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Simple and rapid detection *Aspergillus fumigatus* by loop-mediated isothermal amplification coupled with lateral flow biosensor assay

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Keywords
A. fumigatus, LAMP-LFB, lateral flow biosensor, limit of detection, loop-mediated isothermal amplification.

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Abstract

Aims: We have developed a new diagnostic technique, termed loop-mediated isothermal amplification coupled with lateral flow biosensor (LAMP-LFB), which has been successfully applied to the detection of *Aspergillus fumigatus*.
Material and Methods: A set of six LAMP primers was designed according to the *A. fumigatus*-specific *anxC4* gene, which specifically recognized eight different regions of the target sequence. The LFB was employed for reporting the *A. fumigatus*-LAMP results, and the visual readouts were obtained within 2 min. The strains of *A. fumigatus* species and non-*A. fumigatus* species were used to test the assay's sensitivity and examine the analytical specificity of the target assay. Optimal LAMP conditions were 66°C for 50 min. The limit of detection is 100 fg. No cross-reactions were obtained, and the specificity of LAMP-LFB assay was 100%. The whole process of the assay, including 20 min of DNA preparation, 50 min of constant temperature amplification, and 2 min of detection by the sensor strip, took a total of 72 min (less than 75 min). Among 89 sputum specimens for clinical evaluation, 10 (11·23%) samples were *A. fumigatus*-positive by LAMP-LFB and traditional culture method, 9 (10·11%) samples were *A. fumigatus*-positive by PCR method. Compared with culture method, the diagnostic accuracy of LAMP-LFB method was 100%.
Conclusions: The novel LAMP-LFB detection technology established in the current research is a rapid and reliable detection tool for *A. fumigatus*.
Significance and Impact of the Study: This novel LAMP-LFB assay can quickly, specifically and sensitively detect *A. fumigatus*, thereby speeding up the detection process and increasing the detection rate. In addition, it can also be used as a new molecular method for detection of *A. fumigatus* in clinical and laboratory areas.

Introduction

Aspergillus fumigatus is an opportunistically pathogenic saprophytic fungus. Because of its ability to survive under a wide range of temperatures (12–65°C) and pH, it is distributed throughout the world. It mainly exists in gardens, greenhouse soil and compost heaps (van de Veerdonk *et al.* 2017). *Aspergillus fumigatus* infection is mainly caused by inhalation of airborne conidia, which is more common in indoor and outdoor environments (about 1–100 conidia per cubic meter) (Latgé and Chamilos 2019). Conidia in the air are inhaled into the lungs and reach the alveoli through the respiratory system, and eventually develop into invasive aspergillosis (IA) in patients with immune dysfunction (Voltersen *et al.* 2018). In the past few decades, despite a series of advances in the diagnosis and treatment of IA, the morbidity and mortality of IA are still increasing (Cadena *et al.* 2016).

The poor treatment results are related to the patient's weakened immune status, importantly, *A. fumigatus* pathogen was not identified and detected as early as possible (Tang *et al.* 2016). Therefore, a rapid, simple and sensitive detection technology for *A. fumigatus* is urgently needed to control and manage IA.

At present, the conventional fungal culture method is considered the gold standard test in the diagnosis of IA. But its sensitivity is very low, and it takes several days to observe the growth, which is very laborious and time-consuming. Laboratory diagnostic methods mainly include Galactomannan detection (GM) test, histopathological studies, direct microscopic examination. GM test using bronchoalveolar lavage fluid (BAL) specimens is considered to be of great significance in the diagnosis of IA. However, the limitations are the availability of the test and the high false-positive rate (Clancy *et al.* 2007; Husain *et al.* 2008; Hoenigl *et al.* 2014). Molecular methods include polymerase chain reaction (PCR) and real-time fluorescent PCR can be used for early diagnosis and has strong specificity. However, PCR-based technique operation is complicated, time-consuming and requires specialized high-end equipment and skilled personnel, which is not suitable for use in the grassroots and remote poor areas (Yu *et al.* 2019). Therefore, there is an urgent need for a reliable and rapid detection method for the pathogen of *A. fumigatus*.

So far, a novel technique called loop-mediated isothermal amplification (LAMP) has been successfully established, which can amplify DNA with high efficiency, specificity and rapidity (Notomi *et al.* 2000). The LAMP reaction is usually performed at a constant temperature (60–67°C), requires 4–6 primers to identify distinct regions of the target, and relies on autocycling strand displacing DNA synthesis by the Bst DNA polymerase large fragment (Nagamine *et al.* 2002; Zhang *et al.* 2014). To date, turbidimeters, gel electrophoresis, lab-on-a-chip devices, colorimetric agents and nanoparticle-based lateral flow biosensors (LFBs) have been used to analyse LAMP amplicons (Zhang *et al.* 2014; Li *et al.* 2020). Especially, in view of its simple, fast and low-cost advantages, LFB has been increasingly used as an alternative tool for analysing LAMP products (Wang *et al.* 2017a, 2017b). At present, this detection method has been applied to *Staphylococcus aureus*, *Brucella* and *SARS-CoV-2* (Wang *et al.* 2017a, 2017b; Li *et al.* 2019; Zhu *et al.* 2020).

