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Agreement between RAPD, API20C AUX, CHROMagar Candida and microculture on oral Candida identification

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Abstract

Aim: To measure the agreement of methods for identification of *Candida* species in oral cavity samples, comparing the CHROMagar *Candida*, microculture, API 20C AUX and RAPD techniques. **Methods**: Ninety-one colonies of *Candida* were isolated and presumptively identified in CHROMagar *Candida,* submitted to microculture, API 20C AUX and RAPD techniques. After this, agreement among methods using Kappa test was performed. **Results:** Agreement rates between RAPD and CHROMagar *Candida*, showed significant accuracy for *C. albicans*, *C. tropicalis*, *C. dubliniensis* and *C. krusei* (Kappa: 0.760, 0.640, 0.416 and 0.360, respectively, $p<0.05$). Comparing RAPD results with microculture, the highest agreement was for *C. albicans* (Kappa: 0.851 - $p<0.05$) but no significant agreement for *C. lusitaniae*, *C. krusei* and *C. guilliermondii* was obtained ($p>0.05$). The agreement was significant for all identified species when RAPD (OPE-18) and API 20C AUX ($p<0.05$) were used. Critical levels of agreement between RAPD and microculture were observed when *C. lusitaniae*, *C. krusei* and *C. guilliermondii* were identified. **Conclusions**: API 20C AUX presented the best agreement with molecular random identification and CHROMagar showed good agreement for *C. albicans*, *C. tropicalis*, *C. dubliniensis* and *C. krusei* identification.

Keywords: candida; mouth; methods.

Introduction

Candida species are commensal microorganisms of the oral cavity. They have several virulence factors, which in the presence of local and systemic host failures may result in their transition from commensal to pathogenic organisms[1], causing oral and systemic infections that pose significant public health problems. Their isolation is used in investigations related to salivary disfunction, oral candidiasis, orofacial pathologies, and immune suppressant status[2-4].

There is a variety of methods for identifying *Candida* species from clinical samples in the oral cavity[5]. The CHROMagar *Candida* differential medium is commonly used to isolate and identify presumptive *C. albicans*, *C. dubliniensis*, *C. tropicalis* and *C. krusei*. Their sensitivity and specificity are considered satisfactory for these species[2,6-7].

The microculture analysis has considerable accuracy and presents low cost[2],

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but it requires visual experience, sometimes limited by the resolution of optical microscopy and confused by similarities among species' expressions. The biochemical characterization could be performed using the API® 20C AUX (BioMerieux, France) which relies on variations in the assimilation of carbohydrates[7-9]. However, it presents limitations related to cost and to distinguish between some species[2]. A study of 159 clinical isolates of *Candida* species identified by the very similar kit API® *Candida* AUX (BioMerieux, Marcy l'Etoile, France) reported that 12 isolates (7.5%) were incorrectly identified[10-11].

In recent decades, traditional methods of microorganism phenotyping have been replaced or added by the procedures associated to recombinant DNA[12-14]. Methods based on molecular markers are useful not only for phenotyping, but also for differentiation of *Candida* species[15-16]. The RAPD (Random Amplification Polymorphic DNA) allows the amplification of DNA sequences and is a simple and quick technique that does not require prior knowledge on the genomes to characterize organisms, using one randomly determined (usually a decamer) primer[17]. It is used for genetic characterization of a range of organisms, plants, animals or microorganisms, including *Candida* species, for different purposes[18-21]. The sensitivity, specificity and resolution of the OPE-18 primer for identification of *Candida* species has been reported and could be used for epidemiological *Candida* identification[11,22].

Due to scarce information about presumptive, biochemical and molecular agreement on *Candida* identification, this study aimed to measure the assertive correlation between the presumptive identification of *Candida* species from oral cavity using CHROMagar *Candida*, microculture, API® 20C Aux and OPE-18 genotyping.

Material and methods

Ethical procedures

Ethical considerations in accordance with Helsinki Declaration have been observed. This research was conducted according to the ethical principles of research involving human participants, as stipulated by Resolution 196/96 of the National Health Council of the Ministry of Health of Brazil. The collection and analysis of data in this study were certified by the research ethics committee of the State University of Montes Claros, MG, Brazil, protocol CEP nº. 1111/08.

Origin of samples

The *Candida* isolates resulted from salivary collections of oral cavity of patients irradiated on head and neck due to malignant neoplasms (n=29) and elderly volunteers (n=63). The collection comprised 91 isolates of *Candida* species.

