Molecular and conventional methods for detection of Candida species isolated from a sample of immunocompromised Iraqi patients with pulmonary symptoms

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Objectives Candida species has emerged as a potentially pathogenic fungus rather than benefit mucosal commensal in patients with pulmonary diseases. Therefore, our study was carried out to detect Candida species in sputum samples from patients with pulmonary diseases using conventional and molecular methods.

Methods A total of 100 sputum samples obtained from patients with pulmonary symptoms such as chronic productive cough, shortness of breath, wheezing and fever were included in this study. Sputum samples were dispensed into three specimen parts; the first one was applied for cultured on Sabouraud dextrose agar at 37°C for 48 h and then the purified colony of Candida underwent biochemical tests including API, Candida strips, and germ tube. The second part was undergone direct Gram stain, while the third part was applied for DNA extraction and then molecular diagnosis with PCR technique using specific primers.

Results Culture result revealed 43 positive samples for Candida species out of 100 samples. Among these positive samples, 23 (53.5%) were positive for C. albicans in each of culture and germ tube. API 20 Candida found that (40) samples were positive for Candida species as, 23 (57.5%) represent Candida albicans, 8 (20.0%) Candida glabrata, 4 (10.0%) Candida parapsilosis, 4(10.0%) Candida tropicalis and only one (2.5%) as Candida krusei. Molecular test revealed that forty one samples out of forty three culture isolates of Candida species were positive as follow: twenty three (53.48%), belong Candida albicans, Nine (20.93%) belong Candida glabrata, Six (13.95%) Candida parapsilosis, Four (9.30%) belong Candida tropicalis.

Conclusion Candida albicans is highly prevalent among patients suffering from bronchopulmonary symptoms. The molecular and conventional methods gave concomitant results as detection tools for the diagnosis of such microorganisms.

Keywords Candida albicans, phospholipase B gene, OMNIgene, API 20 AUX

Introduction

Candidiasis is a mycotic infection caused by members of the genus Candida. Chiefly, Candida albicans is responsible for about (70–80%) of all Candida infection. The Candida as an opportunistic yeast pathogen which increases predominantly in patients with predisposing condition, including immunodeficiency such as HIV infections, prolong used of broad-spectrum antibiotics, corticosteroids, diabetic patients and infections with other debilitating disease.

In immunocompromised patients, the clinical appearance of the C. albicans infection is often very complex and identification of the organism is difficult. Therefore, speedy diagnosis and management of candidiasis are crucial for these patients. Candida was frequently isolated from the mucosal surface of normal individuals, is capable of initiating a variety of recurring diseases especially in the vagina, oral and gastrointestinal mucosa. It also can affect different organs of the body, as systemic candidiasis involves major organs including, heart, kidneys, liver, spleen, lungs, brain, peritoneum, joint, and skeletal muscles, and was referred to generalized dissemination of the pathogen.

Candida pneumonia is one of the most challenging of all the Candida infections. Pneumonia due to infection with Candida spp. is extremely rare, but because of contamination with oral flora, these organisms are frequently cultured from respiratory secretions. Candida species are the fourth common cause of lung infections in hospitalized patients, and the most commonly isolated species include C. albicans, C. glabrata, C. tropicalis, and C. parapsilosis.

Methods

Sputum samples have been collected from 100 patients of age group ranged from 10 to 90 years old, with a mean age 47.23 ± 19.51. Some of these patients were suffering from systemic diseases such as tuberculosis, diabetes mellitus, leukemia, while others were with immunocompromised status. Those patients were attending and admitting to Al-Yarmouk Teaching Hospital, Al-Emamain Al-Kadhemain Teaching Hospital and Chest and Respiratory Diseases Institute/Baghdad Medical City during the period from September 2015 to February 2016. Each sputum sample was dispensed into three specimen parts. The first one was applied for culture on Sabouraud dextrose agar at 37°C for 48 hrs. Purified colonies from this culture had undergone biochemical tests including API Candida strips and germ tube. The second part was used in direct Gram stain while the third one was applied for molecular method. Standard strains of C. albicans ATCC 10231, was obtained from the National Institute of Health in Baghdad which was used as a positive control.

