

CANDIDA SP IN THE ORAL CAVITY WITH AND WITHOUT LESIONS: MAXIMAL INHIBITORY DILUTION OF PROPOLIS AND PERIOGARD

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ABSTRACT

Fifty individuals of both sexes aged on average 45.2 years were evaluated at the Semiology Clinic of FORP-USP in order to isolate and identify yeasts from the oral cavity, with and without lesions, and to determine the maximal inhibitory dilution (MID) of the commercial products Propolis (Apis-Flora) and Periogard (Colgate) against the strains isolated. Yeasts of the genus *Candida* were detected in the saliva of 9/19 (47.4%) individuals with a clinically healthy mouth, 18/22 (81.8%) of individuals with oral lesions, and in 4/9 (44.4%) of patients with deviation from normality, and were detected in 19/22 (86.4%) of the lesions. In the group with oral candidiasis, we isolated in tongue and lesion, respectively for each specie: *C.tropicalis* (8% and 10.7%), *C.glabrata* (4% and 3.6%) and *C.parapsilosis* (2% and 3.6%), in addition to *C.albicans* (71.4% and 67.8%) as the only species and the prevalent. The total cfu counts/ml saliva showed a higher mean value in the group with oral candidiasis ($171.5\% \times 10^3$) than in the control group (72.6×10^3) or the group with abnormalities (8.3×10^3). Most of the test strains 67/70 (95.71%) were sensitive to the antiseptics, with Propolis presenting a MID of 1:20 for 54/70/77.1%), and Periogard a MID of 1:160 for 42/70 (60%) strains from healthy sites, results similar to those obtained with strains from oral lesions. Different results were mainly observed among different species. The results indicate the possibility of using the antiseptics Propolis and Periogard (chlorhexidine) for the prevention and treatment of oral candidiasis.

Key words: Oral candidiasis, Propolis, chlorhexidine, yeasts

INTRODUCTION

Yeast-like fungi establish a biological link with the host which guarantees their saprophytic condition by establishing an ecological equilibrium denoted "amphibiosis" (17). When this equilibrium is broken

due to different endogenous and/or exogenous factors these amphibionts behave as opportunistic microorganisms, eventually causing multiple oral infections which, if unresolved, may become generalized, leading to more severe mycoses (2).

The lesions of candidiasis are more frequent on

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the tongue, cheeks and palate (5), sites that may be more frequently and densely colonized in subjects arraying yeasts of the genus *Candida* (24). Although the most frequent etiologic agent of oral candidiasis is *Candida albicans* (9,24), other species of the genus, such as *C.tropicalis*, *C.glabrata*, *C.krusei* and *C.parapsilosis*, among others, may be responsible for this type of mycosis (24).

Antifungal prophylaxis may be indicated for the prevention of colonization or multiplication of *Candida* spp in patients susceptible to primary infection and also for the prevention of recurrence in patients previously submitted to antimycotic treatment (2).

Propolis is widely used in the preparation of medications in different countries in the world, not only in Europe when it has been used as a therapeutic substance for many years, but also in some South American countries such as Brazil and Uruguay (14,21). Its presence has been reported in compounds with antifungal activity against yeasts (6) and filamentous fungi (11). Rojas and Lugo (16) demonstrated the presence of antifungal activity of an alcohol extract of Propolis against 23 strains of yeasts of the genus *Candida* isolated from different biological materials, with a fungistatic action at low concentrations (0.85mg/ml for 2 hours of treatment and 0.55mg/ml for 24 hours of treatment).

Periogard contains chlorhexidine gluconate as the active principle, a substance that has proved to be effective in the chemical control of dental plaque and the consequent prevention of gingivitis, mainly in special patients (3,13), and also as an antifungal agent (4).

Although standard mechanical methods are generally preferred to the use of some chemical product for the routine control of dental plaque, the supervised use of chlorhexidine is indicated in certain situations in which it is difficult and painful to maintain the desirable level of oral hygiene (8), i.e., among older individuals and among patients with mental problems and with Down's Syndrome (13,20) or medicinally compromised (diabetes, anticancer treatment, bone marrow transplant, etc.).

The *in vitro* evaluation of the yeast sensitivity to antiseptics has been little studied even though the application of oral antiseptics deserves to be considered at least as a preventive measure or as an alternative or a complementary procedure in treatment (10,13).

