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# A novel diagnostic method for malaria using loop-mediated isothermal amplification (LAMP) and MinION™ nanopore sequencer

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## Abstract

**Background:** A simple and accurate molecular diagnostic method for malaria is urgently needed due to the limitations of conventional microscopic examination. In this study, we demonstrate a new diagnostic procedure for human malaria using loop mediated isothermal amplification (LAMP) and the MinION™ nanopore sequencer.

**Methods:** We generated specific LAMP primers targeting the 18S–rRNA gene of all five human *Plasmodium* species including two *P. ovale* subspecies (*P. falciparum, P. vivax, P. ovale wallikeri, P. ovale curtisi, P. knowlesi* and *P. malariae*) and examined human blood samples collected from 63 malaria patients in Indonesia. Additionally, we performed amplicon sequencing of our LAMP products using MinION™ nanopore sequencer to identify each *Plasmodium* species.

**Results:** Our LAMP method allowed amplification of all targeted 18S–rRNA genes of the reference plasmids with detection limits of 10–100 copies per reaction. Among the 63 clinical samples, 54 and 55 samples were positive by nested PCR and our LAMP method, respectively. Identification of the *Plasmodium* species by LAMP amplicon sequencing analysis using the MinION™ was consistent with the reference plasmid sequences and the results of nested PCR.

**Conclusions:** Our diagnostic method combined with LAMP and MinION™ could become a simple and accurate tool for the identification of human *Plasmodium* species, even in resource-limited situations.

**Keywords:** Malaria, *Plasmodium ovale curtisi*, *Plasmodium ovale wallikeri*, LAMP, Nanopore sequencer, MinION™, Diagnosis

## Background

Malaria is a severe public health problem prevalent in tropical and subtropical areas worldwide. It is a mosquito-borne infectious disease caused by the following five protozoan parasites: *Plasmodium falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi*. There are emerging differences among first-line treatments for these parasites, which are dependent on the *Plasmodium* species and appropriate selection of anti-malarial drugs based on accurate recognition of the pathogens. Thus, it is important to correctly identify the pathogen after the initial diagnosis and before standard empirical chemotherapy commences. The conventional gold standard method of diagnosing malaria involves microscopic examination of Giemsa-stained thick blood smears. This method is inexpensive and easily implemented, even in remote endemic areas. However, microscopy is not as sensitive as molecular diagnostic methods for the identification of species, and both

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the sensitivity and specificity often depend on the technical skill of the investigator. In fact, compared with molecular diagnostic methods such as polymerase chain reaction (PCR), only half the number of patients with malaria are correctly diagnosed via microscopy in endemic areas [1, 2]. Additionally, some *Plasmodium* species, such as *P. knowlesi* and *P. malariae*, are difficult to distinguish from each other based on morphological features. The presence of *P. knowlesi* has most recently been reported in East Asian countries, especially Malaysia [3]. Although the first confirmed case of human *P. knowlesi* infection was reported in 1965 [4], it has now become clear from the results of a retrospective investigation in 2004 using PCR methods that many *P. knowlesi* human infection cases in Malaysia have been misdiagnosed as *P. malariae* by microscopy [5].

In recent years, *P. ovale* has been classified into two distinct subspecies, *P. ovale curtisi* and *P. ovale wallikeri*, based on molecular investigations [6–8], and it has been suggested that there are potential clinical differences such as symptoms and latency duration between the two subspecies [9, 10]. Thus, further epidemiological studies are required to determine the differences in prevalence and distribution between these two subspecies. Nested PCR or semi-nested PCR methods for detecting human *Plasmodium* spp. are widely used, but none can completely detect and simultaneously separate the five species, including the two *P. ovale* subspecies [11–13]. Therefore, a simple molecular diagnostic method corresponding to the latest classification of *Plasmodium* parasites is urgently needed to discriminate between *Plasmodium* spp.

Loop-mediated isothermal amplification (LAMP), which is an innovative nucleic acid amplification (NAA) technique, is regarded as a more rapid, easy-to-perform, and cost-effective procedure than PCR [14]. LAMP methods have already been used for the clinical diagnoses of several infectious diseases, including malaria, and are promising molecular technologies with high validity, even in resource-limited areas [15–37]. However, the following disadvantages of LAMP methods could be problematic: 1) the sequences of LAMP products are difficult to confirm; 2) LAMP primers are difficult to design because of the long nucleotide sequences of the primers; and 3) the primer sets and reaction tubes must be constructed individually, according to the target genes and samples.

The MinION™ nanopore sequencer is a pocket-sized and USB-connected portable real-time sequencer developed by Oxford Nanopore Technologies (ONT; Oxford, UK). The main advantages of the MinION™ are its portability, small platform, long reads, and real-time sequencing. In particular, sequence data can be made available in real time as they are generated and MinION™ can be easily transported and used away from laboratories under various clinical situations [38, 39]. In recent years, MinION™

has already been used to sequence PCR amplicons [40] or amplicons of RT-LAMP procedures [41].

