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Original article

Multilocus enzyme electrophoresis analysis and exoenzymatic activity of *Candida albicans* strains isolated from women with vaginal candidiasis

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Summary

Twenty-eight *Candida albicans* strains obtained from women with vaginal candidiasis were tested for phospholipase and proteinase production and clustered by multilocus enzyme electrophoresis (MLEE). The proteolytic and phospholipidic activity were considered moderate (0.56 ± 0.12 mm and 0.53 ± 0.09 mm, respectively) for all isolates. The isoenzymes malate dehydrogenase (MDH) and sorbitol dehydrogenase (SDH) showed strong intra-specific discriminatory power. The numerical and genetic interpretation of the bands produced by the isoenzymes tested presented similar discriminatory power. The genetic diversity of the isolates was measured by allelic and genic frequency, perceptual index of polymorphic *loci* (P = 87.5%), average number of alleles per locus, average number of alleles per polymorphic locus, average heterozygosity observed and average heterozygosity expected. We verified that three isoenzymatic loci (*Adh*, *Gdh* and *Sdh-2*) were not in Hardy–Weinberg equilibrium. A dendrogram constructed based on the genetic distance matrix of Nei showed seven clusters; 57.15% (16) of the isolates were considered highly related or indistinguishable, and 42.85% were considered moderately related or unrelated. We did not find a relationship between the clusters and the exoenzymes production.

Key words: Phospholipase, proteinase, multilocus enzyme electrophoresis, *C. albicans*.

Introduction

Candida species are opportunistic pathogens that can cause a wide variety of infections. Vaginal candidiasis is ranked as the second most common cause of gynaecological infections, and it is estimated that 75% of women are affected during their child-bearing years; therefore it is considered one of the most frequent reasons for visiting a gynaecologist.[1,2] Among the species involved in these infections, *Candida albicans* is responsible for the majority of symptomatic episodes, about 90% of cases.[3,4] The severity of these infections is related to virulence factors including phenotypic switching, adhesins, dimorphism and the secretion of hydrolytic enzymes, such as aspartyl proteinases and phospholipases.[1]

For these reasons as well as to improve the response to treatment and prevention of these infections, it should be taken into account whether the infection is caused by a single strain, or if there has been replacement by new, perhaps more resistant, strains with different genotypes. To answer these questions, strain typing by conventional and molecular methods provides

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useful additional information.[5] For this reason, we decided to study phospholipase and proteinase production and molecular (isoenzymes expression) features in this report.

For molecular analysis of strains of *Candida* involved in cases of infection, many techniques have been proposed, such as random amplified polymorphic DNA (RAPD),[6–10] restriction fragment length polymorphism (RFLP),[5,11] real-time PCR,[12,13] microsatellite polymorphism (MSP)[14,15] and multilocus enzyme electrophoresis (MLEE).[5,16–18]

Multilocus enzyme electrophoresis evaluates the polymorphism of isoenzymes or allozymes from strain isolates. Proteins from cell extracts are separated by electrophoresis under native conditions, and the enzymes are visualised by specific enzyme-staining procedures. The main advantage of this method is its high discriminatory power when a sufficient number of enzymes are evaluated. The methodological drawback of MLEE is that at least ten enzymes must be evaluated to provide enough variability among isolates, and it is therefore relatively time consuming.[5]

In this work, we characterised *C. albicans* strains isolated from women with vaginal candidiasis according to their aspartyl proteinases and phospholipase production, and the genetic diversity among the isolates was verified by MLEE. Information obtained in this report can contribute to the understandings about the profile of circulating strains of *C. albicans* involved in vaginal candidiasis cases isolates from patients treated on Centro Integral de Atenção a Saúde da Mulher (CAISM) in Aracaju city, Sergipe, Brazil.

Materials and methods

Isolation and identification of yeast

Fifty patients with vaginal candidiasis between the ages of 17 and 40, treated in CAISM Aracaju city, Sergipe, Brazil were involved in the study. These 28 patients presented colonisation for *C. albicans* and 22 for other species of *Candida*. This present work was approved by the Ethic Committee of the University Hospital in Aracaju, Sergipe, Brazil. Prospective women were enrolled in the study after a written and verbal informed consent was obtained.

In this study we consider only the strains of *C. albicans* as being the most prevalent species involved in cases of vaginal candidiasis. The phenotypic identifications were based on germinative tube and chlamydospore production, growth on CHROMagar® *Candida* (Oxoid, São Paulo, Brazil), thermal tolerance (42 °C), osmotic tolerance (6.5% NaCl) and assimilation of carbon as well as nitrogen sources, as recommended by Kurtzmam and Feel [19]. Molecular identification by semi-nested PCR (snPCR) as described by Milde *et al.* [20] and Ahmad *et al.* [21] was also performed.

