Development of a Multiplex Loop-Mediated Isothermal Amplification Assay for Diagnosis of *Plasmodium* spp., *Plasmodium falciparum* and *Plasmodium vivax*

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Abstract: Malaria, caused by the parasite *Plasmodium* and transmitted by mosquitoes, is an epidemic that mainly occurs in tropical and subtropical regions. As treatments differ across species of malarial parasites, there is a need to develop rapid diagnostic methods to differentiate malarial species. Herein, we developed a multiplex malaria Pan/Pf/Pv/IC LAMP to diagnose *Plasmodium* spp., *P. falciparum*, and *P. vivax*, as well as the internal control (IC), within 40 min. The detection limits of the multiplex malaria Pan/Pf/Pv/IC LAMP were $1 \times 10^2$, $1 \times 10^2$, $1 \times 10^2$, and $1 \times 10^3$ copies/µL for four vectors, including the 18S rRNA gene (*Plasmodium* spp.), lactate dehydrogenase gene (*P. falciparum*), 16S rRNA gene (*P. vivax*), and human actin beta gene (IC), respectively. The performance of the LAMP assay was compared and evaluated by evaluating 208 clinical samples (118 positive and 90 negative samples) with the commercial RealStar® Malaria S&T PCR Kit 1.0. The developed multiplex malaria Pan/Pf/Pv/IC LAMP assay showed comparable sensitivity (100%) and specificity (100%) with the commercial RealStar® Malaria S&T PCR Kit 1.0 (100%). These results suggest that the multiplex malaria Pan/Pf/Pv/IC LAMP could be used as a point-of-care molecular diagnostic test for malaria.

Keywords: *Plasmodium* spp.; *Plasmodium falciparum*; *Plasmodium vivax*; LAMP; multiplex LAMP

1. Introduction

Malaria, which is caused by the *Plasmodium* protozoan and transmitted by mosquitoes, is an epidemic that predominantly occurs in tropical and subtropical regions [1]. Malaria infection has mainly been reported in Africa and several other countries, and more than 40% of the world’s population remains at risk from various malarial species [2]. According to the World Health Organization (WHO), 409,000 malaria deaths were reported worldwide in 2019 [3]. *Plasmodium* species that infect humans include *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium knowlesi* [4]. *P. falciparum* accounts for 99.7% of malaria cases in Africa and causes fatal symptoms. *P. vivax* induces weaker symptoms than *P. falciparum* but is known to occur worldwide and has a 75% infection rate in the Americas [3]. Malaria infections commonly cause frequent fevers, anemia, and an enlarged spleen; however, treatments differ for each species [5]. Malaria treatments are largely divided into chloroquine and artemisinin antibiotics [6]. *P. vivax* and *P. ovale* are treated using chloroquine antibiotics; *P. falciparum*, *P. malariae*, and *P. knowlesi* are treated with artemisinin antibiotics [7]. These five types of human-affecting Plasmodia are responsible for an infectious disease that can be cured if appropriate treatments are employed. However, if *P. falciparum* is not detected early, it can lead to severe
disease and death [8]. Therefore, it is crucial to diagnose malaria promptly during early stages and distinguish among malaria species [9].

For malaria detection, currently available diagnostic methods include blood smears, the application of a rapid diagnostic kit (RDT), and molecular diagnostics. Blood smear, a representative diagnosis sample for malaria, is simultaneously performed with thick- and thin-layer smears, followed by Giemsa staining, in order to identify the presence of *Plasmodium* species under a microscope [10]. However, blood smears are time-consuming and require trained specialists because the staining process takes 45 min [11] and a trained microscopist consumes 15–30 min for microscopic examination of a single blood slide [11]. Furthermore, with thick-layer smears, it is difficult to identify the type of malaria, and with thin-layer smears, it can be challenging to confirm the diagnosis if the infection density is low [12]. RDT results can be confirmed within 20 min, but the sensitivity is lower than that of other diagnostic methods [13]. Polymerase chain reaction (PCR), a molecular diagnostic method, has the highest sensitivity and specificity among malaria diagnosis methods and can identify *Plasmodium*, as well as distinguish between species [14]. However, PCR takes approximately 3 h and requires expensive equipment with thermal cycling capabilities and skilled professionals [15]. Thus, several isothermal amplification methods for malaria diagnoses have been proposed as molecular diagnostic methods to overcome these limitations, including loop-mediated isothermal amplification (LAMP) [16], lateral flow recombinase polymerase amplification (LF-RPA) [17], and quantitative nucleic acid sequence-based amplification (QT-NASBA) [18]. Among these isothermal amplification methods, LAMP is the most extensively investigated method for malaria diagnosis [19–22]. LAMP requires four primers, containing six parts of the target gene sequence and two loop primers, that enhance the performance of the loop structure. This primer set reacts with the target gene to form a loop structure, thus robustly amplifying the target gene at 58–65 °C using Bst polymerase, a displacement DNA polymerase.