Isolation and presumptive identification of *Candida* species

The isolation of yeasts was made in salivary samples collected from the buccal mucosa and tongue with a swab and sterile saline solution (NaCl, 0.85%) as diluent. The isolation and presumptive identification was made by drawing aliquots (100 µL) from each sample and placing them on plates containing CHROMagar *Candida* and incubated at 37 °C for 24 to 48 h, in duplicate. Yeast identification was made by considering the morphology and color of the colonies[2,23]. Each colony of *Candida* was cataloged and then stored at -20 °C in Sabouraud Dextrose Broth (DSB, Oxoid Ltd., London, England) amended with glycerol (40% v/v). ATCC 10231 of *C. albicans* was used as quality control (QC).

Microculture characterization of isolates

Microcultures with Cornmeal Agar-Tween 80 (Rheum, Lenexa, KS, USA)[23] were made to highlight blastospores, chlamydospores, pseudohyphae and true hyphae of the isolates. To differentiate *C. albicans* and *C. dubliniensis* from other *Candida* species, germ tube production was viewed on bovine serum[24-25]. To distinguish *C. albicans* from *C. dubliniensis*, cultivation on Sabouraud Dextrose Agar (Oxoid, Hampshire, England) for 48 h was made at 42 °C, using ATCC 10231 as QC.

Identification by API 20C AUX

The inoculum used to this procedure was obtained from cultured yeast on Sabouraud Agar. The procedures for inoculation and interpretation were performed according to the instructions provided by the manufacturer (BioMerieux, France). Identification list on these indexes was considered as excellent (%ID>99.9, T>0.75), very good (>99.0% ID and T>0.5) or acceptable (%ID>90.0 and T >0.5)[7].

Identification of isolates by RAPD (Random Amplification Polymorphic DNA)

The extraction and purification of DNA from isolates of *Candida* spp was made with the Purelink Genomic DNA® kit (Invitrogen K1820-02, Brazil). The used DNA was obtained from cells grown in YPD broth (1% Malt Extract Powder, 2% bacteriological peptone and 2% dextrose - D-glucose) at 37 °C and shaking (150 rpm for 24 h)[11]. A total of 50 µL of concentrated suspension of each isolate was obtained by centrifugation (3,500 rpm for 30 min). The purification of DNA was made by adding 200 µL of digestion buffer, 20 µL proteinase K and 20 µL RNase. We added to 200 µL of binding buffer and then the tubes were heated for 10 min at 80 °C in a water bath. To neutralize the detergent and to allow the connection with the silica column, 200 µL of absolute ethanol was added (Merck, Darmstadt, Germany). The tubes were centrifuged at 13,000 rpm for 1 min and the pellet was discarded. Subsequently were added 500 µL of the first washing buffer and centrifuged again at 13,000 rpm for 1 min and the precipitate discarded. The column with the silica was passed to the second tube and added 500 µL of the second washing buffer and centrifuged to 13,000 rpm for 1 min and for 3 additional minutes to enhance drying.

For the first extraction, 200 µL of sterile water were added to Milli-Q heated to 60 °C in the column in a second

tube. Then it was centrifuged at 13,000 rpm for 1 min. For the second extraction, 200 µL of elution buffer of the same column were placed in a third tube and centrifuged it at 13,000 rpm for 1 min.

The products of RAPD-PCR were obtained with OPE-18 primer (5'-GGACTGCAGA-3') (Gibco BRL, Grand Island, NY, USA). The preparation of reactions for each isolate was done by adding 1 µL of primer, 5 µL of dNTP mix (dATP, dCTP, and dTTP DGPT - Invitrogen, Brazil), 2.5 µL 10x PCR Buffer Rxn, 1 µL $MgCl_2$ (50 mM), 0.5 µL Taq DNA polymerase (2.5 U - Invitrogen Platinum®, Brazil) and 5.5 µL Milli-Q. The final volume was 25 µL, 15 µL of MIX and 10 ìL of extracted DNA. The amplification consisted of 39 one-minute cycles at 94 ˚C, 1 min at 36 ˚C, 2 min at 72 ˚C followed by a 10 min cycle at 72 ˚C[11].

PCR products were separated by agarose gel eletroforesis (1.4% / v - 5µL ethidium bromide – 10 mg/mL), 80 V for 5 h. We used ATCC 10231 as QC and two molecular weights were incorporated (100 bp and 250 bp - Invitrogen, São Paulo, SP, Brazil). The DNA bands were observed and photographed in transillumination and the images were analyzed considering the literature reports[11,22].

Table 1 shows comparison of the methods used in this study.

