A Comparison of Candida Detection in Sputum by the Conventional Culture and Fluorescent Polymerase Chain Reaction Methods

Xiaoqun Xu*, Huoyang Lv*, Fengwei Zhang, Houyong Zhu, Long Cai

Background: Candida is a pathogenic fungus. In recent years, the increase in immunosuppressive diseases has led to an increase in Candida infections, with the lungs being the most common site. Therefore, the aim of this study was to compare the positive detection rates of Candida in sputum samples by Candida culture and fluorescent polymerase chain reaction (PCR), and to explore a new method for rapid, accurate, and effective detection of Candida in sputum, providing swift evidence of clinical fungal infection.

Material/Methods: From October 2016 to March 2017, 300 sputum samples were collected and detected by the conventional culture method and fluorescent PCR method. The positive rate of Candida detection was compared between the 2 methods.

Results: In the 300 sputum samples, the positive detection rate of Candida was 50% by the culture method and 65.67% by the fluorescent PCR method (P<0.001). Therefore, the positive detection rate of Candida was higher by the fluorescent PCR method.

Conclusions: The conventional culture method for Candida needs a longer duration (24 h to 48 h) and the positive detection rate is low. However, it takes only 3 h to detect Candida in sputum by the fluorescent PCR method, the positive detection rate is high, and can be used as a screening method for Candida in sputum samples. Additional large-scale clinical trials need to be completed to assess the correlation between fluorescent PCR and pulmonary Candida infection.

Keywords: Candida • Culture • Real-Time Polymerase Chain Reaction

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Background

*Candida* is a pathogenic fungus. *Candida albicans* is found on all parts of the human body. It is one of the most common pathogens that causes deep fungal tissue infections [1]. When there is a balance between *Candida* and the body, there is no pathogenic effect on the body [2,3]. When the body’s immunity decreases, *Candida* can cause harm to the human body. In recent years, there has been an increase in immunosuppressive diseases, including acquired immune deficiency syndrome (AIDS) and other factors, including organ transplantation, leukemia, use of broad-spectrum antibiotics, indwelling catheterization, diabetes mellitus, and intravenous drug abuse [4-7]. This has led to a dramatic increase in *Candida* infections worldwide, with the lungs being the most common site [8]. Patients with a pulmonary candidiasis infection have symptoms including cough, expectoration, asthma, and dyspnea. If the infection is serious, there can be systemic symptoms (fatigue, fever, and chills). However, due to the atypical symptoms and signs, non-specific imaging, and often an accompanying mixed bacterial infection, missed diagnoses and misdiagnoses are common. Pulmonary *Candida* infections can greatly increase the mortality rate of immunocompromised patients. Mortality rates vary and can range from 25% to 60% in patients with intra-abdominal infections and from 30% to 50% in patients with bloodstream infections [9]. In critically ill patients, the mortality rates can reach 80% [10]. In a recent population-based survey in the USA, the mortality rate of invasive candidiasis was 29% [11]. In Spain, the mortality rate was 31% [12]. However, mortality can be much higher in other settings, as seen in 2 multicenter studies in Brazil (54% and 72%, respectively), and a hospital in South Africa (60%) [13]. *Candida* infections gradually develop into serious clinical diseases, which can lead to death in severe cases [14]. Therefore, exploring a new method for rapid and accurate detection of *Candida* in sputum is of great importance to study the pathogenicity of *Candida* and prevent and monitor pulmonary *Candida* infection. With new developments in molecular biology technology, the fluorescent polymerase chain reaction (PCR) method can be used to detect and identify clinical pathogens. In this study, the conventional culture method and fluorescent PCR method were used to detect *Candida* in sputum. The positive detection rates between the 2 methods were compared to find the most rapid, accurate, and effective method to detect *Candida* in sputum.

Material and Methods

Collection of Samples

From October 2016 to March 2017, 300 sputum samples were collected from patients in several wards of our hospital. Basic information about the patients, including the name, sample number, age, sex, and hospitalization number were checked. Sputum samples were divided into 2 parts; 1 part was cultured by the conventional method, and the remaining samples were stored for 3 days in the freezer at -20°C for fluorescent PCR detection. Informed consent from the patients was exempted by the Ethics Committee of Zhejiang Provincial People’s Hospital (Ethics Application Ref: 2020QT351). This study’s protocol complied with the requirements of the Helsinki Declaration of the World Medical Association and the International Ethics Guide for Human Biomedical Research of the Council for International Organizations of Medical Sciences (CIOMS).

