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Identification of *Candida dubliniensis* among oral yeast isolates from an Italian population of human immunodeficiency virus-infected (HIV+) subjects

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Candida dubliniensis, an emerging oral pathogen, phenotypically resembles *Candida albicans* so closely that it is easily misidentified as such. The aim of the present study was to evaluate the usefulness of two phenotypic methods, growth at 45°C and 2,3,5-triphenyltetrazolium chloride (TTC) reduction, for confirming presumptive identification of *C. dubliniensis* and *C. albicans* by colony color on CHROMagar *Candida* (CAC) medium. A combination of these methods was used to establish the prevalence of oral *C. dubliniensis* in an Italian population of 45 human immunodeficiency virus (HIV)-infected subjects. Twenty-two samples (48.9%) were positive for yeasts on CAC medium producing a total of 37 fungal isolates. The colony color and 45°C growth ability test correctly identified all *C. dubliniensis* and *C. albicans* isolates (5/37, 13.5%, and 16/37, 43.2%, respectively), while assessment of TTC reduction misidentified one *C. albicans* isolate. The isolation rate of *C. dubliniensis* was 11.1% (5/45 patients). All of the *C. dubliniensis* isolates were highly susceptible to fluconazole (MIC = 0.5 μg/ml). The combination of CAC medium screening with growth at 45°C and TTC reduction tests may represent a simple, reliable and inexpensive identification protocol for *C. dubliniensis*.

Key words: *Candida dubliniensis*; HIV; identification; Italy; oral cavity

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Candida dubliniensis is a recently described species considered to be an emerging opportunistic pathogen (17). This yeast has mainly been associated with oral colonization and infection in human immunodeficiency virus (HIV)-positive individuals, and a number of isolates have been found to be resistant to fluconazole (14, 19). The majority of *C. dubliniensis* isolates have been identified in Western Europe and North America (1, 4, 19) and, to date, a single case of isolation of *C. dubliniensis* has been reported in Italy (3).

C. dubliniensis is phenotypically so similar to *Candida albicans* that identification of the former has proved problematic in clinical samples (11). However, the recent description of phenotypic tests, including the observation of differently colored primary colonies on CHROMagar *Candida* medium (16), detection of inability to grow at 45°C (13), and assessment of triphenyltetrazolium chloride (TTC) reduction (21), may facilitate the identification of this species in oral samples. Accurate *C. dubliniensis* identification can be achieved by the study of carbohydrate assimilation profiles with commercially available kits, such as the API 20C AUX system (bioMérieux, Marcy l'Etoile, France) (12). Furthermore, the use of

C. dubliniensis-specific PCR primers allows rapid genetic identification (5, 6). Unfortunately, commercial kits are quite expensive and genetic identification requires specialized equipment which is not available in many clinical laboratories.

The aim of the present study was to evaluate the usefulness of two phenotypic methods, growth at 45°C and TTC reduction, for confirming presumptive identification by colony color on CAC medium. A combination of these methods was used to establish the prevalence of oral *C. dubliniensis* in an Italian population of HIV-infected subjects. Results were compared to those obtained using the API 20C AUX system for accuracy and cost-effectiveness. Phenotypic identification was confirmed by PCR amplification. The fluconazole susceptibility of *C. dubliniensis* isolates was also assessed.

Materials and methods

Clinical samples

Forty-five oral swabs from 45 unrelated HIV+ subjects managed at the Guadagna Hospital (Palermo, Italy) were evaluated for the presence of yeasts. Oral samples were obtained from the mid-dorsum of the tongue with a sterile swab regardless of clinical symptoms. Of the patients sampled, 12 (26.7%) were female and 33 (73.3%) were male, with an age range of 24–63 years. All of the subjects granted informed consent.

Reference strains

C. albicans ATCC 90029 (American Type Culture Collection, Rockville, MD) and *C. dubliniensis* NCPF 3949 (National Collection of Pathogenic Fungi, Bristol, UK) type strains were used as quality controls in all identification tests.

Culture and CHROMagar *Candida* presumptive identification

Sample swabs were used to inoculate the chromogenic isolation medium CHROMagar *Candida* (Becton Dickinson and Company, Sparks, MD). Plates were incubated at 37°C for 48 h. The yeasts were presumptively identified by both colony color evaluation and tests for detection of the formation of germ tubes at 37°C in calf serum (10, 22). Germ tube-positive isolates producing dark-green colonies were considered suspicious for *C. dubliniensis*, while light-green colonies were presumptively identified as *C. albicans* (16).