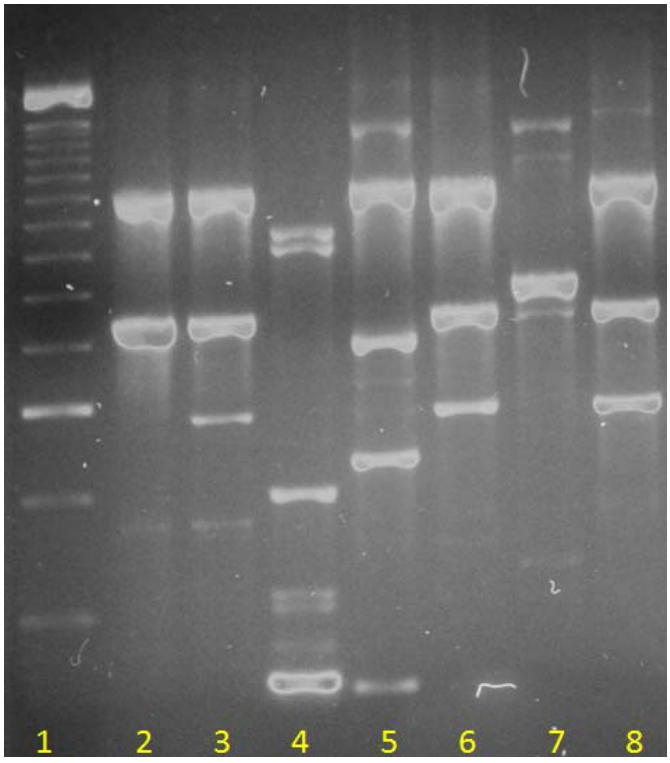

Fig. 1. RAPD showing different species of Candida. (1) Ladder (250 pb), (2) *C. albicans* ATCC,(3) *C. albicans* ATCC, (4) *C. tropicalis;* (5) *C. krusei,* (6) *C. albicans* ATCC, (7) *C. guilliermondii,* (8) *C. albicans.*

Results

Among the 91 isolates, *C. albicans* was the most prevalent, identified presumptively in 35 (38.5%) of colonies by CHROMagar *Candida.* On the confirmatory identification, 31 (34.1%) of these isolates were confirmed on microculture as *C. albicans*, while 30 (32.9%) were confirmed by the API 20C Aux® and 29 (31.9%) by RAPD. RAPD identified 29 (31.9%) *C. albicans*, 4 (4.4%) *C. dubliniensis*, 10 (10.9%) *C. tropicalis*, 9 (9.9%) *C. krusei*, 12 (13.2%) *C. glabrata*, 9 (9.9%) *C. parapsilosis*, 6 (6.6%) *C. guilliermondii*, 6 (6.6%) *C. lusitaniae* and 5 (5.5%) *C. kefyr.* Figure 1 shows RAPD with different species of Candida.

The CHROMagar *Candida*® presumptively identified 28 (30.8%) of isolates as other *Candida* species (*C. dubliniensis, C. tropicalis,* and *C. krusei*). The agreement between genetic typing and CHROMagar *Candida*® was higher for *C. albicans* and lower for *C. krusei.* Table 2 shows the agreement coefficient (Kappa) between RAPD (OPE-18) and CHROMagar *Candida.*

In the RAPD technique only one isolate (1.1%) presented an undefined pattern, followed by five (5.5%) in API 20C AUX® and 8 (8.8%) in microculture. Considering the comparative analysis between the RAPD characterization and microculture evaluation, we can observe that the identifications of *C. albicans*, *C. dubliniensis*, *C. tropicalis*, *C. glabrata*, *C. kefyr* and *C. parapsilosis* were significantly concordant in decreasing levels among the methods, in that order. When RAPD and API 20C AUX® were compared, the species *C. tropicalis*, *C. albicans*, *C. glabrata*, *C. kefyr*, *C. dubliniensis*, *C. lusitaniae*, *C. krusei* and *C. guilliermondii* showed significant decreasing agreement, in that order. Table 3 shows the Kappa coefficient among RAPD, API 20C AUX and microculture.

Discussion

The presumptive identification of yeasts may be crucial in the diagnosis and treatment of fungal infections. It is a fundamental recognition and validation of methods that should be fast, accurate and inexpensive. Bernal et al.[25] (1996) using the CHROMagar *Candida* for presumed identification

Table 1. Comparation of methods used in this study.

