Clin Oral Invest (2013) 17:957–965
DOI 10.1007/s00784-012-0757-6

ORIGINAL ARTICLE

Oral candidosis in lichen planus: the diagnostic approach is of major therapeutic importance

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Received: 23 December 2011 / Accepted: 14 May 2012 / Published online: 15 June 2012

Abstract
Objectives *Candida albicans* is the most common fungal pathogen in humans, but other *Candida* species cause candidosis. *Candida* species display significant differences in their susceptibility to antimycotic drugs. Patients with symptomatic or erythematous oral lichen planus (OLP) commonly have *Candida* infection requiring correct identification of *Candida* species in order to initiate adequate antimycotic therapy. Therefore, conventional cytosmear and culture tests were compared with genetic diagnostics on oral rinse followed by agar culture and material collected by cytobrush from OLP patient mucosal lesion.
Methods The genetic approach was validated on a reference panel of 60 well-defined unrelated fungal species. The study included 37 OLP patients. Oral candidosis (OC) was established based on clinical signs of OC and/or oral mucosal symptoms and at least one hypha in lesional cytosmear. Antimycotic treatment was initiated after OC diagnosis, and symptomatic treatment was initiated in no-candidosis situations.
Results The composition of *Candida* species in oral rinse/culture test was different from that of lesional cytobrush sampling as more non-albicans species were detected by the latter. Unexpectedly, *Candida dubliniensis* was found to be overrepresented among patients with a history of antimycotic treatment indicating unintentional iatrogen selection. Of the 22 OLP patients receiving treatment, 27 % of these should have been offered alternative therapy based on the improved diagnostic approach.
Conclusion This study highlights the importance of lesional sampling in OLP patients with suspected OC.
Clinical relevance Correct fungal identification is critical in order to initiate adequate antimycotic therapy, thus minimizing iatrogen selection of non-albicans species.

Keywords Candidosis · Oral lichen planus · Genetic testing · Antimycotic treatment · *Candida* therapeutics

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Electronic supplementary material The online version of this article (doi:10.1007/s00784-012-0757-6) contains supplementary material, which is available to authorized users.

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Introduction

Candida infection is a common disorder in the oral cavity. Oral candidosis (OC) is subdivided into primary and secondary types, the latter being superimposed on other diseases of the oral mucous membranes. One such disease is oral lichen planus (OLP), a chronic inflammatory disease affecting about 2 % of the Scandinavian population [1]. The reticular and papular elements are the most characteristic manifestations of OLP but erythematous and ulcerative manifestations are frequently seen [2]. The histopathological characteristics of OLP include hyperkeratinization [3] possibly predisposing for candida infection. Erythematous areas in OLP may be a manifestation of the disease itself or

a result of superimposed candidosis, both of which are associated with morbidity as soreness and pain [4, 5]. Additionally, recent studies have been aimed at correlating predisposing genetic factors with OLP [6].

Conventional OC diagnostics are done by microscopic analysis of cytosmears, morphological characteristics on selection agars, or PCR analysis on cultured oral samplings [7]. About 50 % of the population carries *Candida albicans* (*C. albicans*) as part of the oral bioflora [8]. Other *Candida* species such as *Candida glabrata*, *Candida krusei*, and *Candida tropicalis* are isolated from healthy individuals with increasing frequencies [9]. Furthermore, recent findings demonstrate that *Candida dubliniensis* constitutes a prevalence ranging from 1.2–16.6 % among mistakenly identified *C. albicans* patient isolates [10]. Errors in *Candida* species diagnostics may lead to resistance and emergence of non-albicans species after inadequate antimycotic treatment [11]. Studies have shown that OLP patients suffer from OC and that *C. albicans* is most frequently detected [12, 13]. Moreover, recent studies found an increased presence of non-albicans species among OLP patients [14]. These findings may be a result of the use of diagnostic tests capable of identifying a wider range of *Candida* biotypes and/or the changing face of candidemia due to differential *Candida* sensitivity to antimycotic treatment [11, 15, 16]. In this respect, nystatin, azol-containing therapeutics, e.g., fluconazole, miconazole, and ketokonazole, and amphotericin B are the most commonly prescribed drugs for treatment of OC. Alternative drugs such as chlorhexidine possess both antibacterial and antimycotic effects [17] and colostrum-containing products may have an antimycotic effect [18, 19]. *C. krusei* is natively resistant to azol-containing products and *C. glabrata* and *C. dubliniensis* have less susceptibility to this therapeutics [20]. Thus, selection of *Candida* species due to the therapeutic approach has occurred, and resistance has developed [21]. It has been argued that production of nitrosamine by *C. albicans* may be the reason that non-homogeneous leukoplakias have a higher premalignant potential than homogeneous leukoplakias [22]. This, in conjunction with the morbidity, is the reason for initiation of antimycotic treatment in secondary candida infection in OLP.

