Analysis of Parity Between Protein-based Electrophoretic Methods for the Characterization of Oral *Candida* Species

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Electrophoretic studies of multilocus-enzymes (MLEE) and whole-cell protein (SDS-PAGE) were carried out in order to evaluate the parity between different methods for the characterization of five Candida *species commonly isolated from oral cavity of humans by numerical taxonomy methods. The obtained data revealed that sodium dodecyl sulfate polyacrylamide gel electrophoresis is more efficient in grouping strains in their respective species while MLEE has much limited resolution in organizing all strains in their respective species-specific clusters. MLEE technique must be regarded for surveys in which just one species of* Candida *is involved.*

Key words: polyacrylamide gel electrophoresis - multilocus enzyme electrophoresis - *Candida* - numerical analysis

The yeasts pertaining to the genus *Candida* are found dispersed in different epitelial areas of the body, including oral mucosa. In recent years, they have received more attention due to their involvement in a increasing number of cases of opportunist oral infections in patients with Aids and those having immunosuppresive medication. Of epidemiological interest, characterization procedures based on molecular fingerprints have been applied in order to establish possible relationships among *Candida* isolates involved in oral infections (McCullough et al. 1996).

Different types of electrophoretic techniques have been used for the characterization or typing of *Candida* including electrophoretic separation of chromosomes (Monod et al. 1990, Asakura et al. 1991), DNA fragments (Scherer & Stevens 1987), multilocus-enzymes (Lehmann et al. 1989a, Pujol et al. 1993, Reynes et al. 1996), and whole-cell proteins (Shen et al. 1988, Vancanneyt et al. 1991, 1992, Höfling et al. 1998). The two latter methods have been used successfully for yeast characterization. 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 matrices or dendrograms (Kersters 1985).

In this experiment, we compare multilocus-enzyme electrophoresis (MLEE) and polyacrylamide gel electrophoresis (SDS-PAGE) for their ability to discriminate five *Candida* species isolated from saliva of healthy subjects.

MATERIALS AND METHODS

Candida strains - Representative strains of different *Candida* species isolated from human oral cavity and identified by biochemical and physiological tests were obtained from the Microbiology and Immunology Laboratory, Dentistry College of São José dos Campos: *C. albicans* (97.a, F.72, E.37, 17.b, CBS.562^T), *C. guilliermondii* (FCF.405, FCF.152, CBS.566^T), *C. parapsilosis* (21.c, 7.a,CBS.604^T), *C. krusei* (1M.90, 4.c, CBS.573^T), *C. tropicalis* (1.b, FCF.430, CBS.94^T). The superscript T in CBS strains indicates that they are the respective type-strains for each species. *Saccharomyces cerevisiae* type-strain (CBS.1171^T) was included as an extra-generic organism (Costas et al. 1989).

Cell cultivation and whole-cell protein extraction - All strains were grown in 50 ml of Yeast Peptone Dextrose medium (2% dextrose, 2% peptone, 1% yeast extract) in a shaker table under 150 rpm, at 30ºC, overnight. The cells were harvested by centrifugation at 2,000 g for 3 min and the pellets were washed four times with cold sterile water in order to remove either culture medium traces or extra-cellular metabolites (Woontner & Jaehning 1990). The last washed pellets were transferred to 2 ml microcentrifuge tubes and acid-washed glass

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beads (v/v) plus 200 ml of cold sterile water were added. Cells were lysed using a Mini-Bead Beater cell disrupter (Biospec) at 4600 r.p.m., repeating four times of 30 sec at 5-min intervals, and placed in an ice bath. After cell disruption, the micro-centrifuge tubes were centrifuged at 10,000 g for 2 min, and the supernatant's protein concentration were determined according to Bradford (1976) and adjusted to 80 µg/ml (Ames 1974). The MLEE supernatants were applied on Whatman 3 filter paper wicks of 5x12 mm (Selander et al. 1986), and for SDS-PAGE technique equal volumes of supernatant and loading buffer of Bruneau and Guinet (1989) (5mM Tris, 2.5% 2-mercaptoethanol, 1.5% SDS, 0.025% bromophenol blue) were combined and heated in a boiling water bath for 10 min.

