

SUBGINGIVAL DISTRIBUTION OF YEAST AND THEIR ANTIFUNGAL SUSCEPTIBILITY IN IMMUNOCOMPETENT SUBJECTS WITH AND WITHOUT DENTAL DEVICES

Virginia M. Jewtuchowicz^{1,2}, María I. Brusca¹, María T. Mujica², Laura A. Gliosca¹, Jorge L. Finquelievich², Cristina A. Iovannitti², Alcira C. Rosa¹

¹ Microbiology and Parasitology Department, Faculty of Dentistry, University of Buenos Aires. ² Microbiology, Parasitology and Immunology Department, Faculty of Medicine, University of Buenos Aires, Argentina.

ABSTRACT

Yeasts colonize the subgingival biofilm, which becomes a reservoir that favors their reproduction. The purpose of the present work was to determine the prevalence of yeasts of the *Candida* genus in the subgingival biofilm of gingivoperiodontal disease patients, including users and non-users of dental devices, and their susceptibility to fluconazole and voriconazole.

Samples of subgingival pockets of immunocompetent non-smokers showing gingivitis and periodontitis were inoculated in a differential chromogenic medium. Sixty three percent of subjects used dental devices. Yeasts were identified and susceptibility to fluconazole and voriconazole was tested following CLSI M44-A standards.

The prevalence of yeasts in the subgingival biofilm was 40%

CI 95% (30.5-50.3); 10% were patients who did not use dental appliances. The most frequently observed yeasts were *C. albicans*, and *C. parapsilosis*, *C. dubliniensis*, *C. tropicalis* and *C. guilliermondii*. Only *C. dubliniensis* and *C. guilliermondii* showed resistance to azoles. The use of dental devices significantly increased the prevalence of yeasts in periodontal pockets inpatients presenting gingivitis. It is noteworthy that non *albicans* *Candida* species, such as *C. dubliniensis* and *C. guilliermondii*, considered emerging species, which have a diminished susceptibility to antifungal agents were found in the crevicular fluid of immunocompetent patients.

Key words: periodontal disease, subgingival biofilm, *Candida* species, *Candida guilliermondii*, *Candida dubliniensis*.

DISTRIBUCIÓN SUBGINGIVAL DE LEVADURAS Y SU SENSIBILIDAD A ANTIFÚNGICOS EN INDIVIDUOS INMUNOCOMPETENTES CON O SIN APARATOLOGÍA BUCAL

RESUMEN

Las levaduras del género *Candida* colonizan el biofilm subgingival. Su hallazgo constituye un reservorio favorable para su multiplicación. El propósito de este trabajo fue investigar la presencia de levaduras del género *Candida* en el biofilm subgingival de individuos con enfermedad gingivoperiodontal a fin de establecer la prevalencia de especies y los perfiles de susceptibilidad a fluconazol y voriconazol de las mismas.

Se obtuvieron muestras del biofilm subgingival en cien pacientes inmunocompetentes, no fumadores, con salud gingivoperiodontal, gingivitis y periodontitis, con y sin aparatología bucal. Las muestras se sembraron en medio cromogénico diferencial y las levaduras aisladas se identificaron mediante micromorfología y pruebas bioquímicas. Los estudios de sensibilidad a fluconazol y voriconazol se realizaron según las normas CLSI M44-A. La prevalencia de leva-

duras en el biofilm subgingival fue del 40% IC95% (30.5-50.3) siendo el 10% y 30% la frecuencia de las mismas para los pacientes sin y con aparatología bucal respectivamente. *C. albicans* fue la levadura más frecuente. Encontramos otras especies como *C. parapsilosis*, *C. dubliniensis*, *C. tropicalis*, *C. guilliermondii* y *C. sake*. Solo se observó resistencia a los azoles en *C. dubliniensis* y *C. guilliermondii*. El tratamiento con aparatología bucal incrementó la prevalencia de levaduras en bolsa periodontal en forma significativa. Es importante destacar la presencia en el fluido subgingival de especies de *Candida* no *albicans* consideradas emergentes y con sensibilidad disminuida a los antifúngicos como *C. dubliniensis* y *C. guilliermondii*.

Palabras clave: enfermedad periodontal, biofilm subgingival, *Candida spp*, *Candida guilliermondii*, *Candida dubliniensis*.

