Establishment of a rapid diagnosis method for Candida glabrata based on the ITS2 gene using recombinase polymerase amplification combined with lateral flow strips

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Candida glabrata is the second or third most common Candida-associated species isolated from hospital-acquired infections, surpassing even C. albicans in some hospitals. With the rapid progression of the disease course of C. glabrata infections, there is an urgent need for a rapid and sensitive on-site assay for clinical diagnosis. Isothermal amplification is a recently developed method for rapid nucleic acid detection that is being increasingly used for on-site detection, especially recombinase polymerase amplification (RPA). RPA combined with lateral flow strips (LFS) can rapidly amplify and visually detect the target gene within 20 min. The whole detection process can be controlled within 30–60 min by rapid sample pre-treatment. In this study, RPA-LFS was used to amplify the internal transcribed spacer region 2 gene of C. glabrata. The primer–probe design was optimized by introducing base mismatches (probe modification of one base) to obtain a highly specific and sensitive primer–probe combination for clinical sample detection. RPA-LFS was performed on 227 clinical samples to evaluate the detection performance of the RPA-LFS system. Eighty-five samples were identified as C. glabrata, representing a detection rate of 37.5%. The results were consistent with...
qPCR and conventional culture methods. The collective findings indicate a reliable molecular diagnostic method for the detection of C. glabrata, and to meet the urgent need for rapid, specific, sensitive, and portable clinical field-testing.

**KEYWORDS**

*Candida glabrata*, recombinase polymerase amplification, lateral flow strip, ITS2, qPCR

**Introduction**

*Candida* spp. fungi are the most common opportunistic pathogens. The five most common are *Candida albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*, which account for 95% of human pathogenic fungal infections (Sendid et al., 2002; Khodadadi et al., 2017). Although *C. albicans* is still the most common fungus in clinical practice, its isolation rate has decreased in recent years. Compared to non-*C. albicans*, infections caused by *C. glabrata* have the highest morbidity and mortality rates, ranging from 40–70% (Krcmery and Barnes, 2002). The morbidity and mortality rates of *C. glabrata* occurring in oncology patients and bone marrow transplant patients are as high as 50% and 100%, respectively (Li et al., 2007). *C. glabrata* has become the second or third most common pathogen. The number of mucosal and systemic infections caused by *C. glabrata* has increased significantly. *C. glabrata* can cause infection alone or along with other pathogenic fungi, such as *C. albicans*, and there are no specific clinical symptoms (Tati et al., 2016). Therefore, a rapid and accurate diagnostic method for *C. glabrata* has become the key to the prevention and treatment of candidiasis.

At present, identification of *C. glabrata* typically uses the traditional fungal culture method. This approach is time-consuming and cumbersome, which limits the early diagnosis of *C. glabrata* and delays effective treatment. Highly sensitive and specific molecular biology methods used for *C. glabrata* include polymerase chain reaction (PCR) restriction fragment length polymorphism (PCR-RFLP) based on DNA amplification (Neji et al., 2017), real-time PCR (Souza et al., 2012), random amplified polymorphic DNA (RAPD) (Al-Tekreeti et al., 2018), PCR analysis of polymorphism by intron length, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Ferreira et al., 2011), sequencing analysis of pan-fungal markers, and isothermal assays (Guinea et al., 2021). The main isothermal amplification techniques are the loop-mediated isothermal amplification (LAMP) assay (Fallahi et al., 2020), rolling circle DNA amplification (Carinelli et al., 2017), nuclear acid sequence-based amplification (Huang et al., 2019), single primer isothermal amplification (Yang et al., 2021), isothermal amplification dependent on helicase-dependent isothermal DNA amplification (HAD) (Jeong et al., 2009), and strand displaced amplification (Zhang et al., 2017). Recombinase polymerase amplification (RPA) is a recently developed thermostatic amplification technique that can combine the advantages of the above methods and compensate for their shortcomings, which could potentially be valuable as a rapid, specific, sensitive, and portable diagnostic assay. We previously reported a related study on the detection of *C. albicans* using an RPA-lateral flow strip (LFS) protocol, which provides a reference for the detection of *C. glabrata* (Wang et al., 2021).

