Evaluation of CHROM-Pal medium for the isolation and direct identification of *Candida dubliniensis* in primary cultures from the oral cavity

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Candida albicans is the species most frequently isolated from oral specimens, but the recovery of other *Candida* species such as *Candida dubliniensis* is increasing. Differentiation of *C. dubliniensis* from *C. albicans* requires special tests and both species are misidentified in some studies. CHROM-Pal (CH-P) is a novel chromogenic medium used in our laboratory for differentiation between *C. albicans* and *C. dubliniensis* on the basis of colony colour and morphology, and chlamydospore production. The performance of CH-P and CHROMagar *Candida* (CAC) was compared for primary isolation and presumptive identification of yeasts from oral specimens from human immunodeficiency virus (HIV)-infected and uninfected individuals. The identification of *Candida* species on both media was compared with two reference identification methods (API ID 32 C and multiplex PCR). A total of 137/205 oral swabs (66.8%) plated onto CH-P and CAC media were positive by culture and resulted in the growth of 171 isolates of *Candida* species on CH-P, whilst only 159 isolates grew on CAC. *C. albicans* was the most frequently isolated species in both groups of patients, followed by *Candida parapsilosis* in the HIV-negative group, and by *C. dubliniensis* in the HIV-infected group. The other *Candida* species isolated were *Candida guilliermondii*, *Candida glabrata*, *Candida krusei*, *Candida tropicalis*, *Candida famata*, *Candida rugosa*, *Candida kefyr*, *Candida pelliculosa* and *Candida pulcherrima*. The sensitivity and specificity for identifying *C. albicans*, *C. krusei*, *C. tropicalis* and *C. dubliniensis* on CH-P were over 98.5%, always equal to or higher than those obtained when CAC was used. CH-P is a simple reliable medium for primary isolation and presumptive identification of yeast isolates from oral samples. The ability of CH-P to discriminate between *C. dubliniensis* and *C. albicans* was significantly higher ($P < 0.05$) than that of CAC.

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INTRODUCTION

Members of the genus *Candida* are responsible for a variety of infections in humans ranging from superficial to deep-seated candidiasis. The most prevalent presentation is oral candidiasis, which includes erythematous candidiasis, pseudomembranous candidiasis, angular cheilitis, denture stomatitis, chronic mucocutaneous

candidiasis and leukoplakia (Delgado & Aguirre, 1997; Samaranayake & MacFarlane, 1990; Sánchez-Vargas *et al.*, 2005). Most patients with oral candidiasis have some degree of immunosuppression caused by human immunodeficiency virus (HIV) infection, chemotherapy for the treatment of tumours or immunosuppressors used for organ transplantation. Other factors that enhance oral candidiasis are xerostomy and a complex array of virulence factors in the fungus including adhesins that mediate the adhesion of *Candida* to the epithelial cells

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and dentures, filamentation, enzyme production, phenotypic switching and thigmotropism (Aguirre, 2002; Quindós & Pontón, 1996).

Candida albicans is the most pathogenic species of the genus and the most frequent cause of oral candidiasis (Coleman *et al.*, 1997). Recent studies have demonstrated that the incidence of infections caused by other species of the genus such as *Candida glabrata*, *Candida parapsilosis*, *Candida krusei*, *Candida tropicalis* and *Candida dubliniensis* is increasing (Coleman *et al.*, 1997; Hazen, 1995; Kirkpatrick *et al.*, 1998; Pfaller *et al.*, 1996).

The first step in laboratory diagnosis of oral candidiasis is usually the culture of the oral specimen. In recent years, a number of chromogenic media including CHROMagar Candida (CAC; CHROMagar), chromID Candida (bioMérieux), Albicans ID2 (bioMérieux), Candida ID2 (bioMérieux) and CandiSelect 4 (Bio-Rad) have been marketed. These media allow the isolation and presumptive identification of most clinically relevant yeasts based on the colour of the colonies (Beighton *et al.*, 1995; Eraso *et al.*, 2006; Horvath *et al.*, 2003; Hospenthal *et al.*, 2006; Ilkit *et al.*, 2007; Kirkpatrick *et al.*, 1998; Perry & Freydière, 2007; Pfaller *et al.*, 1996; San Millan *et al.*, 1996; Sendid *et al.*, 2007). CHROM-Pal medium (CH-P) is a modification of CHROMagar medium that is supplemented with Pal's agar. This medium has been developed in our laboratory to differentiate *C. albicans* from *C. dubliniensis* based on colony morphology and colour, as well as the production of chlamydospores (Sahand *et al.*, 2005). In this study, we investigated, for the first time, the usefulness of CH-P for the primary isolation and presumptive identification of yeasts from oral specimens.