To date, a variety of LAMP detection methods have been developed, including colorimetric and fluorescence detection, detection using fluorescent probes, and detection using lateral flow devices [23]. Among these, detection methods using fluorescent probes can be adapted to multiplex LAMP assays. Multiplex LAMP methods that use currently available probes include the methylation-specific LAMP (MS-LAMP), assimilating probe-LAMP, fluorescence of loop primer upon self-enriching-LAMP (FLOS-LAMP), detection of amplification by release of quenching (DARQ), quenching of unincorporated amplification signal reporters (QUASR), and molecular beacon LAMP (MB-LAMP) [24–29].

In the present study, we developed a multiplex malaria Pan/Pf/Pv/IC LAMP method to detect all *Plasmodium* species (Pan), *P. falciparum* (Pf), *P. vivax* (Pv), and the internal control (IC; human beta-actin), based on a slightly modified DARQ probe method. The limit of detection (LOD) test for the multiplex malaria Pan/Pf/Pv/IC LAMP assay was performed using four *Plasmodium* species, namely *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*. In addition, the performance of the multiplex Pan/Pf/Pv/IC LAMP assay was compared with that of the commercial RealStar® Malaria S&T PCR Kit 1.0.

2. Materials and Methods
2.1. Clinical Samples and DNA Extraction

Clinical whole blood samples were collected from patients infected with malarial parasites at the Korea University Guro Hospital from June 2000 to July 2017. All clinical samples were diagnosed by microscopic examination of Giemsa-stained blood smears and in-house PCR tests at the Korea University Guro Hospital. In total, 208 whole blood specimens were used in the present study, including 118 positive specimens of four types of malaria (61 *P. falciparum*, 54 *P. vivax*, 2 *P. malariae*, and 1 *P. ovale*) and 90 negative specimens. DNA extraction from the 208 whole blood samples was performed using the NX-48 Viral NA Kit, with a Nextractor NX-48 system (Genolution, Seoul, Korea), according to the manufacturer’s instructions. DNA was stored at −50 °C. All tests were performed in a blinded manner, with the operator unaware of any previous test results.
The study was conducted in accordance with the guidelines of the Declaration of Helsinki
and was approved by the Institutional Review Board of Korea University Guro Hospital
(2017GR0769 (8 March 2017), 2020GR0451 (7 October 2020)).

2.2. Primer Design

Before use in the LAMP assays, *P. falciparum* LAMP primer sets were designed for
the lactate dehydrogenase (LDH) gene of *P. falciparum* and assessed for specificity via a
BLAST search of sequences in GenBank (National Center for Biotechnology Information
[NCBI], Bethesda, MD, USA). *P. falciparum* LAMP primers, including two outer primers
(forward primer F3 and backward primer B3), two inner primers (forward inner primer
FIP and backward inner primer BIP), and two loop primers (forward loop primer LF and
backward loop primer LB), were designed using the Primer Explorer software (Version 4;
Eiken Chemical Co., Tokyo, Japan). To detect all malaria species and *P. vivax*, we used
malaria Pan and *P. vivax* LAMP primer sets, designed by Mohon et al. and Chen et al.,
respectively [30,31]. For the IC, we used the previously designed LAMP primer set for
the actin beta gene [32]. A multiplex LAMP assay was designed using two types of
additional oligonucleotide sequences at the 5′ loop primers for probing and complementary
oligonucleotide sequences for quenching. For the multiplex LAMP assay, FAM, HEX, Texas
Red, and Cy5 fluorescence tagging probes were used for Malaria Pan, *P. falciparum*, *P. vivax*,
and IC, respectively. All LAMP primers and probes were synthesized by Macrogen Inc.
(Seoul, Korea; Table 1).

