Original article

**Speciation and antifungal susceptibility of esophageal candidiasis in cancer patients in a tertiary care hospital in South India**

J. Abirami Lakshmy¹, Radhika Katragadda², J. Balaji³

¹Department of Microbiology, Karpagavinayaga Institute of Medical Sciences, Karpagavinayaga Nagar, Chinnakolambakkam, Palayanoor P.O, Kanchipuram District-603308, Tamil Nadu, India.
²Department of Microbiology, Government Kilpauk Medical college and Hospital, Chennai-600010, Tamil Nadu, India.
³Department of Prosthodontics and Implantology, Indira Gandhi Institute of Dental Sciences, MGMCR1 campus, Pilaaiyarkuppam, Puducherry-607402, India.

**Article history**

Received 30 September 2015
Accepted 05 January 2016
Early online 25 January 2016
Print 31 January 2016

**Corresponding author**

J. Abirami Lakshmy
Assistant Professor,
Department of Microbiology,
Karpagavinayaga Institute of Medical Sciences, G.S.T Road,
Karpagavinayaga Nagar,
Chinnakolambakkam,
Palayanoor P.O., Maduranthakam
Taluka, Kanchipuram District-603308,
Tamil Nadu, India.
Phone: +91-44-27592844
Mobile: +91-9751308239
Email: drabi3285@gmail.com

**Abstract**

Esophageal candidiasis is the most common opportunistic infection in patients with altered immunity such as HIV infection, cancer patients on chemotherapy and radiotherapy. Neutropenia, irradiation and chemotherapy will facilitate deeper mucosal invasion leading to esophageal candidiasis. Empirical treatment of esophageal candidiasis without antifungal susceptibility testing will lead to the emergence of drug resistant species increasing the morbidity and mortality associated with cancer. The present study aimed to study the frequency of esophageal Candida in individuals with cancer, species level identification and antifungal susceptibility pattern. Scrapings of whitish appearing lesions were obtained from a total of thirty five cases of endoscopically identified esophageal candidiasis were obtained from cancer patients. Identification of the Candida isolates were done by cultivation in Sabouraud dextrose agar (SDA), Gram staining, Germ tube test, colony morphology in Chrom agar and Corn meal agar, sugar assimilation and fermentation tests. Antifungal susceptibility was done by Microbroth dilution method for Fluconazole, Itraconazole and Amphotericin B. We found that Candida albicans was the predominant species isolated followed by Candida tropicalis and Candida glabrata. Sensitivity rates were 94%, 96% and 100% for Fluconazole, Itraconazole and Amphotericin B. Species level identification of Candida isolated from esophageal candidiasis and their antifungal sensitivity testing should be performed for early identification of resistant strains and for promptly treating the cases there by preventing the dissemination of infection in case of immune-compromised individuals. Further the susceptibility pattern will facilitate therapeutic guidance especially in individuals prone to relapse.

**Key words:** Antifungal susceptibility pattern, Cancer patients, Esophageal candidiasis

**DOI:** 10.5455/jmas.214436

© 2016 Deccan College of Medical Sciences. All rights reserved.
Candida species were previously thought to be insignificant cause of infection. Gastrointestinal tract serves as a reservoir of Candida. Alteration in immune status will promote the proliferation of endogenous Candida. Candida can cause infections ranging from superficial to deep and disseminated forms involving the internal organs. Within the past two decades Candida species has emerged as a major human pathogen currently ranked as one of the important cause of nosocomial infection.

Esophageal candidiasis is one of the most common opportunistic infections in individuals with impaired cellular immunity such as Human Immuno-deficiency Virus (HIV) infection, carcinoma, individuals on corticosteroid therapy, chemotherapy and radiotherapy. Diabetes, acid suppression, steroids, gastric surgery and esophageal motility disorders were also considered as predisposing factor for esophageal candidiasis. Candidiasis can develop secondary to malignancy due to impaired antifungal host defense due to mucosal damage. Esophageal candidiasis might represent a progressive immunity decline. Esophageal candidiasis has been reported in 1.4% of stomach cancer cases and in 7.8% of other malignancies. Alteration in immune status will promote the proliferation of endogenous Candida and will facilitate deeper mucosal invasion leading to esophageal candidiasis. From the esophagus Candida can spread to distant organs via hematogenous route.