The morphological forms of *C. albicans* are believed to play a role during the pathogenicity. In general, blastospores are the form when there is no excessive growth of the fungus and hyphae are seen when the fungus invades the mucous membrane [23]. However, hyphae may be present in healthy persons without OC [8]. The morphologic phenotype and the quantity can be evaluated in a microscope. In plate culture, the number of colony forming units (cfu) are counted and arbitrary cutoff numbers for OC have been suggested, e.g., in immunocompromised hosts [24].

The scope of this study is to evaluate the value of different *Candida* diagnostic approaches, i.e., cytosmear, plate culture, and genetic analysis of clinical specimens taken from OLP patients and the influence on the therapeutic decision.

Methods

Study group

Thirty-seven patients with OLP participated in the study; 21 females and 16 males. All patients were Caucasians. The participants were recruited consecutively in October and November 2008 among the OLP patients regularly monitored at the Clinic of Oral Medicine, Copenhagen. The clinical diagnosis of OLP was established by at least two specialists in oral medicine on clinical manifestations of reticular and/or papular eruptions during the control period. Seventeen patients had the clinical diagnosis supported by histopathological features of OLP [25]; the remainder did not have a biopsy taken. Exclusion criteria included recent (within 3 months) treatment with antibiotics, antimycotic agents, glucocorticoids, or immunosuppressive agents and patients with possible contact lesions, i.e., lichenoid lesions restricted to the mucosa in contact with dental restorations [26].

The study was approved by the local ethical committee (H-A-2008-068). All participants received verbal and written information about the project, and informed consent was obtained from all.

Interview, oral examination, and medical treatment

All enrolled patients were interviewed using a structured questionnaire about health and lifestyle factors, i.e., smoking and alcohol habits, health status, current and recent medication, and daily intake of vitamins and herbal supplements. Previous systemic or topical antifungals (i.e., nystatin, miconazole, fluconazole, and amphotericin B), glucocorticoids (i.e., triamcinolone, fluocinolon acetonide), or treatment with colostrum-containing oral hygiene products (orabase and tooth paste) were recorded in order to evaluate association to candida load and species differentiation. Local predisposing factors of oral candida carriage were registered, i.e., smoking habits, use of dentures, poor oral hygiene, use of steroid aerosol inhalators, and xerostomia in relation to hyposalivation [5]. The clinical examination was performed by two clinicians (LKK, CKR) and included registration of oral hygiene, stability and retention of dentures, registration of the OLP manifestations, and specific examination of the mucous membranes for signs of oral candidosis defined as the presence of pseudomembranes and/or erythema with or without mucous membrane symptoms, specific changes of the tongue, i.e., fissures, lobulation, and atrophia of filiform papillae, and changes of the palate, i.e., erythema and mucosal hyperplasia. Other

mucosal changes were recorded as well. For *Candida* diagnostics, mucosal cytosmears, cytobrush samples, and oral rinse samples were taken.

An oral pathologist (JR) scored the quantity and evaluated the morphology of PAS-positive blastospores and hyphae in the mucosal smears. The diagnosis of OC was established on clinical signs and/or oral mucosal symptoms and at least one or few hyphae in the cytosmear. In these cases, antifungal treatment was initiated. Standard treatment was topical miconazole 2 % orabase if no risk of drug–drug interactions with the patient's daily medication, and nystatin oral suspension 100,000 IE when there is risk of drug–drug interactions with the patient's daily medication. In this study, the results of the culture were evaluated before initiating the treatment, as rosa cfu would indicate that *C. krusei* is not sensitive to azol treatment that is why nystatin was prescribed. Patients with symptoms and erythema but no candidosis (NC) were offered topical fluocinolon acetonide 0.025 % gel treatment.

