (DBS) with and without RT

The LOD for the *P. genus* qPCR assay was also evaluated using archived DBS from *P. knowlesi* clinical infections with and without RT (Fig 1C). Nucleic acids were extracted from the DBS samples, with 10µl immediately reverse transcribed into cDNA. *P. genus* qPCR was conducted on serial 1:10 DNA and cDNA dilutions run in duplicate with the LOD calculated using the initial enumerated parasitemia divided by the corresponding dilution level.

Evaluation of PCR assay specificity on clinical malaria samples

For the analysis of *P. knowlesi*, *P. falciparum*, and *P. vivax* clinical isolates, in addition to a *P. cynomolgi* macaque-derived isolate, all samples were individually tested using the *P. genus* qPCR assay and the three *P. knowlesi*-specific PCR assays. Results were compared against the reference PCR. Specificity for the *P. genus* assay was evaluated for malaria detection (any *Plasmodium* species) versus malaria-negative controls, and for species-specific assays using the corresponding *Plasmodium* species infection versus other *Plasmodium* species and malaria-negative controls combined. Clinical whole blood samples collected in EDTA likely had RNA degradation upon thawing; therefore, reverse transcription was not performed for this part of the analysis.

Statistical analyses

Parasite counts for each clinical *Plasmodium* species infection were summarised using median and interquartile range (IQR). The median whole blood LOD was calculated with and without reverse transcription for the *P. knowlesi* and *P. vivax* isolates. To calculate the LOD fold-change, the LOD without RT was divided by the LOD with RT. One-way ANOVA was used to test for differences in parasite count distribution across *Plasmodium* species, followed by Student's t-test for pairwise comparisons of log-transformed data. Results of PCR assays evaluated against reference PCR were defined as true positive (TP), false negative (FN), true negative (TN), and false positive (FP), enabling calculation of diagnostic sensitivity (TP/TP+FN) and specificity (TN/TN+FP) with exact binomial 95% confidence intervals. All statistical analyses were performed using Stata version 17.0 (StataCorp, Texas, USA).

Results

Limit of detection of *P. genus* PCR assays

The LOD was performed on *P. knowlesi* (n = 4), *P. vivax* (n = 4) and *P. cynomolgi* (n = 1) whole blood isolates. For the *P. genus* Kamau et al. qPCR assay, without reverse transcription, the median LOD to detect each individual *Plasmodium* species was 2 parasites/µL (Table 2 and Fig 2A). With reverse transcription, the assay sensitivity for *P. knowlesi* improved with a LOD of ≤ 0.0002 parasites/µL for all four isolates (10,000-fold change). The LOD for both *P.*

Table 2. Limit of detection (LOD) of *Plasmodium* genus and species-specific PCR assays.

PCR assay	Target	Tested species	Samples tested	Replicates per sample	LOD without RT	LOD with RT	Fold change post RT (x)
<i>Plasmodium</i> genus detection							
Kamau et al. [19]	<i>P. genus</i>	Pk	4	2–4*	2 (1–2)	≤0.0002 (≤0.0002–0.002)	10000
	<i>P. genus</i>	Pv	4	2–4*	2 (1.1–2)	0.002 (0.002–0.101)	1000
	<i>P. genus</i>	Pc	1	2	2	0.002	1000
Total	<i>P. genus</i>	Pk,Pv,Pc	9	2–4*	2 (2–2)	0.002 (≤0.0002–0.002)	1000
Snounou et al. [21]	<i>P. genus</i>	Pk	4	2	2 (1.1–2)	0.0011 (0.006–0.0016)	1818
	<i>P. genus</i>	Pv	1	2	0.2	0.02	10
	<i>P. genus</i>	Pc	1	2	0.2	0.01	20
Total	<i>P. genus</i>	Pk,Pv,Pc	9	2–4*	1.1 (0.2–2)	0.0016 (0.001–0.01)	677
<i>Plasmodium</i> species-specific detection							
Imwong et al. [27]	Pk	Pk	4	2	2 (1–11)	0.000725 (0.0002–0.0016)	2759
Divis et al. [28]	Pk	Pk	4	2	0.2 (0.11–1.1)	≤0.0002 (≤0.0002–0.0002)	1000
Lubis et al. [29]	Pk	Pk	4	2	0.11 (0.02–0.2)	1.1 (0.2–2)	0.1
Lee et al. [22]	Pk	Pk	4	2	11 (2–20)	11 (1.1–20)	No change

Abbreviations: *P. genus* = *Plasmodium* genus; Pk = *P. knowlesi*; Pv = *P. vivax*; Pc = *P. cynomolgi*; RT = reverse transcription

LOD is the lowest parasitemia (parasites/μL) detected by all replicates for each PCR assay

LOD and fold-change post RT are reported as median (IQR) for *P. knowlesi* (n = 4) and *P. vivax* (n = 4 for Kamau assay)

*2 replicates tested per sample without RT; 4 replicates tested per sample with RT

#*P. vivax* assay cross-reacts with *P. cynomolgi*

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vivax and *P. cynomolgi* improved to 0.002 parasites/μL with RT (1,000-fold change); **Fig 2B**. In comparison, the reference Snounou *P. genus* assay had a slightly higher LOD for *P. vivax* and *P. cynomolgi* without RT (0.2 parasites/μL for both); however, with RT a less pronounced improvement in LOD was demonstrated at 0.02 and 0.01 parasites/μL, respectively.