Fluconazole is the empirical drug of choice for treating esophageal candidiasis. In individuals with cancer there is mucosal disruption facilitating the colonized Candida species to invade deeper tissue leading to esophageal candidiasis or even disseminated candidiasis. Indiscriminately treating all cases of esophageal candidiasis without antifungal susceptibility report can lead to the eradication of drug sensitive species by more inherently resistant species such as Candida krusei and Candida glabrata. Thus the emergence of antifungal drug resistance by the isolates has made in vitro antifungal testing methods essential to choose the appropriate antifungal drug and to predict the outcome of therapy.

Hence the present study was undertaken to isolate, speciate and to study the antifungal susceptibility pattern of esophageal candidiasis in cancer patients.

Materials and methods

This observational study was conducted from July 2012 to June 2013. The study was approved by the Institutional Ethical Committee. Informed consent was obtained from the patients included in the study.

Thirty five cancer patients with endoscopically suspected esophageal candidiasis on treatment with either chemotherapy or radiotherapy and not on any antifungal drugs were included in the study. Esophageal candidiasis was confirmed by the presence of typical coalescent white patches covering the esophageal mucosa. Esophageal scrapings from such lesions were collected in a sterile container and transported to the laboratory as early as possible. Samples were processed for microscopy, culture and characterization of Candida species.

From each specimen, smears were made on a grease free clean glass slide air dried and heat fixed and staining was done by Gram stain. The material was then inoculated immediately into two sets of Sabouraud Dextrose Agar (SDA) culture tubes and incubated at 25°C for 24-48 hours (Growth of the Candida in both the SDA tubes indicate that the strain isolated is not a contaminant but a pathogen). The medium was also supplemented with chloramphenicol (0.05 g/L) to prevent bacterial overgrowth. Growth usually occurs as creamy, white pasty colonies. Gram staining was performed from the colonies for confirmation of presence of Gram positive ovoid 5-7μm sometimes elongated to 4-6 × 6-10 μm in size.

Further species identification was done by Germ tube test, growth in Chrom agar based on the color of colonies (HIMEDIA), growth in Corn meal tween 80 Agar (Dalmau Plate Culture Technique), sugar fermentation test and carbohydrate assimilation test as per standard microbiological guidelines. Antifungal susceptibility testing was performed by Microbroth dilution method as per Clinical and Laboratory Standards Institute (CLSI) guidelines for Fluconazole, Itraconazole and Amphotericin B. Sensitivity, specificity and the diagnostic accuracy (Wilson score) of direct Gram staining was analyzed.

Antifungal susceptibility testing

Antifungal susceptibility testing was performed by Microbroth dilution method using RPMI 1640 with glutamine as per CLSI guidelines (2009). Stock suspension was prepared and diluted with RPMI to obtain an inoculum which contains 1x10³ to 5x10³/ml CFU. Antifungal stock solution was
prepared by dissolving Fluconazole in sterile distilled water. Amphotericin B and Itraconazole were dissolved in Dimethyl sulfoxide. The test was performed in a sterile disposable 96 well microtitre plate. The 2X drug concentration in 100μl volume was dispensed into the wells of row 1 to row 10 of the microtitre plate using a micropipette. Row 1 contained the highest drug concentration and row 10 contained the lowest drug concentration. Each well was inoculated with the 100 μl of 2X inoculum suspension. The growth control well contained 100 μl of sterile drug free medium and the corresponding diluted 2X inoculums suspensions. Row 11 was used to perform the sterility control containing only the drug free medium only. The microtitre plates were incubated at 35ºC for 48 hours. The plates were observed for the presence or absence of visible growth. A numerical score which ranges from 0 to 4 was given to each well.

0 = Optically clear
1 = Slightly hazy or approximately 25% of growth control
2 = Prominent decrease in turbidity or approximately 50% of growth control
3 = Slight reduction in turbidity or approximately 80% of growth control
4 = No reduction in turbidity

End point of MIC

Fluconazole and Itraconazole - Score 2 or less
Amphotericin B - Score 0

Results

A total of 35 endoscopically confirmed cases of esophageal candidiasis among cancer patients were included in the study. The distribution of cases based on the site of malignancy is shown in table 1. Out of the total 35 cases, 15 (42.8%) were suffering from carcinoma esophagus and 14 (40%) were having carcinoma stomach. The age and sex distribution of the patients are depicted in table 2 and 3. Most of the patients (40%) were in the age group of 41-50 years and 60% of the total cases were male patients. Out of the 15 carcinoma esophagus cases, 13 were adenocarcinoma of lower third of esophagus and two were squamous cell carcinoma. In case of carcinoma stomach all 14 were adenocarcinoma.

