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Phenotypic and genotypic identification of *Candida dubliniensis* from subgingival sites in immunocompetent subjects in Argentina

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Jewtuchowicz VM, Mujica MT, Brusca MI, Sordelli N, Malzone MC, Pola SJ, Iovannitti CA, Rosa AC. Phenotypic and genotypic identification of Candida dubliniensis *from subgingival sites in immunocompetent subjects in Argentina.*
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Introduction: It is generally recognized that *Candida dubliniensis* is commonly found in immunocompromised patients, such as those with advanced human immunodeficiency virus infection, at sites of periodontal disease. Since there are no data available for Argentina, the aim of this study was to determine the prevalence of and to identify *C. dubliniensis* in periodontal pockets from immunocompetent subjects living in Buenos Aires, Argentina, through a comparison of phenotypic and molecular assays.
Methods: Yeasts recovered from subgingival plaque samples were studied for 180 immunocompetent non-smoking patients with periodontal disease. Yeasts were identified by conventional mycological methods and by specific polymerase chain reaction (PCR) assay. Fluconazole and voriconazole susceptibility studies were performed in keeping with the Clinical and Laboratory Standards Institute.
Results: Among 76 yeasts isolated, *C. dubliniensis* comprised 10.5% (n = 8; 95% confidence interval 4.7–19.7), which corresponded to 4.4% of patients studied (8/180). *C. albicans* was the most frequently isolated species of yeast. A great majority of *C. dubliniensis* isolates was susceptible with only one isolate resistant to both antifungals.
Conclusion: Micromorphology on Staib agar was the phenotypic method that was most concordant with PCR and it was useful for selecting presumptive *C. dubliniensis*. This is the first report to use PCR to identify *C. dubliniensis* in subgingival fluid from immunocompetent individuals with periodontal disease in Argentina. On the basis of the findings presented here, we confirm that *C. dubliniensis* can colonize periodontal pockets of immunocompetent patients with periodontal disease.

Key words: *Candida dubliniensis*; fluconazol; periodontal disease; subgingival biofilm; voriconazol

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Periodontal disease is characterized by an infectious, inflammatory, and degenerative response in the gingival and underlying connective tissues elicited by microbial colonization and multiplication in periodontal pockets, such as *Tannerella forsythensis*, *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Treponema denticola* (33).

Candida dubliniensis, as described by Sullivan et al., is a yeast species recently identified as an opportunistic pathogen associated with oral candidiasis, particularly in individuals who are positive for human immunodeficiency virus (HIV) and patients with acquired immunodeficiency syndrome, noted for its reduced susceptibility to azole and its enhanced *in vitro* adherence to human buccal epithelial cells (3, 8, 21, 24, 29). Since then, its isolation

has been reported in numerous locations around the world (1, 2, 4–6, 13, 14, 22, 27, 30, 32). Prevalence rates have varied between 15 and 30% in HIV-positive populations worldwide, whereas they have been reported to be below 5% in the oral cavities of healthy individuals (9, 16, 30).

This species shares morphological and physiological characteristics with *Candida albicans*. To differentiate these species, arrays of controversial criteria have been used (5, 9, 32). Therefore, rapid, nucleic acid-based methods that identify the most medically important species of yeasts have been performed using polymerase chain reaction (PCR) assay.

C. dubliniensis has the ability to colonize deep periodontal pockets (10, 23), which supports the importance of studying *C. dubliniensis* in subgingival pockets for further clinical and epidemiological evaluations.

No data are available for Argentina so the aim of this study was to determine the prevalence and to identify *C. dubliniensis* in periodontal pockets from immunocompetent subjects living in Buenos Aires, Argentina, through a comparison of phenotypic and molecular assays. It was also aimed to study the susceptibility of the *C. dubliniensis* isolates to fluconazole and voriconazole.

Materials and methods

Yeasts recovered from subgingival plaque samples were studied from 180 immunocompetent non-smoking patients with periodontal disease (12, 26) who attended the dental clinic of the University of Buenos Aires, Argentina. Periodontal evaluations included clinical examination and radiographs with clinical measurements: clinical attachment level, pocket depth, plaque index (26), gingival index (15), and bleeding on probing. Location of the gingival margin was determined and tooth mobility was assessed. Measurements were made at four sites per tooth (mesial, buccal, distal, and lingual positions) at 15 teeth excluding the third molar.

