



Molecular Characterization of Fungal Colonization on the Provox™ Tracheoesophageal Voice Prosthesis in Post Laryngectomy Patients

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(Received 10 Dec 2020; accepted 15 Feb 2021)

Abstract

Background: Tracheoesophageal voice prostheses (TVPs) have been the gold standard in rehabilitation, after laryngectomy, producing faster and premier voicing towards esophageal speech. Fungal colonization shortens the device's lifetime and leads to prosthesis dysfunction, leakage, and subsequent respiratory infection. Therefore, in the current study, we aimed to investigate the fungal colonization patterns and to propose prophylactic measures that shall increase the longevity of voice prosthesis.

Methods: Failed TVPs were removed - due to leakage and/or aspiration - from 66 post laryngectomy patients and examined. They were referred to Amiralam and Rasoul Hospital, the main centers of Ear, Nose, and Throat in Tehran, Iran from April 2018 to January 2020. Fungal colonization patterns were assessed using DNA sequencing techniques. Furthermore, the susceptibility to fluconazole, amphotericin B, nystatin, and white vinegar was evaluated according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.

Results: Resident fungal species from the upper airways colonized all the 66 TVPs (100%). Diabetes (31%) and smoking (98%) were the predominant underlying disease and predisposing factors, respectively. Among the 79 fungal agents isolated from the 66 TVPs, *Candida glabrata* (n=25, 31.7%) was the most common. A significant reduction in minimum inhibitory concentration (MIC) values were observed for white vinegar when used alone ($P<0.05$).

Conclusion: White vinegar at a very low concentration could decrease the amount of fungal colonization on TVPs without any adverse effects; its wide accessibility and affordability ensure a decrease in the overall health cost.

Keywords: Voice prostheses; Fungal colonization; Antifungal susceptibility test; Candida



Introduction

A total laryngectomy or laryngopharyngectomy is a decisive treatment modality in 15-40% of patients with loco regionally advanced cancer of the larynx or hypopharynx. The most crippling side effect of total laryngectomy is the loss of the patient's speech (1).

After a laryngectomy, the respiratory tract is detached and the patients have to breathe by an artificial hole through the neck - a tracheostoma. A one-way bypass vent is located between the trachea and the esophagus. The patients could speak by closing the tracheostoma with a finger, pushing air into the tracheoesophageal conduit through the esophagus, and the residual muscles function as pseudo-vocal cords (1, 2).

Post laryngectomy patients use silicone rubber voice prostheses to rehabilitate their voices after total laryngectomy (3-5). The average life of these devices is 3-6 months, with considerable variation of several days to several years, depending on the type of patient, their demographic features, characteristics of TVP material such as silicone, and valve resistance (6-11).

Leakages and other problems of the TVP such as displacement, infections, or fistula-formation, including granulation tissue formation force patients to replace the device (12-14). Studies have proposed reasons for the varied device's lifetime that include patient's characteristics (e.g. location of living, dietary template, use of antifungal treatment, manner of cleaning, type of disinfectant), treatment procedure (e.g. prior radiotherapy and number of it, follow-up support), and socio-economic factors (15-18).

The esophagus is a non-sterile environment, due to its proximity to the oral cavity and the inhabiting microflora, providing a steady fount of fungi to colonize TVPs (20). The leading problem that reduces the average life of TVPs is colonization by yeasts. Following colonization, the yeast cells produce either pseudohyphae or hyphae on TVPs, predisposing to leakage, and aspiration through the device or increased airflow resistance during speech (7-9, 19). In 1986, Mahieu et al

described the first *Candida* vegetation on silicone voice prostheses due to fungal colonization (21). Since that time, some studies have recognized the colonization of TVPs by *Candida* species (22,23). *C. albicans* predominates on TVPs, but *C. glabrata* and *C. krusei* have also been frequently isolated. These mentioned species should be a source of concern because of their reported resistance to azoles (22-25).

