Journal de Mycologie Médicale xxx (2018) xxx–xxx

Available online at
ScienceDirect
www.sciencedirect.com

Elsevier Masson France
EM|consulte
www.em-consulte.com

Original article/Article original

Oropharyngeal candidiasis in head and neck cancer patients in Iran: Species identification, antifungal susceptibility and pathogenic characterization

Z. Jahanshiri [a,*], S. Manifar [b], H. Moosa [a], F. Asghari-Paskiabi [a], H. Mahmoodzadeh [c], M. Shams-Ghahfarokhi [d], M. Razzaghi-Abyaneh [a]

[a] Department of Mycology, Pasteur Institute of Iran, 12, Farvardin street, Tehran 13164, Iran
[b] Department of Oral Medicine, School of Dentistry, Tehran University of Medical Sciences, Cancer Institute of Tehran, Imam Khomeini Hospital Complex, Tehran, Iran
[c] Department of General Surgery, Tehran University of Medical Sciences, Cancer Institute of Tehran, Imam Khomeini Hospital Complex, Tehran, Iran
[d] Department of Mycology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

ARTICLE INFO

Article history:
Received 19 September 2017
Received in revised form 9 January 2018
Accepted 11 January 2018
Available online xxx

Keywords:
Oropharyngeal candidiasis
Head and neck cancer
Candida
Antifungal susceptibility
Biofilm

ABSTRACT

Objective. – Oropharyngeal candidiasis (OPC) is the most frequent opportunistic fungal infection in head and neck cancer patients. This study was done to identify the *Candida* species, which cause OPC, and to evaluate their antifungal susceptibility pattern and pathogenic characteristics in Iranian head and neck cancer patients treated by radiotherapy.
Material and methods. – The oral clinical samples were determined by culturing on CHROMagar, carbohydrate assimilation and ITS sequencing methods. Biofilm formation, phospholipase and proteinase activity and antifungal susceptibility were examined too.
Results. – Among 54 patients with confirmed OPC, 39 (72.22%) patients were male and 15 (27.77%) were female. The most frequently *Candida* species from a total of 60 isolates was *C. albicans* (53.3%), followed by *C. tropicalis* (21.66%), *C. glabrata* (15%), *C. kefyr* (5%) and *C. dubliniensis* (1.66%). All the isolates were high-producers of biofilm. All of *Candida* isolates were proteinase positive and 47 isolates (81.04%) represented phospholipase activity. The maximum and minimum rates of antifungal resistance belonged to ketoconazole (93.75% of *C. albicans* and 89.28% of *Candida* non-*albicans*) and fluconazole (62.50% and 42.85% of *C. albicans* and *Candida* non-*albicans*), respectively. The most effective antifungal against all *candida* isolates was fluconazole.
Conclusion. – Our data can estimate abundance of OPC in male and female head and neck cancer patients and is helpful to use effective strategies for antifungal treatment, prophylaxis, and preventive therapies in these patients.

Introduction

Radiotherapy (RT) is one of the major treatment methods among patients with head and neck malignancies. RT in such cases increases oral fungal colonization, which may lead to significantly increased rates of oral fungal infections. Oropharyngeal candidiasis (OPC) is the most frequent cause of morbidity in these patients that began by a stage of colonization of the *Candida* on the oral mucosa. OPC is manifested by various symptoms such as pseudomembranous (thrush), erythematous and angular cheilitis. OPC creates painful infection that may expand to inner sites such as esophagus, leading to life-threatening systemic infections [1,2].

Candida species are commonly present as normal oral flora in humans. Any variation in physiological and pathological conditions, particularly after RT in patients with malignancies, may cause the yeast to switch to its pathogen type. Studies shown that among the *Candida* species, *C. albicans* is the most important species (> 80%) that causes OPC. Also non-*albicans* species are known to be contributed in OPC in patients with head and neck cancer [3]. The possibility of oral fungal colonization can increase up to 74.5% during RT. This increase in percentage of colonization mostly occurs immediately after RT (71.4%). The use of tobacco can also increase the risk of colonization and fungal infection during RT [4,5].

* Corresponding author.
E-mail addresses: z_jahanshiri@pastuer.ac.ir, zjahanshiri@yahoo.com (Z. Jahanshiri).

https://doi.org/10.1016/j.mycmed.2018.01.001
1156-5233/© 2018 Published by Elsevier Masson SAS.

Recent studies have shown that the sensitivity of *Candida* spp. to certain antifungal agents, especially azoles, is decreasing which is due to their extensive use as prophylaxis in cancer patients; this leads to high incidences of ineffective treatments [6,7].

There are few studies regarding to OPC in head and neck cancer patients who are treated by RT in Iran. Here, we studied 54 patients with detectable OPC among head and neck malignancy patients who had been treated by RT in the major cancer treatment institute in Tehran. This study was conducted to identify *Candida* spp. causing OPC, to determine their antifungal susceptibility pattern and pathogenic characteristics among the head and neck cancer patients in Iran. Our data can help to use more effective strategies in antifungal treatments and to design an appropriate prophylaxis program for the benefit of such patients.

Materials and methods

Patients

This study was conducted in Cancer Institute of Imam Khomeini Hospital at Tehran, with the approval of the Ethics Committee of Pasteur Institute of Iran, Tehran (IR.PII.REC.1394.59). In the present study, 160 patients with head and neck cancer who had received radiotherapy for two weeks were selected during a 6-month period (Jan 2016 to Jun 2016). The daily radiation dose was between 1.8 and 2 Gray in six fractions per week; the patients received no antifungal and azole-prophylaxis before and during radiotherapy. The study was informed and patients consent was obtained from every patient. The demographic data including sex, age and type of cancer were documented in patients' sheets. Sampling was carried out using sterile swabs. The patients were examined for OPC infection and those who had verified OPC were selected for this study. OPC was verified by finding white plaques on intraoral mucous layer and confirmed by existence of yeasts and hyphae on KOH 10% preparation and positive culture of oral swabs.