The specific RPA reaction is constructed using the internal transcribed spacer protein-2 (ITS2) gene of *C. glabrata* as the detection target. The amplification product is labeled with fluorescein isothiocyanate (FITC), which permits effective and accurate color development through the lateral flow strips (LFS) (Pienenburg et al., 2006). The assay process is simple and rapid. The reaction time at 37°C is approximately 20 min, and the LFS results can be observed in only 5 min. Because the assay system is less dependent on the laboratory environment, LFS is suitable for on-site accurate diagnosis of diseases. In addition, the assay system can be extended to different areas, such as healthcare and food safety, and used to detect other pathogenic bacteria from different sources. This is important to promote the development of clinical testing and diagnostics and its interdisciplinary aspects.

In this investigation, we obtained primer-probe sets with high specificity and sensitivity by carefully modifying the primer and probe bases. The results obtained using these sets were compared with the experimental results of qPCR and conventional culture methods by testing clinical samples. The aim was to ensure that our established method could be used for the diagnosis of Candida infections caused by *C. glabrata*.

**Materials and methods**

**Ethics statement**

The study protocol was approved by the Medical Ethics Committee of the Second People’s Hospital of Lianyungang City.
(Lianyungang, Jiangsu, China) (permit number 2020013). Informed consent was obtained from patients prior to collection of clinical samples.

Strain acquisition

C. glabrata ATCC 15126/66032/2001/64677 was purchased from Shanghai Covey Chemical Technology Co., Ltd. (Shanghai, China). Twenty strains of C. glabrata were isolated from clinical samples collected from 2020 to 2021. The specificity of the RPA-LFS assay was verified based on the ITS2 gene (GenBank: MF769562.1) of 23 common pathogens stored in our laboratory. These included C. parapsilosis ATCC 22019, C. krusei ATCC 14243, C. tropicalis ATCC 20962, C. albicans ATCC 10231, C. auris, C. dubliniensis, C. neoformans ATCC 14116, Acinetobacter baumannii ATCC 19606, A. calcoaceticus, A. lwoffii, A. haemolytius, A. jundi, Aspergillus fumigatus, Enterococcus faecium, Escherichia coli O157, Staphylococcus aureus, S. capitis, S. epidermidis, S. haemolyticus, S. hominis, S. saprophyticus, S. warneri, and S. maltophilia. In total, 227 clinical samples suspected of fungal infection were collected and pre-processed by the microbiology team of our laboratory in our hospital. The samples included 121 blood, 53 sputum, 35 urine, and 18 other samples.

Genomic DNA extraction

All bacterial strains were boiled at 100°C for 10 min before being used as templates for DNA release. Unless otherwise stated, 1 µL 10^8 colony forming units (CFU)/mL heat-treated cultures were used as templates. For C. glabrata and other fungi, genomic DNA was extracted and purified from cultures or clinical samples using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. An Invitrogen™ Qubit™ 4 Fluorometer (Thermo Fisher Scientific) was used according to the manufacturer’s instructions to quantify the extracted genomic DNA.

Primer and probe design and screening

Two pairs of RPA primers based on the ITS2 gene were designed using Primer Premier 5.0 software (Premier Biosoft International, San Francisco, CA, USA). After entering the sequence of a specific target region, the primers parameters were set as follows: product size of 80–150 bp and primer size of 30–35 bp, with no more than three consecutive bases in the three ends of complementary pairing. The maximum hairpin score, maximum primer-dimer score, and maximum poly-X score were each 5. The species specificity of the primer and probe sequences were confirmed using Primer-BLAST on the NCBI website (https://www.ncbi.nlm.nih.gov/tools/primer-blast). Forward primers were extended 15-23 bp backward at the 5’ ends and the performance of the probes and reverse primers was evaluated using Primer Premier 5 software. Theoretically, the formation of dimer and hairpin structures between the probe and the reverse primer was avoided as much as possible with a probe size of 46–53 bp, GC content of 30–80%, and Tm of 57–80. The 5’ end of the probe was labeled with FITC, the 3’ end was closed with a C3 spacer, and the middle base of the probe was replaced with tetrahydrofuran (THF). There were at least 30 bases before THF and then at least 15 bases following. The 5’ ends of the reverse primers were labeled with biotin.