Based on the polymorphisms of 18S–rRNA sequences in the *Plasmodium* species, we demonstrated a rapid and easy-to-perform diagnostic procedure for malaria, which takes advantage of the specific characteristics of the LAMP method as an NAA and MinION™ nanopore sequencer. We propose that this novel diagnostic approach could help to provide accurate diagnoses and appropriate treatments for malaria patients.

## Methods
### Plasmid construction
The templates used for the analytical LAMP reactions were six sets of pEX-A2J1 plasmids harboring partial sequences within the 18S–rRNA of six human *Plasmodium* parasites, *P. falciparum*, *P. vivax*, *P. ovale curtisi*, *P. ovale wallikeri*, *P. knowlesi* and *P. malariae* (comprising all five human plasmodium species including the two *P. ovale* subspecies) which were constructed by Eurofines Genomics Co., Ltd. (Tokyo, Japan) based on the reference sequences (GenBank accession numbers: *P. falciparum*, HQ283212.1; *P. vivax*, KF018662.1; *P. ovale curtisi*, KF696370.1; *P. ovale wallikeri*, KJ619947.1; *P. knowlesi*, KJ917903.1; and *P. malariae*, LT615260.1). The sequences of the constructed plasmids are summarized in Fig. 1. The plasmids were serially diluted 10-fold and adjusted from $1.0 \times 10^1$ copies/μL to $1.0 \times 10^6$ copies/μL to create 18S–rRNA standard solutions to determine the detection threshold and specificity of the LAMP reactions.

### Design of LAMP primers
The oligonucleotide LAMP primers for the detection of the five *Plasmodium* species were designed based on the sequences of 18S–rRNA, located on their highly conserved sequences, to contain each species-specific region within the F1-B1 primer pairs. Several candidate primer sets were suggested via online LAMP primer design software (PrimerExplorer 5, http://primerexplorer.jp/index.html; Eiken Chemical, Tokyo, Japan), and further refinement of the design was developed manually based on the criteria described in "A Guide to LAMP primer designing" (https://primerexplorer.jp/e/v4_manual/pdf/PrimerExplorer V4_Manual_1.pdf). Each selected primer sequence is given in Table 1, and their positions are shown in Fig. 1.