MLEE and specific-enzyme staining - The electrophoreses were carried out using hydrolyzed corn starch Penetrose 30 (Refinações de Milho Brasil) up to a final concentration of 13% (Val et al. 1981) in 1:30 pH 8.0 Tris-citrate buffer (Selander et al. 1986, Caugant & Sandven 1993). Electrophoreses were carried out at 4ºC and 130 V until the bromphenol blue migration markers had run at least 80 mm from application point. At this time, the electrophoresis was interrupted and the gels were sliced with 1.2 mm thickness. The gel slices were revealed for enzyme active band detection, according to Selander et al. (1986) protocols. Enzymatic systems assayed were: alcohol dehydrogenase (ADH-E.C. 1.1.1.1), lactate dehydrogenase (LDH -E.C. 1.1.1.27), malate dehydrogenase (MDH-E.C. 1.1.1.37), isocitrate dehydrogenase (IDH-E.C. 1.1.1.42), glucose-6-phosphate dehydrogenase (G6PDH-E.C. 1.1.1.49), aspartate dehydrogenase (ASDH-E.C. 1.4.3.x), glucose dehydrogenase (GDH-E.C. 1.1.1.47), mannitol dehydrogenase (MADH-E.C. 1.1.1.67), sorbitol dehydrogenase (SDH-E.C. 1.1.1.14), malic enzyme (ME-E.C. 1.1.1.40), aconitase (ACO-E.C. 4.2.1.3), catalase (CAT-E.C. 1.11.1.6), superoxide dismutase (SOD -E.C. 1.15.1.1), glutamate-oxalacetate transaminase (GOT-E.C. 2.6.1.1), α-esterase (EST-E.C. 3.1.1.1), β-esterase (EST-E.C. 3.1.1.1), leucine aminopeptidase (LAP-E.C. 3.4.1.1), glucosil transferase (GTF-E.C. 2.4.1.11), peroxidase (PO-E.C. 1.11.1.7) e α-amylase (α-AM-E.C. 3.2.1.1).

SDS-PAGE protein analysis - SDS-PAGE protein profiles were obtained after electrophoresis of 50 µl of protein solution in polyacrylamide slab gel with sodium dodecylsulfate (SDS) in a discontinuous buffer system (Laemmli 1970) with 4.5% stacking gel and 12.5% running gel. The electrophoresis was conduced at 125 volts 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.

Computing numerical data - Dendrograms for the different MLEE systems and SDS-PAGE were generated by using the simple matching (S_{SM}) association coefficient (Sokal & Michener 1958, Sneath & Sokal 1973, Naumov et al 1997), based on band positions calculated by the NTSYS software package, version 1.70 (Applied Biostatistics, Inc.). For the present study, a S_{SM} of 1.00 represents identical matches (i.e., all the bands match), a S_{SM} of 0.00 represents no matches, and increasing intermediate values represent increasing proportions of matched bands. Dendrograms, represented by non-rooted trees, based on S_{SM} values were generated by the unweighted pair-group arithmetic average (UPGMA) clustering method (Rohlf 1963, Sneath & Sokal 1973, Naumov et al. 1997).

RESULTS

The application of UPGMA clustering produced two similarity dendrograms shown in Figs 1 and 2, in which several clusters (phenons) could be distinguished. These clusters may be defined by their average similarity values (S_{SM}).

Phenons generated by SDS-PAGE

Phenon I: there is the *S. cerevisiae* type-strain CBS.1171^T

Phenon II: there are three strains of *C. krusei*, with $S_{SM} \geq 0.872$.

Phenon III: there are three strains of *C. tropicalis*, with $S_{SM} \geq 0.897$

Phenon IV: there are three strains of *C. guilliermondii*, with $S_{SM} \geq 0.823$

Phenon V: there are three strains of *C. parapsilosis*, with $S_{SM} \geq 0.833$

Phenon VI: there are five strains of *C. albicans*, with $S_{SM} \geq 0.833$

Interspecific comparison by SDS-PAGE - Among all the species, *C. albicans* (phenon VI) was the most frequently isolated species and its cluster could be grouped to others with $S_{SM} = 0.513$.

C. krusei (phenon II) showed some similarity with *S. cerevisiae* CBS 1171 with $S_{SM} = 0.692$, and both could be isolated from others with $S_{SM} = 0.597$.

C. guilliermondii (phenon IV) and *C. parapsilosis* (cluster V) showed a value of $S_{SM} = 0.7749$, and these two clusters could be grouped with *C. tropicalis* (phenon III) with $S_{SM} = 0.655$.