Isolation and Identification of Candida Species

Gram stain method was applied to each fresh sputum specimen and examined microscopically for detecting Candida species.
Sputum samples were streaked on Sabouraud’s Dextrose Agar (SDA) and incubated at 37°C for 24–48 hrs. The isolates were re-identified by using API 20 C AUX and germ tube production. API 20 C AUX was performed according to the manufacturer’s instructions. (Biomuriex, France) for the confirmatory identification of *C. albicans* and other species. Germ tube production is a diagnostic characteristic method for *C. albicans*. A small part of yeast colony to be tested was emulsified with 0.5 ml of mammalian serum in a small test tube. The tube was incubated aerobically at 37°C in an incubator for 2 hrs. A drop of the serum was removed to a slide and examined microscopically using the ×10 and ×40 objective lenses. A cylindrical filament originating from the blastoconidium without any constriction at the point of origin and without obvious swelling along the length of the filaments indicates a germ tube positive yeast.

**Molecular Method for Diagnosis of Candida Species**

The extraction of DNA was applied from each sample using sporeLYSE, DNA Genotek, purification kit (Canada) with modification by mixing 200 μl of the sputum sediment with the 40 μl of lysis buffer the suspension underwent a freezing–thawing technique by subjecting the samples to liquid nitrogen for 5 min; followed by boiling for 3 minute for five cycles. (Freezing–thawing technique was added to the protocol as an efficient step and enhance the cell lysis). The primer sequences were used for the amplification for PLB genes of *Candida* species were selected according to Nabil S. Harmal et al.,(9) (Table 1). An internal control has been used to measure the efficiency of the DNA extraction process fluids, as well as the impact of external and internal factors on gene amplification process. Since DNA extracted from fluids can be variably degraded and may contain PCR inhibitors. The human beta-globin primers was taken from Saiki et al.,(10) and synthesized in Alpha DNA* (Canada). The thermocycling conditions with a clever scientific thermal cyclers (TC 32/80-UK) were as follows: After initial denaturation at 94°C for 5 min, the 30-cycle amplification profile consisted of 95°C for 30 s, 63°C for 35 s and 72°C for 1 min. Final elongation was occurred at 72°C for 10 min. PCR products were processed into a 2% (wt/vol) agarose gel (Merck-Germany) at 7 V/cm for 1.5 hr. A molecular marker (1-kb DNA ladder; Bioneer) was run concurrently. DNA bands were visualized and photographed under UV light after the gel was stained with ethidium bromide.

### Modifications Step

#### Statistical Analysis

Statistical Analysis System (SAS) software was used for all statistical analysis continuous variables were expressed in mean ± standard deviation (SD). The Pearson’s Chi-square test or Fisher exact test was used for comparing the categorical variable. A two-sided significant level of 0.05 was considered to indicate a statistically significant difference.

#### Results

A total of 100 patients suffering from pulmonary diseases were enrolled in this study. 64 (64%) were males and 36 (36%) were females with a ratio of 1.8:1, Fig. 1. The principal findings were the ages ranged between 10 and 90 years with mean (47.23 ± 19.51) years.

### Table 1. Sequence and product size of PLB genes primers

<table>
<thead>
<tr>
<th>Candida species</th>
<th>Primer name</th>
<th>Primer sequence (5’ → 3’)</th>
<th>Annealing temperature</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. albicans</em></td>
<td>CA F</td>
<td>TTGTGTGTCACATACCAAC</td>
<td>63°C</td>
<td>538 bp</td>
</tr>
<tr>
<td></td>
<td>CA R</td>
<td>TTTGTTGCAACTCTTACACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. glabrata</em></td>
<td>Cg F</td>
<td>TCTCACACTCCATTGTCTCA</td>
<td>50°C</td>
<td>404 bp</td>
</tr>
<tr>
<td></td>
<td>Cg R</td>
<td>AGCAGGTTTACCACATAGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. parapsilosis</em></td>
<td>CPF</td>
<td>TCCATCGAGCAATTTGATG</td>
<td>60°C</td>
<td>252 bp</td>
</tr>
<tr>
<td></td>
<td>CPR</td>
<td>ACCGTTTTGACCTCAAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>CTF</td>
<td>CCCATACGATTTGAGAAT</td>
<td>53°C</td>
<td>501 bp</td>
</tr>
<tr>
<td></td>
<td>CTR</td>
<td>CATTGAGCAAGCATTAC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. Correlation between Candida species and gender

