A Loop-mediated Isothermal Amplification With a Nanoparticle-Based Lateral Flow Biosensor Assay to Detect Pseudomonas aeruginosa in Endophthalmitis

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Purpose: Pseudomonas aeruginosa is the most common bacteria causing endophthalmitis after cataract surgery. Vitreous fluid culture and molecular studies are commonly used in clinical diagnoses, but have disadvantages, such as a long culture cycle and low detection sensitivity. Here, we report a loop-mediated isothermal amplification (LAMP) method combined with the nanoparticles-lateral flow biosensor (LFB) method for rapid and specific detection of P. aeruginosa.

Methods: A set of six primers was designed to target the OprL gene of P. aeruginosa. Genomic DNA extracted from several gram-negative and gram-positive bacteria was used to determine the sensitivity and specificity of the analysis. LAMP reactions were conducted at 65°C for 50 minutes, and results were reported using the LFB method.

Results: The DNA template of P. aeruginosa was specifically recognized by the P. aeruginosa-LAMP-LFB (PA-LAMP-LFB) method as no cross reactions were observed for non-P. aeruginosa templates. The analytical sensitivity of our assay was 100 fg per test for the pure cultured DNA template, and the result obtained using the LFB was consistent with that of colorimetric indicator detection. The whole test could be completed within 1h. This method was used to detect P. aeruginosa, Staphylococcus aureus, and Klebsiella pneumoniae; only P. aeruginosa was positive. The positive rates of P. aeruginosa detected by a traditional culture method, the LAMP-LFB method, and the fluorescence quantitative polymerase chain reaction method were 17.7%, 17.7%, and 13.3%, respectively.

Conclusions: The P. aeruginosa-LAMP-LFB method established here is a rapid, specific, and sensitive method for the detection of P. aeruginosa, which can be widely used.

Introduction

Bacterial endophthalmitis is an infection possibly resulting in blindness caused by bacteria entering the eye. Bacteria enter the aqueous humor or vitreous body through exogenous or endogenous pathways, which are prone to endophthalmitis.¹ The severity of bacterial endophthalmitis depends on complex factors, such as bacterial load, reproduction rate, migration potential, expression of virulence factors, and toxin production levels. Severe bacterial endophthalmitis can cause irreversible retinal damage, vision loss, and the need for eyeball removal in some patients.²

Pseudomonas aeruginosa is a ubiquitous, nonfastidious gram-negative bacteria.³ It is an opportunistic pathogen that can easily infect patients with cystic fibrosis,⁴ burns,⁵ eye trauma, and other immunodeficiencies.⁶ P. aeruginosa is a common bacterium that causes endophthalmitis. If the infection is not treated promptly there is a risk for life-threatening intracranial infection and therefore removal of the infected eye (globe) may be required.²

At present, traditional culture methods, serological detection, and nucleic acid amplification assays are used commonly to diagnose P. aeruginosa in samples. The culture method must be carefully tested at the biosafety level, and usually takes several days to identify and confirm the results.⁷ Some P. aeruginosa strains are nutritionally deficient, and attempts to culture these strains can lead to false-negative results; this phenomenon can be fatal in patients with...
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intraocular infection of *P. aeruginosa*. Serological detection has the disadvantage of being slow and also has a low sensitivity in practical applications. Compared with traditional culture methods, nucleic acid amplification assays, such as conventional polymerase chain reaction (PCR) and real-time fluorescent PCR, are rapid, sensitive, and specific, and are often used to detect bacteria in clinical samples. However, PCR-based testing requires specialized PCR instruments and must be carried out by experienced laboratory personnel, which is not applicable in some small medical institutions and rural areas.

Loop-mediated isothermal amplification (LAMP) has been used widely in microbial diagnosis because of its simple method and short reaction time, among other advantages. However, indicators of LAMP results often depend on complex instruments (such as a real-time turbidimeter), tedious processes (including agarose gel electrophoresis), and special reagents (colorimetric indicators for example). The *OprL* gene of *P. aeruginosa* has been validated with high specificity for target pathogen detection and is a suitable molecular marker for developing *P. aeruginosa*-based diagnostic tests.