INTRODUCTION

Infections in the oral cavity, including periodontitis, periodontal abscesses and stomatitis, and dental procedures (tooth extraction, endodontic treatment, use of irrigation appliances) can trigger systemic

infection as a result of the dissemination of the microorganism from its primary location via the blood stream.

Periodontal disease (PD) is characterized by an inflammatory, degenerative, and necrotic response

in the gingival and underlying connective tissues elicited by microbial colonization in periodontal pockets. The signs and symptoms of PD should be the result of the interaction between the pathogenous microorganism and the inflammatory response of the host (1) that allows different *Candida* species to colonize the subgingival biofilm (2). Yeasts are regularly found in the normal flora of human oral mucosa, representing potential sources of oral Candidiasis, as well as other more serious forms of the disease, such as the esophageal and systemic variants (3). *Candida* species have been recovered from periodontal pockets in a large number (7.1-19.6%) of patients with PD (4-7).

Although the yeast most frequently associated to this type of infection is *Candida albicans*, other less prevalent emerging species have been isolated (8). Some of the latter species are known to be less susceptible to antifungal agents commonly used to treat fungal infection, such as fluconazole (9,10). It is therefore essential to identify the *Candida* species involved in buccal infections, since they may be implicated in periodontal disease and/or constitute infection foci, posing a risk of systemic dissemination of strains that are less susceptible, i.e. resistant, to antifungal drugs of widespread use.

It is well documented that orthodontic/orthopedic treatment increases the levels of yeasts in the mouth (11-13). The use of dental devices is a risk factor for gingivo-periodontal diseases and dental caries, since they facilitate the accumulation of microorganisms, in terms of quantity and type/diversity, altering the oral microbiota. Orthodontic devices can act as traps, since yeasts and other microorganisms adhere to their surface - whether acrylic, glass, composite, sealants, or membranes, and retain microorganisms, thus becoming a niche for colonization by normal or opportunistic microorganisms and facilitating infection.

The purpose of this study was to evaluate the presence of yeasts of the *Candida* genus in the subgingival biofilm of subjects presenting gingivo-periodontal disease, including users and non-users of dental devices in order to establish the prevalence of the different species and their susceptibility profiles to fluconazole and voriconazole.

MATERIALS AND METHODS

Subjects: One hundred adult healthy immunocompetent non-smoker subjects, who were patients of

the dental clinic of Buenos Aires University, were included in the study. These 100 subjects ranged in age from 18 to 75 years (55 were female, and 45 were male). The patients were divided into groups according to their oral health status: healthy (n=8), and presenting gingivo-periodontal disease: gingivitis (n=39) and periodontitis (n=53), as judged by clinical measurements: pocket depth, plaque index (14), gingival index (15), attachment loss. Bleeding on probing was determined after 30 seconds and recorded as positive (1) or negative (0). The location of the gingival margin was determined and tooth mobility was assessed. Periodontal evaluations of all patients included clinical examination and radiographs. Sixty three percent of subjects used dental devices, 43 orthodontic appliances (25 fixed and 18 removable) and 20 prosthetic devices (9 fixed and 11 removable).

Participation in our survey was voluntary and all the patients gave their informed consent.

Sample Collection Method: All study subjects were asked to rinse their mouth with sterile distilled water, after which the dental professional isolated the area by means of cotton rolls and a high speed suction device. Following removal of the supragingival plaque, 4 subgingival plaque samples were taken from each subject at 4 sites (the right upper and lower central incisor, the first right upper molar and the first left lower molar to mimic the more common sites infected in periodontitis) of the periodontal pocket using a 7/8 Gracey curette. Samples were pooled and placed in Eppendorf tubes containing 500 µl of sterile saline solution.

Mycology studies

The samples were analyzed through direct microscopic observations with lactophenol cotton blue and inoculated into agar plates containing differential chromogenic medium (CHROMagar *Candida*, Paris, France). The cultures were incubated at 28°C for 1 week, and fungus growth was evaluated daily.

Identification of yeast species

The isolated yeasts were identified by conventional mycology methods: colony color on the chromogenic medium, micromorphology in agar milk 1% Tween 80 (16), and carbohydrate assimilation tests using the commercially available kit API ID 32D (®BioMérieux, France). Further studies were

performed if green colored strains were found on the CHROMagar *Candida*, including xylose assimilation, growth at 45°C, and chlamyospore formation on Staib agar in order to identify *C. dubliniensis* (17).