| Target                  | Name                      | Sequence (5′-3′)                      | Length (mer) | Reference |
|-------------------------|---------------------------|--------------------------------------|--------------|-----------|
| Malaria pan             | M. pan F3                 | GTA TCA ATC GAG TTT CTG ACC           | 21           | [31]      |
|                         | M. pan B3                 | CTT GTC ACT ACC TCT CTT CT            | 20           |           |
|                         | M. pan FIP                | TCG AAC TCT AAT TCC CCG TTA CTA AC   | 45           |           |
|                         | M. pan BIP                | CGG AGA GGC AGG CTG AGA AAT GGA AT   | 41           |           |
|                         | M. pan FLP                | CTT CAT AGC ATG TTA CGG ACC          | 20           |           |
|                         | M. pan BLP                | AGC TAC CAC ATC TAA GGA AGG CAG      | 24           |           |
| M. pan BLP probe        | [FAM]-CGG GCC CTG ACA AAG GGA ACA CCC ACA CTC CGA GCT ACC | 56           | (Present study) |
| Plasmodium falciparum   | P. f F3                   | AAT TGT AAA CTT ACA TGC ATC AC       | 23           |           |
| (LDH gene)              | P. f B3                   | TCA AAT TTA GCT TTT TCC TCA CT       | 23           |           |
|                         | P. f FIP                  | GCC CTT CTA ACA AGG TGG AGC AGC TGC TGC TAT TAT CGA AAT | 42 |           |
|                         | P. f BIP                  | TAT GGA CAC CCC GAC GAT AT TTA GGT CTA ATT CAA TTA CTA GAA CAC | 48 |           |
|                         | P. f FLP                  | CAA ATC TAG AAG TAG TGC TCT CCA     | 24           |           |
|                         | P. f BLP                  | GGT ACA CCT GTT TTA GGT GCT         | 24           |           |
|                         | [HEX]-CGG GCC CTG ACA AAG GGA ACA CCC ACA CTC CGA CTA CTC TATG CTG TTG TTT TAC ATG | 56 |           |
| Plasmodium vivax        | P. v F3                   | CGA ATG ATG GGA ATT TAA AAC CT      | 23           | [30]      |
| (16S rRNA gene)        | P. v B3                   | AAG GAT TAC CAG TTA TGT GGA T       | 22           |           |
|                         | P. v FIP                  | CTA TGT GAC GTG GAA TTA CGC CTC CCA AAA CTC AAT TGG AGG | 42 |           |
|                         | P. v BIP                  | AAT TGT TGC AGT TAA AAC GCT GGT AAG CTA CTA GAA GGC GGT CTG TCT | 41 |           |
|                         | P. v FLP                  | GCT GCT GCC ACC AGA CTT             | 18           |           |
|                         | P. v BLP                  | AGT ATG TAT TCA AAG AAT CCG          | 20           |           |
|                         | [Texas red]-CGG GCC CTG ACA AAG GGA ACA CCC ACA CTC CGA CGA CGA ATG | 52 |           |
| Human (actin gene)      | IC F3                     | AGT ACC CCA TCC AGC AGC             | 18           | [32]      |
|                         | IC B3                     | AGC CTG CAT AGC AAG GGA TCA A       | 20           |           |
|                         | IC FIP                    | GAG CCA AAC CCA GCA GCT CAT CAT TGT ATT ACC AAG TGG GAC A       | 40           |           |
|                         | IC BIP                    | CTA AAC CCC AAG GCC AGC CTG GGG TGT TGA AGG TC         | 38           |           |
|                         | IC FLP                    | TGT GGT GCC AGA TTT TCT CCA       | 21           |           |
|                         | IC BLP                    | CGA GAA GAT GAC CCA GAT CAT GT     | 23           |           |
|                         | [Cy5]-CGG GCC CTG ACA AAG GGA ACA CCC ACA CTC CGA GAG AAG ATG | 55 |           |
| Quencher probe 1        | GAG TGT GGG TGG TCC CTG TCT GTC ACC CGG CCG -BHQ1 | 30 | | |
| Quencher probe 2        | CCT ACC CTC GTC CTA ACA CCG GAG CCT CCA CGA CTG AGC -BHQ2 | 35 | | |

2.3. Multiplex Malaria Pan/Pf/Pv/IC LAMP Assay

The multiplex malaria Pan/Pf/Pv/IC LAMP assay was performed using a Mmis0 DNA
amplification kit (M monitor, Daegu, South Korea). The LAMP reaction mixture
comprised of 12.5 µL of 2x reaction buffer, 0.625 µL of malaria Pan LAMP primer mix,
0.625 µL of *P. falciparum* LAMP primer mix, 1.25 µL of *P. vivax* LAMP primer mix, 0.6 µL of
IC LAMP primer mix, 720 nM quencher 1 solution, 216 nM quencher 2 solution, 2 µL of enzyme mix, and 5 µL of sample DNA (final reaction volume: 25 µL). The LAMP primer mix was composed of 4 µM of two outer primers (F3 and B3), 32 µM of two inner primers (FIP and BIP), 10 µM of loopF primer, 6 µM of loopB primer, and 4 µM of loopB probe. The LAMP assay was run on a CFX 96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) at 60 °C for 40 min. In addition, a negative control (human serum RNA and distilled water) was used as the baseline. The positive signal was determined by examining whether the signal was steep or gradual, considering the baseline, as the baseline of the LAMP assay was unstable when compared with quantitative PCR (qPCR) or RT-PCR.

2.4. Real-Time Reverse Transcription (RT)-PCR

The RealStar® Malaria Screen & Type PCR Kit 1.0 (Altona Diagnostics, Hamburg, Germany) was performed with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer’s protocol. Thermocycling parameters specified in the kit were as follows: inactivation at 95 °C for 2 min, 45 cycles of denaturation at 95 °C for 15 s, annealing with fluorescence detection at 58 °C for 45 s, and extension at 72 °C for 15 s. The RealStar® Malaria Screen & Type PCR Kit 1.0 was used with two tubes, one tube to diagnose P. falciparum and P. vivax, as well as the other tube to diagnose P. knowlesi, P. malariae, and P. ovale.