METHODS

Patients. Oral swabs were processed from 151 HIV-negative patients attending the outpatient clinic of the Department of Dentistry at the University of Basque Country or a private dental clinic, as well as oral swabs from 54 HIV-positive patients attending the Department of Infectious Diseases in Cruces Hospital, Baracaldo, Spain.

Male and female patients of different age groups, ranging between 10 and 81 years (mean 47.8 years), were included in the study. Thirty-six patients wore removable dentures. The experimental protocols were approved by the Institutional Review Board of the School of Medicine and Odontology at the University of the Basque Country, Leioa, Spain, and subjects gave their informed consent.

Clinical specimens and identification of isolates. Samples were taken from oral lesions and from buccal mucosa, tongue and dentures in the absence of oral lesions by means of sterile swabs. Swabs were replaced into sterile transportation medium and taken to the mycology laboratory for identification. Each swab was first plated on CAC and then on CH-P. The plates were incubated at 30 °C (CH-P) or 37 °C (CAC).

CAC was prepared according to the manufacturer's instructions. CH-P was prepared as described by Sahand *et al.* (2005) by mixing equal volumes of prepared CAC and Pal's agar (50 g unsalted powdered sunflower seeds were added to 1 litre of distilled water, boiled for

30 min and filtered through cheesecloth; the medium was supplemented with 1 g creatinine, 1 g glucose and 1 g KH₂PO₄; the pH was adjusted to 5.5 and, after the addition of 15 g agar l⁻¹, the medium was autoclaved at 110 °C for 20 min). After stirring, the mixture was poured into 90 mm diameter Petri dishes. Plates were inoculated and observed daily for up to 7 days for growth, colony morphology and pigmentation. For CH-P, plates were additionally subjected to microscopical examination to detect chlamydospores to differentiate *C. dubliniensis* from *C. albicans*.

Definitive identification of the yeast strains isolated was carried out by means of their carbohydrate assimilation patterns on API ID 32 C strips (bioMérieux). Multiplex PCR was used for further confirmation of the identification of *C. albicans* and *C. dubliniensis* simultaneously using two sets of species-specific primer pairs. One pair was specific for *C. albicans*, CALB1F and CALB2R (Genotek-Bonsai; CALB1F: 5'-TTTATCAACTTGTCACACCAGA-3'; CALB2R: 5'-ATCCCGCCTTACCACTACCG-3'), and was designed from the internal transcribed spacer regions ITS1 and ITS2 of the rRNA gene, amplifying a band of 273 bp (Luo & Mitchell, 2002). The other pair was specific for *C. dubliniensis*, CDBF28F and CDBR110R (Genotek-Bonsai; CDBF28F: 5'-AAATGGGTTTGGTGCCAAATTA-3'; CDBR110R: 5'-GTTGGCATTGGCAATAGCTCTA-3'), and amplified a region of 809 bp of the topoisomerase II gene (Kanbe *et al.*, 2005).

Statistical analysis. In order to establish the diagnostic value of CAC and CH-P for identification of *Candida* species, the sensitivity and specificity were calculated. The significance of differences between both media in discriminating between *C. albicans* and *C. dubliniensis* was assessed by the χ^2 method of McNemar. A value of $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

A total of 62 (41.1 %) out of 151 samples from non-HIV-infected patients showed no growth on the two chromogenic media, whilst the remaining 89 positive specimens (58.9 %) resulted in growth of 108 isolates of *Candida* species. One hundred and six isolates grew to the same extent on the two media, but two of the isolates were detected only on CH-P. Seventy-three swabs (82.0 %) resulted in pure culture, whilst 16 specimens (18 %) gave rise to mixed cultures. In the majority of the mixed cultures, two different species were isolated, but three different species were isolated from three samples. As shown in Table 1, *C. albicans* was the most frequently isolated species (60.2 %), followed by *C. parapsilosis* (10.2 %), *Candida guilliermondii* (9.3 %), *C. glabrata* (6.5 %), *C. krusei* (3.7 %), *C. dubliniensis* and *C. tropicalis* (both 2.8 %) and one isolate each for *Candida famata*, *Candida kefyr*, *Candida pelliculosa*, *Candida pulcherrima* and *Candida rugosa* (0.9 %). One isolate of *C. parapsilosis* and another one of *C. guilliermondii* failed to grow on CAC, although they grew well on CH-P (Table 2).