Limit of detection of *Plasmodium* species-specific PCR assays

The *Plasmodium* species-specific LOD evaluation was performed on *P. knowlesi* (n = 4), *P. vivax* (n = 1) and *P. cynomolgi* (n = 1) whole blood isolates previously evaluated for *Plasmodium* genus detection. For the *P. knowlesi*-specific assays, the median LOD without and with reverse transcription was 2 and 0.0007 parasites/μL, respectively, for the Imwong et al. reference assay (2759-fold change); 0.2 and 0.0002 parasites/μL, respectively, for Test A (1000-fold); 0.11 and 1.1 parasites/μL, respectively, for Test B (no improvement); and 11 parasites/μL for both (no change) for Test C (**Table 2** and **Fig 2A**).

Without reverse transcription, the LODs using species-specific reference assays were 2 parasites/μL for *P. cynomolgi* and 20 parasites/μL for *P. vivax*. With reverse transcription, the LODs were 0.002 and 0.02 (1000-fold change) for *P. cynomolgi* and *P. vivax*, respectively. However, additional testing of the *P. cynomolgi* isolate using the reference *P. vivax*-targeted rVIV1/rVIV2 primers [21] amplified this target from the macaque-origin *P. cynomolgi* infection both without (0.2 parasites/μL) and with reverse transcription (0.02 parasites/μL), producing a false-positive *P. vivax* result (**Table 2** and **Fig 2B**).

The LOD with reverse transcription of the *P. genus* assay was comparable to the LOD of the best performing species-specific assay for *P. knowlesi* (≤0.0002 parasites/μL) and *P. cynomolgi* (0.002 parasites/μL) detection. In contrast, the *P. genus* assay had a superior LOD compared to the reference species-specific assay for *P. vivax* (0.002 vs 0.02 parasites/μL respectively).