Reproducibility of SDS-PAGE patterns - 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 S_{SM} = 0.853 and 1.000, respectively.

Enzymatic systems - The one-dimensional electrophoreses of protein extracts from 12 *Candida* strains, their respective type-strains, and *S. cerevisiae* type-strain, showed that among twenty assayed enzymes, five did not show any enzymatic activity (ASDH, MADH, SDH, GTF, and α-AM).

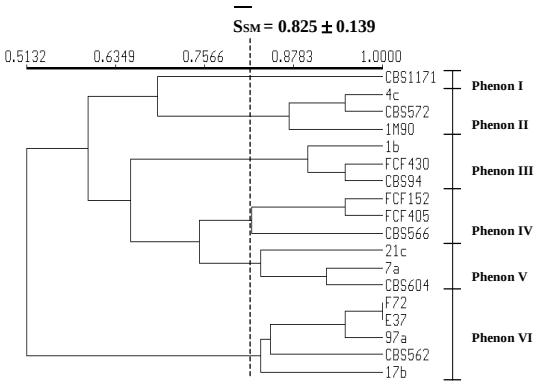

Fig.1: non-rooted dendrogram of similarity among *Candida* strains grouped by simple matching associative coefficient and UPGMA algorithm from sodium dodecyl sulfate polyacrylamide gel electrophoresis profiles.

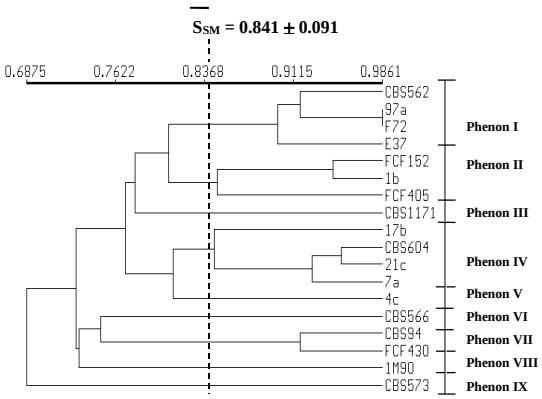

Fig. 2: non-rooted dendrogram of similarity among *Candida* strains grouped by simple matching associative coefficient and UPGMA algorithm from multilocus enzyme electrophoresis profiles.

Phenons generated by MLEE

Phenon I: there are four strains of *C. albicans* (CBS.152^T, 97.a, F.72, and E.37) with $S_{SM} \geq 0.898$

Phenon II: there are two strains of *C. guilliermondii* (FCF.152 and FCF.405) and one *C. tropicalis* (1.b), with $S_{SM} \geq 0.847$

Phenon III: there is the *S. cerevisiae* type-strain CBS.1171^T

Phenon IV: there are three strains of *C. parapsilosis* (CBS.604^T, 21.c, and 7.a) and one *C. albicans* strain (17.b), with $S_{SM} \geq 0.845$

Phenon V: there is a *C. krusei* strain (4.c)

Phenon VI: there is the *C. guilliermondii* type-strain CBS.566^T

Phenon VII: there are two strains of *C. tropicalis* (CBS.94^T, and FCF.430), with S_{SM} = 0.917

Phenon VIII: there is the strain 1M.90 of *C. krusei*

Phenon IX: there is the *C. krusei* type-strain (CBS.573^T)

Interspecific comparison by MLEE - Excluding phenon I, composed only by *C. albicans*, and those in which only one strain were detected (phenons III, V, VI, VIII, and IX), all other clusters had an impure composition with more than one species component.

DISCUSSION

The analysis of electrophoretic profiles of proteins and multilocus-enzymes has allowed the identification, classification of numerous strains, species and genera of yeasts (Baptist & Kurtzman 1976, Okunishi et al. 1979, Yamazaki & Komagata 1981, Maiden & Tanner 1991, Vancanneyt et al. 1991, 1992).