Antifungal susceptibility testing

Antifungal susceptibility studies were performed on forty-nine strains of the *Candida* genus employing the agar disk diffusion method, in keeping with the CLSI (“Clinical and Laboratory Standards Institute”) M44-A standards (18). Petri dishes containing Muller Hinton agar were added with 2% glucose and 0.5 µg/mL of methylene blue. The dishes were inoculated in three directions so as to cover the surface, using a swab impregnated with a suspension of the yeast equivalent to 0.5 Mc Farland scale. A disk containing 25 µg of fluconazole and a second disk containing 1 µg of voriconazole (Becton Dickinson Sparks, Maryland, USA) were then placed on each dish. The samples were incubated at 35°C for 24 hours, and the diameter of the inhibition halo was determined at 80% inhibition using BIOMIC image analyzing system, version 6.0, (Giles Scientific Inc. New York, NY).

The strains were identified as susceptible (S), susceptible dose-dependent (S-DD), and resistant (R) to the antifungal drug, according to the diameter of the inhibition halo: ≥19 mm, 15-18 mm and ≤14 mm respectively in the case of fluconazole and ≥17 mm, 14-16 mm and ≤13 mm respectively in the case of voriconazole. *Candida albicans* ATCC 90029 and *Candida parapsilosis* ATCC 22019 served as quality control.

Statistical Analysis

Statistical analysis was performed using STATADISTICX 7.0 and SPSS version 11.0. Confidence intervals (CI) were calculated at 95% employing the Epi-Info 6.04 program (Atlanta University).

RESULTS

The prevalence of yeasts in the subgingival biofilm of the studied population was 40% CI95% (30.5-50.3). The frequency in patients with and without dental devices was 30 and 10% respectively.

Yeasts with or without pseudohyphae were recognized by microscopic examinations in 95% of subgingival samples with yeasts positive culture.

Table 1 summarizes the species distribution of yeast isolates in subgingival biofilm according to periodontal health status and use of dental devices, as confirmed by morphological and biochemical criteria. The most frequently observed yeast, in both gingivitis and periodontitis samples was *C. albicans* (47.2%; IC95%: 33.5 -61.2). However, the finding of non *albicans Candida* species and other yeasts (*Rhodotorula spp*) is noteworthy. They included *C. parapsilosis* (n=10), *C. dubliniensis* (n=6), *C. tropicalis* (n=4), *C. guilliermondii* (n=3), *C. sake* (n=1) and *Rhodotorula* (n=4) (Table 1).

The occurrence of two or three coisolated species was observed in 20% of samples (n=8) and association was observed most frequently among *C. albicans*, *C. parapsilosis* and *C. tropicalis*.

Fig. 1 shows the number of isolates of yeast species according to periodontal health status and use of dental devices. Prevalence of yeasts in periodontal

TABLE 1. Species distribution of yeast isolates in subgingival biofilm according to periodontal health status and use of dental devices in non smoker patients.

Yeast species	HD* N (%)	HWD* N (%)	GD* N (%)	GWD* N (%)	PD* N (%)	*PWD N (%)	N (%) Total	IC 95%
<i>C. albicans</i>	1(1.9)	0	9(17)	0	6(11.3)	9(17)	25(47.2)	33.5-61.2
<i>C. parapsilosis</i>	1(1.9)	0	2(2.8)	0	2(2.8)	5(9.4)	10(18.9)	9.9-32.4
<i>C. dubliniensis</i>	0	0	2(2.8)	0	3(5.7)	1(1.9)	6(11.3)	4.3-23.0
<i>Rhodotorula spp</i>	2(2.8)	0	1(1.9)	0	0	1(1.9)	4(7.5)	2.1-18.2
<i>C. tropicalis</i>	0	0	0	1(1.9)	3(5.7)	0	4(7.5)	2.1-18.3
<i>C. guilliermondii</i>	0	0	0	0	3(5.7)	0	3(5.7)	1.2-15.7
<i>C. sake</i>	0	0	0	0	0	1(1.9)	1(1.9)	0.1-10.1
Total isolates	4(7.5)	0	14(26.4)	1(1.9)	17(32.1)	17(32.1)	53	

*HD: Healthy with devices; HWD: Healthy without devices; GD: Gingivitis with devices; GWD: Gingivitis without devices; PD: Periodontitis with devices; PWD: Periodontitis without devices.