RPA reaction

RPA reactions were performed using the TwistAmp® Liquid DNA Amplification Kit (TwistDx Inc., Maidenhead, UK) according to the manufacturer’s instructions. The 50 µL reaction system contained 25 µL 2× reaction buffer, 5 µL 10× Basic e-mix, 2.5 µL 20× core mix, 2.4 µL of 10 µM forward primer, 2.4 µL of 10 µM reverse primer, and 9.2 µL of distilled water. A 2.5 µL volume of 280 mM magnesium acetate and 1 µL of template were added to the lid of the reaction tube. After a brief centrifugation, the reaction mixture was incubated for 30 min at 37°C. RPA amplification products were purified by a PCR cleaning kit (Shanghai Meiji Biotechnology Co., Ltd., Shanghai, China) and used for 2% agarose gel electrophoresis.

RPA-LFS assay

RPA reactions were performed according to the manufacturer’s instructions for the Twist Amp DNA amplification nfoil kit (TwistDx Inc.). Each 50 µL volume of reaction mixture contained 2.1 µL of forward and reverse primers (10 µM), 0.6 µL of probe (10 µM), 2.0 µL of template, and other standard reaction components. Primers and probes were synthesized by Anhui General Biotechnology Co., Ltd. (Hefei, China). To start the reaction, 2.5 µL of magnesium acetate (280 mM) was added, and the reaction mixture was incubated at 37°C for 20 min. Then, 5 µL of the amplification product was diluted 20-fold and assayed by LFS (Usta Biotechnology Co., Ltd., Hangzhou, China). The LFS system consists of a sample pad, gold standard antibody pad impregnated with a mouse gold nanoparticle-labeled anti-FITC antibody, test line encapsulated with streptavidin, control line encapsulated with an anti-mouse antibody, and an absorption pad, arranged by solvent migration. The LFS was inserted into 100 µL of solvent for approximately 2 min until the test and control lines were visually detected.
Conventional culture

The 227 clinical samples were incubated on Stachybotrys medium for 24–48 h and then further inoculated on Candida chromogenic medium. The initial identification was based on the color of the medium. *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. krusei* harbor enzymes interact with the substrate and produce green, purple, dark blue, and pink colors, respectively. Using the DL-96IImicrobiological assay system (Zhuhai Deer Biological Engineering Co., Ltd., Guangdong, China), the isolated and cultured fungi were identified according to genus and species. Each fungal suspension was added to the biochemical reaction wells and incubated at 28–30°C. The biochemical reactions directly produced the color changes due to fungal metabolism or color changes after the addition of chromogenic agents.

Quantitative PCR analysis

Primers and probes are listed in Table 1. Specific primers and probes designed in our laboratory were targeted to the ITS2 gene of *C. glabrata* for qPCR detection. The qPCR reaction mixture consisted of 12.5 µL MonAmp™ SYBR Green qPCR mixture (Mona Biotech, Suzhou, China), 0.5 µM forward and reverse primers, 1 µL genomic DNA, and distilled water added to 25 µL. The cycling program was 95°C for 10 min, followed by 40 cycles on a LightCycler 480 qPCR machine (Roche, Basel, Switzerland) at 95°C for 15 s and 55°C for 60 s, respectively.