In this study, LAMP combined with an LFB was used to establish a rapid and sensitive method for the detection of *P. aeruginosa*. This report is the first time this method has been used to detect *P. aeruginosa* in endophthalmitis. The rapidity of this test would enable physicians to commence treatment of their patient promptly, therefore preventing further complications of the infection.

### Methods

#### Reagents and Instruments

To achieve the rapid and simple LAMP test, we used a lateral flow biosensor (LFB) for a simple and objective report of the LAMP results, termed LAMP-LFB. Briefly, the LAMP products were loaded onto the nitrocellulose filter membrane of the LFB and the results were displayed with red lines. The test line is indicated by TL, and CL indicates the control line. The LFB materials, including sample pad, nitrocellulose membrane, conjugate pad, absorbent pad, and backing card were purchased from the Jie-Yi Biotechnology. Co., Ltd. (Shanghai, China). Colorimetric indicator (Malachite Green), universal isothermal amplification kits and biotin-14-dCTP were purchased from Bei-Jing Hai Tai Zheng Yuan. Co., Ltd. (Beijing, China). Dye (crimson red) streptavidin-coated polymer nanoparticles (129 nm, 10 mg mL−1, 100 mM borate, pH 8.5 with 0.1% bovine serum albumin, 0.05% Tween 20, and 10 mM EDTA) were purchased from Bangs Laboratories, Inc. (Fishers, IN). Anti–fluorescein isothiocyanate (rabbit antifluorescein antibody) and biotin BSA (biotinylated bovine serum albumin) were purchased from Abcam Co., Ltd. (Shanghai, China).

#### Primer Design

Based on the specific *OprL* gene of *P. aeruginosa*, a set of six LAMP primers, including two external primers (F3 and B3), two ring primers (FIP and BIP), and two internal primers (LF and LB), was designed with two software packages (PrimerExplorerV4 and Primer Premier 5.0). The specificity of the LAMP primer set was confirmed by sequence alignment analysis using the National Center for Biotechnology Information database. To construct the primer for LFB detection, the 5′ ends of FIP and LF were labeled with fluorescein isothiocyanate and biotin, respectively. The sequences, locations, and modifications of the primers are shown in Figure 1 and Table 1.

Right arrows and left arrows indicate sense and complementary sequences that are used. The positions of the primers are shown in a different color.

#### Bacterial Strains

In this study, 35 strains (Table 2) were used, including 14 *P. aeruginosa* that had been isolated from clinical samples and 21 non–*P. aeruginosa* strains. *P. aeruginosa* American Type Culture Collection (ATCC) was used as the reference strain to evaluate the specificity of LAMP-LFB detection. A QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) was used to extract genomic DNA, according to the manufacturer’s instructions. The extracted DNA was stored at −80 °C until use. The purity and concentration of the extracted genomic DNA were determined using an ultraviolet spectrophotometer (NanoDrop 2000, Thermo, MA).

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**Figure 1.** Sequence and location of *OprL* gene used to design LAMP primers.
Table 1. Primers Used in the Current Report

| Primers | Sequences and Modifications | Length | Genes |
|---------|-----------------------------|--------|-------|
| F3      | 5′-AGCCGGAAGCCATGC-3′        | 15 nt  | OprL  |
| FIP*    | 5′-TGGCCTTTCCACACTACGGCTGTGGACGTCATACACGCAAAG-3′ | 40 mer |       |
| LF      | 5′-TGACCCTGCTGTCTA-3′        | 16 nt  |       |
| LF*     | 5′-FITC-TGACCCTGCTGTCTA-3′   | 16 nt  |       |
| LB      | 5′-CGATACACATGGCTGCTC-3′     | 18 nt  |       |
| BIP     | 5′-ACCGACGAACCGCGC-TAGCGCTGAACGGCCTTG-3′    | 34 nt  |       |
| B3      | 5′-AACGCCCTCGACGACCC-3′      | 16 nt  |       |

*LF*, 5′-labeled with FITC when used in *P. aeruginosa* LAMP-LFB assay.
FITC, fluorescein isothiocyanate; FIP*, 5′-5′-labeled with biotin; mer, monomeric unit; nt, nucleotide.