Growth at 45°C

Ability to grow at 45°C was assessed for all germ tube-positive isolates by removing a small portion of a single colony from the CAC plate and streaking it over the surface of two separate Sabouraud dextrose agar plates (Becton Dickinson and Company), which were incubated at 37 and at 45°C, respectively, for 48 h. Colony formation on the last three quadrants of the plate was scored as good growth, while growth on the first quadrant only was considered poor growth (2).

Triphenyltetrazolium chloride (TTC) reduction

Tetrazolium salt (TTC) agar medium containing 100 μg/ml of 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich, Milan, Italy) was used to assess the ability of all germ tube-positive isolates to reduce tetrazolium salt (21). A heavy inoculum from a 24-h-old culture was streaked over the surface of a TTC agar plate and incubated at 37°C for 24 h.

API 20C AUX system

Substrate assimilation with the API 20C AUX system was used to confirm presumptive identification of all the isolates. The inoculum suspension was prepared according to the instructions of the manufacturer. The API trays were inoculated and incubated for 72 h at 30°C. Cupules showing turbidity significantly greater than that of the negative control cupule were considered positive.

Fluconazole susceptibility testing

The fluconazole susceptibility of all *C. dubliniensis* isolates was evaluated using a broth microdilution susceptibility test carried out by the method outlined in the National Committee for Clinical Laboratory Standards (NCCLS) document M-27 A (9). The medium consisted of RPMI (Sigma–Aldrich) 1640 medium supplemented with 2% glucose, buffered with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma-Aldrich) and adjusted to pH 7. Doubling dilutions of the stock fluconazole solution (Pfizer Central Research, Sandwich, UK) were prepared in RPMI−2% glucose medium, dispensed into 96-well microtiter plates (Biospa, Milan, Italy), and inoculated with the test organism as described in the NCCLS protocol (9).

Preparation of template DNA from yeast isolates

DNA was extracted from yeast isolates using a previously described method (7) with some modification. Briefly, yeast cells were cultured in YEPD broth (1% yeast extract, 2% peptone and 2% dextrose) and incubated overnight at 37°C under shaking conditions. The yeast cells were collected by centrifugation (1.5 ml of the broth culture at 10 000 ***g*** for 5 min), suspended in 1 ml of 1 M sorbitol, centrifuged again, and resuspended in 1 ml of 1 M sorbitol−50 mM phosphate buffer (pH 7.5) containing 2% β-mercaptoethanol and 4 mg of lyticase enzyme (Sigma-Aldrich). After 1 h of incubation at 37°C and microscopic visualization of the spheroplasts, the cells were centrifuged at 8000 ***g*** for 5 min, suspended in 0.5 ml of 50 mM EDTA (pH 8.0)−0.2% sodium dodecyl sulfate, and incubated at 70°C for 30 min. After the addition of 5 M potassium acetate (50 μl; pH 5.4), the suspension was left at 0°C for 30 min and then centrifuged at 10 000 ***g*** for 20 min. The supernatant was treated with RNase (10 μg/ml for 30 min at 37°C), and then an equal volume of ice-cold phenol–chloroform–isoamyl alcohol (25 : 24 : 1) was added for DNA extraction. The tube was mixed by inversion and centrifuged at 10 000 ***g*** for 2 min. The aqueous layer was transferred to a fresh tube, and the extraction procedure was repeated twice. DNA was then precipitated with cold ethanol at −20°C for 30 min and centrifuged at 19 800 ***g*** for 20 min. The pellet was air-dried, then suspended in 100 μl of sterile distilled water and stored at −20°C. DNA concentrations were estimated by spectrophotometric analysis of absorbances at 260 and 280 nm. Template DNA was diluted to a working concentration of 10 ng/μl.

PCR amplification

Two forward species-specific primers for *C. albicans* (CAL5) and *C. dubliniensis* (CDU2) were used in separate PCR amplifications with a common reverse primer (NL4CAL). Amplification pro-