*Candida* Culture

All the samples were cultured on both bacterial and fungal media. The fungal culture media (Yeast Identification Medium, Mellier [Shanghai] Biological Products Co., Ltd., Shanghai, China) were Sabouraud dextrose agar with chloramphenicol and brain-heart infusion agar with antibacterial agents.

Principle of DNA Extraction from *Candida*

DNA was extracted using the centrifugal adsorption column method. DNA is adsorbed by the special silicon matrix adsorption material of the centrifugal tube of the DNA solid extract in an environment of high salt and low pH (Sample treatment solution B of the DNA Extraction Kit [Hangzhou Derlead Biotech Co., Ltd., Hangzhou China]). Then, it is released in a low-salt and high-pH environment (DNA eluent of DL-Sputum [fungus], DNA Extraction Kit, Hangzhou Derlead Biotech Co., Ltd., Hangzhou, China).

Principle of the Fluorescent Polymerase Chain Reaction (PCR)

Fluorescent PCR [15] is a fluorescent chemiluminescence method for the determination of total products after the PCR. TaqMan probes (Pan-Candida Nucleic Acid Detection Kit Fluorescent PCR, Hangzhou Derlead Biotech Co., Ltd., Hangzhou, China) were used in this study; the TaqMan probe includes fluorescence reporting groups and fluorescence quenching groups. When the 2 groups exist at the same time, the fluorescence emitted by the reporting group is quenched. When the PCR is amplified, the probe is degraded by the probe enzyme and the fluorescence reporting groups and fluorescence quenching groups are separated; then, the fluorescence reporting groups can emit fluorescent signals. The monitoring system can receive each fluorescent signal by amplifying a DNA strand and forming a fluorescent molecule; the accumulation of PCR products and the formation of fluorescent signals are synchronized.

After mixing the fluorescein-labeled TaqMan probes with the template DNA, the TaqMan probe pairs complementary to the
template DNA were cut off in the PCR reaction process, and the free fluorescein in the reaction system emitted fluorescence under specific light excitation. With an increase in the number of cycles, the number of target gene fragments increase exponentially, and the copy number of target genes is obtained by real-time detection of the corresponding fluorescent signal intensity using the standard template number of the known concentration (Figure 1A, 1B).

Figure 1. (A) Amplification plots for the fluorescent polymerase chain reaction method. (B) Standard curve for the fluorescent polymerase chain reaction method.
In this experiment, the primers and probes for fluorescent PCR were designed based on the highly conserved sequences of the 18S, 5.8S, and 28S subunits of fungal ribosomal DNA and the specific sequences of the internal transcribed spacer, as well as the specific primers and probes.

**DNA Extraction**

We referred to the study by Ramos et al for DNA extraction [16]. The kit used for DNA extraction was the DL-Sputum (fungus) DNA Extraction Kit (Hangzhou Derlead Biotech Co., Ltd, Hangzhou, China); its composition is shown in Table 1. For DNA extraction, 1.2 mL of sputum digestion solution was added into a 2-mL Eppendorf (EP) tube, then 0.6 mL of sputum solution was added and incubated at room temperature (optimum temperature 37°C) for 15 min to 30 min, and oscillated every 3 min for 15 s, until the mixture became clear and had uniform viscosity. Then, it was centrifuged at 10,000 rpm for 3 min, the supernatant liquid was discarded, and the cells were retained. Then, 1 mL of sample cleaning solution was added, oscillated, and suspended, and it was then centrifuged for 3 min at 10,000 rpm, and the supernatant liquid was discarded. The precipitation was suspended by adding 300 μL of sample treatment solution A. All the suspended samples were transferred to the centrifuge tubes containing solid DNA extracts (1 h before use, the centrifuge tubes were centrifuged to collect solids at the bottom of the tube), oscillated for 5 min, and then instantaneously centrifuged. Then, 200 μL of sample treatment solution B was added, mixed well, and centrifuged at 13,000 rpm for 10 min. The supernatant liquid was transferred to a new 1.5-mL EP tube (taking care not to inhale the oil in the lower layer) and mixed with 0.5 times absolute ethanol by volume. All the liquid in the centrifuge tubes was transferred to the DNA purification column with casing, centrifuged for 1 min at 13,000 rpm, and the centrifugal fluid in the casing was discarded. Then, the purification column was replaced in the casing and centrifuged for 3 min at 13,000 rpm. After centrifugation, the cap of the purification column was opened and aired for 1 min to 2 min to volatilize the ethanol on the filter membrane. The casing was discarded and the purification column was placed in a new 1.5-mL EP tube. Next, 40 μL of DNA eluent preheated at 70°C was dropped carefully over the filter membrane at the bottom of the purification column and allowed to stand for 3 min. Finally, these EP tubes were centrifuged at 13,000 rpm for 2 min. The cap of the EP tube was cut during centrifugation, and the centrifuged DNA recovery solution was transferred to another sterile EP tube for storage. The DNA recovery solution obtained by centrifugation can be used for subsequent experiments. The extracted fungal DNA should not be stored at room temperature; it should immediately be stored at below -20°C.