Candida diagnostics

Cytosmears were taken with a sterile wooden tongue depressor from sites with suspected *Candida* infection or from the dorsum of the tongue with no signs of OC. The cytosmears were fixed on microscope slides and stained by the periodic acid Schiff (PAS) technique [8]. Quantification of blastospores and hyphae was as follows: 1, one or a few; 2, moderate number; and 3, extensive amount. Thin hyphae with atypical *C. albicans* morphology were scored 4 and were not diagnosed as OC. The cytosmears were scored initially as part of the routine diagnostic setting and after a period of 12 months again in order to evaluate the intraobserver reliability. *Cytobrush sampling* was performed using 20 strokes with a sterile cytobrush (Cytotak, sterile, MW & E, UK) on the same locations where the cytosmears was taken. The brushes were kept at −20 °C until DNA extraction. *Oral rinse sampling* was done by collecting 10 ml of sterile phosphate-buffered saline (PBS) after the patient gargled for 1 min. The oral rinse solution was collected in a 50-ml Falcon tube. A 1-ml oral rinse was centrifuged at 3,000 rpm for 10 min., and the pellet was resuspended in 100-μl sterile PBS [27]. A 1-ml oral solution was inoculated onto a BBL™CHROMagar™ Candida (Becton Dickinson and Company, USA) (designated agar plates) and spread out to form single colonies. Agar plates were incubated at 37 °C for 48 h. The number of cfu were counted, and their appearance was noted and digitally imaged (Canon EOS 10D). Agar plate colonies were collected for DNA extraction.

DNA extraction

Several DNA extraction methods were tested for yield, sensitivity, and reproducibility, including conventional phenol–chloroform extraction, QIAamp® DNA Investigator Kit (Qiagen, Germany) and NucleoSpin® Tissue kit (Macherey-Nagel, Dueren, Germany). The NucleoSpin® Tissue kit gave superior and reproducible results (data not shown) and was chosen as the DNA extraction method of choice. *Agar plate colony* DNA extraction was performed as recommended by the manufacturer, with a minor change, by the introduction of an additional 5 μl lyticas (5 U/μl) and 20 μl (20 mg/ml) RNase treatment, ensuring fungal cell wall degradation and RNA removal, respectively. In brief, up to 10 morphological identical CHROMagar plate colonies were picked with a sterile tip and suspended in 180 μl lysis buffer. In cases were multiple nonidentical colonies appeared on chrome agar plates, only morphological identical colonies were pooled. Further processing of the lysates generated was performed as recommended by the supplier. In *oral rinse* extraction, 5-ml oral rinse was centrifuged at 20,000×*g* for 20 min, and pellets were resuspended in 180 μl lysis buffer and processed as recommended by the supplier. In *cytobrush extraction*, the brush was cut off and suspended in 360-μl lysis buffer and processed by NucleoSpin® DNA extraction. DNA was recovered in 100 μl of elution buffer, and 10 μl was hereof evaluated for concentration and integrity by 0.5 % agarose gel electrophoresis. Stained agarose gel was UV light-imaged. Representative examples of the DNA quality obtained from all three extraction procedures (colony, cytobrush, oral rinse) are shown in Supplementary Fig. 1a.

Candida PCR and cloning

Polymerase chain reaction was performed on purified isolated genomic DNA. *Candida*-specific primers were selected using bioinformatic analysis of fungal conserved internal transcribed spacer (ITS) target regions (see Supplementary Fig. 2a). Region 1 is as follows: ITS1 (5′-TCC GTA GGT GAA CCT GCG GAA GG-3′) and ITS2 (5′-TCC TCC GCT TAT TGA TAT GCT TAA G-3′). Region 2 is as follows: ITS10 (5′-AGT CGA GTT GTT TGG GAA TGC AGC T-3′) and ITS12 (5′-GCG ICT TII IIT CIT TIT GCC AIC ATC C-3′). Primers were used to amplify DNA fragments corresponding to 5–8S rRNA genes (see Supplementary Fig. 2a). Reaction mixtures were set up in 25-μl final volume containing 1 ng Candida DNA; 15 ng DNA from cytobrush or 100–200 ng DNA from oral rinse; 0.25 mM dNTP; 0.5 μM primers 1x buffer (Dynabuffer, Thermo Scientific, USA), and 5 U DNA polymerase (Dynazyme, Thermo Scientific, USA). DNA polymerase was added to the reaction mix after preheating the mixture to 90 °C. PCR cycling was carried out in a thermo cycler (Perkin-Elmer TC 480) with the following parameters: region 1: 20–40 cycles of 45 s at 95 °C, 20 s at 53 °C, and 1 min at 72 °C; region 2: 30 cycles of 45 s at 95 °C, 20 s at 45 °C, and 1 min at 72 °C.