Six of the 54 (11.1 %) oral specimens taken from HIV-infected patients showed no growth on either chromogenic medium. The 48 positive specimens (88.9 %) resulted in the growth of 63 isolates of *Candida* species. Twelve oral swabs (25 %) gave rise to mixed cultures of two different species, and in one specimen four different species were

Table 1. Distribution of *Candida* species isolated from oral swabs of HIV-infected and uninfected individuals

Yeast species	Uninfected patients		HIV-infected patients	
	No. of isolates	Percentage of isolates	No. of isolates	Percentage of isolates
<i>C. albicans</i>	65	60.19	33	52.38
<i>C. parapsilosis</i>	11	10.19	7	11.11
<i>C. guilliermondii</i>	10	9.26	2	3.17
<i>C. glabrata</i>	7	6.48	4	6.35
<i>C. krusei</i>	4	3.70	1	1.59
<i>C. dubliniensis</i>	3	2.78	10	15.87
<i>C. tropicalis</i>	3	2.78	4	6.35
<i>C. famata</i>	1	0.93	2	3.17
<i>C. kefyr</i>	1	0.93	–	–
<i>C. pelliculosa</i>	1	0.93	–	–
<i>C. pulcherrima</i>	1	0.93	–	–
<i>C. rugosa</i>	1	0.93	–	–
Total	108	100	63	100

isolated. Fifty-three isolates showed growth to the same extent on the two media, whilst ten isolates were detected only on CH-P medium (four *C. parapsilosis*, three *C. albicans*, one *C. dubliniensis*, one *C. tropicalis* and one *C. famata*; Table 2). As shown in Table 1, *C. albicans* was the most frequently isolated species (52.4 %) in oral samples of HIV-infected patients, followed by *C. dubliniensis* (15.9 %), *C. parapsilosis* (11.1 %), *C. glabrata* and *C. tropicalis* (both 6.35 %), *C. famata* and *C. guilliermondii* (both 3.2 %) and *C. krusei* (1.6 %).

The prevalence of *C. dubliniensis* in our study was 1.99 % for the HIV-negative individuals, but rose to 18.5 % in HIV-infected individuals. These data are in agreement with previously published studies (Faggi *et al.*, 2005; Kirkpatrick *et al.*, 1998; Milan *et al.*, 2001; Tintelnot *et al.*, 2000).

Finding an easy, low-cost and efficient method for identification of yeast isolates is very important in any mycological laboratory. Although identification by means of molecular methods such as PCR is more reliable, they are not affordable in many laboratories. In the present work, final identification of *Candida* isolates was accomplished by ID 32 C and multiplex PCR as a reference to compare the performance of the two chromogenic media under study.

CAC is one of the most widely used media in the mycology laboratory. Colony characteristics presented in Table 2 for identification of *C. albicans*, *C. tropicalis* and *C. krusei* using CAC were in agreement with previously published reports (Beighton *et al.*, 1995; Hospenthal *et al.*, 2006; Pfaller *et al.*, 1996). The sensitivity and specificity of CAC for identifying *C. krusei* and *C. tropicalis* were over 99 %. There is some debate about the ability of CAC to identify *C. glabrata*, as some studies claim that it can be identified by the pink/purple/violet colour of the colonies (Horvath

et al., 2003; Pfaller *et al.*, 1996). However, other *Candida* species including *C. parapsilosis*, *C. guilliermondii*, *C. famata*, *C. kefyr* and *C. pelliculosa* also produce pink/purple/violet colonies on CAC (Beighton *et al.*, 1995; Eraso *et al.*, 2006; San Millan *et al.*, 1996). The data presented in Table 2 confirm the difficulty of identifying *C. glabrata* on CAC. In addition, nearly half of the *C. albicans* isolates grew as light-green colonies on CAC, whilst the remaining isolates grew as green and dark-green colonies. The latter is supposed to be the colour of *C. dubliniensis* colonies on CAC (Fig. 1, Table 2). Our findings are in accordance with previous reports that observed that *C. dubliniensis* shows different tones of green colour that are difficult to differentiate from the green colour produced by *C. albicans* (Eraso *et al.*, 2006; Tintelnot *et al.*, 2000). The diagnostic values of CAC for differentiating *C. dubliniensis* from *C. albicans* in oral swabs of HIV-infected and HIV-negative individuals were low (Table 3).

The discrimination between *C. albicans* and *C. dubliniensis* on CH-P was easier as the majority of *C. albicans* (94.9 %) grew as light-green, smooth colonies (Fig. 1 and Table 2). Only five isolates of *C. albicans* (5.1 %) produced bluish-green colonies, but the morphology of the colonies was smooth and they did not produce chlamydo-spores, making the presumptive identification very simple. In contrast, 100 % of *C. dubliniensis* isolates produced rough, bluish-green colonies on CH-P and produced chlamydo-spores, as described previously by Sahand *et al.* (2005). The presumptive identification of *C. krusei* and *C. tropicalis* on CH-P was similar to that on CAC, as *C. krusei* grew as pink fuzzy colonies and *C. tropicalis* grew as blue colonies, so the diagnostic value of CH-P for identifying *C. krusei* and *C. tropicalis* was very close to that of CAC (over 98 %).