duced a DNA fragment of 175 bp when chromosomal DNA from one of the target species was present (6). Oligonucleotide primers were added, each at a concentration of 0.5 mM, to the PCR mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.0 mM $MgCl_2$, 0.2 mM of dATP, dCTP, dGTP and dTTP, and 5.0 U of Taq DNA polymerase (AmpliTaq Gold; Applied Biosystems, Foster City, CA) per 100 µl. The mixture was divided into 24-µl aliquots, and 1.0 µl of DNA was added (10 ng/µl). PCRs were performed in a Perkin-Elmer GeneAmp PCR System 2400. Tubes were incubated at 94°C for 4 min, and amplifications were performed for 35 cycles, with denaturation at 94°C for 20 s, annealing at 67°C for 1 min, and extension at 72°C for 20 s, followed by 72°C for 4 min. DNA fragments produced by PCR were visualized on 3% ethidium bromide-stained NuSieve 3:1 agarose (BioWhittaker Molecular Applications, Rockland, ME) gels run at 4 V/cm for 3 h in TBE buffer (89 mM Trizma base, 89 mM boric acid and 2.5 mM EDTA-Na_2, pH 8.2). A 20-bp molecular ruler (Bio-Rad Laboratories, Hercules, CA) was used to calculate amplified band sizes.

Results

Phenotypic characterization

Of the 45 swabs, 22 (48.9%) were positive for yeasts on CAC isolation medium. The results of phenotypic identification assays are shown in Table 1. A total of 16 light-green colonies, considered typical for *C. albicans*, and five dark-green colonies, presumptively identified as *C. dubliniensis*, were recovered from the 22 yeast-positive samples. Only two samples grew neither dark- nor light-green colonies and one sample grew both. Three of the five dark-green-colony-positive samples also grew non-green colonies, as did eight of the 16 light-green-colony-positive samples.

All green-colony isolates were confirmed to be germ tube positive and were submitted to tests for detection of inability to grow at 45°C and assessment of TTC reduction. Four of the 21 green-colony isolates were not able to grow at 45°C and one exhibited highly restricted growth. The same five isolates also produced red to dark-pink colonies on TTC agar, as did the *C. dubliniensis*

Table 1. Identification characters of 37 oral yeast isolates from 22 HIV+ subjects

Patient	Isolate	Colony color on CHROMagar *Candida*	GTT	Growth at 45°C	Colony color on TTC agar	API 20C AUX biocode (48 h incubation)	API 20C AUX identification	PCR identification
1	a	light green	+	good	pale pink	2566174	*C. albicans*	*C. albicans*
	b	pink (rough with spreading)	–	ND	ND	6000004[a]	*C. krusei*	ND
5	a	dark pink	–	ND	ND	2000040	*C. glabrata*	ND
7	a	light green	+	good	pale pink	2572174	*C. albicans*	*C. albicans*
9	a	dark pink	–	ND	ND	2000040	*C. glabrata*	ND
	b	dark green	+	no	red	6172134	*C. dubliniensis*	*C. dubliniensis*
10	a	light green	+	good	pale pink	2546174	*C. albicans*	*C. albicans*
11	a	light green	+	good	pale pink	2546174	*C. albicans*	*C. albicans*
	b	pink (rough with spreading)	–	ND	ND	6000104[a,b]	*C. krusei*	ND
	c	dirty pink	–	ND	ND	2040036	*S. cerevisiae*	ND
19	a	light green	+	good	pale pink	2576174	*C. albicans*	*C. albicans*
20	a	dark green	+	no	red	6172134	*C. dubliniensis*	*C. dubliniensis*
	b	pink (rough with spreading)	–	ND	ND	6000104[a,b]	*C. krusei*	ND
	c	dirty pink	–	ND	ND	2040032	*S. cerevisiae*	ND
21	a	light green	+	good	pale pink	2576174	*C. albicans*	*C. albicans*
	b	dark pink	–	ND	ND	2000040	*C. glabrata*	ND
22	a	dark blue with halo	–	ND	ND	2556175	*C. tropicalis*	ND
	b	dark pink	–	ND	ND	2000040	*C. glabrata*	ND
23	a	dark green	+	poor	dark pink	6172134	*C. dubliniensis*	*C. dubliniensis*
24	a	light green	+	good	pale pink	2546174	*C. albicans*	*C. albicans*
30	a	light green	+	good	pale pink	2576174	*C. albicans*	*C. albicans*
31	a	light green	+	good	pale pink	2576174	*C. albicans*	*C. albicans*
34	a	light green	+	good	white	2576174	*C. albicans*	*C. albicans*
	b	pink	–	ND	ND	2000040	*C. glabrata*	ND
35	a	pink (rough with spreading)	–	ND	ND	6000104[a,b]	*C. krusei*	ND
	b	light green	+	good	pale pink	2566174	*C. albicans*	*C. albicans*
	c	dark green	+	no	dark pink	2172134	*C. dubliniensis*	*C. dubliniensis*
36	a	light green	+	good	pale pink	2576174	*C. albicans*	*C. albicans*
46	a	light green	+	good	dark pink	2566174	*C. albicans*	*C. albicans*
	b	pink (rough with spreading)	–	ND	ND	6000104 [a,b]	*C. krusei*	ND
	c	white	–	ND	ND	6040032	*S. cerevisiae*	ND
47	a	light green	+	good	pale pink	2576174	*C. albicans*	*C. albicans*
48	a	light green	+	good	pale pink	2576174	*C. albicans*	*C. albicans*
	b	purple	–	ND	ND	2040032	*S. cerevisiae*	ND
49	a	dark green	+	no	dark pink	2172134	*C. dubliniensis*	*C. dubliniensis*
50	a	light green	+	good	pale pink	2566174	*C. albicans*	*C. albicans*
	b	pink	–	ND	ND	2000040	*C. glabrata*	ND

