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A Sensitive, Colorimetric, High-Throughput Loop-Mediated Isothermal Amplification Assay for the Detection of *Plasmodium knowlesi*

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Abstract. The simian parasite *Plasmodium knowlesi* is now the commonest cause of malaria in Malaysia and can rapidly cause severe and fatal malaria. However, microscopic misdiagnosis of *Plasmodium* species is common, rapid antigen detection tests remain insufficiently sensitive and confirmation of *P. knowlesi* requires polymerase chain reaction (PCR). Thus available point-of-care diagnostic tests are inadequate. This study reports the development of a simple, sensitive, colorimetric, high-throughput loop-mediated isothermal amplification assay (HtLAMP) diagnostic test using novel primers for the detection of *P. knowlesi*. This assay is able to detect 0.2 parasites/ μ L, and compared with PCR has a sensitivity of 96% for the detection of *P. knowlesi*, making it a potentially field-applicable point-of-care diagnostic tool.

Human infection with the simian malarial parasite, *Plasmodium knowlesi* results from transmission from long-tailed and pig-tailed macaques, via the *Anopheles leucosphyrus* mosquito vector,¹ and has been documented in all countries of southeast Asia, except Laos and Timor Leste.¹ In Sabah, Malaysia, while the total number of reported malaria cases has decreased since 1992, the absolute number and proportion due to *P. knowlesi* have increased significantly such that in 2013, 62% of malaria notifications were due to *P. knowlesi*, making it the most common cause of malaria in this region.²

Plasmodium knowlesi malaria is difficult to diagnose by conventional microscopy. Ring and trophozoite stages of *P. knowlesi* appear morphologically very similar to both *Plasmodium falciparum* and *Plasmodium malariae*.¹ Microscopy has been found to be unreliable for diagnosing *P. knowlesi* in regions where it coexists with *P. falciparum* and *Plasmodium vivax*,^{3,4} resulting in inappropriate or delayed administration of anti-malarial treatment. Although high parasitemia has been found to predict severe disease, symptomatic disease frequently occurs at low parasitemia.^{5,6} There are currently no *P. knowlesi*-specific rapid diagnostic tests (RDTs), and those based on parasite lactate dehydrogenase (pLDH) and aldolase have been shown to be inadequately sensitive for clinical diagnosis.^{4,7} Of concern, Grigg and others reported that for the 31% of *P. knowlesi* malaria patients with parasitemia < 1,000 parasites/ μ L, pLDH-based RDTs had a sensitivity of only 12%.⁷

Currently, definitive diagnosis of *P. knowlesi* infection relies on polymerase chain reaction (PCR) performed retrospectively in reference laboratories. Tests that have been reported include nested PCR assays targeting either the 18S ribosomal small subunit⁸ or the cytochrome b mitochondrial gene.⁹ Assays have been configured as single-step PCR,¹⁰ real-time PCR,¹¹ multiplex PCR,¹² or semi-nested multiplex PCR.¹³ However, due to its cost and requirements for infrastructure and technical expertise, PCR remains a reference laboratory confirmation tool and is generally inaccessible in regional health-care facilities, making it unsuitable as a point-of-care diagnostic tool. Of the alternative molecular technologies, loop-mediated isothermal amplification (LAMP) offers much

potential as a field-applicable molecular diagnostic tool. Of the two published *P. knowlesi*-specific LAMP assays, primers targeting the beta tubulin gene using a Loopamp kit (Eiken Chemical Co. Ltd., Tokyo, Japan) are yet to be validated in human clinical samples.¹⁴ A LAMP assay targeting the *P. knowlesi* apical membrane antigen-1 reported a sensitivity of 100% and specificity of 98% in 74 clinical samples.¹⁵ However, it required post-amplification addition of SYBR (Invitrogen, Carlsbad, CA) which risks contamination. Although it has been recommended that LAMP be reserved for surveillance rather than point-of-care diagnostics, in view of the difficulties associated with the identification of *P. knowlesi* using microscopy and RDTs, there is a pressing need for developing point-of-care diagnostic alternatives for the diagnosis of *P. knowlesi* infection.

This report describes a sensitive, high-throughput, colorimetric, field-applicable LAMP assay targeting a mitochondrial gene that can detect *P. knowlesi* infection at low parasitemia.