In this study, a LAMP-LFB assay was established and used to detect *A. fumigatus* by detecting the specific *anxC4* gene of species *A. fumigatus*. *AnxC4* is a new member of the annexin family with an unusually long N-terminal tail and lacks conserved repeats, which is obviously different from human annexin (Khalaj *et al.* 2004). This makes it a meaningful target for fungal detection. The specificity and sensitivity of this LAMP-LFB assay were evaluated, and 89 clinical sputum samples were tested using this assay. Besides, the clinical results obtained from this assay were compared with the results of pure culture and the traditional PCR method.

Materials and methods

Reagents and instruments

Universal isothermal Amplification kits and visual detection reagent (VDR) were purchased from BeiJing-Hai-TaiZhengYuan Technology Co., Ltd. (Beijing, China). The backing card, conjugate pad, sample pad, nitrocellulose membrane (NC) and absorbent pad were obtained from Jie-Yi Biotechnology. Co., Ltd. (Shanghai, China). Biotin-BSA (biotinylated bovine serum albumin) and anti-FITC (rabbit anti-fluorescein antibody) were purchased from Abcam. Co., Ltd. (Shanghai, China). Dye (Crimson red) streptavidin-coated polymer nanoparticles (129 nm, 10 mg ml^{-1}, 100 mmol l^{-1} borate, pH 8·5 with 0·1% BSA, 0·05% Tween 20 and 10 mmol l^{-1} EDTA) were purchased from Bangs Laboratories (IN). DNA extraction kits (QIAamp DNA minikits; Qiagen, Hilden, Germany) were purchased from Qiagen (Beijing, China).

Design of LAMP assay primers

A set of primers for the *anxC4* gene (GenBank accession no. AY598940) of *A. fumigatus* were designed by Primer Explorer V4 (http://primerexplorer.jp/elamp4. 0.0/index.html; Eiken Chemical Co., Ltd., Tokyo, Japan) and primer software PRIMER PREMIER 5.0. After screening experiments, we chose the best pair of primers. Including two inner primers (FIP and BIP), two outer primers (F3 and B3) and two loop primers (LF and LB). In addition, the *anxC4* gene can encode a protein expressed in all *A. fumigatus*. The specific sequences and positions of the designed primers are shown in Fig. 1 and Table 1. All primers were synthesized by TsingKe. Co., Ltd. (Beijing, China) at HPLC purification grade.

Fungi, bacterial strains and genomic template preparation

In this study, we collected 22 fungi strains and 11bacterial strains (Table 2) from clinical and environmental samples, including 16 strains of *A. fumigatus* and 17 non-*A. fumigatus* strains. *Aspergillus fumigatus* (ATCC 16907) was used as a reference strain to optimize the LAMP-LFB assay. According to the manufacturer's instructions, genomic templates were extracted from all isolated strains using the DNA extraction kit (QIAamp DNA Mini Kit)

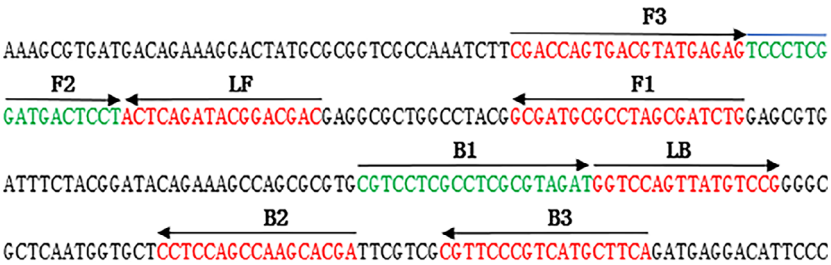

Figure 1 Sequence and location of *anxC4* gene used to design loop-mediated isothermal amplification primers. The nucleotide sequences of the sense strand of *anxC4* are listed. Right arrows and left arrows indicate sense and complementary sequences that are used.

Table 1 The primers used in the current report

Primer's name*	Sequences and modifications	Length	Genes
F3	5′-CGACCAGTGACGTATGAGAG-3′	20nt	anxC4
B3	5′-TGAAGCATGACGGGAACG-3′	18nt	
FIP	5′-CAGATCGCTAGGCGCATCG C-TCCCTCGGATGACTCCT-3′	38mer	
FIP*	5′-FITC-CAGATCGCTAGGCGCATCG C-TCCCTCGGATGACTCCT-3′	38mer	
BP	5′-CGTCCTCGCCTCGCGTAGAT-T CGTGCTTGGCTGGAGG-3′	38mer	
LF	5′-GTCGTCCGTATCTGAGT-3′	17nt	
LF*	5′-Biotin-GTCGTCCGTATCTGAGT-3′	17nt	
LB	5′-GGTCCAGTTATGTCCG-3′	16nt	