**Fluorescent PCR Detection**

The Pan-Candida Nucleic Acid Detection Kit (Fluorescent PCR) (Hangzhou Derlead Biotech Co., Ltd, Hangzhou, China) was used for the fluorescent quantitative PCR, and its composition is shown in Table 2. All the reagents need instantaneous centrifugation before use. Each reaction system is shown in Table 3. The dosage of the above reagents (except the sample/reference) was calculated according to the number of reaction tubes, added to the appropriate centrifuge tubes, and mixed well. The liquid in the centrifuge pipette was blown slowly and repeatedly, taking care to avoid splashing of the liquid or producing a large number of bubbles. After instantaneous centrifugation, 16 μL of PCR reaction solution was separately packed into each PCR reaction tube. The prepared PCR reaction tube, negative control, and positive control were transferred to the sample processing area (sampling area). Then, 4 μL of DNA or negative or positive control samples were added to the prepared PCR reaction tube, and then centrifuged immediately after capping the tube (or fixing the sealing film), and transferred to the sample detection area. Finally, the prepared reaction tube was placed in the PCR instrument, ([StepOne Plus ZJSRM Plus] YY-FZ-QY-03 Hangzhou Boke Co., Ltd., Hangzhou, China). The purified nucleic acid was obtained by centrifugation can be used for subsequent experiments. The extracted fungal DNA should not be stored at room temperature; it should immediately be stored at below -20°C.

| Sequence | Component                          | Storage conditions | Specification and quantity |
|----------|------------------------------------|--------------------|---------------------------|
| 1        | Sputum digestive fluid             | Room temperature   | 50 ml                     |
| 2        | Sample cleaning solution           | Room temperature   | 40 ml                     |
| 3        | Sample treatment solution A        | Room temperature   | 6 ml                      |
| 4        | DNA solid extract                  | Room temperature   | 20 Tubes                  |
| 5        | Sample treatment solution B        | Room temperature   | 4 ml                      |
| 6        | DNA purification column            | Room temperature   | 20 Groups                 |
| 7        | Flushing fluid                     | Room temperature   | 7.5 ml                    |
| 8        | DNA eluent                         | Room temperature   | 1 ml                      |

Table 1. The sputum DNA extraction kit.
China) and the reaction and detection was amplified according to the edited sample information, following the conditions shown in Table 4.

**Setting Analysis Conditions for the Results of Quantitative Fluorescent PCR**

When analyzing the results of the PCR amplification curve, the mapping type can be set as Rn (the fluorescence intensity measured at each point minus the fluorescence baseline intensity) vs cycle. Baseline setting: The baseline value was set between 2 and 3 cycles of PCR with the parameters shown in Table 4. The main purpose of setting the baseline is to calibrate the test results of the samples, so that the experimental results can better reflect the authenticity of the data.

Threshold setting: The threshold line of analysis software was set manually. The threshold line is located in the linear part of the exponential growth period of the amplification curve above the baseline. The point where the amplification curve intersects the threshold line is the CT value, which represents the variable value Rn (Rn=Rn [post-PCR reading]-Rn [pre-PCR reading]) before and after the fluorescence intensity amplification of the standardized reporting group. CT stands for the number of cycles that the fluorescence signal in each sample goes through when it reaches the set field value. The CT value was negatively correlated with the logarithmic value of the initial target DNA fragment (reference value). The standard curve is shown in Figure 1A and 1B.