2.5. Limit of Detection (LOD) Tests of the Multiplex Malaria Pan/Pf/Pv/IC LAMP Assay

The LOD of the multiplex malaria Pan/Pf/Pv/IC LAMP was determined using pTOP Blunt V2 vectors, including the 18S rRNA partial gene sequences of Plasmodium spp., LDH partial gene sequences of P. falciparum, the 16S rRNA gene partial sequences of P. vivax, and beta-actin partial gene sequence of humans. All vectors were constructed by Macrogen, Inc. The vectors were serially diluted 10-fold, from 1.0 × 10^7 copies/µL to 1.0 × 10^1 copies/µL, to determine the LOD of the multiplex malaria Pan/Pf/Pv/IC LAMP assay. In addition, the LOD of malaria Pan/Pf/Pv/IC LAMP was tested with serially diluted clinical samples (10^-1 to 10^-6) infected with P. falciparum, P. vivax, P. malariae, and P. ovale. The LOD of the multiplex malaria Pan/Pf/Pv/IC LAMP for clinical malaria samples was compared with that of the RealStar® Malaria Screen & Type PCR Kit 1.0.

3. Results

3.1. Optimization of the Multiplex Malaria Pan/Pf/Pv/IC LAMP Primer Set

To optimize the multiplex malaria Pan/Pf/Pv/IC LAMP primer set, different concentration ratios of the malaria Pan, Pf, Pv, and IC primer sets (1:1:1, 1:1:2:1, and 1:2:2:1, respectively) were evaluated against the synthetic malaria Pan, P. falciparum, P. vivax, and human actin beta vectors (10^7 copies; ratio of 1:1:1:1) (Figure 1A). Three signals (malaria Pan, Pf, and IC) were detected at a ratio of 1:1:1:1, whereas four signals (malaria Pan, Pf, and IC) were detected at ratios of 1:1:2:1 and 1:2:2:1. Among the three ratios of the LAMP primer set, the ratio of 1:1:2:1 for the multiplex malaria Pan/Pf/Pv/IC LAMP primer set exhibited the lowest Ct values (19.55, 23.83, 18.12, and 18.12) for malaria Pan, P. falciparum, P. vivax, and IC vectors, respectively. Thus, the ratios of malaria Pan, Pf, and IC LAMP primer set (1:1:2:1) were optimum for the multiplex malaria Pan/Pf/Pv/IC LAMP assay. Next, temperature gradient tests (58/60.8/65 °C) were performed to determine the optimal temperature for the multiplex malaria Pan/Pf/Pv/IC LAMP assay (Figure 1B). Among the three different temperatures, the LAMP assay performed at 58 °C showed the lowest Ct values and relative fluorescence unit (RFU) of all fluorescence channels (CT/RFU, malaria Pan: 19.55/8132, Pf: 23.83/1810, Pv: 18.12/2689, and IC: 23.57/4367). Figure 1C presents the multiplex malaria Pan/Pf/Pv/IC LAMP assay, performed using human serum DNA samples spiked with malaria Pan, P. falciparum, and P. vivax vectors (10^7 copies) under optimum conditions. The ratio of the malaria Pan, Pf, Pv, and IC LAMP primer sets was 1:1:2:1, and the LAMP assay was performed at 58 °C.
3.2. Limit of Detection (LOD) of the Multiplex Malaria Pan/Pf/Pv/IC LAMP Assay

The analytical sensitivity of the multiplex malaria Pan/Pf/Pv/IC LAMP assay was compared with that of the corresponding monoplex LAMP primer sets against synthetic pan, Pf, Pv, and IC vectors, ranging from $10^7$ to $10^1$ DNA copies/µL (Figure 2, Table 2). The monoplex malaria Pan, Pf, Pv, and IC LAMP revealed an LOD of $1 \times 10^2$, $1 \times 10^2$, $1 \times 10^2$, and $1 \times 10^3$ copies/µL, respectively. In the multiplex malaria Pan/Pf/Pv/IC LAMP assay, the LOD results for the four channels were the same as those for the corresponding monoplex LAMP assays; the Ct values of the multiplex malaria Pan/Pf/Pv/IC LAMP assay were delayed, when compared with those of the corresponding monoplex LAMP assay. Furthermore, the LOD of the LAMP assay was compared with that of monoplex LAMP and commercial RealStar® Malaria Screen & Type PCR Kit 1.0 (Altona Diagnostics, Hamburg, Germany) using serially diluted *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* clinical samples (clinical whole blood DNA sample dilution range of $10^{-1}$ to $10^{-6}$; Table 3). For the *P. falciparum* clinical samples, monoplex malaria Pan and *P. falciparum* primer sets showed detection limits of $10^{-4}$ and $10^{-3}$, respectively. The multiplex malaria Pan/Pf/Pv/IC LAMP assay showed an LOD of $10^{-4}$ (pan), $10^{-4}$ (Pf), and $10^{-2}$ (IC), whereas RealStar® Malaria Screen & Type PCR Kit 1.0 showed an LOD of $10^{-5}$. For the
P. vivax clinical samples, the monoplex malaria Pan and Pv LAMP primer sets showed LODs of $10^{-4}$ and $10^{-2}$, respectively. The multiplex malaria Pan/Pf/Pv/IC LAMP assay presented LODs of $10^{-3}$ (pan), $10^{-3}$ (Pv), and $10^{-2}$ (IC), and the RealStar® Malaria Screen & Type PCR Kit 1.0 showed the same detection limits ($10^{-3}$). For the P. malariae and P. ovale clinical samples, the monoplex malaria Pan primer set showed LODs of $10^{-4}$ and $10^{-1}$, respectively. The multiplex malaria Pan/Pf/Pv/IC LAMP assay showed detection limits of $10^{-3}$ (pan) and $10^{-1}$ (pan) for P. malariae and P. ovale. The RealStar® Malaria Screen and Type PCR Kit 1.0 showed detection limits of $10^{-5}$ and $10^{-1}$ for P. malariae and P. ovale clinical samples, respectively.