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

Table 2 Fungi and bacterial strains used in the current study

Strains	Strain no. (source of strains)	No. of strains	m-LAMP result
Aspergillus fumigatus	ATCC 16907	1	P
Aspergillus fumigatus	Isolated strains (ZJ)	15	P
Candida parapsilosis	Isolated strains (ZJ)	1	N
Staphylococcus aureus	ATCC 33862	1	N
Streptococcus pneumoniae	Isolated strains (ZJ)	1	N
Candida albicans	ATCC 24433	1	N
Aspergillus flavus	Isolated strains (ZJ)	1	N
Aspergillus versicolor	Isolated strains (ZJ)	1	N
Aspergillus terreus	Isolated strains (ZJ)	1	N
Aspergillus niger	Isolated strains (ZJ)	1	N
Stenotrophononas maltophilia	Isolated strains (ZJ)	1	N
Listeria monocytogenes	Isolated strains (ZJ)	1	N
Pseudomonas aeruginosa	ATCC 35032	1	N
Enterococcus faecium	Isolated strains (ZJ)	1	N
Vibrio cholerae	Isolated strains (ZJ)	1	N
Bordetella pertussis	Isolated strains (ZJ)	1	N
Leptospira interrogans	Isolated strains (ZJ)	1	N
Shigella boydii	Isolated strains (ZJ)	1	N
Plesiomonas shigelloides	Isolated strains (ZJ)	1	N

Only *A. fumigatus* strains could be detected by the LAMP technique, indicating the extremely high selectivity of the method.
A. fumigatus, *Aspergillus fumigatus*; ATCC, American Type Culture Collection; (ZJ), Zhejiang Provincial People's Hospital; P, positive; N, negative.

and measured by a UV spectrophotometer (Nano drop ND-1000, Beijing, China, caliber). For the extraction of fungal DNA, the isolates were cultured on Sabouraud dextrose agar (SDA) plate at 30°C for 3 days, and the plate surface was then flooded with 10 ml fungal saline to harvest the conidia. Then, 1 ml of saline suspension was prepared for centrifugation and the cell pellet was collected. DNA was then extracted according to the QIAamp DNA Kit. For the extraction of bacterial DNA, the isolates were inoculated on a blood plate at 37°C for 18–24 h, and then a sterile cotton swab was used to scrape the colonies on the plate and added to 2 ml of sterile saline. Next, as with fungal DNA extraction, 1 ml saline suspension was prepared for centrifugation, cell pellets were collected and DNA was extracted according to the QIAamp DNA Kit. *Aspergillus fumigatus* DNA templates were diluted 10-fold continuously (100 pg μl^{-1}, 10 pg μl^{-1}, 1 pg μl^{-1}, 100 pg μl^{-1}, 10 fg μl^{-1}, 1 fg μl^{-1}, 0·1 fg μl^{-1}) to determine the sensitivity of LAMP-LFB method for detecting *A. fumigatus*. In addition, 1 μl of each dilution was added as a unit to the standard LAMP reaction. The DNA in this assay was extracted from sputum samples, and the concentration ranges from 5 to 49 ng μl^{-1}.

Preparation of LFB

LFB was prepared on the basis of previous studies. The LAMP result is displayed by LFB in this experiment

(Wang *et al*. 2017a, 2017b, 2018). In brief, LFB is mainly composed of backing card, conjugate pad, sample pad, NC and absorbent pad. SA-PNPs were impregnated on the conjugate pad. Moreover, the anti-FITC (0·15 mg ml^{-1}) and biotin-BSA (2·5 mg ml^{-1}) were immobilized in the reaction area of the biosensor by physical adsorption. Then, test lines (conjugated with anti-FITC) and control lines (conjugated with biotin-BSA) were formed on the NC membrane, respectively. The two visible crimson red lines (TL and CL) on the biosensor indicate a positive result for LAMP product.

Aspergillus fumigatus-LAMP assay

According to previous research (Tang *et al*. 2016; Tone *et al*. 2017; King *et al*. 2019), *A. fumigatus*-LAMP assay was performed in a 25 μl amplification mixture containing outer primer F3 and B3 (0·4 μmol l^{-1} each), inner primers FIP and BIP (1·6 μmol l^{-1} each), loop primers LF and LB (0·8 μmol l^{-1} each), DNA template (1 μl), 8 U Bst DNA polymerase (1 μl) and 2·5 μl 10× of the supplied buffer. All mixtures were heated at 66°C for 1 h, *Streptococcus pneumoniae* (isolate) and *S. aureus* (ATCC 33862) were used as negative control (NC) sample, and double distilled water (DW) was used as the blank control (BC) sample.

LAMP-LFB assay

One microlitre of LAMP products, which were FITC and biotin-labelled LAMP products, was loaded into the sample zone. Subsequently, 60 μl of running buffer was also added to the same area. Then, LAMP products and SA-PNPs can be transferred from the conjugate region to TL and CL. Biotin-labelled LAMP products and SA-PNPs form complex by the interactions of biotin–streptavidin–biotin in the conjugated region. And the biotin/LAMP complexes were immobilized on the test strip through the hapten (FITC) and anti-FITC interaction. SA-PNPs that did not construct complexes were captured at CL through the interaction of streptavidin and biotin. Finally, SA-PNPs/LAMP/FITC complex and non-complex SA-PNP were reported on the visible lines TL and CL of the sensor strip, respectively. Moreover, additional monitoring techniques, mainly including VDR and real-time turbidity (LA-320C) were used to report the *A. fumigatus*-LAMP products in the assay.