PCR amplicons were visualized by 2 % agarose gel electrophoresis. A negative no-template control and a *C. albicans* genomic DNA (0.2–1 ng) control was included in all PCR assays. Representative PCR results using genetic material isolated by the three extraction methods described previously are shown in Supplementary Fig. 1a. Candida specific amplicons of the expected sizes were excised from the gel and purified using QIAquick® Gel Extraction Kit (Qiagen, Germany). Purified amplicons were analyzed and quantified by 2 % gel electrophoresis. In cases where heterogeneous banding patterns were observed after *Candida* PCR, the gel-purified amplicons were Topo-TA cloned (Invitrogen), transformed into XL-1-blue (Stratagene) and plated onto ampicillin, tertracyclin, X-gal, and IPTG-containing LB agar plates. Plates were incubated over night at 37 °C.

DNA sequencing

A 20-ng gel purified *Candida* PCR product was sequenced directly using BigDye terminator V.3.1 sequencing chemistry (Applied Biosystems, USA) as recommended by the supplier and ITS1 or ITS12 as sequencing primers. Topo-TA cloned PCR products were sequenced directly from single white colonies picked directly from the plate. In brief, a well-sized colony was picked and resuspended in 10-μl TE, overlaid with mineral oil and boiled for 5 min in a boiling water bath. Five microliters of bacterial lysate was sequenced directly, as described above, using T7 as sequencing primer, but using 45 cycles instead of 33 cycles normally used. Generated sequencing reactions were analyzed using an Avant 3300 sequencer (Applied Biosystems, USA).

Genetic *Candida* diagnostics—assay validation on reference strains

Different regions have been suggested as optimal targets for unbiased *Candida* amplification [28–30]. Based on bioinformatic analysis of ITS1 and ITS2 sequences from known *Candida* species, a "universal" target region that would allow for unbiased amplification of *Candida* DNA from multiple species was identified. ITS1 and ITS2 primers selected were validated for efficacy using a total of 60 validated reference strains representing five of the most common candidemic subspecies (10 *C. albicans*, 10 *C. glabrata*, 10 *C. tropicalis*, 10 *C. parapsilosis*, 10 *C. krusei*, and 10 *Saccharomyces cerevisiae*) obtained from the Danish certified reference laboratory, Statens Serum Institut, Denmark. This material represented 10 validated patient isolates of each strain that were tested in our genetic assay. The strains were inoculated on BBL™CHROMagar™ Candida (BD) and incubated at 37 °C for 48 h. Colonies were collected for DNA isolation and sequencing. Representative examples of PCR on extracted reference strain DNA are shown in Supplementary Fig. 1b.

Sequencing of amplified ITS1 and IT2 regions from these reference strains showed that both target regions were suitable for un biased amplification and identification of all reference strains included in this study; representative sequence results are shown in Supplementary Fig. 2b, c. In fact, within the reference strains sequenced, subtle intra-reference strain sequence variations were observed in a few strains (Supplementary Figs. 3 and 4). Importantly, the ITS regions selected also allowed for *Saccharomyces cerevisiae* discrimination (data not shown), thus providing evidence for the usefulness of this assay in unbiased identification of *Candida* and other yeast species. Since the diagnostic value of the ITS1 and ITS2 targets analyzed was identical (Supplementary Fig. 2b, c), only the ITS1 region was used in our clinical studies.

Statistics

The SAS software package (Version 9.2) was used for statistical analyses. For not normally distributed data, the Kruskal–Wallis or the Wilcoxon rank sum test was used. Difference in proportion was evaluated by the Chi-Square or Fisher's exact test when expected numbers are less than 10. An unpaired *t* test was used to analyze the age distributions of the groups. The level for statistical difference was set at $p < 0.05$.