White vinegar has been proven as a natural antifungal product that can be used to treat many fungal infections (27). Vinegar had considerable antifungal properties against *Candida* spp. isolated from patients with denture stomatitis thus can be used as a therapeutic alternative in these patients (28). To the best of our knowledge, the present study is the first research in Iran, which aimed to investigate the fungal colonization patterns of TVPs and the susceptibility patterns of isolated agents to commonly used antifungals. The study is also the first in the world to examine the antifungal activity of white vinegar - a low-cost product with minimal side effects - against fungal agents isolated from TVPs.

Materials and Methods

We conducted this study on a randomized group of patients who used TVPs following a total laryngectomy. They were referred to Amiralam and Rasoul Hospital, the main centers of Ear, Nose, and Throat in Tehran, Iran. We investigated 66 TVPs from April 2018 to January 2020. Specialists examined prominent TVP defects including typical leakage or significant objective colonization on the device macroscopically upon removal.

Ethics approval

The ethical committee of Tehran University of Medical Sciences approved this study (the number of Ethics Committee protocol: (IR.TUMS.SPH.REC.1397.002). Written informed consent was obtained from all subjects or

their guardians before sample collection. All data were de-identified.

Statistical analysis

Data are expressed as average, statistical significance between groups was determined by unpaired student's t-test using $p < 0.05$ as a criterion for significance.

Culture and phenotypic examination

Direct microscopic examination was conducted using 10% potassium hydroxide. All samples were cultured on Sabouraud dextrose agar with chloramphenicol (SC, Merck, Germany) and incubated at 30 °C for 15 days. Yeast colonies were sub-cultured on CHROMagar *Candida* (CHROMagar Microbiology, Paris, France) and incubated at 35 °C for two days.

Molecular identification

From the purified extracted DNA, a fragment of the ITS gene was amplified using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') primers under the following thermal conditions: 95°C for 5 min; followed by 35 cycles of 94 °C for 30 s, 45°C for 30 s, and 72°C for 45 s; then, one final extension at 72°C for 5 min. PCR products were subjected to single direction sequencing using forward primer (Bioneer, South Korea). The results were checked visually using Chromas version 3.5.1 (<http://technelysium.com.au/wp>). The species were compared with the GenBank reliable sequences and were identified using the basic local alignment search tool of the National Center for Biotechnology Information (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The phylogenetic dendrogram was constructed using the Molecular Evolutionary Genetics Analysis (MEGA) version X (26).

***In vitro* antifungal susceptibility testing**

Antifungal susceptibility testing (AFST) was performed according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) M27-A3 and M27-S4 documents (28, 29). The antifungal agents tested were fluconazole (FLC), Amphotericin B (AMB), Nystatin (NYS) (Sigma, St. Louis, MO, USA), and white vinegar (4%). Equal volumes of the highest concentrations of vinegar (1% citric acid) and nystatin (32 mg/L) or amphotericin B (16 mg/L) or fluconazole (64 mg/L) were combined and added to the wells. The highest concentration of vinegar in this study was 1% citric acid. *C.parapsilosis* ATCC 22019 was used as quality control strains. Briefly, homogeneous conidial suspensions were spectrophotometrically measured at the 530 nm wavelength and a percent transmission within the range of 75-77%. The final inoculum suspension was adjusted in RPMI-1640 with L-glutamine and without sodium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA), with 0.165 M morpholine propane sulfonic acid (MOPS, Sigma-Aldrich, St. Louis, MO, USA). The 96-wells were incubated at 35°C and examined visually after 24 and 48 h to determine MIC values according to the CLSI recommendations. The geometric mean (GM) MICs, MIC50, MIC90, and MIC ranges were calculated.

Results

We enrolled 66 patients with failed voice prostheses, including 56 (84.4%) males and 10 (15.2%) females, in the current study. The patients were within the age range of 32–89 years, and the highest prevalence was found in the age group of 65-75 years (Table 1). Diabetes was the underlying disease in 31% of patients and smoking was the most prevalent predisposing factor (98%) (Table2). All of the patients had probiotic dairy in their food regime. No antifungal was used as prophylaxis against the fungal colonization of TVPs.

