Acta Dermatovenerol Croat 2019;27(3):137-141 ORIGINAL SCIENTIFIC ARTICLE

Identification and Antifungal Activity Profile of *Candida* Species Isolated from Patients with Pemphigus Vulgaris with Oral Lesions

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ABSTRACT Pemphigus vulgaris is an autoimmune disease that mostly affects the mucosa and oral cavity. *Candida* species can invade the mucosal lesions of these patients and cause diseases. The aim of this study was to identify the fungal agents isolated from mucosal lesions and evaluate antifungal activity profile against the isolates. A total of 25 patients with pemphigus vulgaris with active oral lesions and 25 healthy people serving as a control group were included in this study. Identification of the fungal isolates was performed based on conventional methods and DNA sequence analysis of the internal transcribed spacer (ITS) rDNA gene region. The sequence results were deposited in the NCBI database using the Basic Local Alignment Search Tool. Antifungal activity of fluconazole, itraconazole, ketoconazole, posaconazole, econazole, and amphotericin B against the isolates were evaluated based on the CLSI M-44 A protocol. Oral candidiasis was detected in 20% of the patients. *Candida* species isolated from oral lesions of patients with pemphigus were identified as *Candida albicans* 22/25, *Candida glabrata* 2/25, and *Candida dubliniensis* 1/25. All of the isolates were sensitive to amphotericin and econazole, 96% to fluconazole and posaconazole, and 92% to ketoconazole and itraconazole. One patient showed a profile resistant to fluconazole, posaconazole, and ketoconazole, simultaneously. Ninety six percent of control group isolates were sensitive to six antifungals. *Candida albicans* was the most prevalent species isolated from oral lesions of patients with pemphigus vulgaris and the control group. Amphotericin B and econazole were the most effective antifungals against the isolates.

KEY WORDS: pemphigus vulgaris, *Candida*, antifungal, ITS

INTRODUCTION

Pemphigus vulgaris (PV) is a chronic autoimmune mucocutaneous disease characterized by the formation of erosions, ulcers, and blisters on the mucosa and skin (1). Without corticosteroid therapy disease often leads to death (2). Despite other types of pemphigus such as pemphigus erythematosus,

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foliaceus, and pemphigus vegetans, which rarely affect the oral mucosa, oral lesions are common in pemphigus vulgaris and have a high frequency in patients (3). Oral lesions may associate with blisters on the lips, gum, and gingiva and are mostly painful (4). Corticosteroid therapy (such as prednisolone, 20 to 120 mg/day) is prescribed in all cases to prevent the progression of the disease (5). It has been reported that corticosteroid therapy provides a suitable state for opportunist microorganisms to cause invasion and infections, which is the most common complication in patients with PV (1). Bacterial and viral agents were also reported in association with PV oral infections (6). *Candida* species are the most common cause of opportunistic infections and clinical manifestation due to fungi in patients with PV mostly being present as thrush or stomatitis, pharyngitis, and *Pneumocystis carinii* pneumonia (7).

There are many antifungal drugs used for prevention and therapy in fungal infections. Failure in therapy commonly occurs in the antifungal resistant cases.

The main aim of this study was the molecular identification of the fungal species associated with oral lesions in patients with pemphigus vulgaris by internal transcribed spacer (ITS) sequence analysis and antifungal activity evaluation of six antifungal drugs against the isolates with the disk diffusion method.

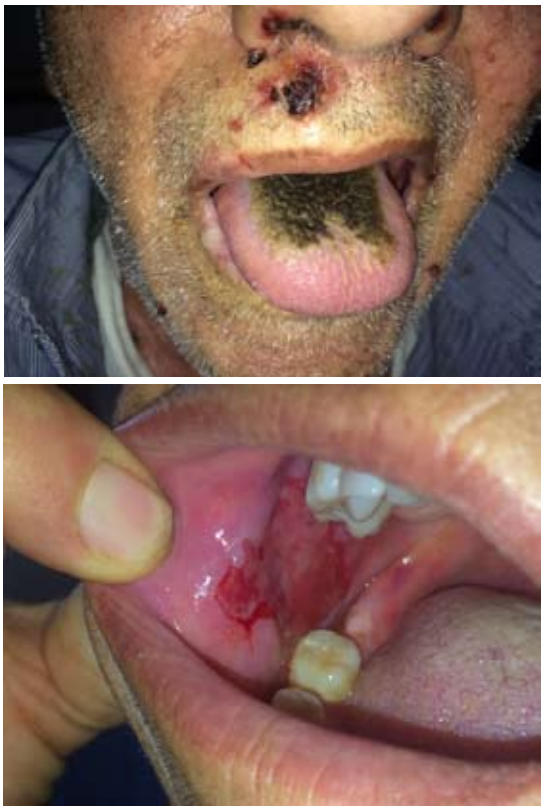

Figure 1. Lesions and oral candidiasis (black hairy tongue) in patients with pemphigus.