The objectives of the present study were: 1) to

isolate, identify and determine the prevalence of yeasts in the oral cavity of individuals with and without lesions, and 2) to test the minimum inhibitory dilution (MID) of Propolis and Periogard against the isolated yeasts.

MATERIALS AND METHODS

The study was conducted on 50 adults of both sexes aged on average 42.56 years, 19 of whom (38.0%) presented oral health (control group), 22 (44.0%) had some type of intraoral lesion with suspected oral candidiasis, and 9 (18.0%) showed deviation from normality (fissured and/or coated tongue).

Approximately 2.0 ml of nonstimulated saliva was collected from each patient into 20 x 150 mm sterilized tubes containing glass beads. The tubes were then shaken in a Mixtron-Toptronix apparatus until a uniform suspension was obtained, to be used for serial decimal dilutions in phosphate buffered saline (PBS).

Aliquots of 0.1 ml of pure saliva from each of three dilutions (10^{-1} , 10^{-2} e 10^{-3}) were added to the center of a 15 x 100 mm Petri dish containing agar Sabouraud plus chloramphenicol (Sba) and then seeded with a sterilized L-shaped glass rod. The plates were then incubated at 37°C for 24-48 h and stored at room temperature for the subsequent tests.

Material was collected aseptically from the dorsum of the tongue of each patient. After clinical examination of the individuals with lesions clinically suspected to be caused by yeasts, material was collected from the lesions with the aid of a flame-sterilized, and cooled platinum loop and directly seeded onto agar (Sba) distributed into different test tubes. After seeding, all tubes containing samples from the tongue, or from the lesions were incubated at 37°C for 3 to 5 days, reisolated in agar Sabouraud, and distributed into different plates by the depletion technique.

The yeasts were identified by classical methods (12,22) using the following tests: formation of germinative tubes, study of micromorphology, assimilation of carbon and nitrogen sources, fermentation, urea hydrolysis, and triphenyltetrazolium reduction.

The inocula for the identification tests were obtained from recent cultures (24 to 48 hours at 37°C) on agar Sabouraud after culture purity was confirmed.

For the detection of the MID of Propolis and Periogard, 70 yeast strains of different species of

the genus *Candida* isolated from the oral cavity of subjects without lesions were tested. The Propolis sample tested was supplied by Apis-Flora (Ribeirão Preto/SP) in the form of an alcohol extract containing quantities corresponding to 10 g% of the soluble solids present. Periogard, a pharmaceutical product launched on the Brazilian market by Colgate (Osasco/SP) which contains 0.12% chlorhexidine was purchased on the local market.

The antifungal activity of the products tested was determined by the technique of dilution on a solid medium (19). Serial dilutions of the antiseptics were prepared in duplicate in sterilized distilled water, corresponding to 1:20 to 1:300 for Periogard and to 1:20 to 1:320 for the alcohol extract of Propolis for which a tube containing only 60% ethyl alcohol was prepared in addition to the control tube. A sufficient amount of Mueller Hinton agar medium (MHA-Difco) to provide a final volume of 20 ml, cooled to approximately 50°C, was added to the serial dilution tubes and to the control tubes, followed by homogenization and distribution among sterilized 15 x 100 mm Petri dishes.

Suspensions in sterilized physiological saline containing approximately 1×10^6 cells/ml were applied with Steers replicator onto the series of agar plates. Incubation were to 24 h at 37°C and the results were read by observing the presence of absence of microorganism growth at the corresponding dilution of the antiseptic tested.

RESULTS

Thirty-two (64.0%) of the samples obtained from the oral cavities of 50 individuals were positive for yeast. Yeasts of the genus *Candida* were isolated from 9/19 (47%) saliva samples from clinically healthy individuals, 18/22 (81.8%) samples from

patients suspected to have oral candidiasis and from 4/9 (44.4%) individuals with deviation from normality (fissured and/or coated tongue), and were detected in 19/22 (86.4%) samples from the oral lesions.