**Figure 2.** Limit of detection (LOD) test for the monoplex and multiplex malaria Pan/Pf/Pv/IC LAMP primer set. (A) The monoplex malaria Pan, *Plasmodium falciparum*, *Plasmodium vivax*, and IC LAMP primer sets (left, middle, and right panels, respectively). (B) Multiplex malaria Pan/Pf/Pv/IC LAMP primer set, respectively. The monoplex and multiplex malaria Pan/Pf/Pv/IC LAMP assays were performed with synthetic malaria Pan, *P. falciparum*, *P. vivax*, or beta-actin vectors, ranging from $10^7$ to $10^1$ RNA copies/µL. Colors (red, orange, green, blue, purple, pink, black, and olive) indicate vector copy numbers/µL ($1.0 \times 10^7$ to $1.0 \times 10^1$ copies/µL) and distilled water (DW; negative control). The LOD test was performed in duplicate. IC, internal control; LAMP, loop-mediated isothermal amplification.

**Table 2.** Limit of detection (LOD) tests of monoplex and multiplex malaria Pan/Pf/Pv/IC LAMP assay for malaria Pan, *Plasmodium falciparum*, *Plasmodium vivax*, and internal control vectors (range: $10^2$–$10^1$).

| Vector Set | Vector | Monoplex LAMP | Multiplex LAMP |  $10^2$ |  $10^3$ |  $10^4$ |  $10^5$ |  $10^6$ |  $10^7$ |  $10^8$ |  $10^9$ |  $10^{10}$ |
|------------|--------|---------------|---------------|---------|---------|---------|---------|---------|---------|---------|--------|
| Pan (FAM)  | Malaria Pan | 8.08 | 9.10 | 10.21 | 11.04 | 12.82 | 16.26 | Neg | 12.15 | 13.78 | 15.55 | 17.28 | 19.10 | 22.81 | Neg |
| Pf (HEX)   | *P. falciparum* | 10.44 | 11.21 | 12.59 | 13.30 | 17.73 | 21.26 | Neg | 17.79 | 19.00 | 20.53 | 26.65 | 31.48 | 31.67 | Neg |
| Pv (Tex)   | *P. vivax* | 12.05 | 14.22 | 15.48 | 16.00 | 19.77 | 22.60 | Neg | 13.26 | 15.65 | 17.03 | 17.93 | 25.73 | 28.58 | Neg |
| IC (Cy5)   | IC | 14.26 | 16.18 | 18.00 | 19.62 | 24.28 | Neg | 15.58 | 18.01 | 20.14 | 22.33 | 23.91 | Neg | Neg |
| Pan (FAM)  | Malaria Pan | 12.15 | 13.78 | 15.55 | 17.28 | 19.10 | 22.81 | Neg | 12.15 | 13.78 | 15.55 | 17.28 | 19.10 | 22.81 | Neg |
| + Pf (HEX) | *P. falciparum* | 17.79 | 19.00 | 20.53 | 26.65 | 31.48 | 31.67 | Neg | 17.79 | 19.00 | 20.53 | 26.65 | 31.48 | 31.67 | Neg |
| + Pv (Tex) | *P. vivax* | 13.26 | 15.65 | 17.03 | 17.93 | 25.73 | 28.58 | Neg | 13.26 | 15.65 | 17.03 | 17.93 | 25.73 | 28.58 | Neg |
| + IC (Cy5) | IC | 15.58 | 18.01 | 20.14 | 22.33 | 23.91 | Neg | 15.58 | 18.01 | 20.14 | 22.33 | 23.91 | Neg | Neg |
Table 3. Limit of detection (LOD) tests for monoplex and multiplex malaria Pan/Pf/Pv/IC LAMP assays and RealStar® Malaria Screen & Type PCR Kit 1.0 using clinical malaria whole blood samples (range: $10^{-1}$-$10^{-6}$).