Table 2. Bacterial Strains Used in This Report

| Bacteria                | Strain no. (Source of Strains) | No. of Strains | PA-LAMP-LFB Resulta |
|-------------------------|---------------------------------|----------------|---------------------|
| *P. aeruginosa*         | ATCC                            | 1              | P                   |
| *P. aeruginosa*         | Isolated strains                | 14             | P                   |
| Non–*P. aeruginosa*     |                                 |                |                     |
| S. aureus               | Isolated strains                | 1              | N                   |
| *Staphylococcus epidermidis*| Isolated strains                | 1              | N                   |
| *Staphylococcus saprophytics*| Isolated strains                | 1              | N                   |
| Bacillus cereus         | Isolated strains                | 1              | N                   |
| *Enterococcus faecalis* | ATCC35667                       | 1              | N                   |
| *Enterococcus faecium*  | Isolated strains                | 1              | N                   |
| Enterotoxigenic *E. coli*| Isolated strains                | 1              | N                   |
| *Campylobacter jejuni*  | Isolated strains                | 1              | N                   |
| *Candida tropicalis*    | Isolated strains                | 1              | N                   |
| *Candida albicans*      | Isolated strains                | 1              | N                   |
| Listeria monocytogenes  | ATCC-EGD-e                      | 1              | N                   |
| *Streptococcus pneumonia*| Isolated strains                | 1              | N                   |
| Salmonella              | Isolated strains                | 1              | N                   |
| *Shigella flexneria*    | Isolated strains                | 1              | N                   |
| *Aeromonas hydrophila*  | Isolated strains                | 1              | N                   |
| *Stenotrophomonas maltophilia*| Isolated strains                | 1              | N                   |
| Bordetella pertussis    | Isolated strains                | 1              | N                   |
| *Proteus mirabilis*     | Isolated strains                | 1              | N                   |
| *Acinetobacter baumannii*| Isolated strains                | 1              | N                   |
| Enterobacter cloacae    | Isolated strains                | 1              | N                   |
| Klebsiella pneumoniae   | Isolated strains                | 1              | N                   |

ATCC, American Type Culture Collection; N, negative; P, positive.
aOnly *P. aeruginosa* strains could be detected by the *P. aeruginosa* LAMP-LFB technique, indicating the extremely high specificity of the assay.

Confirmation and Verification of LAMP-LFB Products

To evaluate the viability of primers the designed for the *OprL* gene of *P. aeruginosa*, a standard LAMP-LFB reaction with a total volume of 25 μL was prepared. This was comprised of 12.5 μL of 2× isothermal amplification buffer (TransGen Biotech), 1 μL of Bst enzyme (8 U), 0.1 μL of F3 and B3, 0.2 μL of FIP*, 0.2 μL of FIP and BIP, 0.1 μL of LF*, 0.1 μL of LF and LB, 1 μL of DNA template, and 9.4 μL of double distilled water. The reaction was carried
out at 65 °C, both with and without the *P. aeruginosa* DNA template, for 1 hour. The amplified products were then detected using a colorimetric, specific nucleic acid amplification indicator and the LFB.

### Analysis of Optimal Amplification Temperature of the LAMP-LFB Method

To determine the optimal reaction temperature of the amplification stage of the LAMP-LFB method, 100 ng of *P. aeruginosa* genomic DNA per assay was used as a template for LAMP detection. This was tested at intervals of 1 °C at eight different temperatures ranging from 61 °C to 68 °C and detected using the LFB and a specific nucleic acid expansion indicator.

### Sensitivity of the LAMP-LFB Method

To evaluate the sensitivity of the LAMP-LFB method, a series of gradient diluents of *P. aeruginosa* DNA templates (1 ng/μL ~ 1.4 × 10^5 copies/μL, 100 pg/μL ~ 1.4 × 10^4 copies/μL, 10 pg/μL ~ 1.4 × 10^3 copies/μL, 1 pg/μL ~ 1.4 × 10^2 copies/μL, 100 fg/μL ~ 14 copies/μL, 10 fg/μL ~ 1.4 copies/μL, and 1 fg/μL ~ 0.14 copies/μL). Using the molecular weight per genome as determined from the whole genome sequence of the *P. aeruginosa* strain (a molecular size of 6.26 Mbp for reference *P. aeruginosa* strain [Genebank No. AE004091.2]), we calculate that 7 fg of *P. aeruginosa* DNA correlates to an estimated genome equivalent of one cell or one copy.13 Each dilution was repeated three times to determine the detection limit of the LAMP-LFB method. The amplification products were detected using the LFB and a specific nucleic acid expansion indicator.