Optimizing the temperature of LAMP-LFB assay

Optimal temperature of *A. fumigatus*-LAMP primers was assessed during the primer amplification stage. The reaction temperature ranges from 61 to 68°C at intervals of 1°C, and the results of each reaction are compared.

Sensitivity of LAMP-LFB assay

We used serial dilution (100 pg μl^{-1}, 10 pg μl^{-1}, 1 pg μl^{-1}, 100 fg μl^{-1}, 10 fg μl^{-1}, 1 fg μl^{-1}, 0.1 fg μl^{-1}) of *A. fumigatus* to determine the sensitivity of the LAMP-LFB assay and added 1 μl of genomic DNA into the amplification mixtures. The limit of detection (LoD) is the minimum genomic template dilution concentration for positive results. In addition, real-time turbidity and VDR were used to detect the sensitivity of LAMP-LFB assay and then compared with the LFB results.

Optimizing the reaction time of LAMP-LFB assay

The reaction time is evaluated from 30 to 60 min, with an interval of 10 min. We use LFB to show the results, and all the tests were repeated three times.

Specificity of LAMP-LFB assay

The specificity of the LAMP-LFB assay was proved by the detection of genomic DNA (at least 10 ng μl^{-1}) from 16 *A. fumigatus* strains and 17 non–*A. fumigatus* strains (Table 2). We used LFB to show the results. In addition, all the assays were repeated three times.

Evaluation of the feasibility of LAMP-LFB assay

We collected 89 sputum samples of suspected *A. fumigatus* from the People's Hospital of Zhejiang Province. (Ethics approval: The research protocol for the current study was approved by The Ethics Committee of the Zhejiang Provincial People's Hospital, Hangzhou, Zhejiang, China.) These samples were tested by culture-based technique, PCR detection and our new LAMP-LFB method for the diagnosis of IA. Generally speaking, traditional cultures were conducted with potato dextrose agar plate (PDA), SDA plate or Czapek–Dox agar plate (CA). The sputum samples were inoculated onto the above plate. The samples were cultured at 37°C for 5–7 days and then selected for cultivation. In addition, suspicious *A. fumigatus* can be identified by colony morphology, microscopic examination and GM experiments. For PCR method, *anxC4*-forward primer (5′-TCGCCAAATCTTCGACCA GT-3′) and *anxC4*-reverse primer (5′-GGTCTGGCATAG CTGGGATG-3′) were designed. The assays were conducted using the PCR settings of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 2 min. In addition, the amplicon size of *anxC4* gene by PCR assay is 275 bp (Khalaj *et al*. 2004). Finally,

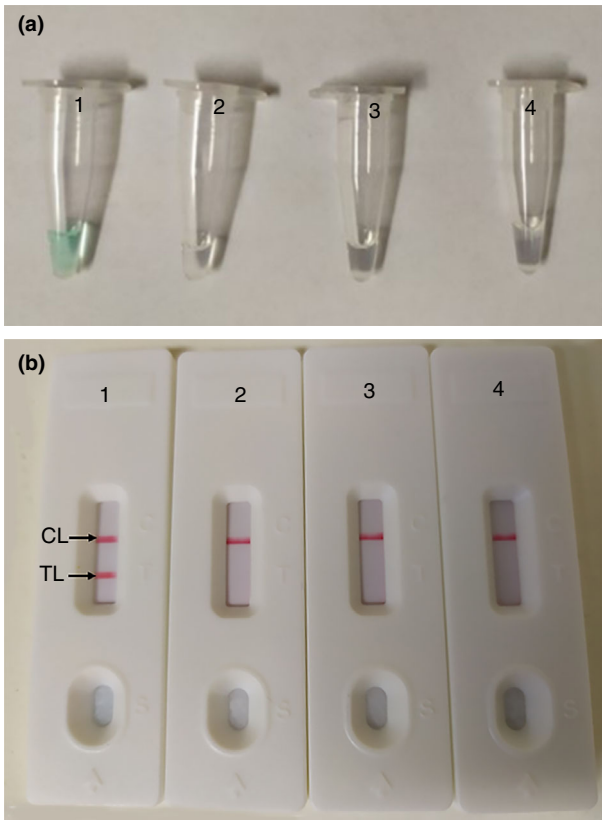

Figure 2 Detection and confirmation of *Aspergillus fumigatus*-LAMP products. (a) Color change of *A. fumigatus*-LAMP tubes; (b) lateral flow biosensor applied for visual detection of *A. fumigatus*-LAMP products. Tube A1 (biosensor B1), positive amplification; tube A2 (biosensor B2), negative amplification (*Pseudomonas aeruginosa*), tube A3 (biosensor B3), negative amplification (*Staphylococcus aureus*), tube A4 (biosensor B4), negative control (DW).

the results of LAMP-LFB method were compared with traditional culture and PCR detection.