Participation in our survey was voluntary and all the patients provided a written informed consent.

All the volunteers were requested to thoroughly rinse their mouths with sterile distilled water. The dental professional then isolated the area using cotton rolls and a high-speed suction device. Following removal of the supragingival plaque, to avoid salivary contamination, four subgingival plaque samples were taken from each patient: the upper right and lower central incisors, the first upper right molar, and the first lower left molar to mimic the more common sites of periodontal pockets infected in periodontitis by using a 7/8 Gracey curette. Samples were pooled and cultured in a differential chromogenic medium (CHROMagar *Candida*, Paris, France). Isolated yeasts were identified by conventional mycological methods: colony color on the chromogenic medium, micromorphology in agar milk−1% Tween-80 (12), and carbohydrate assimilation tests using a commercially available kit API ID 32D (BioMérieux, Lyon, France).

Out of the 76 yeast isolates, 52 *Candida* isolates were selected for this study, based on the production of green colonies on the chromogenic medium, a characteristic of both *C. albicans* and *C. dubliniensis*. Further studies were conducted for phenotypic characterization of *C. dubliniensis*, including xylose assimilation, growth at 45°C, and chlamydospore formation on Staib agar after incubation for 72 h at 28°C (28).

Fluconazole (FCZ) and voriconazole (VCZ) susceptibility studies were performed employing the agar disk diffusion method, in keeping with the Clinical and Laboratory Standards Institute (CLSI) M44-A standards (18). Isolates were identified as susceptible (S), susceptible dose-dependent (S-DD), or 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 FCZ and ≥17 mm, 14–16 mm, and ≤13 mm, respectively, in the case of VCZ. The minimum inhibitory concentration (MIC) was determined according to the CLSI M27-A2 method (17). *C. albicans* ATCC 90029, *Candida parapsilosis* ATCC 22019, and *Candida krusei* ATCC 6258 were incorporated as quality controls.

The MIC interpretive breakpoints for azoles were those suggested by CLSI M27-A2. Isolates with MICs of 8 μg/ml for FCZ and 1 μg/ml for VCZ were considered susceptible. Isolates with MICs of 16 and 32 μg/ml for FCZ, and 2 μg/ml for VCZ were considered susceptible in a dose-dependent manner (S-DD). Isolates with MICs ≥64 μg/ml for FCZ and ≥4 μg/ml for VCZ were considered resistant.

Molecular identification

DNA extraction from selected yeasts was performed according to a previously described technique (11, 25). The same procedures were used for *C. albicans* ATCC 90029 and *C. dubliniensis* NCPF 3949 (provided by CEREMIC, University of Rosario, Argentina), which were used as negative and positive controls of the PCR, respectively. The DNA was quantified and its purity was evaluated to 260 nm (SmartSpec^{TM} 3000 Spectrophotometer; Bio-Rad, Hercules, CA).

Molecular assay was carried out by specific PCR with primers from the *ACT1*-associated intron sequence of *C. dubliniensis*, DUBF (GTATTTGTCCTTCCCCTTTTC) and DUBR (GTGTTGTGTGCACTAACGTC) (5).

Polymerase chain reaction amplification was performed in a total volume of 50 μl containing: 1× buffer 1.5 mM $MgCl_2$, 0.2 mM each of the dNTP, 10 pmol each of the primers, 1.25 U *Taq* DNA polymerase (Invitrogen, Buenos Aires, Argentina), and 50 ng template DNA. The cycling program comprised 3 min at 94°C, 30 cycles of 45 s at 94°C, 45 s at 55°C, and 45 s at 72°C, followed by a final extension of 10 min at 72°C. These steps were carried out in a Minicycler DNA thermal cycler (TM MJ Research Inc. USA). Products were separated by electrophoresis in 2% agarose gels and stained with ethidium bromide. It was visualized under ultraviolet light and digitalized using the image analyzer software (EPI-CHEMI DARKROOM; UVP Laboratory Products).