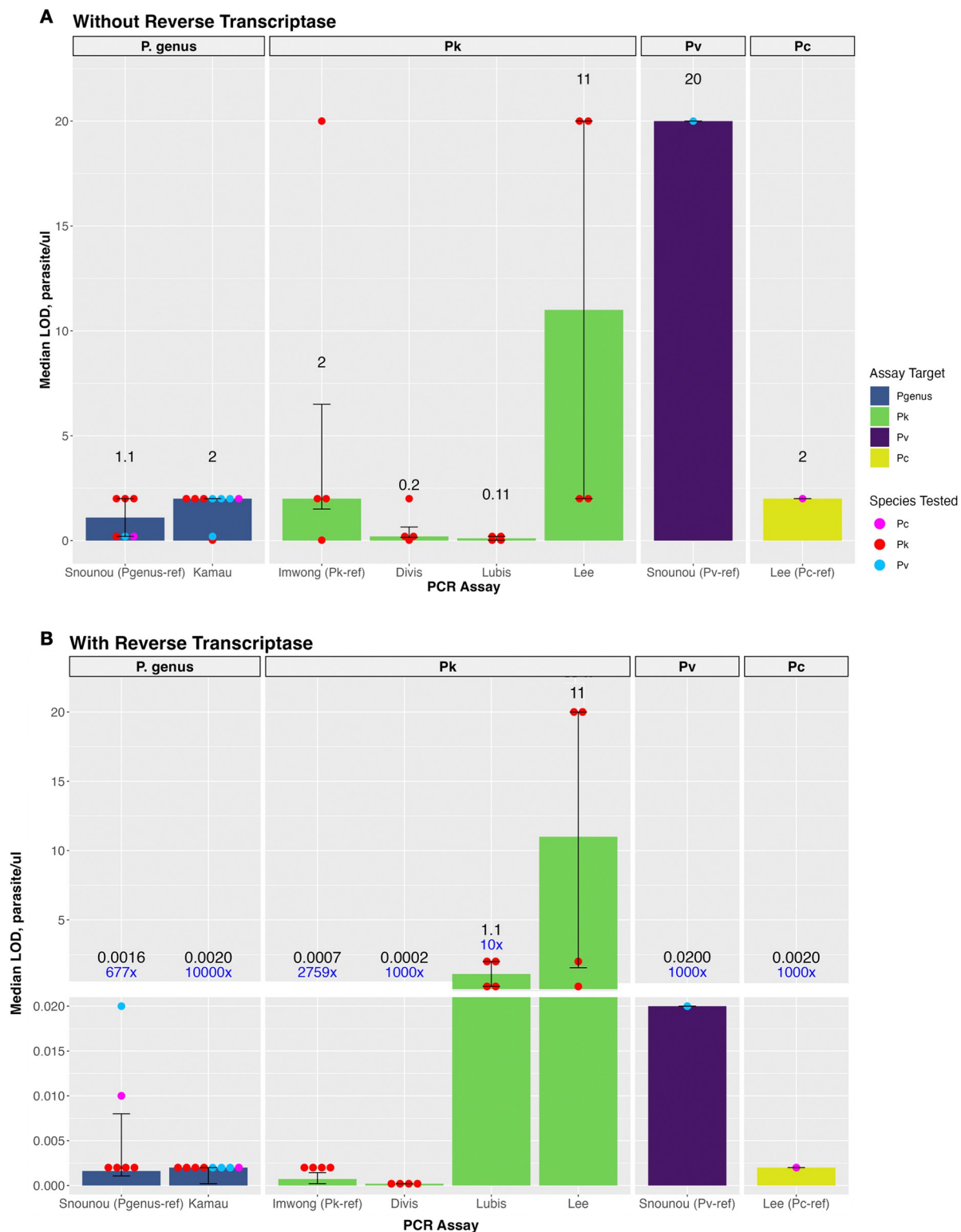


Fig 2. The median LOD and fold-change with and without reverse transcription for PCR assays to detect *Plasmodium* species. (A) The median LOD (parasites/ μ L) for *P. genus* assays using *P. knowlesi*, *P. vivax*, and *P. cynomolgi* samples without reverse transcription; (B) The median LOD (parasites/ μ L) for assays with reverse transcription. Error bars represent the IQR. Abbreviations: *P. genus*, *Plasmodium* genus; *Pk*, *P. knowlesi*; *Pv*, *P. vivax*; *Pc*, *P. cynomolgi*.

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Table 3. Limit of detection of the 18S rRNA *P. genus* qPCR assay for *P. knowlesi* dried blood spot samples.

Species evaluated	Initial parasitemia (/μL)	Time from DBS collection	LOD without RT (DNA)	LOD with RT (cDNA)	Fold change post RT (x)
Recent samples					
Pk	2663	8 months	26.6	0.027	1000
Pk	1377	8 months	13.8	0.138	100
Pk	198	8 months	2.0	0.020	100
Pk	26	8 months	26.0	2.600	10
Median	788	8 months	19.9	0.08	100
(IQR)	(198–1377)		(13.8–26.0)	(0.03–0.14)	(100–100)
Archived samples[†]					
Pk (N = 12)	372 (177–2604)	11 years (8–11)*	20 (2.5–20)	20 (2–20)	1 (1–1.25)

Limit of detection (parasites/μL) with and without RT of each *P. knowlesi* isolate was conducted in duplicate for each target species at each dilution level.

[†] Results are median (IQR)

*Range from 6 to 11 years

Pk = *P. knowlesi*

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Limit of detection of *P. genus* qPCR for dried blood spots (DBS)

The median LOD between DNA and cDNA generated from dried blood spots (DBS) for 4 *P. knowlesi* extracted samples collected 8 months prior to evaluation of the *P. genus* Kamau 2011 assay without and with RT was 19.86 and 0.08 parasites/uL, respectively (249-fold change) (Table 3). Archived DBS samples (n = 12) collected more than 6 years (up to 11 years) prior to extraction demonstrated a similar LOD with and without RT (median 20 parasites/μL).

Specificity of *P. genus* and individual *Plasmodium* species PCR assays

A total of 239 whole blood samples were included in the clinical evaluation of test specificity without RT, consisting of 96 *P. knowlesi*, 50 *P. vivax*, 44 *P. falciparum*, and 1 *P. cynomolgi* infected samples, and 48 malaria-negative controls. The median parasitemia was 1,957/μL (IQR 261–5,762; range 27–210,100 parasites/μL) for *P. knowlesi*, 3,246/μL (IQR 1,588–7,306; range 77–20,064 parasites/μL) for *P. vivax*, and 14,015/μL (IQR 2,193–33,413; range 34–297,000 parasites/μL) for *P. falciparum*.

The *P. genus* qPCR screening assay was both 100% specific and sensitive for the detection of *Plasmodium* species overall compared to the reference PCR, with all 48 samples from uninfected controls confirmed as malaria-negative.

P. knowlesi- assays for Tests A [28] and B [29] correctly identified all 96 *P. knowlesi* and 95 non-*P. knowlesi* samples, resulting in 100% specificity and sensitivity. Test C [22] was negative for a single *P. knowlesi* isolate with a parasite count of 1,535 parasites/μL, resulting in 99% (95% CI 94.3–100.0) sensitivity and 100% specificity. The reference assays for *P. falciparum* and *P. vivax* [21], and for *P. cynomolgi* [22] were negative for all *P. knowlesi* clinical isolates tested (Table 2).

Discussion

Malaria-susceptible countries in most of Southeast Asia, including those approaching or achieving WHO elimination of major human-only *Plasmodium* species, remain at-risk for zoonotic malaria transmission [31,32]. Understanding regional heterogeneity in *P. knowlesi* transmission intensity and disease morbidity will require the selective deployment of highly sensitive and specific molecular detection tools for both diagnostic and surveillance purposes

[23]. Our major finding demonstrated that the use of a reverse-transcription step after extraction of preserved total nucleic acids in clinical samples considerably improves the limit of detection of both the selected *P. genus* RT-qPCR screening assay, and *P. knowlesi*-specific assays (reference and Test A; both >1000-fold) by additionally amplifying ribosomal RNA sequences. The enhanced limit of detection was consistent across both field-stable DNA/RNA Shield samples and to a lesser degree in recent (although not older) dried blood spots. The second key finding of this study was the excellent performance of the *P. genus* screening assay, originally developed and validated for use in an African context for *P. falciparum* [19], to detect previously unvalidated species including *P. knowlesi*, *P. cynomolgi* and *P. vivax*. The specificity of each of the *P. knowlesi*-targeted assays to exclude *P. cynomolgi* and non-zoonotic *Plasmodium* species was confirmed to be excellent. Together these findings highlight the potential utility of incorporating these assays in a molecular surveillance approach to detect both human and zoonotic *Plasmodium* species that are well below the reported parasite count detection limits for current conventional PCR, loop-mediated isothermal amplification, microscopy or parasite lactate-dehydrogenase-based rapid diagnostic tests [23].

