Preliminary characterization and grouping of *Candida* species by numerical analysis of protein profiles obtained by polyacrylamide gel electrophoresis

José Francisco Höfling, Alessandra Silva Campos, Cássio Vicente Pereira, Rosimeire Takaki Rosa & Edvaldo Antonio Ribeiro Rosa

Universidade Estadual de Campinas, Faculdade de Odontología de Piracicaba, Dpto. de Diagnóstico Oral, Laboratorio de Microbiologia e Inmunologia, Piracicaba, Brasil

Summary Whole-cell proteins from isolates of five *Candida* species (*Candida albicans*, *Candida krusei, Candida parapsilosis*, *Candida tropicalis* and *Candida guilliermondii*) were separated by SDS-PAGE and the profiles obtained were converted into a binary data matrix that produced a cophenetic correlation phenogram. The analysis of the phenogram allowed detection of the cophenetic correlation levels existing among these species.

Key words *Candida*, SDS-PAGE, Numerical analysis

Caracterización preliminar y agrupamiento de especies de *Candida* mediante análisis numérico de perfiles proteicos obtenidos por electroforesis en geles de poliacrilamida

Resumen Se separaron proteínas de células completas de aislamientos de cinco especies de *Candida* (*Candida albicans*, *Candida krusei, Candida parapsilosis*, *Candida tropicalis* y *Candida guilliermondii*) mediante SDS-PAGE y los perfiles obtenidos se transformaron en matrices de datos binarios que reproducían un fenograma de correlación cofenética. El análisis del fenograma permitía detectar los niveles de correlación cofenética entre diferentes especies

Candida, SDS-PAGE, Análisis numérico

With the advent of AIDS and the increase of the use of immunossupresive medication, we have observed an increase in the importance of the role of yeasts, mainly that from *Candida* genera, as colonizer and pathogens of the soft tissues in the oral cavity [1,2].

Chromatographic techniques allow the separation of mixtures of compounds, making possible their characterization. Gel electrophoresis allows the separation of different components from a mixture according to molecular mass, molecular conformation, and net charge.

Dirección para correspondencia:
Dr. José Francisco Höfling
Universidade Estadual de Campinas, Faculdade de Odontología de Piracicaba, Dpto. de Diagnóstico Oral, Laboratorio de Microbiologia e Inmunologia
Avda. Limeira, 901, Areiao, CEP 13414-900, CP 052, Piracicaba, SP Brasil.
Tel.: +55 19 430 5321/22/23; Fax: +55 19 430 5218;
E-Mail: hofling@fop.unicamp.br

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Different types of electrophoretic techniques have been used for the characterization or typing of *Candida* including electrophoretic separation of chromosomes [3,4], DNA fragments [5], isozymes [6], cell-wall glycoproteins [7] and whole-cell proteins [8-10]. The latter has been used successfully for bacterial [11-13] and yeast characterization [14-16]. The resulting electrophoretic profiles can be plotted into a binary data matrix that, with computer-assisted support, produces comparative results expressed as similarity or cophenetic correlation matrixes or phenograms [17].

MATERIAL AND METHODS

Yeast strains. A total of 12 *Candida* strains isolated from oral cavity of twelve health subjects were furnished by Dr A. O. C. Jorge from Faculdade de Odontologia de São José dos Campos (São Paulo, Brazil): *Candida albicans* (97a, F72, E37, 17b), *Candida* krusei (1M90, 4c), *Candida parapsilosis* (21c, 7a), *Candida tropicalis* (1b, FCF430), *Candida guilliermondii* (FCF152, FCF405). For each species were added the respective type-strains: *C. albicans* (CBS562), *C. krusei* (CBS573), *C. parapsilosis* (CBS604), *C. tropicalis* (CBS94), *C. guilliermondii*

(CBS566). The type-strain of *Saccharomyces cerevisiae* (CBS1171) also were included in this experiment.