Polymerase chain reaction products of 288 base pairs were purified using a QIAquick PCR purification Kit (Qiagen AG, Basel, Switzerland). Purified amplicons were then sequenced to confirm identification of the species under study.

Statistical analysis

Statistical analysis was performed using STATADISTICX 7.0 and SPSS 11.0 versions. Confidence intervals (CI) were calculated at 95% employing the EPI-INFO 6.04 program (Atlanta University, GA).

Results

Clinical features

The 180 subjects included in the study ranged in age from 18 to 75 years (mean age 37 years), 55% were female (90/180) and had not received any antibacterial or antifungal agents before this treatment. Table 1 shows periodontal clinical parameters (probing depth and clinical attachment level, mean ± SD) of subjects at the time of sampling. Patients were classified into groups according to their periodontal health status, as shown in Table 1: healthy (n = 40), gingivitis (n = 44), and chronic periodontitis (n = 96). Patients with gingi-

Table 1. Periodontal clinical parameters (mean ± SD and 95% confidence interval) of subjects at the time of sampling according to periodontal health status[1]

Clinical parameters	Healthy	Gingivitis	Chronic periodontitis
Pocket depth[2] (mm)	1.5 ± 0.80 (1.2–1.8)	4.0 ± 1.2 (3.6–4.3)	6.2 ± 1.9 (5.8–6.5)
Clinical attachment level (mm)	0	0	6.4 ± 3.6 (5.7–7.1)
Plaque index[3]	0.70 ± 0.18	0.90 ± 0.85	1.70 ± 0.18
Gingival index[4]	0.10 ± 0.24	0.95 ± 0.35	2.10 ± 0.14

[1]Healthy $n = 40$; gingivitis $n = 44$; chronic periodontitis $n = 96$.
[2]Kruskal–Wallis test: (differences in the median values among the groups[1]) H = 902,675 with two degrees of freedom. ($P \leq 0.001$).
[3]Silness and Löe 1964; (26).
[4]Löe and Silness 1963; (15).

vitis or chronic periodontitis were positive for bleeding on probing.

As expected, periodontitis sites showed significantly more signs of disease, including higher mean probing depth ($P < 0.001$) and mean clinical attachment level ($P < 0.001$), when comparing patients with periodontitis with healthy patients.

The carriage of *Candida* spp. and *C. dubliniensis*

Table 2 summarizes species distribution of yeast isolates in subgingival biofilm according to periodontal health status in 180 immunocompetent patients.

Out of the 76 yeasts recovered, *C. dubliniensis* was isolated in eight of 180 (4.4%) patients. *C. albicans* was the most frequent species, corresponding to 24.4% (44/180).

However, other non-*C. albicans* species and other yeasts were found. They included *C. parapsilosis* ($n = 10$), *Candida tropicalis* ($n = 5$), *Candida guilliermondii* ($n = 3$), *Candida sake* ($n = 1$), *Candida glabrata* ($n = 1$), and *Rhodotorula* ($n = 4$), as shown in Table 2.

The occurrence of two or three coisolated species was observed in eight of 180 samples from different patients and association was observed most frequently among *C. albicans*, *C. parapsilosis*, and *C. tropicalis*.

C. dubliniensis isolates were obtained as follows: 6.3% from subjects with chronic periodontitis (6/96), 2.3% from patients with gingivitis (1/44), and 2.5% from healthy individuals (1/40).

Antifungal susceptibility

Among eight *C. dubliniensis* isolates, a great majority was susceptible, whereas only one isolate was resistant to both antifungals by the disk diffusion method. The results were confirmed by MIC, which ranged from 0.06 to 125 μg/ml for FCZ and from 0.06 to 8 μg/ml for VCZ.