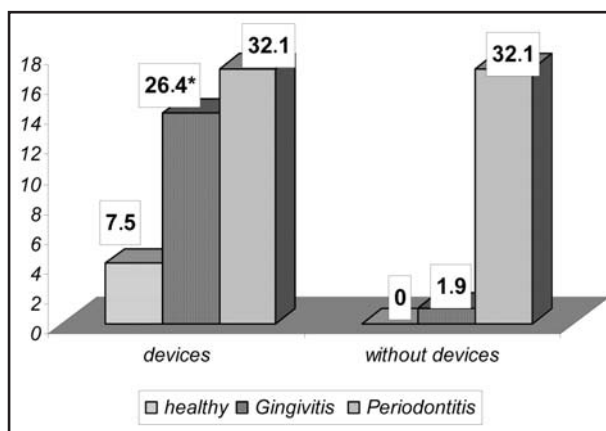


Fig. 1: Number of isolates of yeast species according to periodontal health status and use of dental devices in non-smoker patients.

*: Statistical significance in samples of gingivitis patients who used dental appliances $p = 0.0009$.

pockets of subjects using dental appliances ($n=40$) was significantly higher (Z corrected 1.82 $p=0.0345$) compared to the remaining patients ($n=30$ versus $n=10$ respectively). An increase in yeast prevalence was observed in samples corresponding to gingivitis patients; this increase reached statistical significance in samples of gingivitis patients who used dental appliances ($p(\text{Fisher})= 0.000251$, $\text{CHI}^2(\text{Yates}) = 11.0374$; $\text{GL}= 1$; $p=0.0009$). Fig. 1 shows that the prevalence of yeasts is higher in patients with periodontitis, regardless of whether they use dental devices or not. Table 2 summarizes the in vitro susceptibility of *Candida* species to fluconazole and voriconazole as determined by CLSI disk diffusion testing. All 25 *C. albicans*, 10 *C. parapsilosis*, 4 *C. tropicalis* and 1 *C. sake* isolates were susceptible to fluconazole as well as to voriconazole. Among 6 *C. dubliniensis* isolates, a great majority was susceptible. Only one isolate was resistant to both antifungal agents. All 3 *C. guilliermondii* isolates were found to be S-DD to fluconazole but S to voriconazole. The results were confirmed by MIC (Minimum inhibitory concentration, data not shown).

DISCUSSION

Yeast species of the *Candida* genus play an active role in endoperiodontal processes and periapical abscesses and are present in the crevicular fluid of periodontal disease patients. Their occurrence in gingival and periodontal pockets renders the latter a favorable reservoir for yeast reproduction. Howev-

TABLE 2. In vitro susceptibilities of *Candida* spp. to fluconazole and voriconazole as determined by CLSI disk diffusion testing

Candida species	Fluconazole			Voriconazole		
	%S	%SDD	%R	%S	%SDD	%R
<i>C. albicans</i>	100	0	0	100	0	0
<i>C. parapsilosis</i>	100	0	0	100	0	0
<i>C. dubliniensis</i>	83	0	17	83	0	17
<i>C. tropicalis</i>	100	0	0	100	0	0
<i>C. sake</i>	100	0	0	100	0	0
<i>C. guilliermondii</i>	0	100	0	100	0	0

er, their implication in periodontal disease remains unclear. Jarvensivu et al. demonstrated that *C. albicans* pseudohyphae are involved in adherence to periodontal tissue and in microbial plaque infrastructure during progression of PD (6, 19).

In the present study, a 10% prevalence of yeasts was observed in periodontal pockets of patients not using dental devices. Other authors observed that the prevalence of yeasts at this anatomical site ranged between 7 and 17.5% (7), and reported prevalence above 40% in HIV positive children (5). The prevalence of yeasts in subgingival biofilm was significantly higher in patients using dental devices compared to patients without dental devices both in healthy patients and in patients with gingivitis (Fig. 1). Our results indicate that dental devices serve as an artificial niche, increasing yeast carriage in subgingival plaque.

Direct plating of periodontal pocket samples using CHROMagar *Candida* facilitated isolation of more than one species from a single periodontal pocket subgingival plaque sample as well as the presumptive identification of *C. albicans* and *C. tropicalis*. Similar findings have been reported by other authors (5, 20).