Clinical isolates

Oral samples were cultured on CHROMagar Candida (CHROMagar Company, Paris, France) medium and incubated at 37 °C for 24–48 h, *Candida* species were recognized by the color of the colonies. Yeasts were cultured on Sabouraud Dextrose Agar (SDA, E. Merck, Germany) medium for 48 h at 28 °C for following examinations. All the isolates were examined by standard techniques such as germ tube and growth in corn meal agar. Fresh colonies were picked and carbohydrate assimilation experiment was done by API 20C AUX (bioMérieux, France) kit according to the manufacturer's protocol and the results were analyzed by the apiweb software (bioMérieux, France). All isolates were stored in 15% glycerol at −80 °C until use [8,9].

Molecular identification by ITS sequencing

The isolates were cultured on SDA medium for 48 h at 28 °C. Total genomic DNA of the reference strain and each clinical isolate were extracted as described previously [10]. DNA extracts were stored at −20 °C until use. Amplifications were performed by AmpliTaq Gold PCR Master Mix (Applied Biosystems) in a reaction volume of 50 μL. The sequences of primers for Internal Transcribed Spacer regions (ITS 1 and ITS 4) were 5′- TCCGTAGGTGAACCTGCGG-3′ (forward) and 5′-TCCTCCGCTTATTGATATGC-3′ (reverse). The PCR amplification conditions were 95 °C for 12 min, 30 cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 100 s and a final extension at 72 °C for 10 min. The PCR products were purified and both strands of each gene fragments were sequenced by Applied Biosystems DNA Analyzers Sequencing (Bioneer, Korea). The Sequencing results were analyzed by MEGA6 software and BLASTn at National Center for Biotechnology Information (NCBI) website was used to compare consensus sequences to ITS sequences in the GenBank database [7,11].

Biofilm formation

The ability of biofilm formation was tested on each clinical isolate. Organisms were cultured on Sabouraud dextrose agar plates for 24 h at 37 °C, after incubation, saline washed cell suspension was prepared from each isolate and adjusted to a concentration of 3×10^7 CFU/mL. To each well of polystyrene flat-bottom 96-well microtiter plates, 180 μL Sabouraud Dextrose Broth (E. Merck, Germany) (SDB) and 20 μL cell suspension was added and incubated at 37 °C for 90 min. After incubation time, planktonic cells were removed by discarding of upper medium and each well was washed with sterile phosphate saline buffer (PBS), followed by adding fresh medium to each well and incubating for 48 h at 37 °C. After incubation, biofilm formation was measured by crystal violet method. All wells were washed twice with 200 μL PBS, after air-drying, 100 μL methanol (99%) was added and after 15 min, the supernatant was discarded and the plate was air-dried for 20 min. Staining was performed by adding 100 μL 0.4% crystal violet for each well incubated at room temperature for 45 min. After washing 4 times by distilled water, 150 μL of 33% acetic acid (Sigma) was added and the absorbance was immediately measured at 590 nm. Each isolate was done in triplicate and *C. albicans* strain ATCC10231 was used as a reference strain. To calculate the percent transmission ($\%_T$), the $\%_T$ value of each test sample was subtracted from the $\%_T$ of the reagent blank to obtain $\%_T$ bloc. The ability of biofilm formation of each isolate was scored as negative ($\%_T$ bloc < 10) or graded as 1+ ($\%_T$ bloc $10 - 20$), 2+ ($\%_T$ bloc $20 - 35$), 3+ ($\%_T$ bloc $35 - 50$), and 4+ ($\%\%_T$ bloc ≥ 50). Based on this method of scoring, the isolates were divided into two categories including low biofilm producers (1+) and high biofilm producers (2+, 3+ or 4+) [12,13].

Antifungal susceptibility testing

We used 4 antifungal drugs including fluconazole (FCZ), itraconazole (ICZ), ketoconazole (KCZ) and amphotericin B (AMP-B) according to the CLSI guidelines-document M27-A3 of yeast. Specific antifungal drug concentrations in RPMI 1640 (Sigma Chemical Co., St. Louis, Mo) were dispensed in 96-well microtiter plates. After 48 h, Potato Dextrose Broth (PDB) cultured colonies were picked and fungal suspension in final inoculums sizes of 0.5–2.5×10^3 cell/mL were prepared in RPMI 1640. Aliquots of 100 μL of fungal suspensions were added in wells of a microtiter plate containing 100 μL of various concentrations of antifungals. The plate was incubated at 37 °C and MICs endpoints were determined visually after 24–48 h [8]. *C. albicans* ATCC10231 was used as a quality control. All the experiments were carried out twice in triplicate sets each.

Phospholipase activity

The phospholipase production of *Candida* isolates was evaluated by using the egg yolk agar plate method according to Price et al. and Pereira et al. [14,15]. The test medium consisted of SDA which 57.3 g NaCl, 0.55 g $CaCl_2$ and 8% sterile egg yolk emulsion were added in. Each isolate was spot inoculated in triplicate. The plates were incubated at 37 °C and the diameter of the colonies and the colonies plus precipitation zone (Pz) were evaluated after 3–8 days. The phospholipase activity was calculated according to Price et al. [14]. The average of Pz was measured for each isolates. All the

Please cite this article in press as: Jahanshiri Z, et al. Oropharyngeal candidiasis in head and neck cancer patients in Iran: Species identification, antifungal susceptibility and pathogenic characterization. Journal De Mycologie Médicale (2018), https://doi.org/10.1016/j.mycmed.2018.01.001