GTT: germ tube test; ND: not determined.
[a]Supplementary test: positive glucose fermentation. [b]Supplementary test: absence of growth with actidione.

reference strain. All but one of the remaining 16 green-colony isolates, as well as the reference *C. albicans* strain, produced pale-pink to whitish colonies.

The API 20C AUX system confirmed the presumptive identification as *C. dubliniensis* of the five dark-green colonies and the TTC-reducing isolates that exhibited no or poor growth at 45°C. The remaining 16 green-colony isolates were identified as *C. albicans*. The API 20C AUX system was also used to confirm presumptive identification of the non-green-colony isolates.

Genotypic characterization

Sixteen of 21 green-colony DNA extracts, corresponding to the isolates identified as *C. albicans*, were amplified only in the reaction mixture containing the *C. albicans*-specific PCR primer. The five template DNAs of the isolates identified as *C. dubliniensis* were amplified only in the *C. dubliniensis*-specific reaction mixture (Fig. 1).

Cost-effectiveness of the approach

Cost comparison results were based on the commercial cost of materials in Italy (Table 2), but the cost of the labor associated with testing was not included. Three identification protocols were compared: CAC screening + API 20C AUX system identification, CAC screening + growth at 45°C and TTC reduction, and CAC screening + PCR identification. The three protocols were equally able to identify correctly all of the *C. albicans* and *C. dubliniensis* isolates. An overall reduction of about 60% in the cost of *C. dubliniensis* identification was achieved by the second protocol compared to API 20C AUX. An intermediate cost per identification was calculated for the PCR protocol but availability of molecular biology equipment must be considered (approximately 8000 € is required for basic equipment including: thermal cycler, power supply, electrophoretic chamber and UV transilluminator).

Table 2. Cost per identification with two proposed *C. dubliniensis* identification protocols

Identification protocol	Cost per identification
CHROMagar *Candida* screening + API 20C AUX	7.66 €
CHROMagar *Candida* screening + growth at 45°C and TTC reduction	3.06 €
CHROMagar *Candida* screening + PCR	5.01[1] €

[1]PCR identification requires basic molecular biology equipment (including: thermal cycler, power supply, electrophoretic chamber and UV transilluminator) approximately worth 8000 €.