**Standard of Quality Control**

Unless the negative and positive controls of the kit (Pan-Candida Nucleic Acid Detection Kit Fluorescent PCR, Hangzhou Derlead Biotech Co., Ltd, Hangzhou, China) satisfy the following conditions at the same time, the experiment is considered invalid and needs to be repeated. Negative control: The CT value for negative quality control is \( \geq 34 \), or Undetermined. Positive control: The CT value for positive quality control is \(<34\). A sample CT value of \(<34\) means a positive result and a sample CT value of \(\geq 34\) or Undetermined means a negative result.

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**Table 2. The universal nucleic acid detection kit.**

| Kit     | Components | Storage conditions | Specifications and quantities | Main components                        |
|---------|------------|--------------------|-------------------------------|----------------------------------------|
| A-box   | Primers    | -20°C              | 22 μL × 1 Tube                | Upstream and downstream primers for detection |
|         | Probe      | -20°C, protect form light | 11 μL × 1 Tube                | Probe for detection                    |
|         | Negative control | -20°C | 15 μL × 1 Tube                | Plasmid containing unrelated gene       |
|         | Positive control | -20°C | 15 μL × 1 Tube                | Plasmid Containing Target Gene          |
| B-box   | PCR reaction solution | 2-8°C, freeze prohibited | 220 μL × 1 Tube                | dNTP (containing dUTP), Taq enzyme, UNG enzyme, reference fluorescence, MgCl2, buffer |

**Table 3. Fluorescent PCR detection reaction system.**

| PCR reaction solution | Primer | Probe | Sterilized purified water | Sample/ reference | Total volume |
|-----------------------|--------|-------|---------------------------|-------------------|--------------|
| 10 μL                 | 1 μL   | 0.5 μL| 4.5 μL                    | 4 μL              | 20 μL        |

**Table 4. Detection conditions of fluorescent PCR.**

| Stage | Temperature | Time | Cycle number | Other parameter settings |
|-------|-------------|------|--------------|--------------------------|
| Stage 1 | 50°C | 2 min | 1            | Detection of fluorescein: FAM/TAMRA |
| Stage 2 | 95°C | 10 min | 1            | Reference fluorescence: ROX |
| Stage 3 | 95°C | 15 sec | 40           | Reaction system: 20 μL |
|         | 56°C | 30 sec |              | Fluorescence signal collection: Stage 3 56°C 30 sec |
The diagnosis and treatment of pulmonary candidiasis is difficult to diagnose and treat. The increase in drug-resistance in fungi, resulting in many adverse outcomes for patients, including dysbiosis, liver and kidney function damage, and an increase of the patients' financial burden.

There are some limitations to this study. First, the PCR method is more expensive than sputum culture, which can limit its use for mildly ill patients. However, it will be useful for patients with a high clinical suspicion of Candida infection and a rapidly evolving condition, it is important to obtain microbiological evidence of the Candida infection as soon as possible. The duration of a conventional fungal culture is shown to be negative after 24 h to 48 h, then clinicians need to wait for a second culture result to obtain a positive culture result before using antifungal therapy for suspected infectious patients.

The positive rate of a single sputum culture is low [20,21], and repeated sputum cultures need 4 additional days (average) before antifungal treatment can be started, resulting in an additional 4 days of hospitalization [22]. However, there remain some patients who do not have a definite diagnosis of fungal infection and do not receive antifungal treatment before hospital discharge. Some reasons are that unmedicated individuals have less severe disease and their fungal infection resolves without treatment. However, for those patients with liver cirrhosis, blood tumors, other infections, and immune disorders, there is a clear correlation between the time antifungal treatment is initiated and the length of hospitalization [23-26]. Venkatesh et al [27,28] demonstrated that early precision treatment of a Candida infection could effectively reduce the mortality rate. For patients with a high clinical suspicion of Candida infection and a rapidly evolving condition, it is important to obtain microbiological evidence of the Candida infection as soon as possible. The duration of a conventional sputum culture makes it very difficult to meet this need. The empirical use of broad-spectrum anti-Candida treatments (including amphotericin B, fluconazole, and itraconazole) and the overuse or misuse of antibiotics can cause general drug resistance in fungi, resulting in many adverse outcomes for patients, including dysbiosis, liver and kidney function damage, and an increase of the patients’ financial burden.

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Statistical Analysis

The data were analyzed using SPSS 25.0 (SPSS, Inc., Chicago, IL). The McNemar test was used to analyze the rate according to the amount of data. A bilateral P value <0.05 was considered statistically significant.