<table>
<thead>
<tr>
<th>Sex</th>
<th>Candida albicans</th>
<th>Candida glabrata</th>
<th>Candida parapsilosis</th>
<th>Candida tropicalis</th>
<th>PCR negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Count</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>8</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>20</td>
<td>36</td>
</tr>
<tr>
<td>%</td>
<td>34.8%</td>
<td>44.4%</td>
<td>50.0%</td>
<td>25.0%</td>
<td>34.5%</td>
<td>36.0%</td>
</tr>
<tr>
<td>Male</td>
<td>15</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>38</td>
<td>64</td>
</tr>
<tr>
<td>%</td>
<td>65.2%</td>
<td>55.6%</td>
<td>50.0%</td>
<td>75.0%</td>
<td>65.5%</td>
<td>64.0%</td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>9</td>
<td>6</td>
<td>4</td>
<td>58</td>
<td>100</td>
</tr>
<tr>
<td>%</td>
<td>100.0%</td>
<td>100.0%</td>
<td>100.0%</td>
<td>100.0%</td>
<td>100.0%</td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>0.899</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
The correlations between Candida species infections and gender are shown in Table 2.

Out of 100 patients suffering from pulmonary diseases enrolled in this study, they were categorized according to their underlying diseases as follows; 34 patients with tuberculosis, 26 patients with diabetes mellitus, 14 patients with acute lymphoblastic leukemia, 12 patients with lung cancer, 10 patients with lymphoma, 8 patients with acute myelogenous leukemia, 5 patients with asthma, 3 patients with heart failure, 3 patients with renal failure, 2 patients with liver cancer, 2 patients with ovarian cancer, 2 patients with prostate cancer, 1 patient with chronic bronchitis, 1 patient with chronic myelogenous leukemia and only 1 patient with Rheumatoid arthritis, Fig. 2.

Percentages of Candida Species from Patients with Pulmonary Manifestations and Other Underlying Diseases

This relationship between Candida species isolated from patients with pulmonary manifestations and other underlying diseases are summarized in Table 3, which showed a statistical significant difference ($P < 0.01$).

Cultivation and Gram Stain

A total of 100 sputum samples were cultivated on Sabouraud’s Dextrose agar and incubated for 2 days at 37°C. Forty three samples (43%) were positive for Candida species the colonies were mucoid and have a creamy color. Gram stain confirmed this result in that the 43 samples were gram positive.

Comparison between Molecular and Culture as Detection Methods for Candida Species

Both methods gave positive results for 41 samples. Separately, one sample was negative by culture and positive by PCR method, and two out of 43 samples were negative by PCR and positive by culture method. The sensitivity of culture test was 97.6% with a specificity of 96.6%, while the positive predictive value was 95.4% and negative predictive value was 98.3% with a $P$ value of < 0.001, Table 4.

Germ Tube Formation

A total of 43 culture samples were examined for germ tube. The result revealed that 23 (53%) were positive for Candida albicans, as shown in Fig. 3 and Table 5.

API 20 Candida Kit

A total of 43 culture samples were examined by API 20 AUX Candida strips, it was found that (40) samples were positive for Candida species, 23 (57.5%) represent Candida albicans, 8 (20.0%) Candida glabrata, 4 (10.0%) positive cases of Candida parapslosis, 4 (10.0%) Candida tropicalis and only 1 (2.5%) as Candida krusei (Table 6).
Molecular Detection

Conventional PCR was done for the amplification of PLB gene by using a specific set of primer sequences. The results showed that, this gene (PLB gene) was present in 23 out of 100 sputum samples, PCR product of this gene was 538 bp which represent Candida albicans. Nine Candida glabrata, PCR product of this gene was 404 bp. Six Candida parapsilosis PCR product of this gene was 252 bp. Four Candida tropicalis PCR product of this gene was 501 bp (Figs. 4 and 5).

Discussion

Candida infections are still an important problem, especially for immunosuppressed individuals. Inability or delay in diagnosing fungal infection defers the administration of appropriate therapy. This has grave implications for the prognosis of the patient: reliable and rapid diagnostic tests for systemic mycoses are imperative to improve rates of patient survival.