The community-based detection of submicroscopic *P. knowlesi* and *P. cynomolgi* infections, both symptomatic or asymptomatic, requires ultrasensitive molecular tools to understand the true extent of population-level transmission [23]. Recent studies in areas of both Peninsular Malaysia and the East Malaysian state of Sarawak have reported human infections in local communities living in or near forested areas with other macaque malaria species, including *P. inui*, *P. coatneyi*, *P. fieldi* and possibly *P. simiovale*, in addition to *P. knowlesi* and *P. cynomolgi* [33]. It is unclear whether or to what extent these low-level zoonotic infections may facilitate onward transmission to humans [34,35], as occurs with low parasitemia *P. falciparum* and *P. vivax* infections [36,37], although sustained human-to-human transmission of *P. knowlesi* has not been evident to date [22,38].

The selection of the major RT-qPCR *P. genus* screening assay aimed to maximise the detection limits for low-level zoonotic *Plasmodium* species infections using the known high copy number 18S rRNA target [20], and amplification of both the A- and S- type genes and their RNA transcripts [19]. The excellent LOD demonstrated with the *P. genus* RT-qPCR assay in the current study of <0.0002 parasites/μL for *P. knowlesi* detection is consistent with a previously reported extremely low LOD of ~0.0004 parasites/μL for clinical *P. falciparum* samples [19]. DNA-concentrated packed red blood cell samples have been demonstrated to further improve sensitivity for *P. falciparum* detection in population-based malaria prevalence surveys in elimination areas of Thailand [39]. Comparable performance to this *P. genus* RT-qPCR assay was reported with a separately established ultrasensitive quantitative PCR method (uPCR) with a limit of detection of 0.022 parasites/μL [39], however, a major advantage of the reverse transcriptase qPCR assay is the requirement for comparatively lower blood volumes [18]. Interestingly, in the current study the conventional PCR *P. genus* reference assay also demonstrated a large increase in analytical sensitivity after reverse transcription (~1800-fold) and may provide a more cost-effective option compared to qPCR for surveillance purposes. DNA/RNA Shield was selected as the preferred blood collection preservation method over other media due to its reported ability to stabilise DNA/RNA at ambient temperatures in field settings and compatibility with most DNA and RNA purification kits for subsequent high-throughput workflows including reverse transcription [40].

To date, only a few studies have incorporated reverse transcription in the molecular detection of *Plasmodium* species [41,42]. The reverse transcription step in the present study improved the analytical sensitivity of our selected assays to detect zoonotic *P. knowlesi*, *P. cynomolgi* and other human malaria infections by up to 10,000-fold (*Plasmodium* genus), 2759-fold (*P. knowlesi*), and 1000-fold (*P. vivax* and *P. cynomolgi*), respectively. The *P. knowlesi*-specific

hemi-nested reference assay with reverse transcription demonstrated a comparable limit of detection to the qPCR Test A (which requires an expensive real-time hydrolysis probe), and was superior to both Test B targeting *SICAvar* and Test C targeting the 18S rRNA gene. Without reverse transcription, the lowest limit of detection for *P. knowlesi* was seen with Test B, suggesting that the multiple chromosomal copies of the variant antigen *SICAvar* provide equivalent or better signal amplification than the detection of transcripts from whichever of these gene copies is activated in any particular parasite cell in the peripheral blood. Constraints on the widespread use of reverse transcription include the additional cost, laboratory time, and the usual rapid degradation of RNA molecules in field or laboratory settings. However, the degree of RNA amplification with reverse transcription was aided in our study by collecting blood samples in room-temperature stable RNA preservation media suitable for field-based surveillance, which also allows other potential downstream pathophysiological or transcriptomic analyses dependent on pathogen or host RNA transcripts.

The low reported limit of detection for the RT-qPCR *P. genus* assay conducted on DBS in this study (~0.08 parasites/ μ L with reverse transcription) suggests this type of sample collection would also enable the identification of a large proportion of submicroscopic and/or asymptomatic infections. DBS collection is logistically a more feasible, inexpensive and acceptable option particularly for asymptomatic or younger participants (given the need for finger-prick blood collection rather than venepuncture) for large-scale malaria surveillance surveys. The use of Whatman 3MM filter paper for storage and extraction of DBS with reverse transcription was justified compared to other potential media including Whatman 903 protein saver or FTA classic cards due to the much lower cost and superior detection limits seen when validated using *P. falciparum* mRNA gametocyte transcripts [43,44]. However, the reverse transcription step only improved the LOD in DBS samples that were collected within 8 months; older DBS stored in recommended conditions for more than 6 years did not provide any improvement in the LOD with and without RT due to likely degradation of RNA. Regardless, the *P. genus* LOD of around 2 to 20 parasites/ μ L without RT for DBS samples remains encouraging as a first-line option for surveillance screening purposes. The use of DBS would require further evaluation with *Plasmodium* species-specific PCR assay differentiation, similar to that conducted for the *SICAvar* assay on DBS for *P. knowlesi* which had a very low reported LOD of 0.1 parasites/ μ L [29].