Extraction of whole-cell components. The strains were cultivated on 50 ml YPD medium (yeast extract 1%, peptone 2%, glucose 2%) [3,7] at 37°C, on a shaking incubator (150 rpm), for 18 h. Cells were harvested by centrifugation (3,000 g/5 min) and washed twice with cold saline solution [18]. The pellets were transferred to a mortar with 500 ml of sterile water and liquid nitrogen to freeze the cells that were disrupted by grinding. These preparations were transferred to microfuge tubes that were centrifuged (13,000 g/3 min) and the supernatants were collected. The protein concentration was determined by the Bradford's method [19] and adjusted to 80 mg/ml [20]. Equal volumes of supernatant and loading buffer [9] (5 mM Tris, 2.5% 2-mercaptoethanol, 1.5% SDS, 0.025% bromophenol blue) were combined and heated in a boiling water bath for 10 minutes.

Whole-cell protein electrophoresis. The protein profiles were obtained after electrophoresis of 50ml of protein solution in polyacrylamide slab gel with sodium dodecylsulfate (SDS) in a discontinuous buffer system [21] with 4.5% stacking gel and 12.5% running gel. The electrophoresis was conduced at 125 V in a cold chamber and the gels were stained with Coomassie blue G-250 0.25%. After destaining, the gels were scanned and the profiles of each lane transferred to a densitometry interface in the SigmaGel software (Jandel Software) where the exact position of the protein peaks were determined.

Numerical analysis of the protein profiles. For the establishment of cophenetic levels among the strains, the extracts of the type-strains of each *Candida* species and the type-strain of *S. cerevisiae* (CBS 1171 neotype) were included for the evaluation of reproducibility [14] and as an extrageneric reference [9,13,22], together with Molecular Mass Markers (Bio-Rad Laboratories, USA). The relative mobility (Rm) of each protein band was determined and the matches and mismatches among the bands (originated from presence/absence of such bands) received the representations 1 and 0, respectively. These data allowed to build a binary data matrix that was plotted using the NTSYS 1.70 software (Numerical Taxonomy and Systematics, from Applied Biostatistics, Inc., USA). Calculation of similarity between Rm values was performed by the Pearson product moment correlation coefficient (r) [23,24]. Cluster analysis was obtained with the phenogram by the unweighted pair-group method with arithmetic averages (UPGMA) [14].

RESULTS

Reproducibility. The protein profiles of analyzed strains on different gels were reproducible after three repetitions of each electrophoretic running. Protein extracts of *S. cerevisiae* (CBS 1171) and molecular mass markers were applied in all gels providing mean values r = 0.803 and 1.0, respectively.

Strain clustering. The application of UPGMA clustering method allowed to build the phenogram of cophenetic correlation showed in figure 1, in which seven clusters (phenons) can be distinguished.

These clusters have the following compositions:

- Phenon I: there are three repetitions of molecular mass markers, with r = 1.000.
- Phenon II: there are three repetitions of *S. cerevisiae* CBS 1171, with r ≥ 0.803.
- Phenon III: there are five strains of *C. albicans*, with r ≥ 0.671.

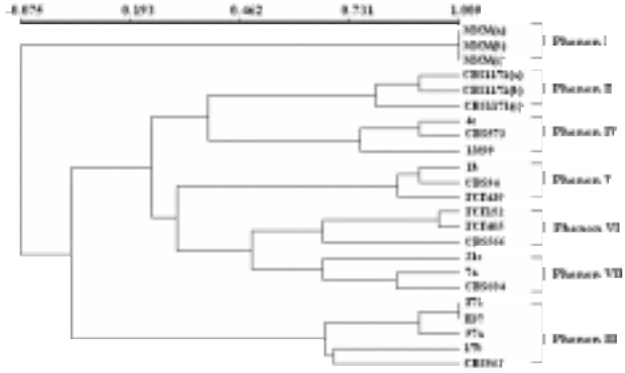

Figure 1. UPGMA phenogram showing the cophenetic correlation among some *Candida* species isolated from human oral cavity, based on their protein profiles obtained by SDS-PAGE.