Novel LAMP primers (Pk101) targeting a *Plasmodium* mitochondrial gene (Table 1) were designed based on modification of previously designed *P. vivax* LAMP primers.¹⁶ Dried blood spot (DBS) samples from patients with PCR-confirmed malaria caused by *P. knowlesi* ($N = 25$; median parasitemia 1,541 parasites/ μ L; range 75–95, 456), *P. vivax* ($N = 15$; median parasitemia 4,630 parasites/ μ L; range 441–110, 460), mixed *P. knowlesi/P. vivax* ($N = 1$; parasitemia 5,767 parasites/ μ L), *P. falciparum* ($N = 4$; median parasitemia 92,938 parasites/ μ L; range 11,024–362,420), and *P. malariae* ($N = 1$; parasitemia 3,354 parasites/ μ L) were used to validate the primers. These patients ($N = 46$) were a subset of patients enrolled in prospective clinical studies, with ethics approval granted by the Malaysian Medical Research Ethics Committee and Menzies School of Health Research, Australia, conducted in Kota Marudu district, Sabah, Malaysia.^{6,17} Approximately 20 μ L DBS were prepared from symptomatic patients presenting to the district hospital with a diagnosis of malaria infection by microscopy. Multiplex PCR was performed as described¹⁸ on DNA extracted using the Qiagen[®] DNA mini kit (Qiagen, Australia) from clinical samples to identify *Plasmodium* genus and human-only species (*P. falciparum*, *P. vivax*, *Plasmodium ovale*, and *P. malariae*), and nested PCR as described⁸ was used for identifying *P. knowlesi*. High-throughput loop-mediated isothermal amplification assay (HtLAMP) was performed on DNA extracted from DBS using a modified chelex

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TABLE 1

Plasmodium knowlesi Pk101 LAMP primer sequences (5' → 3')	
Abbreviation	Primer sequence
F3	GGTACTGGATGGACTTTTATAT
B3	GGTAATGTCAATAATAACATTACAG
LF	GATTACATCTACTGCAACAGG
LB	CTACTGTAATGCATTTAAGATC
FIP	CCAGACACTAAAAGACCAATCCAC CATTGAGTACATCACT
BIP	GCTAGTATTATGTCTTCTTCACTTA GTATACCAAGTGTTAAACC

LAMP = loop-mediated isothermal amplification.

protocol.¹⁹ Briefly, 6 mm DBS samples were incubated in 1 mL of 0.5% saponin in phosphate buffered saline (PBS) for 2 hours at 37°C, centrifuged, washed in PBS, heated at 98°C for 30 minutes in 150 µL of 6% chelex, and centrifuged at 4,000 rpm for 3 minutes. The resultant 100 µL supernatant was stored at -20°C.

To determine the limit of detection (LOD) of the assay, a PCR-confirmed *P. knowlesi* sample with a parasitemia determined by microscopy by two expert microscopists was diluted in *Plasmodium*-negative whole blood to produce a dilution series with parasitemia ranging from 2,000, 200, 20, 2, and 0.2 parasites/µL. The DNA from these samples was extracted using Qiagen[®] DNA mini kit (Qiagen, Australia) and tested in duplicate in the HtLAMP platform using Pk101 primers (HtLAMP-Pk) and published^{14,15} *P. knowlesi* primers (HtLAMP-Pk Iseki and HtLAMP-Pk Lau). HtLAMP-Pk was performed on clinical samples of *P. knowlesi* ($N = 8$), *P. falciparum* ($N = 1$), *P. vivax* ($N = 2$), *P. ovale* ($N = 1$), and *P. malariae* ($N = 1$) to determine species specificity of the primers. HtLAMP reactions were performed in 25 µL total volume containing 1× buffer (20 mM Tris HCl pH 8.8, 10 mM KCl, 8 mM MgSO₄, 10 mM (NH₄)₂SO₄), 1.25 mM each deoxyribonucleoside triphosphate, 1.78 µM each of forward inner primer/backward inner primer, 0.8 µM each of loop forward primer/loop backward primer, 0.2 µM each of forward outer primer/backward outer primer), 120 µM hydroxynaphthol blue (Fluka, Sigma-Aldrich, Australia, CAS number 63451-35-4), 8 units *Bst* polymerase (New England Biolabs, Ipswich, MA), and 5 µL of DNA. The HtLAMP-Pk assay was done in a 96-well microtitre plate, incubated in a water bath at 65°C for 30 minutes, the resultant color change and precipitate recorded visually and confirmed using a portable photo spectrometer at 600 nm with a turnaround time of 6 hours, as previously described.¹⁹ A blue color change with a visible precipitate was a positive result, and purple color without a precipitate was a negative result. PCR was used as the gold standard to calculate the sensitivity and specificity of HtLAMP-Pk. The performance of HtLAMP in a resource-limited setting had previously been validated in a district hospital laboratory in Sabah.¹⁹

The HtLAMP-Pk assay had a LOD of 0.2 parasites/µL, compared with 200–2,000 parasites/µL for HtLAMP-Pk Iseki and 20–2,000 parasites/µL for HtLAMP-Pk Lau (Table 2). It is postulated that the HtLAMP-Pk primers are considerably more sensitive than the other published primers due to the high copy number of the target mitochondrial gene. The HtLAMP-Pk assay did not cross-react with *P. falciparum*, *P. malariae*, and *P. ovale* but did cross-react with *P. vivax*. However, the HtLAMP-Pk assay was more sensitive (96%,