The current study confirmed previous findings detailing cross-reactivity between the nested PCR primers for *P. vivax* (rVIV1/rVIV2) with *P. cynomolgi* [13]. A single mismatch in the 30 nucleotides of the rVIV2 primer sequences was reported to cross-amplify *P. cynomolgi* isolates [13]. The nested *P. vivax*-specific assay using the same primers rVIV1/rVIV2 designed to target the 18S rRNA gene also amplified *P. cynomolgi* in our LOD analysis [21]. The separate *P. cynomolgi* primers remained highly specific and did not erroneously amplify *P. vivax* or other closely related *Plasmodium* species DNA. In practice, the concurrent use of both assays would enable accurate identification of a *P. vivax* mono-infection, however, this approach would not be able to differentiate a *P. vivax*/*P. cynomolgi* co-infection from a *P. cynomolgi* mono-infection. Mis-identification of symptomatic *P. cynomolgi* infections as *P. vivax* would not result in inappropriate treatment, given both have a latent hypnozoite liver life-stage requiring additional radical cure with primaquine. Most other commonly used single-round multiplex [45] and qPCR assays [46] containing *P. vivax*-specific targets have also not been validated against isolates of *P. cynomolgi* or other closely related macaque *Plasmodium* species. However, a variety of sequencing approaches of targeted gene amplicons including mitochondrial COX1 and cytochrome b, *SICAvar* and SSU 18S rRNA followed by sequencing and reference alignment have been used to confirm unknown or mixed zoonotic *Plasmodium* species infections following initial ambiguous PCR results [47,48].

A limitation of this study was the inability to validate submicroscopic clinical *P. knowlesi* infections and other zoonotic species such as *P. fieldi*, *P. inui* and *P. coatneyi* for which samples were not available. Due to the increasing number of published *P. knowlesi* assays, we were not able to evaluate other *P. knowlesi* assays of possibly comparable performance within our selected workflow. There is a small inherent additional risk of contamination with the inclusion of a RT step in this type of ultra-sensitive PCR workflow. We were also unable to evaluate mixed infections of *P. knowlesi*, *P. vivax* and *P. falciparum* despite these cases being reported in certain areas such as Indonesia [29] and Vietnam [49]. Due to sample availability, we were also restricted to only a single *P. cynomolgi* sample to determine the LOD of the *P. cynomolgi*-specific assay [22]. The discrepancy between the LOD for the *P. genus* qPCR screening assay and the species-specific assay for *P. cynomolgi* detection may mean a proportion of very low-level *P. cynomolgi* infections are unable to be identified beyond a *P. genus* threshold using the current protocol.

Conclusions/Significance

The *Plasmodium* genus reverse transcriptase qPCR assay can provide highly sensitive screening for zoonotic and human malaria, including for submicroscopic infections in at-risk populations in endemic areas. The use of this molecular surveillance protocol for either whole blood or DBS collected samples in understudied areas of Southeast Asia would enable improved understanding of the regional disease burden and transmission dynamics of zoonotic malaria. Enhanced molecular tools and future iterative improvements to conventional surveillance protocols are especially critical as Southeast Asia continues to exert considerable public health efforts towards human malaria elimination despite the challenge of additional zoonotic *Plasmodium* species infections at an expanding human-animal-interface.

Disclaimers

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Supporting information

S1 Data. Limit of detection dataset for PCR assays.
(XLSX)

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References

1. Moyes CL, Henry AJ, Golding N, Huang Z, Singh B, Baird JK, et al. Defining the Geographical Range of the *Plasmodium knowlesi* Reservoir. *PLoS Neglected Trop Dis*. 2014; 8(3):e2780. <https://doi.org/10.1371/journal.pntd.0002780> PMID: 24676231
2. Cuenca PR, Key S, Jumail A, Surendra H, Ferguson HM, Drakeley C, et al. Epidemiology of the zoonotic malaria *Plasmodium knowlesi* in changing landscapes. *Adv Parasit*. 2021; 113:225–86. <https://doi.org/10.1016/bs.apar.2021.08.006> PMID: 34620384
3. Barber BE, William T, Grigg MJ, Menon J, Auburn S, Marfurt J, et al. A Prospective Comparative Study of *Knowlesi*, *Falciparum*, and *Vivax* Malaria in Sabah, Malaysia: High Proportion With Severe Disease From *Plasmodium knowlesi* and *Plasmodium Vivax* But No Mortality With Early Referral and Artesunate Therapy. *Clin Infect Dis*. 2013 Feb; 56(3):383–97. <https://doi.org/10.1093/cid/cis902> PMID: 23087389
4. Daneshvar C, Davis TME, Cox-Singh J, Rafa'ee MZ, Zakaria SK, Divis PCS, et al. Clinical and laboratory features of human *Plasmodium knowlesi* infection. *Clinical Infectious Diseases* [Internet]. 2009 Sep 15; 49(6):852–60. Available from: <http://cid.oxfordjournals.org/lookup/doi/> <https://doi.org/10.1086/605439> PMID: 19635025
5. Grigg MJ, William T, Barber BE, Rajahram GS, Menon J, Schimann E, et al. Age-Related Clinical Spectrum of *Plasmodium knowlesi* Malaria and Predictors of Severity. *Clin Infect Dis*. 2018 Aug 1; 67(3):350–9. <https://doi.org/10.1093/cid/ciy065> PMID: 29873683
6. Kotepui M, Masangkay FR, Kotepui KU, Milanez GDJ. Preliminary review on the prevalence, proportion, geographical distribution, and characteristics of naturally acquired *Plasmodium cynomolgi* infection in mosquitoes, macaques, and humans: a systematic review and meta-analysis. *BMC Infect Dis*. 2021; 21(1):259. <https://doi.org/10.1186/s12879-021-05941-y> PMID: 33711940
7. Grigg MJ, Lubis IN, Tetteh KKA, Barber BE, William T, Rajahram GS, et al. *Plasmodium knowlesi* detection methods for human infections—Diagnosis and surveillance. In: *Advances in Parasitology*