Results

Primer validation screening strategy

ITS2 was selected from the *C. glabrata* genome as a target for RPA-LFS detection. Two potential primer pairs were obtained by searching for primers on the ITS2 gene sequence using NCBI primer-BLAST (Table 1). These primers were initially screened by target gene fragment amplification and no-template control. The amplification products were examined by agarose gel electrophoresis to compare the amplification performance of the target gene and primer-dimer formation in the no-template control. The F2/R2 primer pair was selected. This pair displayed the best amplification performance and no primer-dimer formation (Figure 1A). Candidate probes were obtained by extending the 3’ end of the forward primer F2 by 16 bp. All possible dimers generated by the probe and reverse primer were predicted, and then the bases were modified (Table 1) until no cross dimer could be formed (Figure 1B). Finally, five forward primers were designed, screened, and tested upstream of the probe. The LFS results showed that F3/R2/P, F4/R2/P, F5/R2/P, F6/R2/P, and F7/R2/P amplified the target gene fragment efficiently, but F5/R2/P amplified less efficiently, while F3/R2/P, F4/R2/P, and F5/R2/P without template negative controls all showed False-positive results, and only F6/R2/P met the detection requirements (Figure 1C). Therefore, F6/R2/P was used in subsequent experiments.

Sensitivity determination of the PA-LFS assay system

To determine the limit of detection (LOD) of the RPA-LFS system for the detection of Candida, 10-fold gradient dilutions of *C. glabrata* ranging from 10⁶ to 10⁰ CFU/µL were tested. In the 50 µL reaction volume, 1 µL of *C. glabrata* genomic DNA was added. A red band appeared on the test line at 10 CFU/µL and became darker as the concentration of *C. glabrata* solution increased (Figure 2A). To test whether the system can resist interference from other fungal DNA, 10⁵ CFU/µL of genomic DNA from another common pathogen, *C. albicans*, was added to

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**Table 1** Primers and probes.

| Primers/Probes | Primer Sequences | Size (bp) | Reaction name |
|----------------|------------------|-----------|---------------|
| CG-F1          | TGGAGTTTACTTACATTCTTCTTTGTGTTG | 35        | RPA           |
| CG-R1          | TTGTCTACCTGTTGCTGGACATTCTGTTG   | 30        |               |
| CG-F2          | GTGGAATTTACCTTACATTCTTTCTTTGTGTT | 35        |               |
| CG-R2          | GTGTTCTCACTGTTGCTGGACATTCTGTT   | 31        |               |
| CG-P           | FITC-GTGGAGTTTACTTACATTCTTTCTTTCTTTGTTG[TTHF]GGGGAGGCTCTCTTT-C3 spacer | 52        | RPA-LFS       |
| CG-R2B         | Biotin-GTGGTTTACCTTCTTTCTCTTCTCTGTT | 31        |               |
| CG-F3          | AGACGCAACTGGAACGCTAGGATCATTAAAAAAA | 30        |               |
| CG-F4          | TCACGTGATCGCTTCTCTGCGCTGGCTTAA   | 34        |               |
| CG-F5          | CTTGAAATTCGCTTCTCTGCGCTGGCTTAA   | 32        |               |
| CG-F6          | GTGAATGGCTTCTGTTCTGCGCTGGCTTAA   | 30        |               |
| CG-F7          | GGGAGGAAGGCGCAAAAGACCTGGAAGTGT   | 31        |               |
| F              | AGCAAACTGGGAAAG                 | 15        | qPCR          |
| R              | AGCAGAAGGAAATGG                 | 15        |               |

F, forward primer; R, reverse primer; P, probe.
the RPA reaction. *C. albicans* genomic DNA did not interfere with the detection of *C. glabrata* (Figure 2B). The LOD of the RPA-LFS system was 10 CFU/50 μL per reaction. The detection sensitivity was not interfered with by other fungi.

**Interspecies specificity determination of the RPA-LFS assay system**

To confirm the inclusivity and specificity of F6/R2/P, four reference strains, 20 clinical isolates, and other pathogenic bacteria were amplified with RPA-LFS (Table 2). Four reference strains and 20 clinical isolates were positive (Figure 3), and the other pathogenic cultures were negative (Figure 4). The findings indicated that the primer–probe set has good inclusion and specificity for *C. glabrata* and can effectively detect *C. glabrata*, with no cross-reactivity with other pathogenic bacteria.