### LAMP reactions
The reactions were performed using a Loopamp DNA Amplification Reagent Kit (Eiken Chemical). Detection of the LAMP amplicons was performed by real-time measurements of turbidity using Loopamp EXIA (Eiken Chemical) and visual observation of color changes with the naked eye under natural light. The final reaction volume was 25 μL, comprising 80 pmol of the FIP
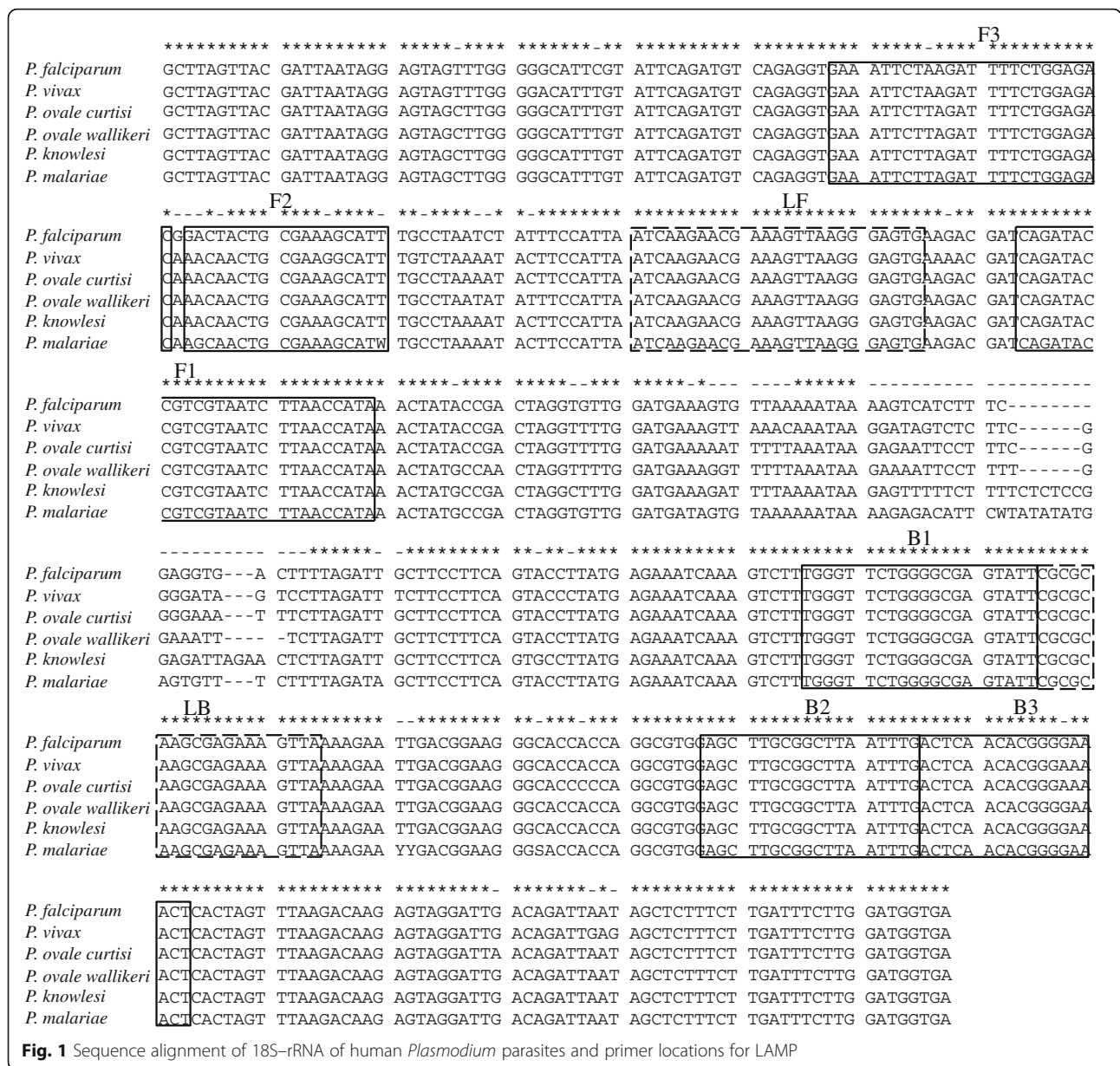
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                        *********  ********  ****-****  *******-**  *********  *********  *****-****  F3
                                                                                                    *********
P. falciparum       GCTTAGTTAC  GATTAATAGG  AGTAGTTTGG  GGGCATTCGT  ATTCAGATGT  CAGAGGTGAA  ATTCTAAGAT  TTTCTGGAGA
P. vivax            GCTTAGTTAC  GATTAATAGG  AGTAGTTTGG  GGACATTTGT  ATTCAGATGT  CAGAGGTGAA  ATTCTAAGAT  TTTCTGGAGA
P. ovale curtisi    GCTTAGTTAC  GATTAATAGG  AGTAGCTTGG  GGGCATTTGT  ATTCAGATGT  CAGAGGTGAA  ATTCTTAGAT  TTTCTGGAGA
P. ovale wallikeri  GCTTAGTTAC  GATTAATAGG  AGTAGCTTGG  GGGCATTTGT  ATTCAGATGT  CAGAGGTGAA  ATTCTTAGAT  TTTCTGGAGA
P. knowlesi         GCTTAGTTAC  GATTAATAGG  AGTAGCTTGG  GGGCATTTGT  ATTCAGATGT  CAGAGGTGAA  ATTCTTAGAT  TTTCTGGAGA
P. malariae         GCTTAGTTAC  GATTAATAGG  AGTAGCTTGG  GGGCATTTGT  ATTCAGATGT  CAGAGGTGAA  ATTCTTAGAT  TTTCTGGAGA

                    *---*-****  F2 ****-****-  **-****-**  *-*******  *********  LF *******  *******-**  *********
P. falciparum       GGGACTACTG  CGAAAGCATT  TGCCTAATCT  ATTTCCATTA  ATCAAGAACG  AAAGTTAAGG  GAGTGAAGAC  GATCAGATAC
P. vivax            CAAACAACTG  CGAAGGCATT  TGTCTAAAAT  ACTTCCATTA  ATCAAGAACG  AAAGTTAAGG  GAGTGAAAC   GATCAGATAC
P. ovale curtisi    CAAACAACTG  CGAAAGCATT  TGCCTAAAAT  ACTTCCATTA  ATCAAGAACG  AAAGTTAAGG  GAGTGAAGAC  GATCAGATAC
P. ovale wallikeri  CAAACAACTG  CGAAAGCATT  TGCCTAATAT  ATTTCCATTA  ATCAAGAACG  AAAGTTAAGG  GAGTGAAGAC  GATCAGATAC
P. knowlesi         CAAACAACTG  CGAAAGCATT  TGCCTAAAAT  ACTTCCATTA  ATCAAGAACG  AAAGTTAAGG  GAGTGAAGAC  GATCAGATAC
P. malariae         CAAGCAACTG  CGAAAGCATW  TGCCTAAAAT  ACTTCCATTA  ATCAAGAACG  AAAGTTAAGG  GAGTGAAGAC  GATCAGATAC