- [Internet]. Academic Press; 2021. p. 77–130. (Drakeley C, editor. *Advances in Parasitology*; vol. 113). Available from: <https://www.sciencedirect.com/science/article/pii/S0065308X21000270>
8. WHO. Malaria Policy Advisory Group (MPAG) meeting (March 2022) [Internet]. 2022 Apr. Available from: <https://www.who.int/publications/i/item/9789240048430>
 9. Barber BE, William T, Grigg MJ, Yeo TW, Anstey NM. Limitations of microscopy to differentiate *Plasmodium* species in a region co-endemic for *Plasmodium falciparum*, *Plasmodium vivax* and *Plasmodium knowlesi*. *Malaria J*. 2013 Jan 8; 12(1):8.
 10. Lee KS, Cox-Singh J, Singh B. Morphological features and differential counts of *Plasmodium knowlesi* parasites in naturally acquired human infections. *Malaria J*. 2009 Apr 23; 8(1):73–73. <https://doi.org/10.1186/1475-2875-8-73> PMID: 19383118
 11. Coutrier FN, Tirta YK, Cotter C, Zarinda I, González IJ, Schwartz A, et al. Laboratory challenges of *Plasmodium* species identification in Aceh Province, Indonesia, a malaria elimination setting with newly discovered *P. knowlesi*. *PLoS Neglected Trop Dis* [Internet]. 2018; 12(11):e0006924. Available from: <https://doi.org/10.1371/journal.pntd.0006924>
 12. Hartmeyer GN, Stensvold CR, Fabricius T, Marmolin ES, Hoegh SV, Nielsen HV, et al. *Plasmodium cynomolgi* as Cause of Malaria in Tourist to Southeast Asia, 2018. *Emerging infectious diseases*. 2019 Oct; 25(10):1936–9. <https://doi.org/10.3201/eid2510.190448> PMID: 31538931
 13. Ta TH, Hisam S, Lanza M, Jiram AI, Ismail N, Rubio JM. First case of a naturally acquired human infection with *Plasmodium cynomolgi*. *Malaria J*. 2014; 13(1):68. <https://doi.org/10.1186/1475-2875-13-68> PMID: 24564912
 14. Barber BE, William T, Grigg MJ, Piera K, Yeo TW, Anstey NM. Evaluation of the Sensitivity of a pLDH-Based and an Aldolase-Based Rapid Diagnostic Test for Diagnosis of Uncomplicated and Severe Malaria Caused by PCR-Confirmed *Plasmodium knowlesi*, *Plasmodium falciparum*, and *Plasmodium vivax*. *J Clin Microbiol*. 2013 Apr 1; 51(4):1118–23. <https://doi.org/10.1128/JCM.03285-12> PMID: 23345297
 15. Grigg MJ, William T, Barber BE, Parameswaran U, Bird E, Piera K, et al. Combining Parasite Lactate Dehydrogenase-Based and Histidine-Rich Protein 2-Based Rapid Tests To Improve Specificity for Diagnosis of Malaria Due to *Plasmodium knowlesi* and Other *Plasmodium* Species in Sabah, Malaysia. *J Clin Microbiol*. 2014 Apr; 52(6):2053–60. <https://doi.org/10.1128/JCM.00181-14> PMID: 24696029
 16. Tan AF, Sakam SS binti, Rajahram GS, William T, Isnadi MFAR, Daim S, et al. Diagnostic accuracy and limit of detection of ten malaria parasite lactate dehydrogenase-based rapid tests for *Plasmodium knowlesi* and *P. falciparum*. *Front Cell Infect Microbiol* [Internet]. 2022; 12:1023219. Available from: <https://www.frontiersin.org/articles/10.3389/fcimb.2022.1023219> PMID: 36325471
 17. Lee PC, Chong ETJ, Anderios F, Lim YA, Chew CH, Chua KH. Molecular detection of human *Plasmodium* species in Sabah using PlasmoNex™ multiplex PCR and hydrolysis probes real-time PCR. *Malaria Journal*. 2015 Jan 28; 14(1):28.
 18. Christensen P, Bozdech Z, Watthanaworawit W, Renia L, Malleret B, Ling C, et al. Reverse transcription PCR to detect low density malaria infections. *Wellcome Open Res*. 2021; 6:39. <https://doi.org/10.12688/wellcomeopenres.16564.2> PMID: 35592834
 19. Kamau E, Tolbert LS, Kortepeter L, Pratt M, Nyakoe N, Muringo L, et al. Development of a Highly Sensitive Genus-Specific Quantitative Reverse Transcriptase Real-Time PCR Assay for Detection and Quantitation of *Plasmodium* by Amplifying RNA and DNA of the 18S rRNA Genes. *J Clin Microbiol* [Internet]. 2011; 49(8):2946–53. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/21653767> <https://doi.org/10.1128/JCM.00276-11> PMID: 21653767
 20. Mercereau-Puijalon O, Barale JC, Bischoff E. Three multigene families in *Plasmodium* parasites: facts and questions. *Int J Parasitol* [Internet]. 2002; 32(11):1323–44. Available from: <https://www.sciencedirect.com/science/article/pii/S002075190200111X> [https://doi.org/10.1016/s0020-7519\(02\)00111-x](https://doi.org/10.1016/s0020-7519(02)00111-x) PMID: 12350369
 21. Snounou G, Viriyakosol S, Jarra W, Thaithong S, Brown KN. Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. *Molecular and biochemical parasitology*. 1993 Apr; 58(2):283–92. [https://doi.org/10.1016/0166-6851\(93\)90050-8](https://doi.org/10.1016/0166-6851(93)90050-8) PMID: 8479452
 22. 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. Kazura JW, editor. *PLoS Pathog*. 2011; 7(4):e1002015. <https://doi.org/10.1371/journal.ppat.1002015> PMID: 21490952
 23. Anstey NM, Grigg MJ. Zoonotic Malaria: The Better You Look, the More You Find. *J Infect Dis* [Internet]. 2019; 219(5):679–81. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/30295775> <https://doi.org/10.1093/infdis/jiy520> PMID: 30295775