C. albicans, prevalent yeast in buccal mucosa (21), was also found to be prevalent in subgingival pocket samples corresponding to gingivoperiodontal disease patients, accounting for 47.2% of the 53 isolated yeast species. This result is in agreement with a number of reports (2, 5, 19). In addition, the yeast most frequently isolated in association with other yeasts was *C. albicans*. Conversely to findings reported by other authors, *C. albicans* was found in 6 of the 8 samples exhibiting two or three coisolated species, and was found to be associated mainly with *C. parapsilosis* and *C. tropicalis*. Portela et al.

found no yeast coisolates in samples corresponding to 42 immunocompetent subjects (5), and Jabra-Rizk et al. found only one of 20 samples obtained from HIV negative patients to present more than one yeast species (2). It would seem that the use of dental appliances increases the likelihood of finding more than one yeast species.

C. parapsilosis was the second most frequently encountered species, accounting for 18.9% of isolates (Table 1). Other studies in the literature did not report the occurrence of this species in crevicular fluid samples of immunocompetent subjects (2). In the present study, *C. dubliniensis* was found in subgingival fluid of immunocompetent subjects (Table 1). Conversely to our findings, Portela et al. (5) reported *C. dubliniensis* in subgingival sites only in HIV positive children. Based on our results, it would seem relevant to test subgingival pockets of HIV negative patients for *C. dubliniensis*, given its capacity to adhere to bacteria in the oral microbiota, such as *Fusobacterium nucleatum*, an anaerobic gram negative bacillus, and one of the most prevalent in the subgingival plaque of periodontal disease patients. The capacity of this species to coaggregate enables it to colonize the depth of subgingival biofilm (22, 23). *C. guilliermondii*, which has been defined as an emerging yeast species, was isolated from crevicular

fluid samples corresponding to 3 periodontal disease patients. Although to the best of our knowledge there are no previous reports on the occurrence of this species in subgingival pockets, it was found to play a role as a copathogen in a case report of dento-alveolar infection (24).

Both Fluconazole and voriconazole were highly active against almost all the yeasts. Our results are in agreement with those of the literature on susceptibility to fluconazole (25). There are no studies on in vitro susceptibility of yeasts found in periodontal pockets to voriconazole.

In conclusion, the use of dental appliances increased carriage and coisolation of yeast species in the subgingival biofilm.

It must be pointed out that the occurrence of emerging species, such as *C. guilliermondii* and *C. dubliniensis*, was observed in crevicular fluid samples of healthy patients, and that both these microorganisms exhibited decreased susceptibility to the azoles studied herein.

Epidemiologic vigilance is essential in order to establish the prevalence of yeast species in periodontal pockets, since they act as a reservoir of opportunistic microorganisms which, under certain clinical conditions, may play an active role in gingivoperiodontal diseases and infection spread.

ACKNOWLEDGEMENTS

This research was supported by Grant UBACYT 0002, Universidad de Buenos Aires. The authors are grateful to Lic. Alejandra Swarts for support with the statistical analysis.

CORRESPONDENCE

Dra. Virginia Marta Jewtuchowicz
M. T. Alvear 2142, Piso 2º B.
(C1122AAH) Buenos Aires, Argentina.
Telephone: 54 11 4292-7671, Fax: 54 11 4508-3708
e-mail: minivirjg@gmail.com

REFERENCES

1. Tonetti MS, Claffey N on behalf of European workshop in Periodontology Group. Advances in the progression of periodontitis and proposal of definitions of a periodontitis case and disease progression for use in risk factor research. *J Clin Periodontol* 2005; 32: 210-21.
2. Jabra-Rizk MA, Ferreira SMS, Sabet M et al. Recovery of *Candida dubliniensis* and other Yeasts from Human Immunodeficiency Virus-associated periodontal lesions. *J Clin Microbiol* 2001; 39: 4520-4522.
3. Urizar JM. Candidiasis orales. *Rev Iberoam Micol* 2002; 19: 17-21
4. Patel M, Coogan M, Galpin JS. Periodontal pathogens in subgingival plaque of HIV-positive subjects with chronic periodontitis. *Oral Microbiol Immunol* 2003; 18: 199-201.
5. Portela MB, Souza PR, Costa EMMB et al. Differential Recovery Of *Candida* Species from Subgingival Sites In Human Immunodeficiency Virus-Positive And Healthy Children From Rio de Janeiro, Brazil. *J Clin Microbiol* 2004; 42: 5925-5927.
6. Dahlen G, Wikstrom M. Occurrence of enteric rods, *Staphylococci* and *Candida* in subgingival samples. *Oral Microbiol and Immunol* 1995; 10: 42-46.
7. Reynaud AH, Nygaard-Osby B, Boygard GK et al. Yeasts in periodontal pockets. *J Clin Periodontology* 2001; 28: 860-864.
8. Martinez M, Lopez-Ribot JL, Kirkpatrick WR et al. Replacement of *Candida albicans* with *C. dubliniensis* in Human Immunodeficiency Virus-Infected Patients with Oropharyngeal Candidiasis Treated with Fluconazole. *J Clin Microbiol* 2002; 40: 3135-3139.
9. Listgarten MA, Lai CH, Young V. Microbial composition and pattern of antibiotic resistance in subgingival microbial samples from patients with refractory periodontitis. *J Periodontol* 1993; 64: 155-161.