Comparison of different *C. dubliniensis* characterization techniques

The results of the tests used for identification of *C. dubliniensis* and *C. albicans* are shown in Table 3. All of the 52 *Candida* isolates studied and grown as green colonies produced chlamydospores on agar milk−1% Tween-80. Typical chlamydospore formation of *C. dubliniensis*, abundant chlamydospores often in clusters or contiguous pairs at the tip of the hypha, was found in seven of the eight isolates (87.5%, 95% CI: 47.3–99.7), which was suggestive of *C. dubliniensis*. This feature was also observed in 18 of 44 *C. albicans* (40.9%, 95% CI: 26.7–56.7).

All *C. dubliniensis* isolates recovered in this study failed to grow at 45°C (95% CI: 63.1–100). However, five *C. albicans* isolates did not grow at this temperature (11.4%, 95% CI: 3.8–24.6) either.

Only one of eight *C. dubliniensis* isolates analyzed in this study (12.5%, 95% CI: 0.3–52.7) assimilated xylose, whereas 43 isolates of *C. albicans* grew in the presence of this carbohydrate (97.72%, 95% CI: 88–99.9).

All *C. dubliniensis* isolates produced abundant pseudohyphae and chlamydospores on Staib agar (Fig. 1), giving the colonies a rough appearance. In contrast, all 44 *C. albicans* grew as smooth colonies, without producing pseudohyphae or chlamydospores, and only yeasts were observed (Fig. 2).

Isolates identified presumptively as *C. dubliniensis* showed a DNA fragment of approximately 288 base pairs by PCR assay using DUBR and DUBF primers (Fig. 3). Sequencing of PCR products confirmed the PCR specificity. Comparative GenBank data demonstrated that the

Table 2. Species distribution of yeast isolates in subgingival biofilm according to periodontal health status[1] in 180 immunocompetent patients

Yeast species	Healthy *n* (%)	Gingivitis *n* (%)	Chronic periodontitis *n* (%)	Total *n* (%)	95% Confidence interval
Candida albicans	7 (17.5)	11 (25.0)	26 (27.1)	44 (24.4)	18.5–31.5
C. parapsilosis	1 (2.5)	2 (4.5)	7 (7.3)	10 (5.6)	2.8–10.3
C. dubliniensis[2]	1 (2.5)	1 (2.3)	6 (6.3)	8 (4.4)	1.9–8.6
C. tropicalis	0	2 (4.5)	3 (3.1)	5 (2.8)	0.9–6.4
C. guilliermondii	0	0	3 (3.1)	3 (1.7)	0.3–4.8
C. sake	0	0	1 (1.0)	1 (0.6)	0–3.1
C. glabrata	0	1 (2.3)	0	1 (0.6)	0–3.1
Rhodotorula spp	2 (5.0)	1 (2.3)	1 (1.0)	4 (2.2)	0.6–5.6

[1]Healthy $n = 40$; gingivitis $n = 44$; chronic periodontitis $n = 96$.
[2]Kruskal–Wallis test (differences in the median values among the groups[1]); $P = 0.667$.

Table 3. Comparison of *Candida dubliniensis* and *C. albicans* identification tests in percentage (%) and number (*n*)

Candida species	Typical[1] *C. dubliniensis* chlamydospores on agar milk−1% Tween-80	Growth at 45°C	D-xylose assimilation	Chlamydospore production on Staib medium	PCR with primers DUBF/DUBR identification
C. dubliniensis ($n = 8$)	87.5 (7)	0	12.5 (1)	100 (8)	*C. dubliniensis* 100 (8)
C. albicans ($n = 44$)	40.9 (18)	95% (5)	97.72 (43)	0	0

[1]Abundant chlamydospores and often in clusters or contiguous pairs at the tip of the hyphae.

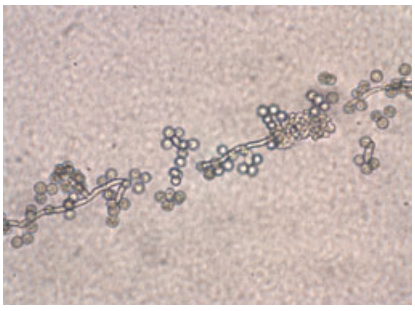

Fig. 1. Microscopic observation (400×) of chlamydospores on Staib agar in *Candida dubliniensis* isolated from subgingival biofilm.