24. Christensen P, Racklyeft A, Ward KE, Matheson J, Suwanarusk R, Chua ACY, et al. Improving in vitro continuous cultivation of *Plasmodium cynomolgi*, a model for *P. vivax*. *Parasitol Int*. 2022; 89:102589. <https://doi.org/10.1016/j.parint.2022.102589> PMID: 35470066
25. WHO. Microscopy for the detection, identification and quantification of malaria parasites on stained thick and thin blood films in research settings. World Health Organisation [Internet]. 2015; Available from: <https://apps.who.int/iris/handle/10665/163782>
26. Zainabadi K, Adams M, Han ZY, Lwin HW, Han KT, Ouattara A, et al. A novel method for extracting nucleic acids from dried blood spots for ultrasensitive detection of low-density *Plasmodium falciparum* and *Plasmodium vivax* infections. *Malar J* [Internet]. 2017; 16(1):377. Available from: <https://doi.org/10.1186/s12936-017-2025-3>
27. Imwong M, Tanomsing N, Pukrittayakamee S, Day NPJ, 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* [Internet]. 2009; 47(12):4173–5. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/19812279> <https://doi.org/10.1128/JCM.00811-09> PMID: 19812279
28. Divis PC, Shokoples SE, Singh B, Yanow SK. A TaqMan real-time PCR assay for the detection and quantitation of *Plasmodium knowlesi*. *Malar J* [Internet]. 2010; 9(1):344. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/21114872>
29. Lubis IND, Wijaya H, Lubis M, Lubis CP, Divis PCS, Beshir KB, et al. Contribution of *Plasmodium knowlesi* to Multispecies Human Malaria Infections in North Sumatera, Indonesia. *J Infect Dis* [Internet]. 2017; 215(7):1148–55. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/28201638> <https://doi.org/10.1093/infdis/jix091> PMID: 28201638
30. Nuin NA, Tan AF, Lew YL, Piera KA, William T, Rajahram GS, et al. Comparative evaluation of two commercial real-time PCR kits (QuantiFast™ and abTES™) for the detection of *Plasmodium knowlesi* and other *Plasmodium* species in Sabah, Malaysia. *Malaria J*. 2020 Aug; 19(1):306.
31. Shearer FM, Huang Z, Weiss DJ, Wiebe A, Gibson HS, Battle KE, et al. Estimating Geographical Variation in the Risk of Zoonotic *Plasmodium knowlesi* Infection in Countries Eliminating Malaria. Churcher TS, editor. *PLoS Neglected Trop Dis* [Internet]. 2016; 10(8):e0004915. Available from: <https://doi.org/10.1371/journal.pntd.0004915>
32. Tobin RJ, Harrison LE, Tully MK, Lubis IND, Noviyanti R, Anstey NM, et al. Updating estimates of *Plasmodium knowlesi* malaria risk in response to changing land use patterns across Southeast Asia. *PLOS Neglected Trop Dis*. 2024; 18(1):e0011570. <https://doi.org/10.1371/journal.pntd.0011570> PMID: 38252650
33. Yap NJ, Hossain H, Nada-Raja T, Ngui R, Muslim A, Hoh BP, et al. Natural Human Infections with *Plasmodium cynomolgi*, *P. inui*, and 4 other Simian Malaria Parasites, Malaysia—Volume 27, Number 8—August 2021—Emerging Infectious Diseases journal—CDC. *Emerg Infect Dis* [Internet]. 2021; 27(8):2187–91. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/34287122>
34. Fornace KM, Nuin NA, Betson M, Grigg MJ, William T, Anstey NM, et al. Asymptomatic and Submicroscopic Carriage of *Plasmodium knowlesi* Malaria in Household and Community Members of Clinical Cases in Sabah, Malaysia. *J Infect Dis*. 2016 Mar 1; 213(5):784–7. <https://doi.org/10.1093/infdis/jiv475> PMID: 26433222
35. Noordin NR, Lee PY, Bukhari FDM, Fong MY, Hamid MHA, Jelip J, et al. Prevalence of Asymptomatic and/or Low-Density Malaria Infection among High-Risk Groups in Peninsular Malaysia. *Am J Trop Med Hyg*. 2020; 103(3):1107–10. <https://doi.org/10.4269/ajtmh.20-0268> PMID: 32618263
36. Schneider P, Bousema JT, Gouagna LC, Otieno S, Vegte-Bolmer MV de, Omar SA, et al. Submicroscopic *Plasmodium falciparum* gametocyte densities frequently result in mosquito infection. *American Journal of Tropical Medicine and Hygiene*. 2007; 76(3):470–4. PMID: 17360869
37. Almeida ACG, Kuehn A, Castro AJM, Vitor-Silva S, Figueiredo EFG, Brasil LW, et al. High proportions of asymptomatic and submicroscopic *Plasmodium vivax* infections in a peri-urban area of low transmission in the Brazilian Amazon. *Parasites Vectors*. 2018; 11(1):194. <https://doi.org/10.1186/s13071-018-2787-7> PMID: 29558985
38. Fornace KM, Topazian HM, Routledge I, Asyraf S, Jelip J, Lindblade KA, et al. No evidence of sustained nonzoonotic *Plasmodium knowlesi* transmission in Malaysia from modelling malaria case data. *Nat Commun*. 2023; 14(1):2945. <https://doi.org/10.1038/s41467-023-38476-8> PMID: 37263994
39. Imwong M, Hanchana S, Malleret B, Renia L, Day NPJ, Dondorp A, et al. High-throughput ultrasensitive molecular techniques for quantifying low-density malaria parasitemias. Gilligan PH, editor. *Journal of clinical microbiology* [Internet]. 2014 Sep; 52(9):3303–9. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4313154/pdf/zjm3303.pdf>
40. Eberhardt E, Hendrickx R, Kerkhof MV den, Monnerat S, Alves F, Hendrickx S, et al. Comparative evaluation of nucleic acid stabilizing reagents for RNA- and DNA-based *Leishmania* detection in blood as