Results

Confirmation and demonstration of LAMP products

The DNA for pure cultures was detected by LAMP reaction at 66°C for 1 h. Then, as shown in Fig. 2a, the *A. fumigatus*-LAMP amplification products were light green, whereas the negative ones remained colourless. In addition, further verification by the biosensor showed that two crimson bands (TL and CL) appeared in the *A. fumigatus*-LAMP amplification products, whereas only one band (CL) appeared in the negative ones (Fig. 2b). Therefore, the LAMP primer set, which was designed for *A. fumigatus* detection, is a good candidate for developing the *A. fumigatus*-LAMP-LFB assay.

Optimization of the temperature for LAMP primer set

To verify the optimal amplification temperature of the LAMP reaction, we use *A. fumigatus* genomic DNA (10 pg μl^{-1}) to perform LAMP reactions at different reaction temperatures (61–68°C, with 1°C interval), and all reactions were analysed by real-time turbidity detection. Kinetic curves were obtained at all reaction temperatures (Fig. 3), and the amplification speed was faster at 66°C compared with the others. Finally, 66°C was selected as the optimal amplification temperature and used as the optimal amplification conditions for the rest of the LAMP reactions.

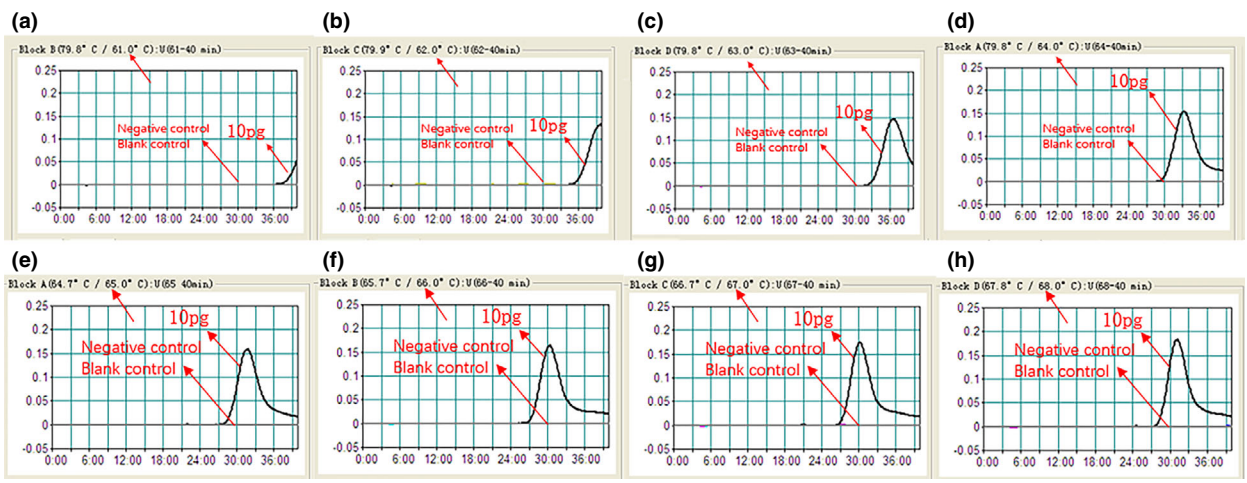

Figure 3 Optimal temperature for *Aspergillus fumigatus*-LAMP primer sets. The LAMP amplifications for detection of *anxC4* gene sequence were examined by real-time measurement of turbidity. The corresponding curves of concentrations of templates were marked in the figures. Eight kinetic graphs (a–h) were generated at various temperatures (61–68°C, 1°C intervals) with target pathogens DNA at the level of 10 pg per vessel (The threshold value was 0·1, and the turbidity of >0·1 was considered to be positive). The graphs from (e) (65°C) to (g) (67°C) showed robust amplification.

Sensitivity of *A. fumigatus*-LAMP assay

Serial dilutions of *A. fumigatus* genomic DNA (100 pg μl^{-1}, 10 pg μl^{-1}, 1 pg μl^{-1}, 100 fg μl^{-1}, 10 fg μl^{-1}, 1 fg μl^{-1}, 0·1 fg μl^{-1}) were prepared to determine the sensitivity of the LAMP-LFB assay. The results showed that the LoD of LAMP-LFB assay for purely cultured *A. fumigatus* was 100 fg. As shown in Fig. 4, two visible crimson red lines (TL and CL) appeared on the LFB, indicating positive results (Fig. 4, C1—C4), whereas only a crimson red line (CL) appeared on the LFB, indicating negative results (Fig. 4, C5–C8). In addition, the detection results of LAMP products using real-time turbidity (Fig. 4a), and VDR (Fig. 4b) was consistent with those obtained by LFB.