                    F1 ********  *********  ****-****  *****--***  *****-*---  ----*****-  ----------  ----------
P. falciparum       CGTCGTAATC  TTAACCATAA  ACTATACCGA  CTAGGTGTTG  GATGAAAGTG  TTAAAAATAA  AAGTCATCTT  TC--------
P. vivax            CGTCGTAATC  TTAACCATAA  ACTATACCGA  CTAGGTTTTG  GATGAAAGTT  AAACAAATAA  GGATAGTCTC  TTC------G
P. ovale curtisi    CGTCGTAATC  TTAACCATAA  ACTATACCGA  CTAGGTTTTG  GATGAAAAAT  TTTTAAATAA  GAGAATTCCT  TTC------G
P. ovale wallikeri  CGTCGTAATC  TTAACCATAA  ACTATGCCAA  CTAGGTTTTG  GATGAAAGGT  TTTTAAATAA  GAAAATTCCT  TTT------G
P. knowlesi         CGTCGTAATC  TTAACCATAA  ACTATGCCGA  CTAGGCTTTG  GATGAAAGAT  TTTAAAATAA  GAGTTTTTCT  TTTCTCTCCG
P. malariae         CGTCGTAATC  TTAACCATAA  ACTATGCCGA  CTAGGTGTTG  GATGATAGTG  TAAAAAATAA  AAGAGACATT  CWTATATATG

                    ----------  ---*******-  -*********  **-**-****  *********  *********  ********** B1  *********
P. falciparum       GAGGTG---A  CTTTTAGATT  GCTTCCTTCA  GTACCTTATG  AGAAATCAAA  GTCTTTGGGT  TCTGGGGCGA  GTATTCGCGC
P. vivax            GGGATA---G  TCCTTAGATT  TCTTCCTTCA  GTACCCTATG  AGAAATCAAA  GTCTTTGGGT  TCTGGGGCGA  GTATTCGCGC
P. ovale curtisi    GGGAAA---T  TTCTTAGATT  GCTTCCTTCA  GTACCTTATG  AGAAATCAAA  GTCTTTGGGT  TCTGGGGCGA  GTATTCGCGC
P. ovale wallikeri  GAAATT----  -TCTTAGATT  GCTTCTTTCA  GTACCTTATG  AGAAATCAAA  GTCTTTGGGT  TCTGGGGCGA  GTATTCGCGC
P. knowlesi         GAGATTAGAA  CTCTTAGATT  GCTTCCTTCA  GTGCCTTATG  AGAAATCAAA  GTCTTTGGGT  TCTGGGGCGA  GTATTCGCGC
P. malariae         AGTGTT---T  CTTTTAGATA  GCTTCCTTCA  GTACCTTATG  AGAAATCAAA  GTCTTTGGGT  TCTGGGGCGA  GTATTCGCGC

                    LB *******  *********  --********  **-***-***  *********  ********** B2  **********  B3 ***-**
P. falciparum       AAGCGAGAAA  GTTAAAAGAA  TTGACGGAAG  GGCACCACCA  GGCGTGGAGC  TTGCGGCTTA  ATTTGACTCA  ACACGGGGAA
P. vivax            AAGCGAGAAA  GTTAAAAGAA  TTGACGGAAG  GGCACCACCA  GGCGTGGAGC  TTGCGGCTTA  ATTTGACTCA  ACACGGGAAA
P. ovale curtisi    AAGCGAGAAA  GTTAAAAGAA  TTGACGGAAG  GGCACCCCCA  GGCGTGGAGC  TTGCGGCTTA  ATTTGACTCA  ACACGGGAA
P. ovale wallikeri  AAGCGAGAAA  GTTAAAAGAA  TTGACGGAAG  GGCACCACCA  GGCGTGGAGC  TTGCGGCTTA  ATTTGACTCA  ACACGGGGAA
P. knowlesi         AAGCGAGAAA  GTTAAAAGAA  TTGACGGAAG  GGCACCACCA  GGCGTGGAGC  TTGCGGCTTA  ATTTGACTCA  ACACGGGGAA
P. malariae         AAGCGAGAAA  GTTAAAAGAA  YYGACGGAAG  GGSACCACCA  GGCGTGGAGC  TTGCGGCTTA  ATTTGACTCA  ACACGGGGAA