The reproducibility of electrophoretic profiles on different slab SDS-PAGE gels was evaluated by the inclusion of molecular mass markers, besides protein extract of a organism from a non-correlated genus (Costas et al. 1989, Bruneau & Guinet 1989) and gave similarity correlation values S_{SM} = 0.853 for three repetitions of *S. cerevisiae* and S_{SM} = 1.000 for three repetitions of molecular mass markers. These values are in agreement with the minimum acceptable proposed by Sneath and Johnson (1972) that was 0.800. The data obtained from grouping of *Candida* strains based on their electrophoretic profiles showed high level of agreement with the inter-specific classification established by conventional methods. Moreover, the isolates of each species 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 shown in Fig. 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 similarity coefficients $S_{SM} \geq 0.833$ for *C. albicans*, $S_{SM} \geq 0.833$ for *C. parapsilosis*, $S_{SM} \geq 0.823$ for *C. guilliermondii*, $S_{SM} \geq 0.897$ for *C. tropicalis*, and $S_{SM} \geq 0.872$ for *C. krusei*.

Shechter et al. (1972), using non-denatured acid and basic protein electrophoresis and association coefficient of Jaccard (S_J), 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 from 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 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 better taxonomic relationships (Kersters 1985).

The systematic proximity between *C. krusei* and *S. cerevisiae* (S_{SM} = 0.692) assessed by SDS-PAGE technique was also observed by Barns et al. (1991) in their analyses based on phylogenetic analysis of 18S ribosomal sub-units RNA genes. Hendricks et al. (1989) support that *Candida* and *Saccharomyces* should have a close phylogenetic relationship, detectable by 18S rRNA sequence analysis.

According to Fig. 2, the MLEE technique grouped most *C. albicans* strains into a single phenon, except for 17.b strain that was shown to be the less related. These enzymes were able to group all strains of *C. parapsilosis* with strain 17.b of *C. albicans*. Such aspect of multispecific cluster generated from MLEE was already observed by Smith et al. (1990), that characterizing different species of *Brettanomyces* and *Dekkera*, obtained a phenogram in which some strains could not be grouped with high similarity values in their respective species-specific clusters and with interference of some strains in other clusters. Jones and Noble (1982) established electrophoretic comparisons among species of dermatophytes based on MLEE technique showing the inclusion of isolates from certain species inner taxa of other species or even of other genera. These authors pointed out that this fact may occur when only a few isolates of each species are included in the surveys. Boerlin et al. (1995) used 16 enzymatic systems for characterizing 21 genetically atypical strains of chlamydospore-forming and germ tube-positive *C. albicans* recovered from human immunodeficiency virus-positive drug users, and demonstrated that some of these strains were grouped in different clusters, showing high diversity on allelic composition.

Extensive enzyme heterogeneity among *Candida* or other yeast genera had already been observed by other groups of researchers that pointed out that it may occur increasing the possibility of distributing such specimens in various groups or clusters (Lehmann et al. 1989a, 1989b, Caugant & Sandven 1993, Naumov et al. 1997). Lehmann et al. (1991) related the phenomenon of isoenzymatic patterns changing of *C. albicans* during its conservation in laboratories, what could increase the apparent polymorphism. Pujol et al. (1997) found atypical strains of *C. albicans* in Aids patients, showing diverse allelic polymorphism.

When comparing the results assessed by SDS-PAGE and MLEE, it can easily be seen that the first one is more useful for grouping isolates in their respective species, maybe due to the expression of species-specific bands while the second one perhaps better explores the variability at a sub-specific level, being useful for analyses of genetic polymorphism among strains of a certain *Candida* species.

In order to ensure whether or not the UPGMA algorithm assesses resemblance between two OTUs in the dendrogram constructions, a product-moment correlation coefficient was computed between the elements S_{JK} of the original similarity matrix S and cophenetic values C_{JK} of the matrix C derived from the dendrogram. The cophenetic correlation coefficient is a measure of the agreement between similarity values implied by the dendrogram and those of the original similarity matrix (Sokal & Rohlf 1962). These coefficient had values r_{CS} = 0.928 for SDS-PAGE and r_{CS} = 0.932 for MLEE, that range between 0.60 and 0.95 (Sneath & Sokal 1973) or higher than 0.90 (Sokal & Rohlf 1970), considered acceptable, corroborating by this way, with the finds of Farris (1969), that pointed out the fact that UPGMA algorithm always maximizes r_{CS} values.

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 brings relevant information in systematic evaluation of related species. We propose that the grouping of *Candida* species by MLEE patterns from the assayed enzymes is not efficient when only based on a few isolates from more than one species, regarding such resource for surveys conduced with a single species of *Candida*, for what, the MLEE technique had already proved to be a useful method for systematic or epidemiological purposes.

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