PATIENTS AND METHODS

Case and control group

A total of 25 cases with oral lesions from 40 patients with PV referred to pemphigus therapeutic clinic in Shiraz were enrolled in our study. The research project was approved by the Ethics Committee of Departmental Review Board (Ethical code: IR.SUMS.REC.1396.S665) of the Shiraz University of Medical Sciences, Shiraz, Iran. The oral cavity of the patients was examined for any erosions, blisters, lesions, and localized plaques on the oral mucosa, and a sterile swap was taken from the suspected area. The swaps were speared on plates containing Sabouraud dextrose agar (Merck, Germany) supplemented with antibiotics (pen-strep, Norbrook, UK) and incubated at 30°C. A total of 25 healthy people were chosen for the control group and matched with the case group in terms of age and sex. Swabs were taken from the oral cavity of the control group and cultured on the above media.

Conventional identification methods

Primary identification of isolates was based on micromorphological analysis based on yeast shape using a teased mount smear (round, ovoid, or rectangular shape) and colony color on CHROMagar *Candida* media (Paris, France).

Molecular method

DNA preparation

Genomic DNA was extracted through the boiling method described by Makimura *et al.* (8) with small modifications. Briefly, a small amount of the yeast colony was suspended in 100 µL of lysis buffer containing 100 mM Tris–HCl, 0.5% SDS, and 30 mM EDTA and boiled for 15 min at 100°C. A solution of potas-

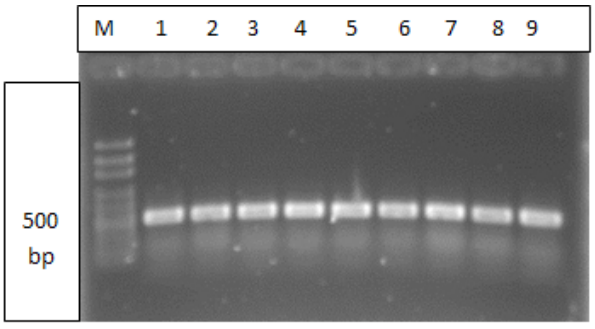

Figure 2. Results of PCR electrophoresis: line M: 100 bp Molecular Marker; line 1: standard positive control; lines 2-9: Candida spp.

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sium acetate (2.5 M) was then added to the lysate, held on ice for 1 h, centrifuged at 12,000 rpm for 5 min, and transferred to a new tube. The yeast DNA in the supernatant was washed twice with ethanol and air dried and re-suspended in 50 µL of distilled water prior to use for PCR. The purity and quantity of DNA were evaluated with absorbance ratio of A260/A280 and nano-drop reading results.

Amplification and sequencing of the ITS regions

A set of universal primers (ITS1, 5′-TCCGTAGGTGAACCTGCG-3′, and ITS4, 5′-TCCTCCGCTTATTGATATGC-3′) (Meta-bion International, Martinsried, Germany) were employed for amplification of the internal transcribed spacer (ITS) region of the ITS1-5.8S-ITS2 segment of the ribosomal DNA gene. PCR amplification was carried out in a final volume of 50 µL consisting of 5 µL of ×10 PCR buffer, 1.5 mM MgCl2, 0.8 mM deoxynucleoside triphosphates (0.2 mM each), 1.2 U of Taq DNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany), 0.5 µM of each primer, 2 µL of DNA template, and eventually with distilled water to a final volume. An initial denaturation step at 95°C for 5 min was followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 64°C for 50 sec, and extended at 72°C for 1 min, with a final extension step at 72°C for 7 min, and kept at 4°C for 5 min. Negative controls were also used in each set of reactions. The PCR product was electrophoresed on 1.2% agarose gel and stained with ethidium bromide. The PCR products were then purified and sequenced (Bioneer Company, South Korea). The sequence results were processed using the web-based blasting program and basic local alignment search tool (http://www.ncbi.nlm.nih.gov/BLAST). Finally, the obtained data were compared with those in the NCBI/Genebank database.

Antifungal susceptibility test

Antifungal activity of six antifungal drugs including fluconazole, itraconazole, ketoconazole, posaconazole, econazole, and amphotericin B were evaluated against the isolates according to the CLSI M-44 A protocol. Standard strains of *C. albicans* (ATCC10261) and *C. tropicalis* (ATCC750) were used as controls.

Statistical analysis:

The Fisher's exact test was used for data statistical analysis and a P value of ≤0.05 was considered significant.

RESULTS

Twenty percent of the patients presented with oral candidiasis (Figure 1). *Candida albicans & dubliniensis, C. tropicalis, C. krusei, C. parapsilosis*, and *C. glabrata* presented green, blue, white-pink, white, and purple colony colors on chromogenic media, respectively. The ITS region of extracted DNA was completely amplified in all yeast isolates (Figure 2). The sequence results of PCR products demonstrate 3 species of *Candida* in the patient group and 4 species of *Candida* in the control group.