C.albicans was the most prevalent among the species isolated and was the only one detected in all types of samples analyzed, occurring at a frequency of 48% (24/50) in saliva, at frequencies of 28.0% (07/20), 14.3% (01/07) and 12.5% (01/08) on normal, fissured and coated tongues, respectively, and at a frequency of 67.8% (19/28) in samples from lesions. Other species were also isolated, mainly from saliva and lesions, at a higher frequency for *C.tropicalis*: 8.0% (04/50) of saliva samples and 10.7% (03/28) of lesion samples, and *C.glabrata*: 4.0% (02/50) of saliva samples and 3.6% (01/28) of lesion samples, with emphasis on the fact that the latter species was isolated from fissured tongues: 28.6% (02/07).

The study of the salivary levels of yeasts of the genus *Candida* by the methods of serial decimal dilution showed a mean number of total cfu/ml saliva of 171.5×10^3 in the group of patients with lesions (range: 0.42×10^3 to $2,760.0 \times 10^3$ cfu/ml). It can be seen that these levels were higher than in the groups of patients with no lesions (control) or with deviation normality, with respective values of 72.64×10^3 cfu/ml and 8.6×10^3 cfu/ml. Among the species isolated from the 3 study groups, *Candida albicans* was the most frequent, with a mean level of 227.52×10^3 cfu/ml in the groups with lesions, 72.28×10^3 cfu/ml in the group without lesions and 11.02×10^3 cfu/ml in the third group. Yeasts of the species *C.tropicalis* and *C.glabrata* were isolated only from patients with lesions, at respective levels of 14.7×10^3 cfu/ml and 48.25×10^3 cfu/ml saliva. *C.guilliermondii* (0.06×10^3 cfu/ml) and *C.parapsilosis* (4.70×10^3 cfu/ml)

Table 1. Number and frequency of species of the genus *Candida* isolated from oral cavity.

Specie	Saliva (50)		Normal (20)		Fissured (7)		Saburrough (8)		Lesion (28)	
	Nº	%	Nº	%	Nº	%	Nº	%	Nº	%
<i>C.albicans</i>	24	48.0	07	28.0	01	14.3	01	12.5	19	67.8
<i>C.tropicalis</i>	04	8.0	-	-	-	-	-	-	03	10.7
<i>C.glabrata</i>	02	4.0	-	-	02	28.6	-	-	01	3.6
<i>C.guilliermondii</i>	01	2.0	-	-	-	-	-	-	-	-
<i>C.parapsilosis</i>	01	2.0	-	-	-	-	-	-	01	3.6
<i>C.krusei</i>	02	4.0	-	-	-	-	-	-	-	-

Legend: (-) non detected.

were only detected in control individuals, from whom *C.krusei* was not isolated from the oral cavity.

In the determination of the action of the antiseptics Periogard and Propolis against the 70 strains of yeasts of the genus *Candida* isolated from the oral cavity of individuals with a clinically healthy mouth or a mouth with lesions, we observed that most strains 67/70 (95.71%) were sensitive to the two antimicrobial agents.

With respect to the action of the different dilutions of the antiseptics tested, we noted that a large number of strains was inhibited by the 1:20 dilution of Propolis 54/70 (77.1%), a MID corresponding to the final concentration of 10.0 mg soluble solids per ml of the commercial product, and by the 1:160 dilution of Periogard 42/70 (60%), which contains a final concentration of chlorhexidine gluconate of 0.0075 mg/ml. We emphasize that all strains tested grew when submitted to Propolis dilutions higher than 1:40.

Of all strains tested, 29/70 (41.42%) were simultaneously sensitive to a MID of 1:160 of Periogard and to a MID of 1:20 of Propolis. Of these strains, 28/29 (96.55%) were of the species *C.albicans*.

Different results were obtained for different species, when they were tested with Periogard, with the MID ranging from 1:100 to 1:200 for this species and from 1:200 to 1:300 for the remaining ones. With respect to Propolis, *C.albicans* and *C.tropicalis* presented strains that were sensitive to the same

concentrations (1:20 or 1:40), whereas the other species were sensitive to a MID of 1:20 (Table 2).

With respect to the *Candida* species, the MID of Periogard for *C.albicans*, the species most often detected 24/31(77.4%), was 1:100 to 1:160, whereas the MID against *C.tropicalis* 4/31 (12.9%) ranged from 1:200 to 1:300. With respect to Propolis, *C.albicans* was sensitive to a MID of 1:20 - 18/24 (75.0%) or 1:40 - 5/24 (20.8%), whereas the remaining species were sensitive to a MID of 1:20 (Table 3).