- Phenon IV: there are three strains of *C. krusei*, with r ≥ 0.754.
- Phenon V: there are three strains of *C. tropicalis*, with r ≥ 0.808.
- Phenon VI: there are three strains of *C. guilliermondii*, with r ≥ 0.666.
- Phenon VII: there are three strains of *C. parapsilosis*, with r ≥ 0.664.

Interspecific comparison. Among all the species, *C. albicans* (cluster III) was the most frequently isolated species and its cluster could be grouped to others with r = 0.050. *C. krusei* (cluster IV) showed correlation with Saccharomyces cerevisiae CBS 1171 with r = 0.385, and both could be isolated from others with r = 0.245. *C. guilliermondii* (cluster VI) and *C. parapsilosis* (cluster VII) showed a value of r = 0.490, and these two clusters could be grouped with *C. tropicalis* (cluster V) with r = 0.309. Molecular mass markers showed correlation with the strains with r = -0.076.

DISCUSSION

The analysis of electrophoretic profiles of proteins associated with computer-statistics techniques has allowed the identification, classification, and even the reclassification of numerous strains, species and genera of bacteria [11-13,22,25] and yeast [10,14,15].

The reproducibility of electrophoretic profiles on different slab gels was evaluated by the inclusion of molecular mass markers, together with the inclusion of the protein extract of yeast from a non-correlated genus [9,12] and gave cophenetic correlation values r=0.803 for the three repetitions of *S. cerevisiae* and r=1.0 for the three repetitions of molecular mass markers. These values are in agreement with the minimum acceptable proposed by Sneath & Johnson [26] that was 0.8. The data obtained from grouping of *Candida* strains based on their electrophoretic profiles showed high level of agreement with the interspecific classification established by conventional methods. Moreover, the isolates of each specie showed identical or very similar profiles when compared. This fact suggests that these protein profiles obtained by SDS-PAGE are relatively stable taxonomic characteristics.

As it can be observed in figure 1, the use of type-strains allowed the identification of clusters at the species level, since the *Candida* isolates were grouped with their respective type-strains. With regard to cluster compositions, the SDS-PAGE technique allowed the organization

of all isolates in distinct clusters, with cophenetic correlation r ≥ 0.671 for *C. albicans*, r ≥ 0.664 for *C. parapsilosis*, r ≥ 0.666 for *C. guilliermondii*, r ≥ 0.808 for *C. tropicalis*, r ≥ 0.754 for *C. krusei*, and r ≥ 0.803 for *S. cerevisiae*.

Shechter *et al.*[27], using non-denatured acid and basic protein electrophoresis and association coefficient of Jaccard (SJ), that excludes negative matches, obtained a phenogram in which the species *C. albicans*, *C. krusei* and *C. parapsilosis* combined among them with 40% of similarity. The species *C. guilliermondii* clustered to this group with 32% and *C. parapsilosis* was the last one to group, with approximately 25% of similarity. This behavior, different of that found in our research, is due to the fact that non-denatured proteins migrate through the gel according to their molecular mass, structural conformation and net charge. In contrast, SDS denatured proteins that migrate according to molecular mass only. As molecular mass is more conserved than net charge, electrophoretic profiles based on this criterion should, in theory, detect higher taxonomic relationship [17].

The systematic proximity detected between *C. krusei* and *S. cerevisiae* (r=0.385) was also observed by Barns *et al.* [28] in their analyses with 18S ribosomal subunites. Hendricks *et al.* [29] support that *Candida* and *Saccharomyces* should have close phylogenetic relationship, detectable by 18S rRNA sequence analysis.

The protein profile analysis by SDS-PAGE improves the knowledge about the taxonomic relationships among oral yeasts. This method shows good reproducibility and allows collection of useful information for numerical analysis. This methodology could prove important information in systematic and epidemiological evaluations involving oral yeasts.

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