Out of the 35 cases, 33 (94.2%) were culture positive. Candida albicans was the predominant species isolated 31 (93.3%) followed by one isolate of Candida tropicalis and Candida glabrata each. Direct gram staining was done to look for the presence of Gram positive pseudohyphae and budding yeast cells and it showed a sensitivity and specificity of 100% in comparison with culture. The diagnostic accuracy of direct gram staining was 100% by Wilson score. Since Candida can also be present as a commensal in the gastrointestinal tract, direct Gram staining of the esophageal scrapings can be a valuable tool in the identification of invasive infection. All the culture positive isolates were further identified by Gram staining, Germ tube test (Fig 1), colony morphology on Chrom agar, growth on Corn meal agar, sugar fermentation and assimilation tests as per standard microbiological techniques. On Chrom agar, Candida was speciated on the basis of color of the colonies: light green color (C. albicans), metallic blue color (C. tropicalis) and pink color colonies (C. glabrata)₁³. On Corn meal agar speciation was done by presence of terminal chlamydospores (C. albicans) (Fig 2), oval blastoconidia arranged along the hyphae (C. tropicalis) and only the presence of blastoconidia without hyphae (C. glabrata). Sugar fermentation and assimilation tests were interpreted as per standard microbiological techniques (Fig 3).

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinoma esophagus</td>
<td>15 (42.8%)</td>
</tr>
<tr>
<td>Carcinoma stomach</td>
<td>14 (40%)</td>
</tr>
<tr>
<td>Carcinoma pancreas</td>
<td>1 (2.85%)</td>
</tr>
<tr>
<td>Others</td>
<td>5 (14.28%)</td>
</tr>
<tr>
<td>Total</td>
<td>35 (100%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Age group (in years)</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>31-40</td>
<td>3 (8.5%)</td>
</tr>
<tr>
<td>41-50</td>
<td>14 (40%)</td>
</tr>
<tr>
<td>51-60</td>
<td>11 (31.4%)</td>
</tr>
<tr>
<td>61-70</td>
<td>7 (20%)</td>
</tr>
<tr>
<td>Total</td>
<td>35 (100%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gender</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>21 (60%)</td>
</tr>
<tr>
<td>Female</td>
<td>14 (40%)</td>
</tr>
<tr>
<td>Total</td>
<td>35 (100%)</td>
</tr>
</tbody>
</table>
Antifungal susceptibility testing was done by Microbroth dilution method as per CLSI guidelines for Fluconazole, Itraconazole and Amphotericin B and interpreted as per table 4. Fluconazole and Itraconazole showed 6% and 4% resistance respectively. Both the non-Candida albicans isolated were resistant to Fluconazole. All the isolates were sensitive to Amphotericin B as seen in figure 4. By Microbroth dilution method Fluconazole, Itraconazole, Amphotericin B showed a sensitivity of 93.9%, 96.9% and 100% respectively (Fig 5).

Discussion

Esophageal candidiasis is the most common opportunistic infections in patient with altered immunity such as HIV, individual on corticosteroid therapy chemotherapy and radiotherapy. Development of esophageal candidiasis is a two step process consisting of colonization and subsequent invasion of epithelial layer. Once colonization has been established impaired cellular immunity permits invasion of epithelial layer. Neutropenia, irradiation, chemotherapy will lead to mucosal disruption facilitating deeper invasion of esophagus by Candida. Naito et al\textsuperscript{15} and Underwood et al\textsuperscript{16} had reported the prevalence of esophageal candidiasis to be 0.71% and 1.17% respectively. Most cases of esophageal candidiasis remain silent and invasion of esophageal wall is usually limited to the superficial epithelium leading on to extensive tissue necrosis and ulceration resulting in esophageal perforation. Candidiasis can develop secondary to malignancy possibly due to impaired antifungal host defense due to mucosal damage\textsuperscript{5,17}. Candida esophagitis is an important problem in cancer patients. The diagnosis can be missed in some cases leading to more invasive form of infection which can be recalcitrant to treat. In the present study cancer patients with endoscopically diagnosed esophageal candidiasis were included and Candida species causing infection were isolated, identified, characterized and antifungal susceptibility testing was done by Microbroth dilution method.
Table 4: Antifungal susceptibility testing by Microbroth dilution method - Interpretive criteria\textsuperscript{11,14}