The 3 species that were resistant to all dilutions of Propolis tested were *C.albicans*, *C.tropicalis* and *C.glabrata* isolated from oral lesion.

DISCUSSION AND CONCLUSION

The density of yeasts in oral is usually high and more than one species is frequently isolated that it is difficult to determine the relative role of individual species in the disease process (1,2).

Total quantitation of yeasts of the genus *Candida* varied in the three groups studied here, with emphasis on the mean level detected in patients with oral candidiasis (171.5×10^3 cfu/ml saliva) compared to the control group (72.64×10^3 cfu/ml) and to the group with deviation from normality (8.26×10^3 cfu/ml).

With respect to the species isolated, *C.albicans* showed the highest levels (72.28×10^3 , 227.52×10^3 and 11.02×10^3 cfu/ml) in the group with lesions and in the group with deviation from normality,

Table 2. Number and frequency of species of the genus *Candida* isolated from oral cavity. Maximal inhibitory dilution (MID) using Periogard and Propolis.

MID	Specie		<i>C.albicans</i> (55)		<i>C.tropicalis</i> (8)		<i>C.glabrata</i> (2)		<i>C.parapsilosis</i> (2)		<i>C.krusei</i> (2)		<i>C.guilliermondii</i> (1)		Total
	N°	%	N°	%	N°	%	N°	%	N°	%	N°	%	N°	%	
1:100	02	3.6	0		0		0		0		0		0		02
1:120	10	18.2	0		0		0		0		0		0		10
1:160	41	74.6	0		0		0		0		0		01	100.0	42
1:200	02	3.6	04	50.0	01	50.0	01	50.0	01	50.0	01	50.0	0		09
1:240	0		03	37.5	0	50.0	0		01	50.0	01	50.0	0		05
1:300	0		01	12.5	01		01	50.0	0		0		0		02
1:20	42	76.4	06	75.0	01	50.0	02	100.0	02	100.0	02	100.0	01	100.0	54
1:40	12	21.8	01	12.5	0		0		0		0		0		13
R	01	1.8	01	12.5	01	50.0	0		0		0		0		03

R = resistant to Propolis at the dilutions studied.

Table 3. Number and frequency of species of the genus *Candida* isolated from oral lesions. Maximal inhibitory dilution (MID) using Periogard and Propolis.

MID	Specie	<i>C.albicans</i> (24)		<i>C.tropicalis</i> (4)		<i>C.glabrata</i> (2)		<i>C.parapsilosis</i> (1)		Total
		Nº	%	Nº	%	Nº	%	Nº	%	
1:100		02	8.3	0		0		0		02
1:120		04	16.7	0		0		0		04
1:160		18	75.0	0		0		01	100.0	18
1:200		0		02	50.0	01	50.0	0		04
1:240		0		01	25.0	0		0		01
1:300		0		01	25.0	01	50.0	0		02
1:20		18	75.0	03	75.0	01	50.0	01	100.0	23
1:40		05	20.8	0		0		0		05
R		01	4.2	01	25.0	0	50.0	0		03

R = resistant to Propolis at the dilutions studied.

respectively, among individuals with oral candidiasis. *C.tropicalis* and *C.glabrata* presented a mean of 14.7×10^3 and 48.25×10^3 cfu/ml, respectively, in individuals with oral candidiasis.

The results obtained for the genus *Candida* in saliva samples from individuals with suspected oral candidiasis (18/81.8%) were lower than those obtained by Davenport (7) (98.0%) and higher than those obtained by Jorge Junior *et al.* (9) (45.5%). The results for lesion samples (86.4%) were lower than those obtained by Rindum *et al.* (15) (98.1%). *C.albicans* was detected in 59.1% of the saliva samples and in 67.8% of the lesion samples, a result lower than that obtained by Davenport (7) in saliva (70.0%) and by Rindum *et al.* (15) in oral mucosa lesions (94.3%).

The results of the present study are reported as MID because we tested a commercial preparation both for chlorhexidine (chlorhexidine gluconate/ Periogard) and Propolis (alcohol extract). Since these preparations might contain substances that inhibit the yeasts tested in addition to the active principle itself of the antiseptics studied, this would invalidate the calculation of minimum inhibitory concentration for the products employed.