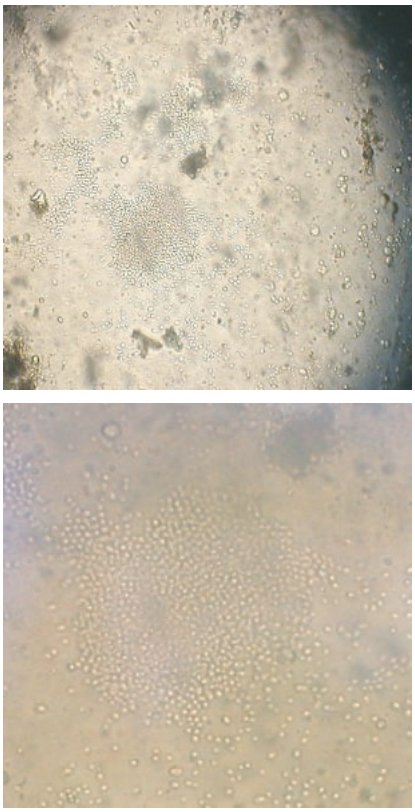

Fig. 2. Observation of yeasts on Staib agar in *Candida albicans* isolated from subgingival biofilm (left, 100× and right, 400×).

fragments corresponded with *C. dubliniensis* (AJ236897_*C. dubliniensis*_act1.txt). *C. albicans* DNA did not amplify with the specific primers used.

Discussion

The prevalence of *C. dubliniensis* in periodontal pockets from immunocompetent patients with periodontal disease in Argentina has not yet been evaluated and information about this species in the rest of the world is scarce.

In the present study, *C. dubliniensis* was found in the subgingival fluid of immunocompetent subjects. Eight isolates of *C. dubliniensis* (4.4%) were isolated from subgingival plaque samples of 180 immunocompetent non-smoking subjects with periodontal disease who attended the dental clinic of the University of Buenos Aires, Argentina.

It is generally recognized that *C. dubliniensis* is commonly found in immunocompromised patients such as individuals with advanced HIV infections with periodontal disease. Portela et al. reported *C. dubliniensis* at subgingival sites in HIV-positive children but not in immunocompetent children (23). Among 54 adult patients with positive histories of HIV infection, Jabra-Rizk et al. found 48% of *C. dubliniensis* at subgingival sites whereas this species was not recovered from the 20 HIV-negative patients under study (10).

In our sample, the *C. dubliniensis* species was found in both healthy subjects and in patients with and periodontal disease, but an increase in its prevalence was observed in patients with chronic periodontitis as compared to those with gingivitis or healthy mouths, but there was no statistically significant difference (Table 2).

On the basis of the findings presented here, we confirm that *C. dubliniensis* can colonize the periodontal pockets of immunocompetent patients who have periodontal disease. Yet it is not known whether this subgingival *C. dubliniensis* colonization or infection participates in the pathogenesis of periodontal disease. 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 frequent in the subgingival plaque of patients with periodontal disease. The capacity of this species to coaggregate enables it to colonize the depth of the subgingival biofilm (7, 8, 10, 23).

In our study, 90% of *C. dubliniensis* isolates presented dark green colonies on the CHROMagar medium and all the *C. albicans* isolates were light green. Other authors have reported dark green colonies in *C. albicans*. Colony color is not enough to discriminate between the two species, except when it is used in primary growth (19, 32).

Neither chlamydospore production on agar milk−1% Tween-80, growth at 45°C or xylose assimilation was useful to differentiate between the species. These results were concordant with others (9, 29).

Micromorphology on Staib agar was the phenotypic method that was most concordant with the PCR results and it was useful for selecting presumptive *C. dubliniensis* (Fig. 1). Our findings demonstrate that PCR identification based upon the *ACT1*-associated intron sequence is a method yielding an accurate and rapid technique for the identification of *C. dubliniensis*. PCR can be an effective and rapid tool for elucidating the epidemiological and clinical significance of this species and it was useful to discriminate both species, given that the DNA of *C. albicans* did not amplify with the specific primers used.