In the patient group, *Candida albicans* was the most common agent 22 (88%), followed by *C. glabrata* (2; 8%) and *C. dubliniensis* (1; 4%).

The results of antifungal activity against the isolates are presented in Table 1. In the patient group, all of the isolates were sensitive to econazole and amphotericin B.

One patient affected with *C. glabrata* showed a profile resistant to fluconazole, itraconazole, and posaconazole and an intermediate profile to itraconazole, simultaneously.

In the control group, *Candida albicans* 16 (64%) was the most common agent. There was no significant difference between two groups regarding *C. albicans* as most common causative agent. The other species were *C. krusei* (5; 20%), *C. tropicalis* (2; 8%) and *C. glabrata* (2; 8%).

Ninety six percent of isolates were sensitive to six antifungal drugs. One isolate (*C. glabrata*) was resistant to fluconazole and showed an intermediate profile to itraconazole and ketoconazole. This isolate had no history of previous encounters with antifungal drugs.

There was no significant relation between etiological agents and resistance to antifungal drugs in the 2 groups.

DISCUSSION

Pemphigus vulgaris is an autoimmune bullous disease characterized by blister formation on the skin

Table 1. Results of disk diffusion test on *Candida* spp isolates in patient and control groups

Itra			Flu			Eco			Ket			Pso			Ampho			Disc
S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	
23	1	1	24	-	1	25	-	-	23	2	-	24	-	1	25	-	-	**Patients (25)**
23	2	-	24	-	1	25	-	-	24	1	-	25	-	-	25	-	-	**Controls (25)**

and oral mucosa. It affects mainly adults with an approximate incidence of 0.5-3.2 per 1,000,000 people annually (3). The best treatment for patients with PV is corticosteroid therapy, which often leads to several complications associated with a high dose of immunosuppressive drugs (1). This condition may represent a risk factor for cutaneous and oral disorders, lead to periodontitis and microbial infections (6,9), and may sometimes mimics dermatophytosis (10). Kiran *et al.* (11) reported a wide range of bacteria colonized on the skin of patients with PV. Al-Dwibe *et al.* (12) reported superficial fungal infections affecting the nails and skin among patients with bullous diseases. They isolated many genera of fungi including *Candida, Trichophyton, Fusarium,* and *Rodotorola* from patients. Candidiasis is the most common opportunist fungal diseases and *Candida* species comprise 25-50% of the oral cavity microbiota of healthy individuals (13). *Candida albicans* is the most common, followed by *Candida glabrata* as the second most frequent cause of candidiasis, accounting for approximately 15-25% of clinical cases (14).

C. glabrata is an opportunistic fungal pathogen the forms part of the normal microbial flora in humans and in immunocompromised patients, and as such presents a significant problem. *C. glabrata* is commonly found in the human gastrointestinal tract and can disseminate to cause invasive candidiasis and serious infections (15).

Oral candidiasis is the most common human fungal infection and *C. albicans* is the most prevalent agent as a normal commensal of the mouth and generally causes no problems in healthy people (16). To our knowledge, there have been no studies on oral cavity mycoflora in patients with PV. We matched the patient (case) and control groups for age and sex. *C. albicans* was the most prevalent etiological agent in both groups. The variation of species in the control group was higher than the patient group. It seems that oral cavity conditions in patients with PV are the same as that of healthy people, and we were thus unable to observer higher relative prevalence of any specific *Candida* species.

The pharmacological treatment of candidiasis can be divided into two procedures: topical therapy that is applied to the affected area and systemic drugs that are used when the infection is more widespread (17). Azole and allylamine antifungal agents have added greatly to the therapeutic options for the treatment of fungal infections. Disk diffusion method is a commonly used method for antifungal susceptibility testing (17). Pfaller *et al.* performed a global study on *Candida* species in 41 countries and found that increased number of isolation in *C. glabrata* (18). In that study, analyses of in vitro susceptibility of 31 *Candida* species to fluconazole were evaluated by disk diffusion method and an increase in fluconazole resistance was seen in *C. glabrata* and *C. albicans*. In the present study, 96% of species in the control group were susceptible to six antifungal drugs, similarly to the patient group. *C. glabrata* have an inherently elevated tolerance to azole antifungals, and clinical isolates generally exhibit a high inherent tolerance level to azole drugs (14).

In one patient with thrush infection, *C. glabrata* was identified as a causative agent and showed multidrug resistance to three antifungals. Similarly, *C. glabrata* was isolated in the control group and showed resistance to fluconazole and intermediate profile to the other azoles. This case had no history of previous encounters with azoles. It appears that inherently reduced azole susceptibilities in most clinical *C. glabrata* isolates could be involved.

CONCLUSION

In this study, various species of *Candida* were collected from patients with oral lesions, and *C. albicans* was identified as the most common isolate. Because most of the isolates were sensitive to antifungals, multiple drug choices are available for treatment in patients with pemphigus vulgaris in case of oral infection or probable systemic candidiasis.

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