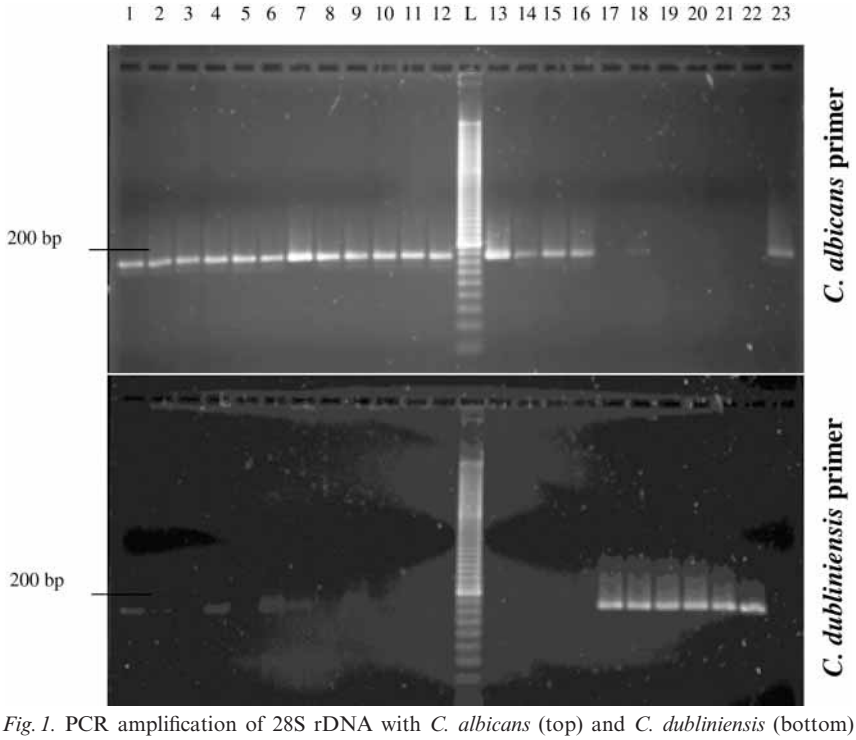

Fig. 1. PCR amplification of 28S rDNA with *C. albicans* (top) and *C. dubliniensis* (bottom) specific primers. L: 20-bp molecular ruler (Bio-Rad Laboratories, Hercules, CA); 1–16: isolates 1a, 7a, 10a, 11a, 19a, 21a, 24a, 30a, 31a, 34a, 35b, 36a, 46a, 47a, 48a and 50a; 17–21: isolates 9b, 20a, 23a, 35c and 49a; 22: *C. dubliniensis* NCPF 3949 type; 23: *C. albicans* ATCC 90029 type strain.

Fluconazole susceptibility

All of the five isolates identified as *C. dubliniensis* were susceptible to fluconazole (MIC = 0.5 μg/ml) by the NCCLS protocol.

Discussion

A major obstacle to clarification of the role of *C. dubliniensis* as an oral pathogen is the lack of an easy-to-perform screening method capable of selective identification of this yeast. *C. dubliniensis* and *C. albicans* have many similarities, including the ability to form germ tubes in serum (21). Since the majority of clinical laboratories use the germ tube test as their sole method for the identification of *C. albicans*, isolates of *C. dubliniensis* have been misidentified as the former species (1, 11).

CAC medium allows presumptive identification of several clinically important *Candida* species (10). On this medium, colonies of *C. dubliniensis* have a dark-green color on primary isolation, while *C. albicans* colonies are light green (16). Variations in shades of green, however, are scored subjectively. Moreover, the shade of green proved to be unreliable after subculturing and storage of *C. dubliniensis* isolates (11, 16). Thus, the use of CAC medium as the unique criterion for differentiating between *C. albicans* and *C. dubliniensis* may underestimate the frequency of *C. dubliniensis* isolates. It may be wiser to use it only for screening green colonies from primary cultures, regardless of their shade, before submitting them to further identification tests.

The ability to grow at 45°C proved to be a suitable phenotypic marker for *C. dubliniensis* (13). Nevertheless, one should be aware that some *C. albicans* strains are also unable to grow at 45°C, as the maximum growth temperature of *C. albicans* varies from 43 to 46°C (20).

Positive TTC reduction as an identification parameter is also valuable, as it is not usually encountered in *C. albicans* (21). Moreover, differences in the assimilation of glycerol, methyl-α-D-glucoside, D-trehalose and D-xylose detected by the API 20C AUX system have been shown to be useful in differentiating *C. dubliniensis* from *C. albicans* (12). However, routine use of the API 20C AUX system might be considered too expensive for many clinical laboratories. Analysis of the ribosomal DNA subunits represents a reliable identification procedure for medically important yeasts (6). The genetic approach allows the difficulties encountered with phenotypic markers to be overcome, but PCR methods, although easy to use, are unfortunately still not available in all laboratories.

The main aim of this investigation was to evaluate the effectiveness of an identification protocol for *C. dubliniensis* consisting of a preliminary screening by colony color on CAC, followed by tests for ability to grow at 45°C and TTC reduction.

In our study, the CAC medium allowed the detection of five *C. dubliniensis* isolates on the basis of the typical dark-green colonies, even in mixed cultures and in the presence of *C. albicans* (Table 1), and also when the colony counts were low (data not shown). The dark-green-colony isolates also showed a red or dark-pink color on TTC medium, and were not able to grow, or grew poorly, at 45°C. In contrast, all of the light-green-colony isolates, identified as *C. albicans*, grew well at this temperature and produced pale-pink or whitish colonies on TTC medium, with the exception of a single isolate atypically showing a dark-pink color. Assimilation profiles with the API 20C AUX system identified the five dark-green-colony isolates as *C. dubliniensis*. The specific primer set used for PCR confirmed the identification by API 20C AUX of isolates of the two species.