<table>
<thead>
<tr>
<th>Antifungal drug</th>
<th>Susceptible</th>
<th>Susceptible dose dependent</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluconazole</td>
<td>≤ 8μg/ml</td>
<td>16-32 μg/ml</td>
<td>≥ 64 μg/ml</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>≤ 0.125 μg/ml</td>
<td>0.25-0.5 μg/ml</td>
<td>≥ 1 μg/ml</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>≤ 1 μg/ml</td>
<td>-</td>
<td>&gt;1 μg/ml</td>
</tr>
</tbody>
</table>

In this study all the patients have underwent chemotherapy, steroid therapy and antibiotic treatment. In a study by Sajith et al\textsuperscript{14} at Christian Medical College, Vellore, 60 patients with endoscopically suspected esophageal candidiasis with associated risk factors were studied. In that study they have documented that out of the 60 patients eleven individuals were on immunosuppressant therapy and ten had neoplasia.

In this study we observed that Candida albicans was the predominant species isolated which is consistent with the findings of Wilheim et al\textsuperscript{6}, Sajith et al\textsuperscript{14} (97.4%) and Badarinarayanan et al\textsuperscript{18} 87.5%.

In present study single isolate of Candida tropicalis and Candida glabrata were identified (3.03%). Reports from various parts of the world have indicated the emergence of non-Candida albicans species as a cause of infections\textsuperscript{16,17}. Candida albicans and non-Candida albicans differ in their antifungal susceptibility pattern. Few Candida species show innate resistance to drugs like azoles which are usually prescribed as the first line of drug for treating esophageal candidiasis. Empirical treatment with drugs like Fluconazole might eliminate the susceptible species like Candida albicans and promote more discerning growth of species that are naturally resistant like Candida krusei. Prophylactic treatment with antifungal drugs like Fluconazole without analyzing the antifungal susceptibility pattern may escalate the chances of colonization of esophagus by drug resistant non-Candida albicans species. Such colonization may lead to invasion of the epithelial layer and development of esophageal candidiasis with drug resistant strains when mucosal disruption occurs such as in the case of malignancy, cancer chemotherapy and irradiation\textsuperscript{17}. Antifungal susceptibility testing was done by broth microdilution method. Resistance rates were 6% and 4% for Fluconazole and Itraconazole respectively which was consistent with Goldman et al\textsuperscript{19} (4%) and Pfaller et al\textsuperscript{20}. Wilheim et al\textsuperscript{6} has reported Fluconazole resistance as 6 (14.28%). All the isolates were susceptible to Amphotericin B. Sajith et al\textsuperscript{14} has reported Fluconazole resistance as 59.4% and among the patients with neoplasia resistance to Fluconazole was 50%. Other studies have reported resistance rates varying between 10% and 64% for Fluconazole\textsuperscript{20}. In our study the two non-Candida albicans isolated were resistant to Fluconazole. Non-Candida albicans isolation in esophageal candidiasis is on the rise. Few species like C. krusei are inherently resistant to azoles, which is of significant concern since they are being used as the first line of drug for the treatment of esophageal candidiasis. In individuals with cancer and those who are on therapy like radiation and chemotherapy mucosal disruption may lead to development of esophageal candidiasis with drug
resistant isolates. Various studies have documented the prevalence of esophageal candidiasis, its association with different risk factors, species distribution and antifungal susceptibility pattern. In the present study we have studied the species distribution and antifungal susceptibility pattern among a single associated risk factor implicated in esophageal candidiasis i.e. cancer patients on chemotherapy and radiotherapy with altered immune status. For treating such isolates antifungal susceptibility pattern will provide us with an idea to choose the appropriate antifungal drug to prevent further dissemination of the infection which might have serious consequences in immunosuppressed individuals such as in cancer patients.

The emergence of antifungal resistance within Candida species, particularly in cancer patients, necessitates routine investigations into antifungal resistance pattern. Such type of studies will facilitate us to get a competent awareness about their drug resistance pattern and may help the physician in selecting the appropriate antifungal agent for empirical therapy.

Conclusion

Non-Candida albicans is emerging as an important pathogen causing esophageal candidiasis. The finding that significant proportion of Candida albicans showed reduced susceptibility to azoles may have implications for changes in the antifungal drug regimens for treatment. Species level identification and antifungal susceptibility testing is of importance in cancer patients with esophageal candidiasis to prevent invasiveness of the infection. Antifungal susceptibility testing must be done for all the isolates to prevent the emergence of drug resistant isolates.

Conflict of interest: Nil

Acknowledgements: Nil

References