                    *********  *********  *********-  *******-*-  *********  *********  ********
P. falciparum       ACTCACTAGT  TTAAGACAAG  AGTAGGATTG  ACAGATTAAT  AGCTCTTTCT  TGATTTCTTG  GATGGTGA
P. vivax            ACTCACTAGT  TTAAGACAAG  AGTAGGATTG  ACAGATTGAG  AGCTCTTTCT  TGATTTCTTG  GATGGTGA
P. ovale curtisi    ACTCACTAGT  TTAAGACAAG  AGTAGGATTA  ACAGATTAAT  AGCTCTTTCT  TGATTTCTTG  GATGGTGA
P. ovale wallikeri  ACTCACTAGT  TTAAGACAAG  AGTAGGATTG  ACAGATTAAT  AGCTCTTTCT  TGATTTCTTG  GATGGTGA
P. knowlesi         ACTCACTAGT  TTAAGACAAG  AGTAGGATTG  ACAGATTAAT  AGCTCTTTCT  TGATTTCTTG  GATGGTGA
P. malariae         ACTCACTAGT  TTAAGACAAG  AGTAGGATTG  ACAGATTAAT  AGCTCTTTCT  TGATTTCTTG  GATGGTGA
```

**Fig. 1** Sequence alignment of 18S–rRNA of human *Plasmodium* parasites and primer locations for LAMP

**Table 1** Nucleotide sequences of constructed LAMP primers for 18S–rRNA

| Primer | Sequence (5′ - 3′) | Length |
|---|---|---|
| F3 | GAAATTCTAAGATTTTCTGGAGAC | 24 |
| B3 | AGTTTTCCCGTGTTGAGT | 18 |
| FIP | TATGGTTAAGATTACGACGGTATCTG-RCAACTGCGAAGGCATW | 43 |
| BIP | TGGGTTCTGGGGCGAGTATT-CAAATTAAGCCGCAAGCT | 38 |
| LF | CACTCCCTTAACTTTCGTTCTTGAT | 25 |
| LB | CGCGCAAGCGAGAAAGTTA | 19 |

F3 and B3, outer primers; FIP and BIP, inner primers; LF and LB, loop primers
The FIP primer consisted of F2 and the complementary strand of F1 (F1c)
The BIP primer consisted of B2 and the complementary strand of B1 (B1c)

primer and 40 pmol of the BIP primer, 20 pmol of each loop primer, 5 pmol of F3 and B3 primers, 1 μL of the provided *Bst* DNA polymerase, reaction buffer (20 mM Tris-HCl, 10 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 0.8 M betaine, and 1.4 mM each dNTP), and 1 μL of plasmid DNA or 1–8 μL of genomic DNA samples. Reactions were carried out at 62 °C, 63 °C, 64 °C, and 65 °C in duplicate to find the optimum temperature for LAMP amplification.

### Detection limit and specificity of the LAMP assay

Ten-fold serial dilutions of each plasmid DNA, ranging from $1 \times 10^1$ to $1 \times 10^5$ copies/μL, were obtained and stored in duplicate at −20 °C until use, to determine the

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detection limit of the LAMP assay. Additionally, to evaluate the specificity of the test, we examined seven kinds of protozoa (*Toxoplasma gondii, Cryptosporidium parvum, Giardia intestinalis, Entamoeba histolytica, Leishmania donovani, Trypanosoma brucei rhodesiense,* and *Trypanosoma cruzi*) isolated by the National BioResource Project (http://www.nbrp.jp/).

### Clinical samples and ethics

Clinical samples were obtained from Sam Ratulangi University in Manado and Bitung, North Sulawesi, Indonesia, from August to December 2010. All patients were diagnosed based on the symptoms of malaria (fever, chills, sweats, fatigue, headache, nausea, vomiting, and general malaise) and blood smears stained with Giemsa stain by medical staff at the hospital. Peripheral blood was collected by FTA Elute cards (GE Healthcare Life Sciences, Little Chalfont, UK) from each patient and stored at room temperature. All samples were confirmed as harboring *Plasmodium* species by species-specific nested-PCR assay targeting the 18S rRNA gene to identify the five human *Plasmodium* species, according to previously reported protocols [42, 43].

Both the design and protocol of this study conformed to the Helsinki Declaration and were approved by the Institutional Ethics Committee. All samples were collected after obtaining written informed consent. The methods of collection and analysis of the human samples were approved and cleared by the Institutional Ethical Review Board of Sam Ratulangi University and the University of Tokyo (approval number 10–49).

### DNA extraction from clinical samples

Genomic DNA was extracted by following the manufacturer's instructions for FTA Elute cards. Briefly, each disk with a diameter of 3.0 mm was cut from the blood spot areas and washed three times with 200 μL of distilled water. DNA was eluted in 30 μL of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) with a heat block at 95 °C for 30 min. Finally, 1.0 μL of supernatant was used as template DNA for the LAMP reactions. If no amplification product was observed, 8 μL of supernatant was also used as template DNA, and we confirmed the final diagnosis by LAMP.