Table 1: The distribution of 66 patients with leakage through the tracheoesophageal voice prostheses based on their gender in different age groups

Patients (n=66)	Gender	Age ranges (years)					
		32-42	43-53	54-64	65-75	75-85	>85
	Male	2 (3.03)	7 (10.60)	19 (28.78)	22 (33.33)	4 (6.06)	1 (1.51)
	Female	2 (3.03)	2 (3.03)	2 (3.03)	3 (4.54)	1 (1.51)	0 (0)
Total (n) (%)	-	4 (6.06)	9 (13.63)	21 (31.81)	25 (37.87)	5 (7.57)	1 (1.51)

Table 2: Underlying diseases and predisposing factors of total laryngectomy patients

Underlying diseases and predisposing factors	Percentage of patients
Tobacco smoker	98
Alcohol intemperance	38
Diabetes mellitus	31
HIV positive	2

All the 66 TVPs had positive results for yeast colonization upon microscopic examination. They were screened based on their colony colors on CHROMagar *Candida* after 48 h of incubation at 37°C (Table 3).

Fungal colonization by single or polyspecies, including both *C. albicans* and non-albicans, was confirmed in all the 66 TVPs examined. All of the sequences have been deposited in GenBank under the accession number.

Table 3: Distribution of the colony colors within each yeast species using CHROMagar *Candida* medium

Species	Colony colors
<i>Candida glabrata</i>	Pink(12) Purple(29)
<i>Candida albicans</i>	Green (22)
<i>Candida krusei</i>	Pale pink (10) Purple(2)
<i>Candida tropicalis</i>	Dark blue (11)

Based on the analysis of the ITS gene sequences, the majority of isolates were identified as *C. glabrata* (25/79, 31.7%), *C. tropicalis* (19/79, 24.0%), and *C. albicans* (18/79, 22.8%). Therefore, the prevalence of *C. glabrata* in this study was higher

than that of *C. albicans*. The spectrum of fungal species and their frequency are presented in Table 4.

Table 4: The spectrum of isolated fungi from 66 failed tracheoesophageal voice prostheses

Fungi	Frequency	Percent
<i>C. glabrata</i>	25	31.7
<i>C. tropicalis</i>	19	24
<i>C. albicans</i>	18	22.8
<i>C. krusei</i>	13	16.5
<i>C. pseudotropicalis</i>	2	2.5
<i>Geotrichum candidum</i>	2	2.5
Total	79	100

Furthermore, mixed fungal-bacterial colonization was observed in 59 (89%) of 66 TVPs.

Table 5 lists the geometric mean (GM), MIC, MIC ranges, MIC50, and MIC90 of three anti-

fungal agents and the combination of these drugs with vinegar against 85 isolated *Candida* species.

Table 5: The geometric mean, MIC ranges, MIC50 and MIC90 values obtained by testing the susceptibility of 79 *Candida* spp. and *Geotrichum* isolates to the antifungal agents and vinegar