**Analysis of clinical samples**

To verify the practical application of RPA-LFS, 227 clinical samples were collected for RPA-LFS, qPCR, and culture. The results are shown in Table 3. Eighty-five samples were identified as *C. glabrata*, representing a detection rate of 37.5%. The results were consistent with qPCR and conventional culture methods. The results showed that RPA-LFS had the same accuracy as qPCR and was consistent with the results of the traditional culture method.
### TABLE 2 Bacterial strains used in this study.

| Species                | Source                  | Strain designation |
|------------------------|-------------------------|--------------------|
| C. glabrata            | Reference strain        | ATCC 15126         |
| C. glabrata            | Reference strain        | ATCC 66032         |
| C. glabrata            | Reference strain        | ATCC 2001          |
| C. glabrata            | Reference strain        | ATCC 64677         |
| C. glabrata            | Sputum isolated strain  | #1 #2 #3 #4 #5 #6 #7 #8 #9 #10 #11 #12 #13 #14 #15 #16 #17 #18 #19 #20 |
| C. parapsilosis        | Reference strain        | ATCC 22019         |
| C. krusei              | Reference strain        | ATCC 14243         |
| C. tropicalis          | Reference strain        | ATCC 20962         |
| C. albicans            | Reference strain        | ATCC 10231         |
| C. auris               | Sputum isolated strain  | N/A                |
| C. dubliniensis        | Sputum isolated strain  | N/A                |
| C. neoformans          | Reference strain        | ATCC 14116         |
| A. baumannii           | Reference strain        | ATCC 19606         |
| A. fumigatus           | Sputum isolated strain  | N/A                |
| A. calcoaceticus       | Sputum isolated strain  | N/A                |
| A. lwoffi              | Sputum isolated strain  | N/A                |
| A. haemolytica         | Sputum isolated strain  | N/A                |
| A. jundi               | Sputum isolated strain  | N/A                |
| E. faecium             | Sputum isolated strain  | N/A                |
| E. coli O157           | Sputum isolated strain  | N/A                |
| S. aureus              | Sputum isolated strain  | N/A                |
| S. capitis             | Sputum isolated strain  | N/A                |
| S. epidermidis         | Sputum isolated strain  | N/A                |
| S. haemolyticus        | Sputum isolated strain  | N/A                |
| S. hominis             | Sputum isolated strain  | N/A                |
| S. saprophyticus       | Sputum isolated strain  | N/A                |
| S. warneri             | Sputum isolated strain  | N/A                |
| S. maltophilia         | Sputum isolated strain  | N/A                |

ATCC, American Type Culture Collection.

**FIGURE 3**
Validation of primer pair F4/R2/P specificity against C. glabrata. (A) #1–#20 are the 20 C. glabrata isolated from clinical samples. NTC denotes negative control. The positions of the test and control lines are marked on the right side of the bar graph. Reactions were performed at 37°C for 20 min. Images represent the results of three independent experiments.
Discussion

Acquired immunodeficiency syndrome, organ transplantation, radiotherapy, and chemotherapy cause the body’s resistance to decline. This can increase the risk of opportunistic infection by Candida spp., as well as causing or complicating endocarditis, pneumonia, and other serious invasive fungal disease. In recent years, the proportion of Candida among the pathogenic fungi isolated clinically has been increasing in China and elsewhere. The clinical isolation rate of C. albicans has decreased, and the infections caused by C. glabrata have increased significantly (Hernando-Ortiz et al., 2021).

Among the traditional diagnostic methods of C. glabrata, the isolation and culture method is time-consuming and cumbersome. True or pseudo filaments are difficult to resolve in C. glabrata by the pathological tissue observation method. The yeast generally exists in the form of budding spores or cells, which are not easy to identify in sections. Thus, diagnosis can be missed, resulting in a low diagnostic rate (Yong et al., 2008; Asadzadeh et al., 2019). Serological methods based on antigen detection can only determine the presence of fungal infections. These methods cannot accurately identify the specific causative fungus, as the cell wall antigens of the cell wall of the pathogenic fungus (mainly dextran, galactose, and mannose) are similar (Sendid et al., 2002).