Optimization of the duration time for LAMP-LFB assay

At the optimal amplification temperature (66°C), we evaluated the effect on LAMP products at four different

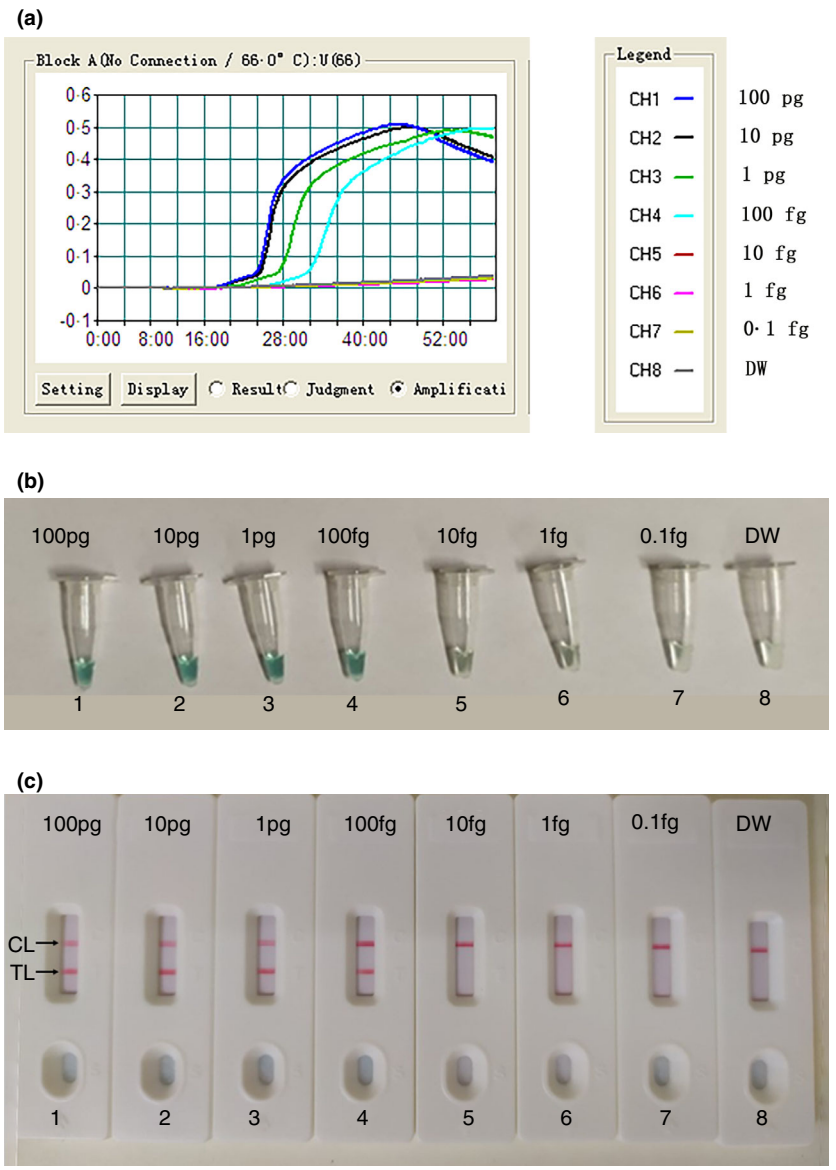

Figure 4 Sensitivity of LAMP-LFB assay using serially diluted genomic templates of *Aspergillus fumigatus*. Signals (a)/Tubes (b)/Biosensors (c). CH1–CH8, B1–B8, C1–C8 represented the DNA levels of 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, 0.1 fg per reaction and blank control (DW), respectively. The genomic DNA levels of 100 pg to 100 fg per reaction showed the positive results. (a) CH1 (——), CH2 (——), CH3 (——), CH4 (——), CH5 (——), CH6 (——), CH7 (——), CH8 (——).

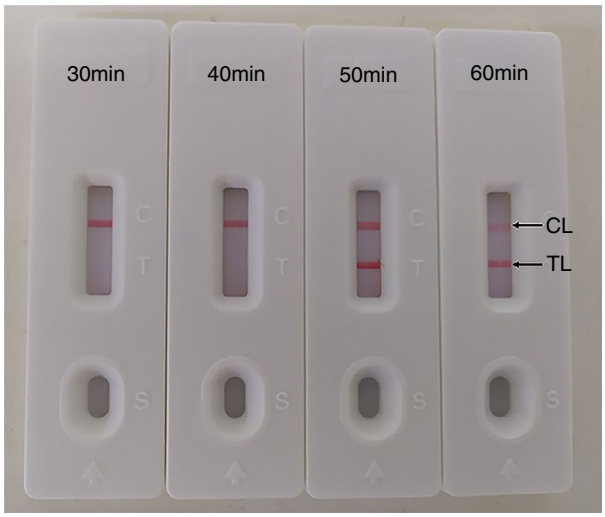

Figure 5 Optimal duration of time required for *Aspergillus fumigatus*-LAMP assay. Four different reaction times (a, 30 min; b, 40 min; c, 50 min; d, 60 min) were tested and compared at 66°C. Biosensors 1, 2, 3, and 4 represent DNA levels of 100 fg μl^{-1} (LoD level). The best sensitivity was seen when the amplification lasted for 50 min (c).

reaction durations (from 30 to 60 min, with an interval of 10 min). And the target DNA at the LoD level (100 fg) was detected when LAMP reaction lasted only for 50 min (Fig. 5). Therefore, the 50-min amplification time was selected as the best reaction time for our LAMP-LFB assay.