Different results were obtained for different species tested with Periogard (0.12% chlorhexidine gluconate). The action of the product against *C.albicans* occurred at a MID of 1:200 to 1:300, indicating that the species isolated at highest frequency from individuals with and without oral lesions had the lowest sensitivity.

In contrast, the antifungal action of Propolis was more uniform against all species tested, with a MID

of 1:20 for most of them, whether or not they were isolated from lesions.

Periogard, whose active principle is chlorhexidine, has been shown to be active against bacteria and fungi (4,13), a fact that was confirmed in the present study, in which strains of the yeasts tested were inhibited by a concentration ranging from 4 to 12ug with an intermediate concentration of 7.5ug/ml for a MID of 1:160 inhibiting 60% of the strains. This result is similar to that obtained by Candido *et al.* (4) who obtained 90% inhibition of the strains at a concentration of 6.4ug/ml, indicating that the commercial product can be used diluted.

Most of the strains tested (77.1%) were sensitive to Propolis at a dilution of 1:20 corresponding to 10 mg/ml of the product (soluble solids) present in the product (soluble solids) present in the alcohol extract. Of these, 18.6% were sensitive to a higher dilution of 1:40, results that are difficult to compare since other authors have tested the action of Propolis using a pure Propolis extract (PPE) and/or yeasts obtained from different sites.

Ota *et al.* (14), in a study of *C.albicans* sensitivity to PPE, observed that the minimum fungicidal concentrations against the 15 strains tested ranged from 3 to 7 mg/ml, indicating their possible use in the prevention of oral diseases.

Rojas and Lugo (16) demonstrated the antifungal activity of the alcohol extract of Propolis against 23 yeast strains isolated from different sites in the human body and observed that the product was fungistatic at a concentration of 0.55 mg/ml.

The diversity of the results obtained with Propolis may be related to the methods employed, the strains

tested and mainly the fact that the antimicrobial effect is directly proportional to its concentration and also depends on its origin since its chemical composition and consequently its antimicrobial effect vary according to such origin (18).

Even considering the fact that *in vivo* cannot be directly extrapolated to *in vivo* effects (20), our results indicate that yeasts of different species of the genus *Candida* could be inhibited by the application of the commercial products Periogard and Propolis to the oral cavity for therapeutic and/or preventive purposes against oral candidiasis.

RESUMO

***Candida* sp na cavidade bucal com e sem lesão: diluição inibitória máxima de Própolis e Periogard**

Foram avaliados 50 indivíduos, de ambos os sexos e faixa etária média de 42,5 anos, da clínica de Semiologia da FORP-USP, objetivando-se isolar e identificar leveduras na cavidade bucal, com e sem lesão, e determinar a DIM dos produtos comerciais Própolis (Apis-Flora) e Periogard (Colgate) frente às cepas isoladas. Leveduras do gênero *Candida* foram detectadas na saliva de 9(47,4%) indivíduos com boca clinicamente sadia, 18/81,8% portadores de lesões bucais e de 4/44,4% pacientes com desvio de normalidade; sendo detectadas em 19/86,4% lesões. No grupo com candidose bucal, respectivamente de língua e lesão, isolou-se *C.tropicalis* (8% e 10,7%), *C.glabrata* 4% e 3,6%) e *C.parapsilosis* (2% e 3,6%), além de *C.albicans* (71,4% e 67,8%) como espécie única e prevalente. A contagem de ufc total/ml de saliva demonstrou um valor médio no grupo com candidose bucal ($171,5\% \times 10^3$) maior do que no controle ($72,6 \times 10^3$) e portadores de anormalidade ($8,3 \times 10^3$). A maioria das cepas testes 67/70 (95,7%) foi sensível aos anti-sépticos, sendo que a Própolis apresentou uma DIM igual a 1:20 para 54/70 (77,1%) e, o Periogard uma DIM de 1:160 para 42/70 (60%) cepas de nichos sadios; semelhante ao obtido com cepas de lesões bucais. Resultados diferentes ocorreram, principalmente, entre espécies diferentes. Os resultados indicam a possibilidade de se empregar os anti-sépticos Própolis e Periogard (clorexidina), na prevenção e na terapêutica da candidose bucal.

Palavras-chave: Candidose bucal, Própolis, Clorexidina, leveduras.

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