Recent studies have suggested that *C. dubliniensis* becomes resistant to FCZ during the course of therapy, making it a relatively more resistant pathogen and possibly more difficult to treat (20, 24). In the present investigation, both FCZ and VCZ were highly active against almost all *C. dubliniensis* isolates (87.5%), only one strain was resistant to both antifungals. One explanation for high sensitivity *in vitro* may be that the study population had not received previous antifungal treatment to differentiate from HIV-infected individuals who usually received treatment for recurrent infections.

There are no *in vitro* studies on yeast susceptibility to VCZ found in periodontal pockets. The definitive identification of *C. dubliniensis* remains a technical problem in routine diagnostic laboratories. It is

Fig. 3. Results of polymerase chain reaction (PCR) amplification with primers DUBF and DUBR: lane 1, 100-bp ladder; lanes 2–9, results of PCR of *Candida dubliniensis* isolated from biofilm subgingival; lanes 10 and 11, results of PCR of *C. albicans* ATCC 90029; lanes 12 and 13, negative and positive controls, respectively.

therefore necessary to determine phenotypic and genotypic characteristics of the isolates to obtain a final characterization.

Based on our results, both phenotypic, based on micromorphology on Staib agar, and specific PCR methods allowed the identification of *C. dubliniensis* in periodontal pockets of immunocompetent subjects living in Buenos Aires, Argentina and determined a prevalence of 4.4%. This is the first report using PCR assay to identify *C. dubliniensis* in subgingival fluid from immunocompetent individuals with periodontal disease in Argentina.