Results

Morphological and colorimetric *Candida* diagnostics

Thirty patients were included in this study. Sixteen patients were diagnosed with oral candidosis on the basis of the presence of hyphae in the cytosmear and clinical signs and/or symptoms, and 21 were diagnosed with no candidosis (Table 1). Two patients had hyphae in the mucosal smear but no clinical signs of OC or any oral symptoms and were included among the NC group. Both were heavy smokers and one also had full dentures.

The mean age±the standard deviation (age range) was 61.5±14.3 years (32–82 years) and 65.4±7.4 years (53–80 years) in the OC and NC group, respectively. Females accounted for 44 % of the OC group and 67 % of the NC group. There was no statistical difference in distribution of the age or gender between the OC and NC group. Eighty-one percent ($n=13$) of the patients in the OC group reported symptoms vs. 48 % ($n=10$) in the NC group (Fisher's exact test, $P=0.047$). Eighty-one percent of the patients in the OC group showed clinical signs of oral candidosis vs. 38 % ($n=8$) in the NC group (Fisher's exact test, $P=0.0178$).

Table 1 Diagnostic tree: association between diagnosis, cytosmear, symptoms, and clinical signs of oral candidosis

Hyphae	Hyphae≥1				Hyphae=0				*P* value
	Symptoms and clinical signs	Symptoms	Clinical signs	No symptoms or clinical signs	Symptoms and clinical signs	Symptoms	Clinical signs	No symptoms or clinical signs	
	Oral candidosis			No candida infection					
Number of patients	10	3	3	2	6	4	2	7	
Cfu mean (range)	860.7 (10; 2,848)	496.3 (137; 788)	289.7 (105; 640)	1,013.5 (27; 2,000[a])	17 (0; 100)	0	189.5 (1; 378)	3.7 (0; 25)	
Cfu (mean range)	685.3 (10;2,848)			120.7 (0;2,000)					$P<0.001$

[a] Patient is a heavy smoker and has full dentures

The total number of cfu was significantly higher in the OC group (Wilcoxon, $P<0.0001$) (Table 1). In eight NC patients, no morphological forms resembling any kind of candida were found in the cytosmear.

Colorimetric discrimination of candida species was done after BBL™CHROMagar™ Candida (BD) inoculation allowing identification of *C. albicans*, *C. tropicalis*, and *C. krusei*. *C. dubliniensis* has been reported to produce dark green colonies on these agar plates, with a distinct color different from the green color produced by *C. albicans.* However, CHROMagar-cultured *C. dubliniensis* is known to lose the characteristic dark green color [10], and in this study, the colonies formed by either of these species were indistinguishable from each other, and thus, it was not possible to distinguish *C. dubliniensis* from *C. albicans*. Moreover, *C. tropicalis* from one NC patient produced green cfu. *C. intermedia* and *C. glabrata* produced indistinguishable dark mauve colonies (Tables 2 and 3).

Genetic *Candida* diagnostics—*Candida* species identification and prevalence

Using our genetic diagnostic *Candida* assay, *C. albicans* was identified as the most prevalent species in our patient samples, followed by *C. dubliniensis*, *C. krusei*, *C. glabrata*, *C. tropicalis*, and *C. intermedia.* More non-albicans species and less *C. albicans* were detected by the cytobrush when compared with plate culture, but it was not statistically significant (Fisher's exact test, $P=0.12$) (Tables 2 and 3).

Among the 21 NC patients, eight had positive plate culture; however, only five had *Candida* DNA detected in oral rinse analysis and seven in cytobrush analysis. In two patients, *C. krusei* or *C. glabrata* were detected by cytobrush analysis, but not in oral rinse (see Table 2).

Among the OC patients, no candida DNA was detected in two patients in oral rinse and in three patients in cytobrush. Only one candida species was detected in oral rinse, whereas in cytobrush 38 % of the patients had more than one candida species detected. Genetic analysis on patient-derived agar plate cultures with green-colored colonies identified *C. albicans* in 75 % (18/24) of the cases and *C. dubliniesis* in 21 % (5/24).

Intraobserver reliability

Intraobserver reliability of the cytosmears was 92 % 1 year after the first scoring of the cytosmears. Inconsistency was seen in three patients. All patients had thin hyphae. At first, the cytosmears were scored as one or few thin hyphae (thinner than normal candida hyphae), but after 1 year, they were scored as one or few hyphae with additionally thin hyphae.