Specificity of *A. fumigatus*-LAMP assay

The DNA of all strains listed in Table 2 was tested to determine the specificity of this method. The results are shown in Fig. 6. Only the DNA from *A. fumigatus* showed the positive results. Two visible crimson red lines (TL and CL) appeared on the biosensors, indicating positive results for *A. fumigatus* (Fig. 6, B1–B2). By contrast, only one red line (CL) appeared on the biosensors, indicating negative results for non–*A. fumigatus* strains and blank control (DW) (Fig. 6, B3–B20), and the result of VDR was consistent with LFB (Fig. 6a).

Application of LAMP-LFB for the detection of *A. fumigatus* by sputum samples

We examined 89 sputum samples of suspected *A. fumigatus* infection by traditional culture, PCR and LAMP-LFB method, respectively. As shown in Table 3, the PCR method showed that 9 (10·11%) sputum samples were positive, and the other 80 (89·89%) sputum samples were negative. Results of LAMP-LFB detection showed that 10 (11·23%) sputum samples were positive (covering nine positive sputum samples by PCR method). This detection result was consistent with the traditional culture method, which confirmed that 10 (11·23%) sputum samples were positive. Based on the above results, it can be considered that the LAMP-LFB assay designed in this report is an excellent detection tool and has advantages over the traditional PCR method.

Table 3 Comparison of LAMP-LFB, culture-biotechnical and PCR methods for the detection of *Aspergillus fumigatus* in 89 sputum samples

	Whole sputum samples (n = 89)	
Detection methods	Positive	Negative
LAMP-LFB	10	79
Culture	10	79
PCR	9	80

Discussion

Aspergillus fumigatus is a highly lethal saprophytic fungus and the main cause of severe life-threatening IA (Kwon-Chung and Sugui 2013). Mortality caused by IA exceeds 50%, reaching 95% in some cases (Balloy and Chignard 2009). The rapid detection of clinical specimens for *A. fumigatus* is very important. In this report, we designed a novel type of LAMP-LFB detection method, which has the advantages of simplicity, high sensitivity and specificity. To make the detection technology more efficient and accurate, we successfully designed a set of six LAMP primers targeting the *anxC4* gene of *A. fumigatus* based on the LAMP principle (Fig. 1). These primers can specifically identify eight different nucleic acid regions of *A. fumigatus* with high selectivity. To determine the specificity of the LAMP-LFB assay, we successfully examined genomic templates extracted from 16 *A. fumigatus* strains and 17 non–*A. fumigatus* strains (Fig. 6 and Table 2). The two crimson red lines on the biosensor represent positive reactions only in all *A. fumigatus* strains. By contrast, one crimson red line represents negative reactions in all non–*A. fumigatus* strains.

In addition to its excellent specificity, the LAMP-LFB method is highly sensitive to *A. fumigatus* detection. The LoD is as low as 100 fg of DNA templates extracted from purely cultured *A. fumigatus* strains. And the LoD of the LAMP experiment detected by LFB was consistent with the real-time turbidity and VDR (Fig. 4). Besides, the results of the LAMP assay can be displayed by some simple instruments (such as a conventional heating instrument or a laboratory water bath instrument) providing a constant temperature of 66°C, without the need of other complicated instruments.