Molecular detection techniques require the selection of diagnostic amplification targets to effectively detect specific species. Many studies have evaluated various methods for detecting C. glabrata infections. We designed a pair of primer probes targeting the ITS2 gene to detect C. glabrata. False-positive results were avoided in this study by appropriate base mismatches to reduce the generation of cross-dimers between primers and probes. We designed two pairs of forward and reverse primers based on the target gene and obtained specific RPA amplification primer pair F2/R2. The 16 bases after forward primer F2 were pulled down as the probe. The probe and downstream primers may form cross-dimers, as predicted by Premier 5.0. Only one base at position 41 of the probe eliminated the dimerization; the LOD of base mismatch was not significantly affected. Subsequently, five forward primers were designed upstream of the probe and combined to obtain the best primer-probe sets. Modifications should be made with care to avoid mismatches at the 3’ end of the primer and probe, as this tends to reduce amplification performance. Although the RPA reaction can tolerate seven base mismatches, fewer base substitutions can maximize the detection performance established by the RPA-LFS system. Base modifications are crucial in laying the foundation for screening suitable primers (Piepenburg et al., 2006; Deng et al., 2020). RPA-LFS can accurately detect C. glabrata. The LOD of RPA-LFS was 10 CFU, which is higher than that of qPCR, i.e., 1–10 CFU per reaction (Ogata et al., 2015). In addition, the RPA-LFS method is simple and rapid for detecting C. glabrata and can be completed within 30 min. This method requires only an isothermal temperature of 37°C. In contrast, PCR and qPCR require temperature-controlled equipment and have relatively long reaction times. Conventional PCR techniques, including nested

Table 3: Prevalence of ITS2 in 227 clinical isolates of C. glabrata with the RPA-LFS and qPCR assays.

| RPA-LFS assay | Positive | Negative | Total |
|---------------|----------|----------|-------|
| qPCR Positive | 85       | 0        | 85    |
| qPCR Negative | 0        | 142      | 142   |
| Total         | 85       | 142      | 227   |
PCR, reverse PCR, and others, share the characteristics of rapidity, accuracy, specificity, and sensitivity. However, they require post-electrophoretic analysis, which increases the number of steps and prolongs the detection time (Dudiuk et al., 2014; Garcia-Salazar et al., 2022). The evaluation of clinical samples revealed a very high accuracy of RPA-LFS measurements. Results with different patient samples showed that positive samples from the RPA-LFS assay were comparable to positive samples from the qPCR assay, indicating that the RPA-LFS assay provides an alternative detection method. The main disadvantage with RPA-LFS is the problem of laboratory contamination generated by aerosols. Solving this problem will be challenging and will involve strict partitioning of the spiking, reaction, and test areas.

The foregoing findings demonstrate that the RPA-LFS assay is simple, rapid, accurate, and does not require laboratory facilities. It can be combined with a simple and rapid DNA extraction method for home testing of C. glabrata infections (Hernandez-Carreon et al., 2021). Timely diagnosis can facilitate early treatment of this fungal infection. The establishment of this rapid diagnostic method provides an effective means for early diagnosis and rapid identification of C. glabrata infection, and can be used for epidemiological investigations, disease prediction, and health assessments. It is important for correct clinical diagnosis and treatment, rational drug use, avoiding empirical drug use, and reducing drug-resistant strains.

The rapid clinical visualization molecular diagnosis technique of C. glabrata based on RPA-LFS developed in this study can obtain detection results within 30 min, with high specificity, high sensitivity, little dependence on instruments. This method has no need of specially trained laboratory personnel, high operability, and on-site detection, which can meet the conditions of remote areas weak primary hospitals or bedside diagnosis needs. The method should prove to be valuable for the rapid detection of C. glabrata.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Author contributions

LW, YW, and KW designed the experiments and wrote the manuscript. LZ and YL collected the clinical samples. KW and LW performed the main experiments. YW analyzed the data. All authors reviewed and approved the final version of the manuscript.

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Conflict of interest

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