Strains	Antifungals	GM ^a	MIC ^b range	MIC ^c 50	MIC ^d 90
<i>C. glabrata</i> (n:25)	FLC ^e	23.35	8-32	16	64
	FLC/V ⁱ	1.28, 0.08	1-4/0.063-0.25	1/0.063	2/0.25
	AMB ^g	1.31	1-2	1	2
	AMB/V ⁱ	3.31/0.20	2-4/0.125-0.25	4/0.25	4/0.25
	NYS ^h	2.7	2-4	2	4
	NYS/V ^k	0.23/0.06	0.25-0.5/0.063-0.125	0.25/0.063	0.25/0.063
<i>C. albicans</i> (n:18)	V ^f	0.5	0.5	0.5	0.5
	FLC	0.78	0.125-4	1	4
	FLC/V	1.07/0.015	0.25-4/0.004-0.032	1/0.016	2/0.032
	AMB	0.97	1-2	1	2
	AMB/V	5.2/0.38	2-8/0.125-0.5	4/0.25	8/0.5
	NYS	1.73	0.5-4	2	4
<i>C. krusei</i> (n:13)	NYS/V	1.21/0.10	1-2/0.063-0.125	1/0.063	2/0.125
	V	0.5	0.5	0.5	0.5
	FLC	12.66	8-16	8	16
	FLC/V	9.12/0.19	8-16/0.125-0.25	8/0.125	16/0.25
	AMB	0.88	0.5-2	1	2
	AMB/V	1.61/0.11	1-4/0.063-0.25	1/0.063	2/0.125
<i>C. tropicalis</i> (n:19)	NYS	1.57	1-2	2	2
	NYS/V	1.74/0.08	1-2/0.063-0.125	2/0.063	2/0.125
	V	0.25	0.25	0.25	0.25
	FLC	7.88	4-16	8	16
	FLC/V	6.27/0.125	4-16/0.063-0.25	8/0.125	16/0.25
	AMB	1.08	0.5-4	1	2
<i>C. pseudotropicalis</i> (n:2)	AMB/V	2/0.125	2/0.063-0.25	1/0.125	2/0.25
	NYS	1.41	0.5-2	1	2
	NYS/V	1.24/0.09	0.5-2/0.063-0.25	1/0.125	2/0.25
	V	0.5	0.5	0.5	0.5
	FLC	4	4	4	4
	FLC/V	8/0.125	8/0.125	8/0.125	8/0.125
<i>Geotrichum candidum</i> . (n:2)	AMB	1	1	1	1
	AMB/V	4/0.25	4/0.25	4/0.25	4/0.25
	NYS	4	4	4	4
	NYS/V	4/0.125	4/0.125	4/0.125	4/0.125
	V	0.5	0.5	0.5	0.5

^a GM: Geometric mean; ^bMIC: Minimum inhibitory concentration; ^cMIC50: minimal concentration that inhibits 50% of isolates; ^d MIC90: minimal concentration that inhibits 90% of isolates; ^e FLC: Fluconazole, ^f V: Vinegar; ^gAMB: Amphotericin B, ^h NYS: Nystatin, ⁱ FLC/V: combination of fluconazole and vinegar, ^jAMB/V: combination of amphotericin B and vinegar, ^k NYS/V: combination of nystatin and vinegar

Discussion

There is a lot of interest in the performance of post laryngectomy vocal rehabilitation. The highest quality of voice rehabilitation after total laryngectomy can be accomplished with TVP that functions as walls separating the trachea from the esophagus (3, 4). On the other hand, the oral cavity and oropharynx are believed to be the continuous fount of bacteria and yeast species to the prostheses located in the tracheoesophageal shunt. The bacterial and fungal growth on the TVPs can significantly decrease their life span (6, 7). The microbiological analysis in our study revealed the simultaneous presence of bacteria and fungi in 89% of failed TVPs removed from our patients.

In India, a combination of yeast and bacteria in approximately 55% of culture samples was related to TVPs (30). In addition, the bacteria and fungi were the main part of the microbial plaque on TVPs including normal oral or periodontal flora (8,23,31-33). The bacteria and fungi might have been acting synergistically by enhancing damage to the prosthesis, s surface, in addition to promoting colonization. Moreover, in the current study, we recovered pure yeast cultures from 11% of prostheses. The prevalence of yeasts in the oral cavity is estimated to vary between 20% and 40% in healthy individuals (31).

Irradiation to the head and neck regions, combined with surgical therapy, may further alter the oral flora and saliva composition to favor oral yeast colonization. In the current study, *C. glabrata* was the most common fungi isolated. This finding contradicts the previous studies, which showed a predominance of *C. albicans* in the majority of fungal isolates (21, 32-35, 36). However, it corroborates the results of another study that confirmed a high proportion (79%) of *Candida* contamination on malfunctioning voice prosthesis, with a dominance of non-*albicans* strains, which is consistent with the results of our study (1).