- proxy for visceral burdens. *J Microbiol Methods*. 2020; 173:105935. <https://doi.org/10.1016/j.mimet.2020.105935> PMID: 32376283
41. Adams M, Joshi SN, Mbambo G, Mu AZ, Roemmich SM, Shrestha B, et al. An ultrasensitive reverse transcription polymerase chain reaction assay to detect asymptomatic low-density *Plasmodium falciparum* and *Plasmodium vivax* infections in small volume blood samples. *Malar J*. 2015 Dec 23; 14(1):520. <https://doi.org/10.1186/s12936-015-1038-z> PMID: 26701778
 42. Bousema T, Banman SL, Taylor BJ, Rijpma SR, Morlais I, Yanow SK, et al. A Direct from Blood Reverse Transcriptase Polymerase Chain Reaction Assay for Monitoring *Falciparum* Malaria Parasite Transmission in Elimination Settings. *Am J Trop Med Hyg*. 2017; 97(2):533–43. <https://doi.org/10.4269/ajtmh.17-0039> PMID: 28722583
 43. Jones S, Sutherland CJ, Hermesen C, Arens T, Teelen K, Hallett R, et al. Filter paper collection of *Plasmodium falciparum* mRNA for detecting low-density gametocytes. *Malar J*. 2012; 11(1):266. <https://doi.org/10.1186/1475-2875-11-266> PMID: 22873569
 44. Pritsch M, Wieser A, Soederstroem V, Poluda D, Eshetu T, Hoelscher M, et al. Stability of gametocyte-specific Pfs25-mRNA in dried blood spots on filter paper subjected to different storage conditions. *Malar J*. 2012; 11(1):138. <https://doi.org/10.1186/1475-2875-11-138> PMID: 22545954
 45. Padley D, Moody AH, Chiodini PL, Saldanha J. Use of a rapid, single-round, multiplex PCR to detect malarial parasites and identify the species present. *Ann Trop Med Parasitol* [Internet]. 2003; 97(2):131–7. Available from: <https://doi.org/10.1179/000349803125002977> PMID: 12803868
 46. Rougemont M, Saanen MV, Sahli R, Hinrikson HP, Bille J, Jatton K. Detection of Four *Plasmodium* Species in Blood from Humans by 18S rRNA Gene Subunit-Based and Species-Specific Real-Time PCR Assays. *J Clin Microbiol* [Internet]. 2004; 42(12):5636–43. Available from: <https://journals.asm.org/doi/abs/https://doi.org/10.1128/JCM.42.12.5636-5643.2004> PMID: 15583293
 47. Imwong M, Madmanee W, Suwannasin K, Kunasol C, Peto TJ, Tripura R, et al. Asymptomatic Natural Human Infections With the Simian Malaria Parasites *Plasmodium cynomolgi* and *Plasmodium knowlesi*. *J Infect Dis* [Internet]. 2019; 219(5):695–702. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/30295822> <https://doi.org/10.1093/infdis/jiy519> PMID: 30295822
 48. Putaporntip C, Kuamsab N, Seethamchai S, Pattanawong U, Rojrung R, Yanmanee S, et al. Cryptic *Plasmodium inui* and *Plasmodium fieldi* Infections Among Symptomatic Malaria Patients in Thailand. *Clin Infect Dis*. 2021; 75(5):805–12.
 49. Marchand RP, Culleton R, Maeno Y, Quang NT, Nakazawa S. Co-infections of *Plasmodium knowlesi*, *P. falciparum*, and *P. vivax* among Humans and *Anopheles dirus* Mosquitoes, Southern Vietnam—Volume 17, Number 7—July 2011—Emerging Infectious Diseases journal—CDC. *Emerg Infect Dis*. 2011; 17(7):1232–9.