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References

1. Binolfi A, Biasoli MS, Luque AG, Tosello ME, Magaró HM. High prevalence of oral colonization by *Candida dubliniensis* in HIV-positive patients in Argentina. Med Mycol 2005: **43**: 431–437.
2. Blignaut E, Pujol C, Joly S, Soll DR. Racial distribution of *Candida dubliniensis* colonization among South Africans. J Clin Microbiol 2003: **41**: 1838–1842.
3. Coleman DC, Sullivan DJ, Bennett DE, Moran GP, Barry HJ, Shanley DB. Candidiasis: the emergence of a novel species, *Candida Dubliniensis*. AIDS 1997: **11**: 557–567.
4. Diaz-Guerra TM, Mellado E, Cuenca Estrella M, Laguna F, Rodriguez-Tudela JL. Molecular characterization by PCR-fingerprinting of *Candida dubliniensis* strains isolated from two HIV-positive patients in Spain. Diagn Microbiol Infect Dis 1999: **35**: 113–119.
5. Donnelly SM, Sullivan DJ, Shanley DB, Coleman DC. Phylogenetic analysis and rapid identification of *Candida dubliniensis* based on analysis of *ACT1* intron and exon sequences. Microbiology 1999: **145**: 1871–1882.
6. Fotedar R, Al-Hedaithy SS. *Candida dubliniensis* at a university hospital in Saudi Arabia. J Clin Microbiol 2003: **41**: 1907–1911.
7. Jabra-Rizk MA, Falkler WA, Merz WG et al. Coaggregation of *Candida dubliniensis* with *Fusobacterium nucleatum*. J Clin Microbiol 1999: **37**: 1464–1468.
8. 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.
9. Jabra-Rizk MA, Falker WA, Merz WG, Baqui AA, Kelly JL, Meiller TF. Retrospective identification and characterization of *Candida dubliniensis* isolates among *Candida albicans* clinical laboratory isolates from human immunodeficiency virus (HIV)-infected and non-HIV-infected individuals. J Clin Microbiol 2000: **38**: 2423–2426.
10. 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.
11. Jewtuchowicz VM, Finquelievich JL, Durán E, Mujica MT, Iovannitti CA. Comparación de métodos de extracción de ADN de sangre para detectar ADN fúngico por PCR. Rev Arg Microbiol 2007: **39**: 15–18.
12. Jitaurong S, Klamsiri S, Pattararagron N. Milk medium for germ tube and chlamydoconidia production by *Candida*. Mycophatologia 1993: **123**: 95–98.
13. Kamei K, McCullough MJ, Stevens DA. Initial case of *Candida dubliniensis* infection from Asia: non-mucosal infection. Med Mycol 2000: **38**: 81–83.
14. Kirkpatrick WR, Revankar SG, McAtee RK et al. Detection of *Candida dubliniensis* in oropharyngeal samples from human immunodeficiency virus-infected patients in North America by primary CHROMagar *Candida* screening and susceptibility testing of isolates. J Clin Microbiol 1998: **36**: 3007–3012.
15. Löe H, Silness J. Periodontal disease in pregnancy. I. Prevalence and severity. Acta Odontol Scand 1963: **21**: 533–551.
16. Moran GP, Sullivan DJ, Coleman DC. Emergence of non-*Candida albicans Candida* species as pathogens. In: Calderone RA, ed. Candida and Candidiasis. Washington, DC: ASM Press, 2002: p. 37–53.
17. National Committee for Clinical Laboratory Standards. Reference Method for Broth Dilution Antifungal Susceptibilities using of Yeast. Approved Standard M27-A2. Wayne, PA: National Committee for Clinical Laboratory Standards, 2002: **22**: 1–30.
18. National Committee for Clinical Laboratory Standards. Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts: Approved Guidance M44-A. Wayne, PA: National Committee for Clinical Laboratory Standards, 2004.
19. Odds FC, Bernaerts R. CHROMagar *Candida*, a new differential isolation medium for presumptive identification of clinically important *Candida* species. J Clin Microbiol 1994: **32**: 1923–1929.
20. 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 yeast species to fluconazole and voriconazole by standardized disk diffusion testing. J Clin Microbiol 2005: **43**: 5848–5859.
21. Pfaller MA, Messer SA, Gee S et al. *In vitro* susceptibilities of *Candida dubliniensis* isolates tested against the new triazole and echinocandin antifungal agents. J Clin Microbiol 1999: **37**: 870–872.
22. Polacheck I, Strahilevitz J, Sullivan D, Donnelly S, Salkin IF, Coleman DC. Recovery of *Candida dubliniensis* from non-human immunodeficiency virus-infected patients in Israel. J Clin Microbiol 2000: **38**: 170–174.
23. 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.
24. Ruhnke M, Schmidt-Westhausen A, Morschhauser J. Development of resistance to fluconazole in *Candida albicans* and *Candida dubliniensis* in a patient with AIDS. J Antimicrob Chemother 2000: **46**: 291–295.
25. Scherer S, Stevens DA. Application of DNA typing methods to epidemiology and taxonomy of *Candida* species. J Clin Microbiol 1987: **25**: 675–679.
26. Silness J, Löe H. Periodontal disease in pregnancy. II. Correlation between oral hygiene and periodontal condition. Acta Odontol Scand 1964: **22**: 131–135.
27. Silva V, Cabrera M, Diaz MC, Abarca C, Hermosilla G. Prevalence of *Candida albicans* serotypes in blood isolates in Chile, and first report of *Candida dubliniensis* candidemia. Rev Iberoam Micol 2003: **20**: 46–51.
28. Staib P, Morschhauser J. Chlamydospore formation on Staib agar as a species-specific characteristic of *Candida dubliniensis*. Mycoses 1999: **42**: 521–524.
29. Sullivan DJ, Coleman D. *Candida dubliniensis*: characteristics and identification. J Clin Microbiol 1998: **36**: 329–334.
30. Sullivan DJ, Haynes K, Bille J et al. Widespread geographic distribution of oral *Candida dubliniensis* strains in human immunodeficiency virus-infected individuals. J Clin Microbiol 1997: **35**: 960–964.
31. Tamura M, Watanabe K, Mikami Y, Yazawa K, Nishimura K. Molecular characterization of new clinical isolates of *Candida albicans* and *C. dubliniensis* in Japan: analysis reveals a new genotype of *C. albicans* with group I intron. J Clin Microbiol 2001: **39**: 4309–4315.
32. Tintelnot K, Haase G, Seibold M et al. Evaluation of phenotypic markers for selection and identification of *Candida dubliniensis*. J Clin Microbiol 2000: **38**: 1599–1608.
33. 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**: 210–221.