| Clinical Samples | Whole Blood Clinical Sample | Dilution * | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ |
|------------------|-----------------------------|------------|-----------|-----------|-----------|-----------|-----------|-----------|
|                  |                             | CT         | RFU       | CT         | RFU       | CT         | RFU       | CT         | RFU       |
| **P. falciparum**| Monoplex LAMP Pan (FAM)     | 9.86       | 15,145    | 10.83      | 16,032    | 12.68      | 17,075    | 14.43      | 17,203    |
|                  |                             | 9.71       | 16,795    | 10.82      | 16,581    | 13.73      | 17,218    | Neg        | Neg       |
|                  | Monoplex LAMP Pf (HEX)      | 16.70      | 7543      | 18.24      | 7629      | 20.70      | 8057      | 30.85      | 8118      |
|                  |                             | 21.16      | 3928      | 22.88      | 3969      | 24.72      | 4108      | 38.28      | 4020      |
|                  | Multiplex LAMP Pan (FAM)    | 16.70      | 7543      | 18.24      | 7629      | 20.70      | 8057      | 30.85      | 8118      |
|                  |                             | 21.16      | 3928      | 22.88      | 3969      | 24.72      | 4108      | 38.28      | 4020      |
|                  | Multiplex LAMP Pf (HEX)     | 21.16      | 3928      | 22.88      | 3969      | 24.72      | 4108      | 38.28      | 4020      |
|                  | RealStar® Malaria S&T PCR Kit 1.0 Pf (FAM) | 22.61 | 11,985 | 27.09 | 12,807 | 30.82 | 12,548 | 35.41 | 11,329 | 39.26 | 7558 |
|                  |                             | 22.61      | 11,985    | 27.09      | 12,807    | 30.82      | 12,548    | 35.41      | 11,329    |
| **P. vivax**     | Monoplex LAMP Pan (FAM)     | 16.12      | 13,226    | 18.05      | 14125     | 32.51      | 13,617    | 37.25      | 12,230    |
|                  |                             | 12.80      | 9561      | 14.59      | 9454      | Neg        | Neg       | Neg        | Neg       |
|                  | Monoplex LAMP Pf (HEX)      | 18.10      | 5398      | 20.08      | 5360      | 21.99      | 5541      | Neg        | Neg       |
|                  |                             | 21.16      | 3928      | 22.88      | 3969      | 24.72      | 4108      | 38.28      | 4020      |
|                  | Multiplex LAMP Pan (FAM)    | 19.36      | 7347      | 20.90      | 7527      | 25.27      | 7546      | Neg        | Neg       |
|                  |                             | 22.20      | 7706      | 22.92      | 7741      | 26.23      | 7531      | Neg        | Neg       |
|                  | Multiplex LAMP Pf (HEX)     | 18.10      | 5398      | 20.08      | 5360      | 21.99      | 5541      | Neg        | Neg       |
|                  | RealStar® Malaria S&T PCR Kit 1.0 Pf (FAM) | 24.56 | 13,326 | 28.16 | 12,145 | 31.22 | 1875 | 34.67 | 11,985 | 36.90 | 5049 |
|                  |                             | 24.56      | 13,326    | 28.16      | 12,145    | 31.22      | 1875      | 34.67      | 11,985    |
| **P. malariae**  | Monoplex LAMP Pan (FAM)     | 11.11      | 15,757    | 12.61      | 17,887    | 14.87      | 16,582    | 15.80      | 17,883    |
|                  |                             | 12.61      | 15,757    | 12.61      | 17,887    | 14.87      | 16,582    | 15.80      | 17,883    |
|                  | Monoplex LAMP Pf (HEX)      | 32.60      | 6815      | 37.41      | 6486      | 38.92      | 6449      | Neg        | Neg       |
|                  |                             | 32.60      | 6815      | 37.41      | 6486      | 38.92      | 6449      | Neg        | Neg       |
|                  | Multiplex LAMP Pan (FAM)    | 26.27      | 6097      | 20.08      | 6097      | 21.99      | 6097      | Neg        | Neg       |
|                  | RealStar® Malaria S&T PCR Kit 1.0 Pf (FAM) | 24.56 | 13,326 | 28.16 | 12,145 | 31.22 | 1875 | 34.67 | 11,985 | 36.90 | 5049 |
|                  |                             | 24.56      | 13,326    | 28.16      | 12,145    | 31.22      | 1875      | 34.67      | 11,985    |
| **P. ovale**     | Monoplex LAMP Pan (FAM)     | 16.60      | 15,880    | Neg        | Neg       | Neg        | Neg       | Neg        | Neg       |
|                  |                             | 22.20      | 7885      | Neg        | Neg       | Neg        | Neg       | Neg        | Neg       |
|                  | Monoplex LAMP Pf (HEX)      | 18.10      | 5398      | 20.08      | 5360      | 21.99      | 5541      | Neg        | Neg       |
|                  | Multiplex LAMP Pan (FAM)    | 22.20      | 7885      | Neg        | Neg       | Neg        | Neg       | Neg        | Neg       |
|                  | RealStar® Malaria S&T PCR Kit 1.0 Pf (FAM) | 24.56 | 13,326 | 28.16 | 12,145 | 31.22 | 1875 | 34.67 | 11,985 | 36.90 | 5049 |
|                  |                             | 24.56      | 13,326    | 28.16      | 12,145    | 31.22      | 1875      | 34.67      | 11,985    |

* The test was performed with whole blood clinical DNA samples, which were serially diluted with nuclease-free water.