### Sequencing of LAMP products by MinION™

We performed sequencing of the amplicons obtained by LAMP with the MinION™ nanopore sequencer to identify the *Plasmodium* species. Six amplicons of plasmid DNA harboring the partial sequences of 18S–rRNA of each *Plasmodium* parasite from LAMP were used to validate the performance of MinION™. We prepared the DNA library for MinION™ by using the Ligation Sequencing Kit 2D (R9.4) (ONT, Oxford, UK) for each LAMP amplicon. Amplicons (each 2 μg) were processed for end-repair using the NEBNext Ultra™ II End Repair/dA-Tailing Module (New England Biolabs, Ipswich, MA), followed by purification with Agencourt AMPure XP beads (Beckman Coulter, Brea, CA). Each end-prepped DNA was subsequently subjected to adapter ligation with Blunt/TA Ligase Master Mix (New England Biolabs) to obtain the final DNA library following the ONT protocol of the Ligation Sequencing Kit 2D R9.4 version (SQK-LSK 208). Dynabeads MyOne™ Streptavidin C1 (Invitrogen, Carlsbad, CA) was used to elute the library in the pre-sequencing mix. After the MinION™ Platform QC run, the DNA library was loaded into the MinION™ flow cell (FLO-MIN 105 of 106 R9 version) and the standard 48-h sequencing protocol was initiated using the MinKNOW ONT software.

We used the Rapid Barcoding Sequencing Kit (SQK-RBK001) (ONT) for multiplex sequencing of LAMP amplicons from clinical samples, and thus were able to simultaneously sequence 12 samples on a single flow cell. Each LAMP amplicon (derived from one clinical sample) was purified by using Agencourt AMPure XP beads; 200 ng of each LAMP amplicon was processed for MinION™ barcoding and library preparation. Finally, a mixture of 12 barcoded DNA libraries was loaded into another MinION™ flow cell (FLO-MIN 105 of 106 R9 Version or FLO-MIN107 R9.4 version) and the standard 48-h 1D sequencing protocol was initiated using the MinKNOW ONT software.

### Data analysis

Data analysis was performed according to the protocol described in previous reports [44–46]. Raw read data were obtained by the standard 48-h sequencing protocol in the MinKNOW software (v1.1.21) and basecalled via the '2D Basecalling plus Barcoding' or '1D Basecalling plus Barcoding' workflow (v1.25) of Metrichor (v2.43.1). FASTQ sequences of 2D or 1D pass reads were extracted using Poretools (v0.6.0). 2D or 1D pass data were mapped by BWA-MEM (v0.7.15) using six sequences of *Plasmodium* 18S–rRNA between a pair of LAMP primers, F1c (5′-CAGATACCG TCGTAATCTTAACCATA-3′) and B1c (5′-TGGGTT CTGGGGGCGAGTATT-3′). Mapped data were applied in IGVsoftware (v2.3.80) after filtering with mapQuality 60 by BamTools (v 2.3.0). Based on the mapped data, several mapped reads at each of the sequences of *Plasmodium* 18S–rRNA per total number of mapped reads >0.2 were selected as a "consensus" for each sample. Consensus alignment was obtained using SAMtools (v1.4) and BCFtools (v1.4), following BlastN (v2.5.0) for queries against the database nucleotide collection (nr/nt).

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## Results

### Detection limit and specificity of LAMP

To determine the lower detection limit, 10-fold serial dilutions of each plasmid DNA were amplified and the optimal temperature observed was 62 °C. The results of real-time turbidity detection with LA-200 and amplification of the target DNA are indicated by the rising curve (Fig. 2). The minimum amounts of plasmid DNA on real-time turbidities with LA-200 were $1.0 \times 10^1$/reaction (*P. falciparum, P. ovale curtisi, P. knowlesi, P. malariae*) and $1.0 \times 10^2$/reaction (*P. vivax, P. ovale wallikeri*) within 60 min (Fig. 2). Furthermore, these results resembled those detected visually based on color changes within 60 min with the naked eye (data not shown). Additionally, 1.0 ng of the genomic DNA, derived from seven kinds of human protozoan parasites, was not completely amplified by our LAMP method (data not shown).

### Identification of plasmodium species by LAMP assay and MinION™

Table 2 shows the results obtained by MinION™ sequencing for the identification of *Plasmodium* species. LAMP amplicons targeting 18S–rRNA from six plasmid DNA molecules containing each species-specific 18S–rRNA were sequenced by MinION™. The *Plasmodium* species identified by MinION™ sequencing were consistent with the sequencing data of the constructed plasmids and the highest homology of reference sequences for *Plasmodium* parasite sequences from the NCBI gene databases.

The results of nested PCR and our LAMP method combined with the MinION™ sequencer are summarized in Table 3 and Additional file 1: Table S1. A total of 63 blood samples were obtained from patients with suspected malaria in a clinical setting and parasitemia was confirmed in 54 samples by nested-PCR assay (36 samples had

*P. falciparum*, 17 had *P. vivax,* and 1 had a combination of *P. falciparum* and *P. vivax*). All these samples were found to be positive by our LAMP method, and the results of species identification by MinION™ sequencing were consistent with those of nested PCR. Our LAMP method confirmed the negative status of 8 of the 9 negative samples identified by nested PCR. One sample was found to be positive by our LAMP method and subsequent data analysis of MinION™ sequences identified *P. falciparum*.