Table 2 Patients diagnosed with *no* candidosis: comparison of culture, oral rinse, and cytobrush diagnostics (n=21)

CHROMagar™	Culture color (cfu≥1) *n*	PCR	Culture (cfu≥1) *n*	Oral rinse	Cytobrush	Culture compared with cytobrush
Cfu color	Color→PCR		PCR	PCR	PCR	
Negative	13		13[p26]	12	14	
Green	7 (1)	*C. albicans*	6[p34]	3	3 (1)[p34]	−2
		C. dubliniensis	1	1	1	−
		C. tropicalis	(1)[p32]	1	(1)[p32]	–
Rosa		*C. krusei*	0	0	(1)[p34]	+1
Dark mauve	(1)	*C. glabrata*	(1)[p32]	0	1[p26] (1)[p32]	+1
		C. intermedia	0	0	0	
		Missing		4[a]		

Multiple species detected are enclosed in parenthesis

p patient code

[a] No PCR product

Changes in treatment after supplementary diagnostics

In total, six patients would have been offered another therapy after supplementary diagnostics (culture and DNA-diagnostics), which is 27 % of the 22 patients that should have been offered treatment (Table 4).

In the NC group, candida DNA was detected in cytobrush in four of the eight patients with clinical signs of OC. *C. albicans* was detected in two patients with both symptoms and sign of OC. One had 100 green cfu identified to be *C. albicans* but no blastospores, and one had no cfu but few blastospores. Both would have been treated with miconazole from these results and not fluocinolone acetonide, as they were offered initially. Two had clinical sign of candidosis, thin hyphae but no symptoms: *C. albicans* and *C. krusei* were detected in one and *C. dubliniensis* in the other. Both would have been treated with nystatin from these data instead of no treatment. One patient diagnosed with OC had changed therapy from miconazole to nystatin after culture revealed rosa cfu indicating *C. krusei*, which was confirmed by PCR. Another patient had *C. glabrata* and *C. albicans* detected in cytobrush but only *C. albicans* in culture. As *C. glabrata* has less sensibility to azol drugs, the treatment would have been changed from fluconazole to nystatin.

Candida species related to previous mucosal treatments

Sixty-eight percent of the patients (n=25) had received previous treatment with antifungals (n=18), glucocorticoids (n=16), and/or colostrum-containing oral hygiene products (n=13). Of these, 42 % (n=11) had received one type of treatment; 31 %, two; and 27 %, three or four different types. There was no association between the number of different treatments, and the candida species detected in the cytobrush. Fifty percent of both the patients detected with *C. krusei* (n=2) and *C. dubliniensis* (n=3) in

Table 3 Patients diagnosed *with* oral candidosis: comparison of culture, oral rinse, and cytobrush diagnostics (n=16)

CHROMagar™ cfu color	Culture color (cfu≥1) *n*	Species DNA identification	PCR on culture *n*	PCR oral rinse *n*	PCR cytobrush *n*	Culture compared with cytobrush
Negative	0		0	2	3	
Green	13 (3)	*C. albicans*	10[p20,p27,p33] (2)[p2,p19]	8	5[p2] (4)[p19,p20,p27,p33]	−3
		C. dubliniensis	3 (1)[p15]	1	3 (2)[p15,p19]	+1
Blue	(1)	*C. tropicalis*	(1)[p19]	0	(1)[p19]	−
Rosa	(1)	*C. krusei*	(1)[p15]	1	(3)[p15,p27,p33]	+2
Dark mauve	(1)	*C. glabrata*	0	0	(2)[p20,p27]	+2
		C. intermedia	(1)[p2]	0	0	−1
		Missing		4[a]		

Multiple species detected are enclosed in parenthesis

p patient code

[a] No PCR product

Table 4 Changes in treatment after supplementary diagnostics

Therapeutic approach	No candida infection			Oral candidosis		
	Treatment number		Changes from initial to final number	Treatment number		Changes from initial to final number
	Initial	Final		Initial	Final	
No treatment	17	15	−2			
Fluocinolone acetonide 0.025 %	4	2	−2			
Miconazole	0	2	+2	3	2	−1
Nystatin	0	2	+2	10	12	+2
Fluconazole	0			1	0	−1
Chlorhexidine 0.12 %	0			2	2	
Total change in therapeutic approach			4			2

cytobrush, but none of the patients detected with *C. glabrata* had received previous treatment with azol-containing products. There was no association to the different candida species detected in the cytobrush and topical glucocorticoid treatment.