### Analysis of Optimal Amplification Time of the LAMP-LFB Method

A total of six time points were evaluated at 65 °C to determine the optimal duration of the LAMP reaction. These time points ranged from 10 minutes to 60 minutes at intervals of 10 minutes. The results were reported using the LFB. All samples were repeated twice to evaluate the feasibility of the LAMP-LFB method at different durations.

### Specificity of the LAMP-LFB Method

The specificity of LAMP-LFB was demonstrated using genomic DNA (≥10 ng/μL) from 14 *P. aeruginosa* strains and 21 non-*P. aeruginosa* strains (Table 2), and a 1-μL aliquot of genomic DNA was used as a template for LAMP reactions. All LAMP results were indicated using biosensor. All samples were repeated two times.

### Application of *P. aeruginosa*-LAMP-LFB (PA-LAMP-LFB) Method in Clinical Samples

To evaluate the applicability of LAMP-LFB method in clinical specimens, vitreous fluid samples from 13 patients with endophthalmitis were detected by LAMP-LFB method and compared with fluorescence quantitative PCR and traditional culture. Owing to the low incidence of endophthalmitis, the sampling period is long. Therefore, we also collected oral and pharyngeal swabs from 32 patients with acute respiratory tract infection for this study.

### Results

#### Confirmation and Feasibility of the PA-LAMP-LFB Amplification Method

Positive amplification detected by colorimetric indicator showed as light blue, whereas in the absence of the *P. aeruginosa* DNA template, and in the blank control, the indicator remained colorless (Fig. 2). Two red lines are shown in the positive reaction by the LFB (TL and CL), and only one red line (CL) in negative results and blank controls (Fig. 2B). These results show that the *P. aeruginosa* LAMP primers designed for this study can specifically amplify the target sequence and can be used to establish a method for LAMP-LFB detection of *P. aeruginosa*.

**Color change of *P. pseudomallei* LAMP-LFB tubes (A); LFB applied for visual detection of *P. aeruginosa* LAMP products (B). Tube A1 (biosensor B1), positive amplification; tube A2 (biosensor B2), negative amplification (*Staphylococcus aureus*), tube A3 (biosensor B3), negative amplification (*Klebsiella pneumoniae*), tube A4 (biosensor B4), negative control (DW).**

#### The Optimum Reaction Temperature for LAMP Analysis

Eight typical kinetic curves corresponding to each temperature were obtained using a specific nucleic acid amplification indicator. As shown in Figure 3, the fastest amplification efficiency occurred at 65 °C. Therefore, 65 °C was chosen as the optimal temperature for PA-LAMP-LFB detection in this study.

Eight kinetic graphs (1–8) were obtained at various temperatures (61 °C–68 °C, 1 °C intervals) with target pathogens DNA at the level of 1 ng per tube. The
graphs from 64 °C to 66 °C showed robust amplification. The threshold value was 0.1 and a turbidity of more than 0.1 was considered positive.

Sensitivity of LAMP Detection

As shown in Figure 4, the lowest detection limit of the PA-LAMP method was 100 fg (~14 copies). TL and CL showed positive LAMP results for the OprL gene on the biosensor (Fig. 4A). The sensitivity of the biosensor for the analysis of P. aeruginosa LAMP was consistent with that of the specific nucleic acid amplification indicator (Fig. 4B).

Biosensors (A)/tubes (B) 1 to 8 represented the DNA levels of 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg per reaction and blank control (DW). The genomic DNA levels of 1 ng (~1.4 × 10^5 copies) to 100 fg (~14 copies) per reaction produced the positive reactions.