## Discussion

In this study, we constructed a novel tool for the identification of six *Plasmodium* parasites, including two *P. ovale* subspecies, using MinION™ portable sequencer and the LAMP method. We demonstrated that the method can achieve a comprehensive diagnosis of malaria even in resource-limited endemic regions. In particular, the LAMP method employs a set of four specially designed primers that recognize six distinct sequences on the target DNA, and relies on an auto-cycling procedure under isothermal conditions. In addition, our method enabled rapid library preparation (within 30 min), with multiplex sequencing and streaming analysis of real-time sequencing data via the Rapid Barcoding Sequencing Kit. Therefore, our LAMP method combined with the MinION™ sequencer would be convenient to use in various clinical settings.

Recently, Loopamp™ MALARIA Pan/Pf Detection Kit (Eiken Chemical) has become commercially available for the diagnosis of malaria by the LAMP method. This LAMP kit has good specificity and a lower detection limit of 25 parasites/μL that exceeds the detection sensitivity of rapid diagnostic test (RDT) kits for *P. falciparum*-specific histidine-rich protein II, which often yields false-negative
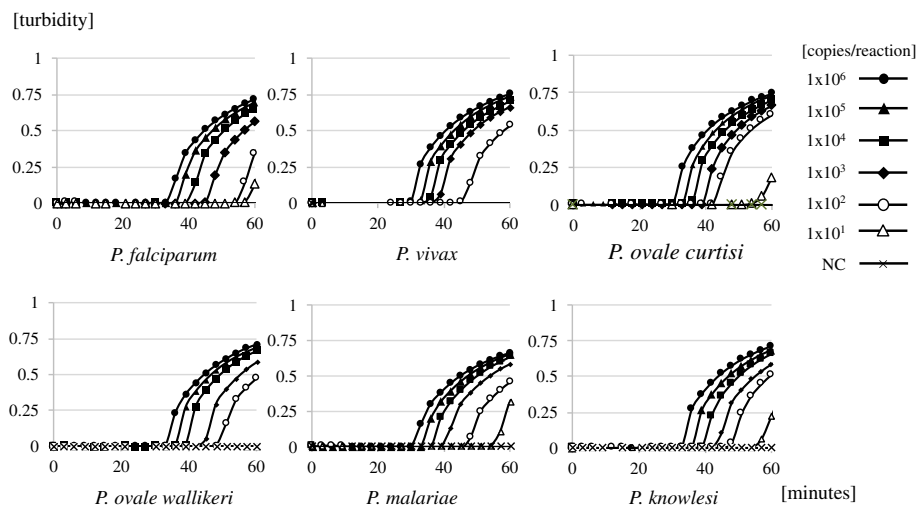


**Fig. 2** Detection limit of LAMP for the six human *Plasmodium* species with 10-fold serial dilutions of each plasmid DNA

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**Table 2** Summary of the MinION™ sequencing of LAMP amplicons

| Samples | Mapped reads | Aligned bases | Mean coverage | % Identity | Identification of species with MinION™ |
|---|---|---|---|---|---|
| Plasmids | | | | | |
| *P. falciparum* | 97,290 | 7,321,826 | 91,522.825 | 100 | *P. falciparum* |
| *P. vivax* | 105,097 | 8,164,884 | 99,571.7561 | 100 | *P. vivax* |
| *P. ovale curtisi* | 58,084 | 4,963,139 | 54,539.98901 | 100 | *P. ovale curtisi* |
| *P. ovale wallikeri* | 1054 | 77,098 | 963.725 | 100 | *P. ovale wallikeri* |
| *P. knowlesi* | 3254 | 244,871 | 2986.231707 | 100 | *P. knowlesi* |
| *P. malariae* | 627 | 51,135 | 581.0795455 | 100 | *P. malariae* |

*Clinical evaluation of LAMP assay and MinION™*

results when the parasite density is low, especially when asexual parasitemia is below 200 parasites/μL [47, 48]. RDTs are the most convenient tools for the diagnosis of *P. falciparum* parasites as point-of-care testing in the endemic field because they do not require electricity or special equipment. However, *plasmodium* lactate dehydrogenase or aldolase-based RDT kits cannot detect non-*P. falciparum* adequately because of their low sensitivity [49–53]. In this study, the detection limit of our LAMP method for all *Plasmodium* species was $1.0 \times 10^1$ to $1.0 \times 10^2$ copies/reaction (1–8 μL of sample DNA could be loaded per reaction); therefore, the theoretical detection limit of the LAMP assay was equivalent to that of the previously described Loopamp™ MALARIA Pan/Pf Detection Kit. Additionally, our LAMP method combined with the MinION™ sequencer has the following advantages: (a) equally high detection sensitivity for all *Plasmodium* species using our protocol, (b) multiplex sequencing and streaming analysis of real-time sequencing data [54] can be achieved, and (c) detection of mixed parasite infections is possible.

Nested-PCR and real-time PCR methods for malaria have previously demonstrated higher sensitivity and specificity than microscopy and RDTs [55]. However, the analytical positive and negative rate of our LAMP method was equivalent to that of nested-PCR methods,

showing its potential as a highly practical diagnostic tool. Among the 63 clinical samples, the results of only 1 were discordant between nested PCR and our LAMP method; nested PCR gave a negative result while our LAMP method gave a positive result. The species was identified as *P. falciparum* based on the LAMP amplicon sequencing by MinION™. It is thought that the parasite density was very low in this sample and below the detection limit of nested PCR. Furthermore, our technology has the advantage of being able to comprehensively and simultaneously distinguish and identify six human malaria parasites including two *P. ovale* subspecies, whereas both PCR-based protocols and RDTs cannot comprehensively detect and separate all species. Adams et al. reported that the LAMP primer set does not require stringent reaction conditions, and the reaction is successful at a broad temperature range of 60–66 °C. They demonstrated that the set should be suitable for use in the LAMP assay even in water baths where the temperatures may fluctuate widely [56]. However, nested-PCR and real-time PCR methods remain unsuitable for routine diagnostic methods in hospitals and field clinics in resource-limited endemic regions, because they require expensive devices such as thermal cyclers or real-time PCR systems for accurate temperature control.