Statistically significant, more patients detected with *C. dubliniensis* in the cytobrush (4/6) (83 %) had a history of treatment with colostrum-containing oral hygiene products vs. eight of 31 (26 %) of the patients without *C. dubliniensis* in the cytobrush (Fisher's exact test, $P=0.014$). Interestingly, all the patients detected with *C. dubliniensis* had received previous treatment of some kind (N.S., Fisher's exact test; $P=0.15$). Thirty-eight percent ($n=5$) of the patients with *C. albicans* detected in the cytobrush had a similar history of treatment with colostrum-containing oral hygiene products. Of these, one had a coexisting infection with *C. krusei, and* the other, with *C. dubliniensis* and *C. tropicalis*.

Discussion

Recent studies have shown an increased occurrence of non-albicans species among OC patients [11, 14]. These findings may be a result of inadequate antimycotic treatment which may change the prevalence of the different *Candida* species, emphasizing the importance of specific *Candida* diagnostic testing in order to initiate adequate therapies. In addition, these findings imply that conventional testing (cytosmear and culture) is inadequate for detailed *Candida* species identification.

The results obtained in this study support the fact that the diagnostic method used strongly influences precise diagnosis and thus has great therapeutic impact. An unexpected result of this study was the high prevalence of *C. dubliniensis* in a group of Danish OLP patients. Furthermore, the differences between the compositions of *Candida* species found in the diagnostic approaches used have therapeutic significance. Thus, 27 % of the patients were offered suboptimal therapeutic treatment and would have received more adequate treatment with this additional knowledge.

In this study, a noninvasive sampling procedure combined with DNA-based identification was found to be suboptimal for diagnosing *Candida* infection in OLP patients since the diversity of candida species found in culture and cytobrush was not reflected in oral rinse, where only one candida species could be detected. Inadequate amounts and quality of DNA obtained from oral rinse sampling could explain why several patients were found negative by this method, in spite of the fact that they were positive on CHROMAgar. Candida DNA could not be detected from oral rinse in eight patients, categorized as “missing” in Tables 2 and 3. We find this surprising since the mean cfu in the NC group was 0.5 and the range 0–1 cfu, and for the OC group the mean cfu was 97.5 and the range was 24-137. Multi candida-species infections were not detected by oral rinse in this study, although *Candida* detection from oral rinse sampling has been demonstrated by others [31].

In three patients with OC (hyphae in cytosmear), *Candida* DNA could not be detected by cytobrush sampling. Two patients presented symptoms, but no signs of OC, but presented 137 and 564 cfu in the culture, respectively. The third presented both symptoms and signs of OC, but the culture only presented 10 cfu of *C. albicans*. This might be due to the study design and sampling order since the cytosmear was taken from the lesion before cytobrush sampling whereby *Candida* could be removed from the lesion after the former procedure.

Four patients with NC and no cytosmear hyphae were found positive on *Candida* cytobrush. Two had blastospores detected in the cytosmear, and two had no detection of any morphological forms of *Candida* in the cytosmear. The cfu of these patients were 1, 2, 25, and 27, which support the clinical judgment of NC but emphasizes the fact that PCR diagnostics do not differentiate between *Candida* carriage and candidosis.

Cytosmear and cytobrush diagnostics are influenced by the clinician's selection of sampling site. In our study, OC was established on clinical signs and/or oral mucosal symptoms and at least one or few hyphae. Morphological deviation of the hyphae may cause diagnostic considerations for the pathologist and depends on the diagnostic experience with non-candida yeast and other candida species than *C. albicans*. Thin hyphae were described in samples from four NC patients but not in OC patients. Two NC patients with thin hyphae had no candida DNA detected by the cytobrush and no candida cfu. However, one of them had 17 not identified non-candida cfu. Two patients with thin hyphae had *Candida* DNA detected by cytobrush, one had *C. dubliniensis* (378 cfu), and the other had *C. albicans* (1 cfu) and *C. krusei* (0 cfu), but only the latter of the two had blastospores in the cytosmear. In three of the four patients diagnosed with thin hyphae, non-candida or non-albicans species were detected by PCR. This highlights the limit of the cytosmear diagnostics, as it is not possible to unerringly identify different fungi by morphology.