3.3. Comparison of Clinical Performance between the Multiplex Malaria Pan/Pf/Pv/IC LAMP and RealStar® Malaria Screen & Type PCR Kit 1.0 Using Clinical Samples

To confirm the clinical performance of the multiplex malaria Pan/Pf/Pv/IC LAMP assay, the assay sensitivity and specificity were compared with those of RealStar® Malaria Screen & Type PCR Kit 1.0 for *P. falciparum* (n = 61), *P. vivax* (n = 54), *P. malariae* (n = 2), *P. ovale* (n = 1), and healthy patient (n = 90) whole blood specimens (Table 4). For *P. falciparum* clinical samples (n = 61), the sensitivities for *P. falciparum* and IC were 100% and 95.08%, respectively, for the RealStar® Malaria Screen and Type PCR Kit 1.0. The sensitivity of the multiplex malaria Pan/Pf/Pv/IC LAMP assay was 100% in the malaria Pan channel (FAM), 100% in the Pf channel (HEX), and 75.41% in the IC channel (Cy5). The cross-reactivity of the Pv channel of the multiplex malaria Pan/Pf/Pv/IC LAMP assay was 100%, whereas that of the Pv channel of the RealStar® Malaria Screen & Type PCR Kit 1.0 was 98.36% against *P. falciparum* clinical samples (n = 61). For *P. vivax* clinical samples (n = 54), the sensitivities for *P. vivax* and IC were 100% with the RealStar® Malaria Screen...
and Type PCR Kit 1.0; the sensitivities of the multiplex malaria Pan/Pf/Pv/IC LAMP assay were 100% in the malaria Pan channel (FAM), 100% in the Pv channel (HEX), and 81.48% in the IC channel (Cy5). The Pf channels of the two assays showed no cross-reactivity with the P. vivax clinical samples. For the P. malariae (n = 2) and P. ovale clinical samples (n = 1), the sensitivities and specificities of both assays were 100%.

Table 4. Comparison of clinical performance between the multiplex malaria Pan/Pf/Pv/IC LAMP assay and RealStar® Malaria Screen & Type PCR Kit 1.0 for Plasmodium species (P. falciparum, P. vivax, P. malariae, and P. ovale) using clinical samples.

| Clinical Samples | Multiplex Malaria Pan/Pf/Pv/IC LAMP Assay | RealStar® Malaria Screen & Type PCR Kit 1.0 |
|------------------|------------------------------------------|-------------------------------------------|
|                  | Pan (FAM) | Pf (HEX) | Pv (HEX) | IC (Cy5) | Pf (FAM) | Pv (HEX) | IC (HEX) | Pm (FAM) | Po (Cy5) | IC (HEX) |
| P. falciparum (n = 61) | P/N | Sensitivity | Specificity | | | | | | | |
| | 61/0 | 61/0 | 0/61 | 46/15 | 61/0 | 1/60 | 58/3 | - | - | - |
| | 100% | 100% | - | 75.41% | 100% | - | 95.08% | - | - | - |
| | - | - | 100% | - | - | 98.36% | - | - | - |
| P. vivax (n = 54) | P/N | Sensitivity | Specificity | | | | | | | |
| | 54/0 | 0/54 | 54/0 | 44/10 | 0/54 | 54/0 | 54/0 | - | - | - |
| | 100% | - | 100% | 81.48% | - | 100% | 100% | - | - | - |
| | - | - | 100% | - | - | - | - | - | - |
| P. malariae (n = 2) | P/N | Sensitivity | Specificity | | | | | | | |
| | 2/0 | 0/2 | 0/2 | 2/0 | - | - | 2/0 | 0/2 | 2/0 | - |
| | 100% | - | - | 100% | - | - | 100% | - | 100% | - |
| | - | - | 100% | - | - | - | 100% | - | - |
| P. ovale (n = 1) | P/N | Sensitivity | Specificity | | | | | | | |
| | 1/0 | 0/1 | 0/1 | 1/0 | - | - | 1/0 | 0/1 | 1/0 | - |
| | 100% | - | - | 100% | - | - | 100% | - | 100% | - |
| | - | - | 100% | - | - | - | 100% | - | - |
| Non-infection (n = 90) | P/N | Sensitivity | Specificity | | | | | | | |
| | 0/90 | 0/90 | 0/90 | 90/0 | 0/90 | 0/90 | 90/0 | - | - | - |
| | 100% | - | - | 100% | - | - | 100% | - | 100% | - |
| | - | - | 100% | - | - | - | 100% | - | - |

P/N: positive/negative ratio.