The Optimal Reaction Time for LAMP-LFB Detection

The target DNA (100 fg ~14 copies) at the limit of detection was detected when the P. aeruginosa LAMP reaction lasted for 50 minutes (Fig. 5E). In this study, a magnification time of 50 minutes was considered the optimal reaction time for P. aeruginosa LAMP.

Six different reaction times (Biosensor A, 10 minutes; Biosensor B, 20 minutes; Biosensor C, 30 minutes; Biosensor D, 40 minutes; Biosensor E, 50 minutes; and Biosensor F, 60 minutes) were examined and compared at 65 °C. P. aeruginosa LAMP reactions were performed using the LoD level of templates (100 fg per reaction ~14 copies per reaction), and the templates at the LoD level can be detected when the isothermal amplification only lasted for 50 minutes (Biosensor E).

Specificity of the LAMP-LFB Method

P. aeruginosa standard strain ATCC (Fig. 6, Biosensor 1), P. aeruginosa isolates 001 (Fig. 6, Biosensor 2), P. aeruginosa isolates 002 (Fig. 6, Biosensor 3), and non-P. aeruginosa strains, such as Staphylococcus aureus (Fig. 6, Biosensors 4–24) were used to determine the specificity of P. aeruginosa LAMP method. As shown in Figure 6 and Table 2, all P. aeruginosa strains were detected specifically by the P. aeruginosa LAMP method, but no non-P. aeruginosa strains were detected. By biosensor detection, both TL and CL appeared in the detection area of the LFB, indicating that the test result was positive for P. aeruginosa (Fig. 6, Biosensors 1–3). All non-P. aeruginosa strains were negative on the LFB (Fig. 6, Biosensors 4–24), and only one red band (CL) was present in the detection area. No cross-reactions between P. aeruginosa and non-P. aeruginosa strains were observed, which indicated that the LAMP-LFB method had 100% specificity for the detection of P. aeruginosa.

The PA-LAMP-LFB was evaluated using different genomic DNA templates: Biosensor 1, P. aeruginosa (ATCC); biosensor 2, P. aeruginosa (isolated strain 001); biosensor 3, P. aeruginosa (isolated strain 002); and biosensors 4–24, S. aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus, Burkholderia cepacia, Enterococcus faecalis, Enterococcus faecium, Enterotoxigenic E. coli, Campylobacter jejuni, Candida tropicalis, Candida albicans, Listeria monocytogenes, Streptococcus pneumonia, Salmonella, Shigella flexneria, Aeromonas hydrophila, Stenotrophomonas maltophilia, Bordetella pertussis, Proteus mirabilis, Acinetobacter baumannii, Enterobacter cloacae, and Klebsiella pneumoniae.
Figure 3. Optimal temperature for *P. aeruginosa* LAMP primer set.
Figure 4. Analytical sensitivity of *P. aeruginosa* LAMP-LFB assay using serially diluted genomic templates with *P. aeruginosa* strain (ATCC).

Figure 5. Optimal duration of time required for *P. aeruginosa* LAMP-LFB assay.

### Evaluation of the PA–LAMP–LFB Assay Using Clinical Specimens

Among the 45 samples, 8 were positive by the LAMP-LFB method (17.7%), 7 samples were positive by fluorescence quantitative PCR (13.3%), and 8 samples were positive by traditional culture biotechnology method (17.7%). The results showed that the P-LAMP-LFB method was more sensitive than the real-time PCR method for the detection of *P. aeruginosa* (Table 3).

### Discussion

*P. aeruginosa* is a common pathogen infecting the cornea. It is able to produce toxins and proteases, trigger a strong inflammatory response, affect the surrounding flora, and may lead to the destruction of eye tissue. Endogenous endophthalmitis caused by *P. aeruginosa* is generally rare, but it is common in postoperative intraocular inflammation. If it is not diagnosed promptly and treated, it will lead to a loss of vision. In a joint analysis of 342 cases of endophthalmitis infection by Jackson et al., *P. aeruginosa* infection accounted for 6% of these, indicating that it is a common bacterial cause of endophthalmitis infection. The early diagnosis of *P. aeruginosa* endophthalmitis is difficult because patients cannot determine whether the pain is caused by trauma or infection. Muna et al. found that a large proportion (73.4%) of patients undergoing vitrectomy developed endophthalmitis within 1 week after surgery, which is consistent with previous studies. This finding means that patients must be reexamined in the first week after a vitrectomy to detect this complication as soon as possible. Traditional methods for the detection and diagnosis of *P. aeruginosa*, such as cultures of the bacteria and PCR-based techniques, are time consuming and laborious. Bista et al. in their series of studies on endophthalmitis after cataract surgery, found that only 32% of the water samples were positive, whereas 61% of PCR tests were positive. A combination of cultures and PCR detected 71% of the positive cases.