In the present study, results indicated that there is no relationship between the infection rate with Candida species and gender. The percentage of infected males were 26 out of 43 (60.4%) and females were 17 out of 43 (39.5%), hence there is no significant difference between male and female infection rate upon existing both in the same environment.

This result was disagreed with other study done by Saba Sabeeh, who found that Candida infection was more frequent among females than males, and disagreed with that done by Ibrahim, who found that infection was more frequent in males than in females. The possible explanation for such discrepancy may be due to nature of the societies and duration of time for sample collection.

In the present study, a relatively high percentage of Candida species infection was found among patients with hematological malignancies, solid tumor, asthma, diabetes mellitus and from patients with tuberculosis. These results are in accordance with those obtained by Lindau et al. and Ansari et al. who proved that a fungal infection represents a growing problem in patients with hematologic malignancies particularly during chemotherapy induced neutropenia and other chronic debilitating diseases.

Microscopic examination of sputum using staining methods, remain popular in the diagnosis of pulmonary infection especially in low-income countries, due to its rapidity, low cost, relatively easy to perform and high positive predictive value. On the other hand, culture is considered to be the “gold standard” method for the diagnosis of pulmonary infections but require 20 to 100 viable organisms per sample, and this is a cumbersome in partially treated patients. Culture also labor intensive and time consuming.

In this study, positive cultures were tested by germ tube and biochemical API 20. Results of germ tube revealed that 53.4% of positive culture were C. albicans. All germ tube samples were positive for C. albicans by API 20 AUX. Regarding other candida species tested by biochemical API 20, results revealed that 8 (20.0%) Candida glabrata, 4 (10.0%) positive cases of Candida parapsilosis, 4 (10.0%) Candida tropicalis and only 1 (2.5%) as Candida krusei. That's mean API 20 Candida and germ tube technique provides a convenient and reliable method for identification of Candida species.

Molecular Method for the Detection of Candida Species

The PLB gene of Candida species is a novel target which shows a high variability of sequences among Candida species. The nucleotide sequence variability between the different species of Candida can reach 95%. Thus, it is possible through designing a specific set of primers to target the unique sequence of PLB gene.

| Table 5. Percentages of germ tube formed by Candida albicans |
|---------------------------------|-------|----------------|
| Germ tube                      | Number| Percentage (%) |
| Negative                       | 77    | 77.00          |
| Positive                       | 23    | 23.00          |
| Total                          | 100   | 100%           |
| Chi-square (χ²)                |       | 12.792**       |
| P-value                        |       | 0.0001         |

**(P < 0.001).
Being used the same set of primers as in the current study, Harmal et al.17 proved that species-specific PCR assay could identify and differentiate between the four most common Candida species isolated from clinical specimens namely, C. albicans, C. glabrata, C. parapsilosis and C. tropicalis. Distinctive product size for each of these 4 species allows specific identification directly from the gel electrophoresis without the need for further genotyping. Based on the molecular weight of the ampiclon product from that PCR product of this gene, it was 538 bp in Candida albicans, 404 bp in Candida glabrata, 252 bp in Candida parapsilosis and 501 bp in Candida tropicalis.18,19

In this study, 23 samples were positive for PLB gene which is specific for Candida albicans, nine belong to Candida glabrata, six belong Candida parapsilosis, and four belong Candida tropicalis. A study done by Cheang Pey Shyuan (19) strongly suggest that PLB is a significant virulence determinant of albicans species. However, the data generated here would provide the vital groundwork for elucidating the intrinsic functional role of PLBs in the virulence and pathogenesis of the Candida albicans and non-albicans Candida species.

The results of the current study showed that the PLB gene provides a novel target that could be used for the identification and detection of medically important Candida species from the clinical samples. From this study, we concluded that Candida albicans is the most dominated isolates from patients suffering from pulmonary manifestations. Culture method is still the gold standard one in comparison with the molecular method.

Acknowledgement
The authors are grateful to all staff member of Medical Microbiology Department College of Medicine AL-Nahrain University for their help and cooperation. DNA Genotek kindly provided spore.LYSE DNA extraction kits free of charge for evaluation.

Conflict of Interest
The authors declare that they have no competing interests.

References