The majority of *C. glabrata* isolates (10/11) grew on CH-P as smooth, white colonies and this appearance was not shared by any other *Candida* species in the present work. Although this is an interesting finding, further studies with more *C. glabrata* strains and other *Candida* species would be needed to validate this observation. For the rest of the *Candida* species, no specific characteristics were detected on CH-P.

The identification of *Candida* species growing on CH-P was based on the colour and morphology of the colonies and, in the case of discrimination between *C. albicans* and *C. dubliniensis*, on chlamydo-spore production of the isolates. The presence of at least two of these characteristics on CH-P guided the presumptive identification of the clinically most relevant *Candida* species to a similar extent as on CAC, but CH-P improved the ability to discriminate between *C. albicans* and *C. dubliniensis* (differences between CAC and CH-P were statistically significant, $P < 0.05$).

In addition to these findings, CH-P was superior to CAC for the primary isolation of *Candida* species. Twelve isolates (*C. albicans*, *C. dubliniensis*, *C. famata*, *C. guilliermondii*, *C. parapsilosis* and *C. tropicalis*) that grew on CH-P failed to grow on CAC, although swabs were streaked first on CAC and then on CH-P. The two media

Table 2. Comparison of the performance of the two chromogenic media with *Candida* species isolated from oral swabs of HIV-infected and uninfected individuals

Yeast species	Total no. of isolates	CAC		CH-P	
		No. of isolates	Colony colour	No. of isolates	Colony colour, morphology and chlamydospore production
<i>C. albicans</i>	98	46	Light-green	93	Smooth, light-green colonies and no chlamydospores
		37	Green		
		12	Dark-green	5	Smooth, bluish-green colonies and no chlamydospores
		3*			
<i>C. parapsilosis</i>	18	5	Purple	9	Rough, purple colonies
		4	Violet	5	Smooth, purple colonies
		3	Beige	3	Smooth, beige colonies
		1	Pink	1	Rough, light-violet colonies
		5*			
<i>C. dubliniensis</i>	13	7	Dark-green	13	Rough, bluish-green colonies and chlamydospore production
		3	Light-green		
		2	Green		
		1*			
<i>C. guilliermondii</i>	12	5	Purple	9	Smooth, pink colonies
		4	Violet	3	Smooth, light-violet colonies
		2	Pink		
		1*			
<i>C. glabrata</i>	11	9	Violet	10	Smooth, white colonies
		1	Purple	1	Smooth, light-violet colonies
		1	Beige		
<i>C. krusei</i>	5	5	Pink†	5	Fuzzy, pink colonies
<i>C. tropicalis</i>	7	6	Blue	3	Rough, blue colonies
		1*		4	Smooth, blue colonies
<i>C. famata</i>	3	1	Violet	2	Rough, beige colonies
		1	Beige	1	Light-violet, rough colonies
		1*			
<i>C. rugosa</i>	1	1	Pink/violet	1	Smooth, light-violet colonies
<i>C. kefyr</i>	1	1	Pink	1	Smooth, pink colonies
<i>C. pelliculosa</i>	1	1	Violet	1	Rough, pink colonies
<i>C. pulcherrima</i>	1	1	Purple	1	Smooth, beige colonies
Total	171	171		171	

*No growth.

†Pink and fuzzy.

Fig. 1. Colony colour and morphology of yeasts isolated on CH-P. Cal, *C. albicans*; Cdu, *C. dubliniensis*; Cgl, *C. glabrata*; Cgu, *C. guilliermondii*; Ckr, *C. krusei*; Cpa, *C. parapsilosis*; Ctr, *C. tropicalis*.

Table 3. Diagnostic values of growth on CAC and CH-P for discrimination between *C. dubliniensis* and *C. albicans*, isolated from oral swabs of HIV-infected and HIV-negative individuals

CI, Confidence interval.

Statistic	CAC		CH-P	
	Value	95 % CI	Value	95 % CI
Sensitivity	0.58	0.30–0.86	1	1–1
Specificity	0.87	0.81–0.94	1	1–1
Positive predictive value	0.37	0.15–0.59	1	1–1
Negative predictive value	0.94	0.89–0.99	1	1–1
Accuracy	0.841		1.037	

are basically the same – CHROMagar Candida – but CH-P is supplemented with a sunflower seed extract, glucose and creatinine, which may help some strains to grow. Incubation temperature could be another influencing factor, but species that failed to grow usually develop adequately at 37 °C.

In conclusion, we recommend the use of CH-P as a primary isolating medium in mycology laboratories because it is easy to prepare, is less expensive than CAC, yields more isolates, allows simple interpretation of colony characteristics and is highly efficient for the presumptive discrimination between *C. albicans* and *C. dubliniensis* (100 % accuracy) and the identification of some other medically important *Candida* species. CH-P facilitates the study of oral yeast microbiota, providing information that may be useful for the treatment of oral candidiasis.

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