As an isothermal amplification technique, LAMP only uses Bst polymerase and is not affected by known Taq polymerase inhibitors, such as NaCl, hemoglobin, ethylenediaminetetraacetic acid, n-acetylcysteine, or
Figure 6. Analytical specificity of *P. aeruginosa* LAMP-LFB assay using different bacterial strains.

Table 3. Comparison of Culture-biotechnical and *P. aeruginosa* LAMP-LFB Assays for the Detection of *P. aeruginosa* in Clinical Samples

| Detection Methods | Endophthalmitis Samples (n = 13) | Oropharyngeal Swab Samples (n = 32) |
|-------------------|----------------------------------|------------------------------------|
|                   | Positive | Negative | Positive | Negative |
| LAMP-LFB          | 2        | 11       | 6        | 26       |
| Culture           | 2        | 11       | 6        | 26       |
| PCR               | 1        | 12       | 5        | 27       |

bile salts. So far, LAMP tests have been successfully applied for the detection of a variety of pathogens. However, these LAMP tests require agarose gel electrophoresis, color indicators (such as hydroxynaphthol blue light, SYBR green, and Calcein dye) or real-time turbidimetric equipment to amplify the results. The horizontal mobile LFB for the combined detection of *Enterococcus faecalis* and *S. aureus* has been successfully developed, and this greatly facilitates the visual detection of human pathogens without need for instrumentation. In this study, clinical samples were collected and several *P. aeruginosa* strains were isolated and cultured. By targeting the *P. aeruginosa*-specific *OprL* gene, a LAMP combined with the nanoparticle LFB detection method for *P. aeruginosa* was successfully established and verified. Under pure culture conditions, the sensitivity of each reaction was found to be as low as 100 fg (Fig. 4). The LAMP-LFB test showed high specificity for the identification of 21 strains of *P. aeruginosa*, and no positive reaction was observed with any other pathogens (Fig. 6). It was proved that the LAMP-LFB method for target detection was reliable. To further evaluate the practicability of the LAMP-LFB detection method for target pathogens, a commercial fluorescent quantitative PCR method for the *OprL* gene of a standard *P. aeruginosa* strain was selected as the control method. The results showed that the detection rate of LAMP-LFB was 17.7%, which was higher than that of real-time fluorescence PCR (Table 3).
LAMP-LFB detection is therefore an alternative to PCR-based methods, with the following advantages. First, it has a high speed and efficiency. The amplification process can be completed in 30 to 60 minutes. Second, compared with the PCR method, the LAMP-LFB method has high specificity because the four primers targeting the *OprL* gene of *P. aeruginosa* can accurately identify the corresponding regions of the target sequence. In addition, compared with culture-based detection and PCR methods, the PA-LAMP-LFB technology requires only a simple instrument with a constant temperature of 65 °C, avoiding long cycle times and the use of expensive analyzers. This point means it can be used as a rapid diagnostic tool for *P. aeruginosa* infection in basic, clinical, and field laboratories.

This study is limited by only using a small number of clinical samples. In the future, more and varied types of clinical samples should be collected to verify the effectiveness of PA-LAMP-LFB detection in multiple scenarios.

In this study, using pure cultures and clinical samples, we successfully developed and verified the combination of efficient LAMP technology based on the *OprL* gene and portable LFB detection as a method for the detection of *P. aeruginosa*. The PA-LAMP-LFB test is a rapid and simple diagnostic method that can be completed within 1h, with excellent sensitivity and specificity. These advantages make it a useful diagnostic tool for the immediate detection of *P. aeruginosa* from eye samples of patients with acute endophthalmitis.

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