When green-colony isolates were tested for growth at 45°C and TTC reduction, instead of carbohydrate assimilation by API 20C AUX, an overall reduction of about 60% in the cost of *C. dubliniensis* identification was achieved without loss in accuracy. The cost per identification of the PCR protocol was intermediate but availability of expensive molecular biology equipment must be considered. The use of commercial identification kits or molecular biology tools, when available, could be restricted to confirmation of *C. dubliniensis* identification obtained by the protocol we evaluated (Fig. 2).

The study we performed also allowed assessment of the prevalence of oral *C. dubliniensis* in a population of Italian HIV+ subjects. The first isolation of this species in Italy has been recently reported (3), but prevalence studies are not yet available. In our HIV+ sample population, *C. dubliniensis* was recovered from the oral cavities of 5/45 subjects (11.1%). This isolation rate is lower than that reported by Coleman et al. (1), possibly because of the small number of subjects studied. *C. dubliniensis* as the sole identified yeast species cultivated in just two oral samples, while the other three *C. dubliniensis* were isolated in mixed cultures. Three of the five subjects harboring *C. dubliniensis* in the oral cavity showed signs of mucosal candidiasis at the moment of sampling, and one of them had received fluconazole therapy in the past. However, induction of fluconazole resistance after exposure to the drug, as reported both *in vitro* and *in vivo* (8, 14, 15), seemed not to have occurred in the isolate from this patient.

In conclusion, the main problem in the detection of *C. dubliniensis* in oral samples is the lack of a simple, reliable, and inexpensive phenotypic identification protocol. Growth of dark-green colonies on CAC medium proved to be useful for presumptive identification in our study, but might not be sufficient alone. The presence of *C. dubliniensis* in green-colony isolates can be reliably confirmed by a combination of no or highly restricted growth at 45°C and the reduction of TTC. If just one of the two tests is to be used, the first is preferred due to ease of interpretation and high accuracy. The API 20C AUX assimilation patterns also allow reliable identification of *C. dubliniensis*, but the identification cost rises considerably. The identification protocol shown in Fig. 2 might represent a cost-effective approach for the detection of *C. dubliniensis*, or at least could facilitate selection of strains to submit to commercial identification kits. This protocol allowed confirmation of the presence of this novel species in the oral cavity of HIV+ subjects in Italy. The use of simplified but accurate identification protocols, such as the one we propose, would help to elucidate the epidemiology of *C. dubliniensis* and establish its clinical significance.

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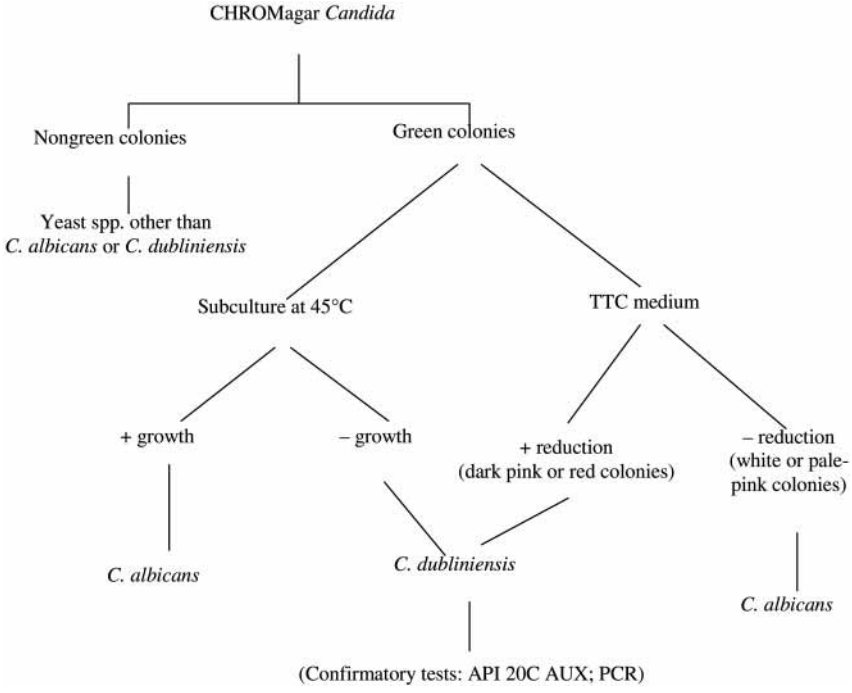

Fig. 2. Proposed flow scheme for the identification of *C. dubliniensis* isolates from oral samples.

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