Method	RAPD	Microculture	CHROMagar	API20C AUX
Method of identification	Random Aleatory Polymorphic DNA. Molecular identification.	Culture and morphological evaluation.	Culture and evaluation by color.	Assimilation of Carbohidrates.
Sensibility	High	Intermediary(some species)	Intermediary (some species)	High

Table 2. Kappa coefficient between the presumptive identification of *Candida* species by CHROMagar Candida and RAPD (OPE18)

	CHROMagar *Candida* *Candida* species			
	C. albicans	*C. dubliniensis*	*C. tropicalis*	*C. krusei*
Kappa(*p**)	0.760 (0.000)	0.416 (0.000)	0.640 (0.000)	0.360 (0.000)

* Kappa coefficient probability.

Table 3. Kappa coefficient applied to comparative identification between API® 20C AUX and microculture analysis with reference to the products of RAPD (OPE 18)

Candida spp	Microculture Kappa (*p**)	API 20C AUX Kappa (*p**)
C. albicans	0.851 (0.000)	0.925 (0.000)
C. dubliniensis	0.852 (0.000)	0.657 (0.000)
C. tropicalis	0.806 (0.000)	0.946 (0.000)
C. krusei	0.059 (0.565)	0.474 (0.000)
C. glabrata	0.712 (0.000)	0.897 (0.000)
C. parapsilosis	0.588 (0.000)	0.732 (0.000)
C. guilliermondii	0.056 (0.587)	0.323 (0.000)
C. lusitaniae	0.004 (0.908)	0.578 (0.000)
C. kefyr	0.739 (0.000)	0.883 (0.000)
Undefined species	0.020 (0.724)	0.019 (0.808)

** Kappa coefficient probability.*

of 593 colonies, revealed 341 (57.5%) *C. albicans*, 339 (57.2%) of them featuring green characteristic color. All 35 (5.9%) *C. krusei* and 73 (12.3%) of *C. tropicalis* presented specific characteristics identified on CHROMagar *Candida*. In the present study, among the 91 isolates, 35 (38.46%) were pale green, 18 (30.7%) pale pink with white halo and 5 (5.5%) were blue on CHROMagar *Candida*. Using RAPD, 29 (31.8%) were identified as *C. albicans*, 8(8.8%) as *C. krusei* and 9 (9.9%) as *C. tropicalis* (Kappa coefficient 0.760, 0.360 and 0.640 respectively - $p<0.05$), showing a good accuracy of CHROMagar *Candida* identification of these species.

Studies with OPE-18 primer[11,22] showed different monomorphic bands for the species *C. glabrata*, *C. guilliermondii* and *C. lusitaniae*. Baires-Varguez et al.[11](2007) using OPE 18 by RAPD-PCR with 92 clinical isolates revealed 20 (21.7%) *C. albicans*, 14 (15.2%) *C. glabrata*, 10 (10.9%) *C. guilliermondii*, 11 (11.95%) *C. lusitaniae* and 15 (16.3%) *C. tropicalis* with a 91% sensitivity for the total isolates, being very specific and sensitive for the *C. glabrata*, *C. guilliermondii,C. tropicalis*, *C. pelliculosa*, *C. albicans*, *C. krusei* and *C. lusitaniae* species. Among the 91 isolates in the analysis using the same technique and the same primer, were obtained 29 (31.9%) *C. albicans*, 12 (13.2%) *C. glabrata*, 6 (6.6%) *C. guilliermondii* and 6 (6.6%) *C. lusitaniae*. The sensitivity and specificity in the present study was respectively 96% and 97% for *C. albicans*, 80% and 100% for *C. glabrata*, 89% and 95% for *C. parapsilosis* and 100% and 98% for *C. tropicalis*.

Several studies used the API® 20C AUX as identification and confirmation of *Candida* species[26-28]. Silva and Candido[26] (2005) using the API® 20C AUX identified 92% (46) of yeasts used in their study, 76% (38) did not require additional tests and 16% (8) required some additional analysis. The results are closer to Sand and Rennie[27] (1999), who found 96.5% accuracy after 72 h. Good results were also obtained by Smith et al.[9] (1999), who found 95.6% of identification without extra tests. In this analysis, among the 91 isolates, RAPD identified 12 (13.2%) *C. glabrata*, 9 (9.9%) *C. parapsilosis*, 6 (6%) *C. guilliermondii*, 6 (6%) *C. lusitaniae* and 5 (5.5%) *C. kefyr*. When the same species were submitted to the API® 20C AUX, the agreement was statistically significant ($p<0.05$).

The agreement between RAPD (OPE-18) and API® 20C AUX is evidently higher. Critical levels of agreement between RAPD and microcultive were observed when *C. lusitaniae*, *C. krusei* and *C. guilliermondii* were identified. For presumptive identification, CHROMagar *Candida* is adequate for *C. albicans*, *C. dubliniensis*, *C. tropicalis* and *C. krusei* identification.

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