**Table 3** Summary of the results of nested PCR and our LAMP method combined with MinION™ sequencer

| | | Sequence confirmation using MinION™ sequencer | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *P.f.* | *P.v.* | *P.f./P.v.* | *P.o.w.* | *P.o.c.* | *P.k.* | *P.m.* | Neg |
| Nested PCR | *P.f.* (*n* = 36) | 36 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | *P.v.* (*n* = 17) | 0 | 17 | 0 | 0 | 0 | 0 | 0 | 0 |
| | *P.f./P.v.* (n = 1) | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | *P.o.* (*n* = 0) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | *P.k* (n = 0) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | *P.m.* (n = 0) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Neg (*n* = 9) | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 8 |
| | Total (*n* = 63) | 37 | 17 | 1 | 0 | 0 | 0 | 0 | 8 |

*P.f., P. falciparum; P.v. P. vivax; P.o., P. ovale; P.o.w., P. ovale wallikeri; P.o.c., P. ovale crutisi; P.k., P. knowlesi; P.m., P. malariae;* Neg, negative

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Techniques for genotyping gene polymorphisms without sequence analysis have been previously developed using the high specificity of the LAMP assay, including LAMP with FIP and/or BIP primers, which were designed to contain a single-nucleotide polymorphism at each 5′ end and prevent the strands from annealing completely with the mutant allele to form the essential dumbbell-like structure [57–59]. Alternatively, peptide nucleic acid-locked nucleic acid mediated LAMP and real-time LAMP combined with fluorescent probes and melting or annealing curve analyses have been reported for genotyping polymorphisms [60, 61]. However, the design of LAMP primers for the above-mentioned protocol is not only quite difficult and limited, but is less sensitive than conventional LAMP assays as a result. In contrast, by our methods with MinION™ sequencing, LAMP primer design for genotyping polymorphisms for the absolute identification of *Plasmodium* parasite species is possible without limitations and could be applicable to simple mutation analysis or genotyping as the next step.

The MinION™ has been widely used to sequence various pathogens in many clinical situations because of its palm-sized platform, USB-3 interfaced equipment, real-time accessibility of sequenced data, and very low initial costs. In addition, the MinION™ can read multiplexed samples (up to 96) in one flow cell via the MinION™-specific barcoding primers (PCR Barcoding Kit 96 (R9), ONT); therefore, the running costs and time taken for analyses are reduced [62]. However, we do not have clinical samples to confirm the detection limits of *P. ovale*, *P. knowlesi,* and *P. malariae;* thus, we have not demonstrated the utility of our method for detecting these *Plasmodium* infections in clinical samples. An accurate evaluation of the sensitivity and specificity of our method requires further investigation in a clinical setting.

## Conclusion

In this study, we demonstrated an innovative diagnostic technology for *Plasmodium* infection that is rapid, simple, and highly sensitive using the LAMP assay combined with MinION™ nanopore sequencer. Our method could contribute to the provision of appropriate treatment for malaria and could also be applied to further analyses of sequence polymorphisms or genotyping. To refine this procedure, the next step will be to conduct a clinical evaluation to ensure sufficient and consistent sensitivity and specificity in resource-limited endemic regions.

## Additional file

**Additional file 1:** The result of nested-PCR, LAMP method and MinIONTM sequencing of LAMP amplicons. (XLSX 19 kb)

**Availability of data and materials**
The datasets supporting the conclusions of this article are included within the article.

**Authors' contributions**
JT YS JY TMa, study concept and design; KI NT KM LRR JS TMa data gathering, data analysis, manuscript drafting, and editing; KH YE RM TMu SM YS JY, study supervision and manuscript revision; YS JY TMa, project administration. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**
Ethical clearances were obtained from Institutional Ethical Review Board of Sam Ratulangi University and The University of Tokyo (approval number 10–49). The purpose of the study was explained in detail and written, informed consent was obtained from all participants. Confidentiality was maintained by avoiding the use of names and other identifiers.

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare that they have no competing interests.

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