Following the widespread use of immunosuppressive therapy and broad-spectrum antimycotic

prophylaxis, *C. glabrata* has emerged as an important opportunistic pathogen in the oral mucosa. The use of dentures, immunosuppression, antibiotic therapy, and aging are risk factors for oral colonization or infection with *C. glabrata*. Compared to *C. albicans*, *C. glabrata* exhibits higher prosthesis -surface-adherence ability (37).

Several studies have discussed using probiotics as an alternative treatment for candidiasis (38-41). Nevertheless, our study showed that probiotic consumption is ineffective on *Candida* colonization of the oral mucosa because all patients included in this study used probiotic dairy in their food regime and had fungal colonization on their TVPs.

Most of the patients in the current study (37.87%) were between the age of 65 and 75 years old. Due to immunosenescence, a high proportion of elderly persons are prone to many diseases including laryngeal cancer. At old age, the immune system functions poorly and this can make individuals in these age groups more susceptible to *Candida* colonization and/or infection (41).

In this study, the male-to-female ratio was 5:1 and there was a significant difference in the prevalence of laryngeal cancer between genders. Laryngeal cancer in male patients is associated with more tobacco and alcohol usage. The large sex difference in patients with laryngeal cancer was highlighted as male: female ratio of 14.9: 1 (42). The male to female ratio diminished considerably in the last century because more women smoke cigarettes in the cancer age group (43).

32% of patients in the current study were diabetic. Previous studies of oral mucosa in diabetic groups have shown *Candida* colonization rates between 21.0% and 77.0% (44- 46). Based on the available evidence to date, clinical practice guidelines state that the most effective treatment of *Candida* colonization on TVPs is removal and replacement of the device, however, the devices are costly, so decontamination is more economical than replacement of the devices. To date the most commonly accepted decontamination methods are antifungals. However, antifungal resistance among yeasts has been a major clinical

challenge. The latter is worrisome since the non-*albicans* strains are less susceptible to antifungals (e.g. *C. glabrata* to fluconazole), have intrinsic resistance to some agents (e.g. *C. krusei* to fluconazole), or show resistance to echinocandins (e.g. *C. tropicalis*) (31). Besides, antifungal drugs may be limited by drug-drug interactions and serious adverse effects/toxicities that prevent their prolonged use or dosage escalation (47). Therefore, there is still a need to embark on methods, which are cost-effective and easy to incorporate into the patient's daily lifestyle.

In this study, we tested the in vitro activities of fluconazole, amphotericin B, nystatin, and edible vinegar against the *Candida* spp. Furthermore, fluconazole, amphotericin B, and nystatin were combined with vinegar. The results of the current study showed that all the isolated *Candida albicans* and non-*albicans* species displayed extremely low MIC for edible vinegar. Especially *C. krusei*, which is most noteworthy for its innate resistance to the antifungal agent fluconazole. Besides, reduced susceptibility to other drugs, *C. krusei* displayed extremely low MIC for edible vinegar. Raj et al. evaluated the effectiveness of vinegar, lime, and saltwater as potential household decontaminants for toothbrushes and concluded that commonly used household materials can be potential decontaminants for toothbrushes and showed that vinegar was the most effective decontamination agent followed by lime and saltwater (48). Solitary use of some compounds (clove oil, onion juice, thyme oil, H₂O₂, NaCl, Dettol, and apple cider vinegar) showed a high antifungal response against *C. albicans*, but the efficacy was reduced when used in combination (49). In addition, Jabir et al. reported apple cider vinegar and acetic acid to have significant antifungal activity against *Aspergillus niger*, *A. flavus*, fluconazole-resistant *C. albicans* and non-*albicans* species (50).

Conclusion

Resident fungal species from the upper airways unavoidably colonize the silicone surfaces and the use of prophylaxis with minimal adverse ef-

fects shall be an effective strategy. *Candida non- albicans* was the most common fungal agents isolated from the failed TVPs. Our study confirmed that white vinegar at a very low concentration could decrease the amount of fungal colonization on TVPs without any adverse effects.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Acknowledgments

The funding from Tehran University of Medical Sciences, Tehran, Iran, supported this work (Grant No: 45247).

Conflict of interest

The authors declare that there is no conflict of interests.

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