10. Pfaller MA, Diekema DJ, Rinaldi MG et al. Results from the ARTEMIS DISK global antifungal surveillance study: a 6.5-Year analysis of susceptibilities of *Candida* and other species to fluconazole and voriconazole by standardized disk diffusion testing. *J Clin Microbiol* 2005; 43: 5848-5859.
11. Menzaghi N, Saletta M, Garattini G et al. Changes in the yeast flora in patients in orthodontic treatment. *Pres Assist Dent* 1991; 17: 26-30.
12. Arendorf T, Addy M. Candidal carriage and plaque distribution before, during and after removable orthodontic applications therapy. *J Clin Periodontol* 1985; 12: 360-368.
13. Addy M, Shaw WC, Hansford P, Hopkins M. The effect of orthodontic applications on the distribution of *Candida* plaque in adolescents. *Br J Orthod* 1982; 9: 158-163.
14. Silness J, L oe H. Periodontal disease in pregnancy. II. Correlation between oral hygiene and periodontal condition. *Acta Odontol Scand* 1964; 22: 131-135.
15. L oe H, Silness J. Periodontal disease in pregnancy. I. Prevalence and severity. *Acta Odontol Scand* 1963; 21: 533-551.
16. Jitaurong S, Klamsiri S, Pattararagron N. Milk medium for germ tube and chlamydoconidia production by *Candida*. *Mycopathologia* 1993; 123: 95-98.
17. Staib P, Morschhauser J. Chlamydospore formation on Staib agar as a species-specific characteristic of *Candida dubliniensis*. *Mycoses* 1999; 42: 521-524.
18. National Committee for Clinical Laboratory Standards. 2004. Method for antifungal disk diffusion susceptibility testing of yeasts: approved guidance M44-A. National Committee for Clinical Laboratory Standards, Wayne, Pa.
19. Jarvensivu A, Hietanen J, Rautemaa R et al. *Candida* yeasts in chronic periodontitis tissues and subgingival microbial biofilms in vivo. *Oral Diseases* 2004; 10: 106-112.
20. Beighton D, Ludford R, Clark DT et al. *Use Of Chromagar Candida Medium For Isolation Of Yeasts From Dental Samples*. *J Clin Microbiol* 1995; 33: 3025-3027.
21. Mujica MT, Finquelievich JL, Jewtuchowicz VM et al. Prevalencia de *Candida albicans* y *Candida no albicans* en diferentes muestras cl nicas. Per odo 1999-2001. *Revista Argentina de Microbiolog a*. 2004; 36: 107-112.
22. Jabra-Rizk MA, Falkler WA, Merz WG et al. Cell Surface Hydrophobicity associated adherence of *Candida dubliniensis* to human buccal epithelial cells. *Rev Iberoam Micol*. 2001; 18: 17-22.
23. Jabra-Rizk MA, Falkler WA, Merz WG et al. Coaggregation Of *Candida dubliniensis* with *Fusobacterium nucleatum*. *J Clin Microbiol* 1999; 37: 1464-1468.
24. McManners J, Samaranayake LP. Suppurative oral candidosis. Review of the literature and report of a case. *Int J Oral Maxillofac Surg* 1990; 19: 257-259.
25. Pizzo G, Barchiesi F, Falconi Di Francesco L et al. Genotyping and antifungal susceptibility of human subgingival *Candida albicans* isolates. *Arch Oral Biol* 2002; 47: 189-196.