4. Discussion

The accurate classification of malaria species during the early stage of diagnosis is valuable for the proper treatment of infected patients and prevention of overexposure to antimalarial drugs, as treatment policies for different species tend to vary, such as artemisinin-based combination therapy (ACT) for P. falciparum and chloroquine for non-falciparum species [7,33]. Currently, microscopy is the gold standard diagnostic method for malaria (including the classification of malaria species); however, an expert with a high level of training and experience is needed for accurate diagnosis, especially for species identification [34,35].

In the present study, we developed multiplex differential diagnostic tools to classify the malaria Pan, P. falciparum, and P. vivax. Developing multiplex LAMP assays lacking non-specific signals is substantially more difficult than developing multiplex qPCR, as one LAMP assay uses six or seven primer/probe mixtures, and combinations of 2–4 LAMP sets can potentially result in the non-specific amplification of several primers. In particular, in the case of LAMP with the DARQ probe method, the probe sequence corresponding to the binding region of the LAMP product needs to be designed with consideration of the low homology with sequences of other primers and LAMP products, given that it induces non-specific signals by probe-primer or probe-LAMP product interaction. Herein, the multiplex malaria Pan/Pf/Pv/IC LAMP assay was designed by combining a newly designed Pf LAMP primer set with Malaria pan and P. vivax LAMP primer sets, designed by Mohon et al. and Chen et al., respectively [30,31]. This combination of four LAMP primer sets (malaria Pan, Pf, Pv, and IC) exhibited no cross-reactivity with each other, as well as 100% specificity for non-infected samples and distilled water. In the LOD test for P. falciparum and P. ovale, the multiplex malaria Pan/Pf/Pv/IC LAMP showed a LOD 10-fold and 100-fold higher than that of the commercial RealStar® Malaria S&T PCR Kit 1.0, respectively. For P. vivax and P. malariae, the LOD of the LAMP assay was the same.
as that obtained with the commercial kit. Notably, the multiplex malaria Pan/Pf/Pv/IC LAMP assay showed a slightly higher LOD than the commercial PCR kit. However, after assessing 208 clinical samples, the multiplex malaria Pan/Pf/Pv/IC LAMP assay revealed sensitivity (100%) and specificity (100%) similar to those detected with the commercial RealStar® Malaria S&T PCR Kit 1.0 (100%).

As the isothermal amplification method does not require an expensive temperature control device to change the temperature for target gene amplification, the isothermal amplification method has the advantage of easy application for on-site diagnosis, which is difficult to implement in general PCR [15,36]. Unfortunately, the multiplex LAMP assay developed using fluorescence is limited, in terms of field applications [37]. Isothermal amplification, or a portable PCR device for detecting multiple fluorescence signals, is required to utilize the multiplex LAMP assay for point-of-care testing (POCT). Most available field isothermal amplifiers have been developed for single or two fluorescence channels [38]. However, Bio Molecular Systems (BMS, Australia) recently developed a 2 kg portable magnetic induction cycler (Mic) PCR machine with four real-time channels available. Thus, along with the Mic PCR Machine, the multiplex malaria Pan/Pf/Pv/IC LAMP assay, developed in the present study, can be used for POCT for diagnosing malaria species.

In the present study, we developed a multiplex malaria Pan/Pf/Pv/IC LAMP assay, which can classify malaria Pan, *P. falciparum*, and *P. vivax* within 40 min. On evaluating 118 malaria-infected clinical samples, the multiplex malaria Pan/Pf/Pv/IC LAMP assay revealed a performance similar to the commercial RealStar® Malaria Screen & Type PCR Kit 1.0, which is based on the qPCR method. Thus, with portable multi-channel isothermal equipment, such as the Mic PCR Machine, the multiplex malaria Pan/Pf/Pv/IC LAMP assay can be applied in resource-limited settings for point-of-care diagnostics.

**Author Contributions:** Conceptualization, W.S.J. and C.S.L.; formal analysis, D.H.L., Y.C., H.J., K.C.M., C.K., M.C. and I.S.P.; investigation, W.S.J., D.H.L. and K.C.M.; methodology, D.H.L., Y.C., H.J., K.C.M., C.K., M.C. and I.S.P.; project administration, C.S.L.; supervision, C.S.L.; writing—original draft, W.S.J.; writing—review & editing, C.S.L. All authors have read and agreed to the published version of the manuscript.

**Funding:** This study was supported by a grant from the Korea Health Technology R&D Project, through the Korea Health Industry Development Institute (KHIDI), and funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HR20C0021), as well as by a government-wide R&D fund for infectious disease research (HG18C0012).

**Institutional Review Board Statement:** The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board of Korea University Guro Hospital (2017GR0769 (8 March 2017), 2020GR0451 (7 October 2020)).

**Informed Consent Statement:** Patient consent was waived by the Institutional Review Board of Korea University Guro Hospital, as the identities of the subjects are completely anonymous, and there is minimal risk involved in the study.

**Data Availability Statement:** Data is contained within the article.

**Conflicts of Interest:** The authors declare no conflict of interest.

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