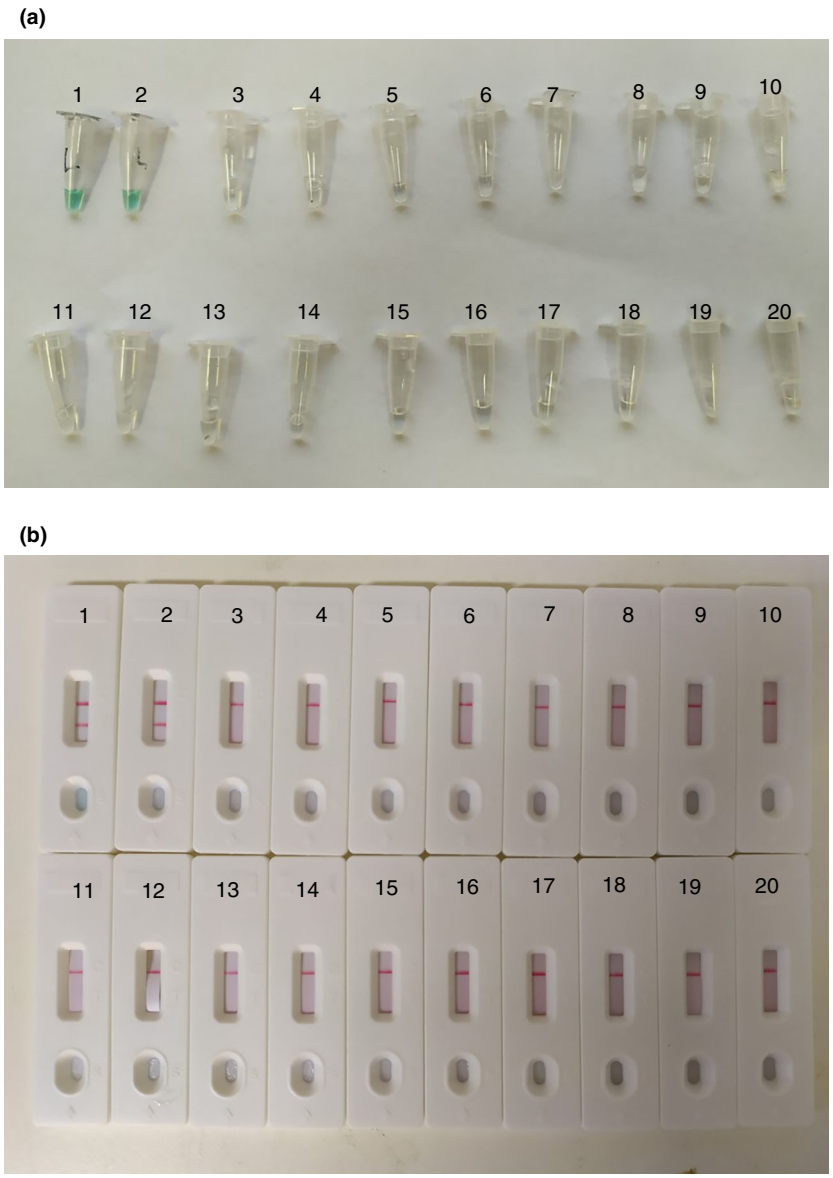

Figure 6 Analytical specificity of *Aspergillus fumigatus*-LAMP-LFB assay using different bacterial strains. The *A. fumigatus* -LAMP-LFB amplifications were tested using different genomic DNAs and were monitored by means of visual format. Tube A1–A2 and Biosensor B1–B2, *A. fumigatus* (isolated strain); Tube A3–A19 and Biosensor B3–B19, *Candida parapsilosis; Staphylococcus aureus*; *Streptococcus pneumoniae*; *Candida tropicalis*; *Aspergillus flavus*; *Aspergillus versicolor*; *Aspergillus terreus*; *Aspergillus niger*; *Stenotrophononas maltophilia*; *Listeria monocytogenes*; *Pseudomonas aeruginosa*; *Enterococcus faecium*; *Vibrio cholerae*; *Bordetella pertussis*; *Leptospira interrogans*; *Shigella boydii*; *Plesiomonas shigelloides*; Tube A20 and Biosensor B20, negative control (DW).

In this report, we used LFB to report LAMP products (Figs 2–6). Compared with real-time turbidity (Figs 3 and 4) and VDR (Fig. 2, 4 and 6), LFB has the advantages of fast results, simple operation and low error rate. And LFB does not require the complicated instrument, expensive reagents and professional technicians (Wang *et al*. 2019). Lastly, the LAMP-LFB method for detecting *anxC4* gene of *A. fumigatus* was successfully established. This method has high specificity and sensitivity. In this experiment, LAMP products were obtained by constant temperature heating at 66°C for 50 min, and then the products were detected directly by LFB.

The novel LAMP-LFB detection technology established in the current research is a rapid detection tool for *A. fumigatus*, which can detect the *anxC4* gene of *A. fumigatus*. And all tests were completed within 75 min, including 20 min of genomic template preparation, 50 min of LAMP constant temperature heating reaction and 2 min

of LFB analysis. Therefore, compared with the traditional culture and PCR diagnostic method, LAMP-LFB method is a time-saving and convenient molecular technique for *A. fumigatus* detection, and the detection time is shortened. In addition, 89 sputum samples collected clinically were tested and examined by traditional culture, PCR and LAMP-LFB methods to evaluate the feasibility of this new method. One sputum sample was tested positive by traditional culture method and LAMP-LFB method but tested negative by PCR method (Table 3). This result indicated that the diagnostic rate of PCR method is lower than that of the new LAMP-LFB detection method, which may be because the target gene templates copy numbers in the sample is lower than the detection limit of the PCR method.

In summary, we have successfully established a novel LAMP-LFB detection method for detecting the *anxC4* gene of *A. fumigatus*. This method shows a high specificity for detecting *A. fumigatus* and can accurately distinguish *A. fumigatus* and non–*A. fumigatus* strains. This method also has excellent sensitivity and can effectively detect a minimum 100fg of gene template (LoD). The whole detection process can be completed in 75 min, and the results of LAMP can be displayed rapidly in 2 min through the biosensor (LFB). Moreover, the clinical feasibility of this assay was satisfactorily demonstrated using clinical samples. The *A. fumigatus*-LAMP-LFB detection method is simple, fast, efficient, reliable and highly specific. Therefore, the LAMP-LFB assay established in this study could be widely used for the rapid detection of *A. fumigatus* in clinical and laboratory areas.

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Conflict of Interest

The authors declare that they have no conflict of interest in this work.

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