TABLE 2

Comparative LOD for three different *Plasmodium knowlesi* LAMP primers performed in the HtLAMP platform (each dilution was tested in duplicate using each of the three primers)

<i>P. knowlesi</i> parasitemia (parasites/µL)	HtLAMP-Pk	HtLAMP-Iseki	HtLAMP-Lau
2,000	Positive	Positive	Positive
200	Positive	Positive*	Positive*
20	Positive	Negative	Positive*
2	Positive	Negative	Negative
0.2	Positive	Negative	Negative
0	Negative	Negative	Negative

HtLAMP = high-throughput loop-mediated isothermal amplification; LAMP = loop-mediated isothermal amplification; LOD = limit of detection. "Positive" values indicate dilutions for which both duplicates were positive by HtLAMP. "Positive*" indicates samples with a single positive HtLAMP result, and "Negative" indicates samples for which both duplicates were negative by HtLAMP.

$N = 25/26$) for the detection of *P. knowlesi* compared with a cross-reacting *P. vivax* HtLAMP assay¹⁶ (sensitivity 42%, $N = 11/26$) and HtLAMP-Pk Iseki¹⁴ (sensitivity 81%, $N = 21/26$) (Table 3). The HtLAMP assay, validated in a district hospital laboratory in Kota Marudu, Sabah, Malaysia, was able to be performed in a resource-limited setting with chelex-extracted DNA from DBS samples, using a water bath, centrifuge, and portable photospectrometer.¹⁹

The main limitation of the HtLAMP-Pk assay is the cross-reactivity with *P. vivax*, which resulted in a low species specificity. Given the 97% sequence identity between these species at this mitochondrial target, it may be challenging to further modify primer sets for this target site to improve species specificity. Although artemisinin combination therapy is an effective treatment for both of these *Plasmodium* species,¹⁷ *P. vivax* infection would need to be confirmed and glucose-6-phosphate dehydrogenase deficiency should be excluded before the administration of primaquine. Furthermore, the LOD of HtLAMP-Pk on chelex-extracted DNA is yet to be determined. The diagnostic sensitivity of the HtLAMP-Pk assay also requires further validation in a larger set of samples from both microscopy positive and negative, and symptomatic and asymptomatic patients with *P. knowlesi* malaria to establish a possible role for the assay as a test for detecting low level parasitemia in cross-sectional surveys. This is warranted due to evidence of significant asymptomatic, submicroscopic *P. knowlesi* carriage amongst household contacts of symptomatic patients.²⁰ In addition, although the HtLAMP platform performed in a resource-limited laboratory setting demonstrated good diagnostic capability for identifying *Plasmodium* genus, *P. falciparum*,¹⁹ *P. vivax*,¹⁶ and *P. knowlesi* (using HtLAMP-Pk Iseki, data not shown), identification of *P. knowlesi* using the more analytically sensitive HtLAMP-Pk assay is yet to be similarly validated.

Nevertheless, it is well recognized that *P. knowlesi* parasitemia can rise rapidly and cause severe disease. Given the diagnostic limitations of currently available tests, there is a need to develop more sensitive diagnostic tools to facilitate early identification of infection with this parasite. Although microscopy may be the main stay of diagnosis for individual symptomatic patients, albeit without specificity,³ the HtLAMP-Pk assay offers a good analytical sensitivity, rapid turnaround time, capability of the platform to be performed in resource-limited settings, and potential for high throughput when multiple samples require testing. Therefore, with further refinement and validation, HtLAMP-Pk may have a potential role to

TABLE 3
Sensitivity and specificity of HtLAMP-Pk compared with PCR on symptomatic, microscopy positive clinical DBS samples

	Sensitivity (N = 26)	Specificity (N = 20)	Specificity, excluding <i>Plasmodium vivax</i> (N = 5)
HtLAMP-Pk	96% (25), 95% CI 80–99	30% (6), 95% CI 12–54	100% (5) 95% CI 48–100
HtLAMP-Pk Iseki	81% (21), 95% CI 61–93	100% (20), 95% CI 83–100	–
HtLAMP-Pv	42% (11), 95% CI 23–63	30% (6), 95% CI 12–54	–

CI = confidence interval; DBS = dried blood spot; HtLAMP = high-throughput loop-mediated isothermal amplification; LAMP = loop-mediated isothermal amplification; PCR = polymerase chain reaction. The diagnostic accuracy of HtLAMP using three different LAMP primers was compared with PCR on DBS samples that were PCR positive for *Plasmodium knowlesi* (N = 26) and PCR negative for *P. knowlesi* (N = 20).

play as a diagnostic tool for early detection of *P. knowlesi* in resource-limited settings.

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