Results

All 300 samples collected were used for Candida detection by both methods (culture and fluorescent PCR), as they were not contaminated or excluded for any other reasons. The results for both methods are shown in Table 5. The positive detection rate for Candida was 50% and 65.67% by the culture method and the fluorescent PCR method, respectively. The fluorescent PCR method significantly increased the positive detection rate compared to the culture method (P<0.001).

Discussion

Candida exists in the normal human body and usually does not manifest pathogenicity. However, when human immunity is low (including patients receiving surgical treatment, minimally invasive surgery, patients with malignant tumors or AIDS), the Candida infection is often secondary. In recent years, the annual incidence of fungal infections has increased due to the long-term use of broad-spectrum antibiotics and treatments using glucocorticoids and immunosuppressants. Candida spp. is the most common pathogen in fungal infections, and the lungs are the most common infection site. The clinical symptoms of pulmonary candidiasis are atypical and early infection is difficult to diagnose and treat. The increase in drug-resistant strains make diagnosis and treatment of pulmonary candidiasis even more difficult. The mortality rate of pulmonary candidiasis is increasing annually and seriously threatens human health [17,18]. Early diagnosis and treatment of pulmonary candidiasis can greatly improve patient prognosis and decrease the mortality rate.

The diagnosis and treatment of pulmonary candidiasis is complex. Clinicians need to find microbiological evidence to confirm the diagnosis before administering antifungal treatment. Confirmed pulmonary candidiasis according to the 2008 European Organization for Research and Treatment of Cancer/Mycosis Study Group’s (EORTC/MSG) revised definition [19] is to test for the microorganisms in pathological tissue or blood samples from sterile material, and suspected pulmonary candidiasis was defined as a positive sample with at least 1 sputum culture. If a patient presents with clinical symptoms or risk factors associated with pulmonary candidiasis and a conventional fungal culture is shown to be negative after 24 h to 48 h, then clinicians need to wait for a second culture result to obtain a positive culture result before using antifungal therapy for suspected infectious patients.

Table 5. Detection results of culture method and fluorescent PCR.

| Detection method | Fluorescent PCR |  | P value |
|------------------|-----------------|---|--------|
|                  | –               | + |        |
| Culture method   | 102             | 48 | <0.001 |
|                  | 1               | 149 |        |
| Total            | 103             | 197 | 300    |

The data were analyzed using SPSS 25.0 (SPSS, Inc., Chicago, IL). The McNemar test was used to analyze the rate according to the amount of data. A bilateral P value <0.05 was considered statistically significant.
with severe infection, when antifungal treatment needs to be considered and the PCR method can quickly obtain evidence, and for patients in whom Candida infection is highly suspected but there is no basis for antifungal treatment. Second, as the aim of this study was to investigate the detection rate of Candida in sputum by the fluorescent PCR and conventional culture methods, the current results do not determine colonization, contamination, and misdiagnosis. Further analysis by fluoroscopic methods is needed to observe the changes in the Candida hyphae to identify Candida infections in the lungs; this is the future research goal of our team. Third, this study did not have sufficient basic patient information (specific symptoms and reasons for hospitalization), which may have affected the results. In addition, nucleic acid detection in this study was performed in collaboration with Hangzhou Derlead Biotech Co., Ltd. under confidentiality agreements; therefore, the names and sequences of all the primers and probes adhere to the principles of nondisclosure. However, the sponsor did not play any role in the study design, data collection and analysis, or the decision to submit the article for publication.

In this study, 300 sputum samples were collected and tested by the conventional culture method and fluorescent PCR method to compare their positive detection rates. In the 300 sputum samples, the positive rate of Candida by the culture method was 50%, and 65.67% by the fluorescent PCR method. The positive detection rate of fluorescent PCR was higher than that by culture, and the difference was statistically significant.

Conclusions

A conventional culture of Candida takes 24 h to 48 h and the positive detection rate is low, while it only takes 3 h to detect Candida in sputum by fluorescent PCR, and the positive detection rate is high, which makes it a better screening method for Candida in sputum. By combining the results of the fluorescent PCR with the patient’s symptoms and other auxiliary examinations, clinicians can progress to a rapid final diagnosis, followed by treatment. However, additional large-scale clinical trials need to be completed to assess the correlation between the fluorescent PCR and pulmonary Candida infection.

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Department and Institution Where Work Was Done

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Conflicts of interest

None.
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