Interestingly, and statistically significant, two species were detected in cytobrush in patients with only one or few hyphae detected in cytosmear, whereas only one species was detected from lesions with moderate and extensive hyphae (Fisher's exact test, $P=0.0030$). This might indicate that favorable conditions for one species suppress the growth of other species.

The cfu was higher in the OC group, but the range was 0–2,000 in the NC group. In this study, it was not possible to make an arbitrary cutoff for candidosis. Thus, in OLP patients, it was not possible to differentiate between carriers of candida and patients with lesional candida infection. In this study, the importance of incorporating predisposing factors in the therapeutic management of OLP patients was demonstrated by the two patients with hyphae but no symptoms or clinical signs of OC. The results in this study demonstrate superior species identification when lesional cytobrush sampling is performed compared with cytosmear and culture diagnostics and oral rinse sampling.

In this study, *C. dubliniensis* was detected in 16 % (6/37) of the patients, and in the study by Al-krarawi et al. [32], *C. dubliniensis* was isolated from 11 % (4/37) of the oral rinse from OLP patients without signs of OC and from 5 % of 220 non-OLP patients without clinical signs of OC. The BBL™CHROMagar™ Candida (BD) plate culture could not differentiate *C. albicans* from *C. dubliniensis*. It has been suggested that *C. dubliniensis* causes more symptoms than *C. albicans*, is associated with infections in immune-compromised patients, e.g., HIV, and is less sensitive to azol therapeutics [20, 33]. In our study, 21 % of the green colonies were *C. dubliniensis* and would have been mistakenly identified as *C. albicans* without additional PCR diagnostic.

It is noteworthy that more *C. krusei*, *C. glabrata*, and *C. dubliniensis* were detected from the lesions than from the culture (Tables 2 and 3).

Oral administration and topical application of bovine colostrum-containing products have been reported as having the capacity to reduce the oral candida load based on culture and smears [17, 18], but there are no reports on whether *C. dubliniensis* is affected. Interestingly, a recent study found significant nutrient regulation differences between *C. albicans* and *C. dubliniensis* [34]. One patient in this study had extensive OC and was found positive for *C. krusei* and *C. dubliniensis.* This patient had a long history of recurrent OC treated with 2 % miconazole. However, to our knowledge there are no reports on miconazole resistence in *C. dubliniensis*. Replacement of *C. albicans* with *C. dubliniensis* after extensive fluconazole treatment in HIV patients has been reported, suggesting that change in the oral ecology might favor *C. dubliniensis* [15].

Generally, the results of this study highlight the importance of correlating clinical signs and symptoms with the laboratory findings. Careful consideration regarding the lesional sampling site is of great importance, in particular in oral mucous membrane diseases like OLP. Our results are in line with that of other studies showing the superior efficiency in precise subspecies identification using gene-based tests [9, 13]. Thus, inclusion of genetic detection methods in routine diagnostics, such as fluorescent in situ hybridization or oligonucleotide microarray detection will enable precise identification and quantification of the species detected. Moreover, the clinician must consider the risk of unintended selection of oral microorganisms by iatrogen changes in oral ecology by oral hygiene products and antimicrobial agents.

Taken together, this study emphasizes that current antimycotic treatment regimens offered to patients may changes the face of candidemia. To our knowledge, this is the first report of high prevalence of *C. dubliniensis* colonization among OLP patients with a history of antimycotic treatment. The results demonstrate the importance in the ability to identify *Candida* species in order to engage adequate antimycotic treatment regimens to prevent therapeutic failure and development of antimycotic drug resistance.

Acknowledgments The authors wish to thank statistician Claus Jensen, UNI C Statistics & Analysis for statistical assistance, Maiken Cavling Arendrup, DMS, PhD, SSI for access to all *Candida* reference strains included in this study, and Camilla Andersen for technical assistance. This study was supported by funds from the Danish Agency for Science Technology and Innovation and Dansk Tandlægeforening.

Conflict of interest The authors declare that they have no conflict of interest.

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