The strains studied were deposited in the culture collection of the Laboratory of Applied Microbiology at Sergipe Federal University, in register numbers 4.2, 8.1, 38.4, 40.1, 74v1, 8.3, 8.2, 10.4, 13.6, 74e1, 71e1, 73e1, 39.3, 38.1, 18.2, 33.1, 73v1, 32.1, 73e2, 39.1, 39.2, 47.1, 73v3, 74e2, 35.1, 38.2, 38.5 and 15.2. The strains are maintained at −80 °C.

Phenotypic characterisation

Proteinase and phospholipase assays

Proteinase and phospholipase assays were performed according to Price *et al.* [22] and Samaranayake *et al.* [23] using the semiquantitative egg-yolk plate method. The inoculated egg-yolk plates were incubated at 37 °C, and daily readings were taken from day 7 to day 12. The formation of zones of precipitation around the colony was considered indicative of enzyme production. The zones of precipitation (as reported using the coefficient, *Pz*) were measured, and from these, the proteinase and phospholipase activities were determined according to the protocol of Price *et al.* [22]. Each strain was tested in triplicate, and the average of the three *Pz* values was reported. A *Pz* value of 1 indicated that no activity of the investigated enzyme was detected in the strain. A low *Pz* value indicated high production of the enzyme[24].

Multilocus enzyme electrophoresis analysis

Enzyme extraction and electrophoresis in polyacrylamide gel

The isoenzymes were extracted according to the protocol of Alfenas [25]. After extraction, the material was electrophoresed on polyacrylamide gels and selectively stained for the following metabolic enzyme activities: Alcohol dehydrogenase (ADH – E.C. 1.1.1.1), glucose dehydrogenase (GDH – E.C. 1.1.1.47), aspartate dehydrogenase (ASD – E.C. 1.4.3.x), sorbitol dehydrogenase (SDH – E.C. 1.1.1.14.), malate dehydrogenase (MDH – E.C. 1.1.1.37) and peroxidase (PO – E.C. 1.11.1.7). Electrophoresis was performed in a vertical, continuous system at 130 V and 4–8 °C for 3 h.

Interpretation of numerical and genetic patterns

Numerical pattern interpretation was based on relative mobility values (*Rf*), using the equation $Rf = d/D$ (where d represents the enzymatic molecule running

distance and *D* represents the stain running distance). The *Rf* values were converted to binary values (1 and 0) (data not shown) that represent the presence and absence of bands, respectively. A numerical pattern was obtained from the combinations of bands for all enzymatic activities. Then, the distinct combinations of the polymorphic bands were designated as electrophoretic types (ETs).[18]

Genetic interpretation pattern was performed following commonly accepted rules for the deduction of the allelic composition and genotype for diploid organisms (Alfenas) [25]. The allelic combination of eight loci showed distinct allelic combinations of polymorphic loci; these combinations were called electrophoretic types (ETs), as in Boriollo *et al.* [18]. The observed allelic frequency and the observed and expected genotypic frequencies (Hardy–Weinberg equilibrium test – EHW) from the *C. albicans* strains were calculated for eight loci (*Adh*, *Gdh*, *Asd*, *Sdh-1*, *Sdh-2*, *Mdh-1*, *Mdh-2* and *Mdh-3*). Heterozygosity was determined from the equation $H = 1 - \Sigma i\ (pi^2)$, where *pi* represents the average frequency of allele '*i*' in the population.

Cluster analysis

The set of data furnished by genetic interpretation of the MLEE patterns was submitted to cluster analysis based on the Nei index (d_{ij}) of genetic distance [26] for all of the isolates using NTSYS-pc (version 2.1). Dendrograms, based on the matrix (d_{ij}), were generated by the SAHN method (sequential, agglomerative, hierarchic and non-overlapping clustering) and the UPGMA algorithm (unweighted pair-group method using an arithmetic average). In addition, we used criteria proposed by Tenover *et al.* [27] for the determination of genetically related and unrelated strains.

Results

Identification and phenotypic characterisation

We did not observe differences in identification by the phenotypic vs. the molecular (snPCR) parameters (data not shown).

Phospholipase and proteinase activity were verified in 75.7% and 42.4%, respectively, of the *C. albicans* isolates obtained from vaginal secretions. The average values of *Pz* were 0.56 ± 0.12 mm and 0.53 ± 0.09 mm, respectively. The average values of *Pz* were low, based on the criteria we chose, showing high production of the tested enzymes by the studied isolates.

Numerical and genetic interpretation of the enzymatic patterns

Tables 1 and 2 present, respectively, the numerical and genetic interpretations of the isoenzymatic pattern in the *C. albicans* population. These results allowed the identification of polymorphisms in 87.5% (7 of 8 isoenzymatic loci) of the studied isoenzymatic loci with 2.14 alleles per locus. Polymorphic *loci* (P) are defined as structural gene loci in which the frequency of the most common allele is lower than 0.99 (99%). In the present study, the frequency of the more common alleles at the polymorphic loci ranged from 0.53 to 0.90. The number of fragments (bands) shown by each isoenzymatic system in the *C. albicans* strains ranged from 5 to 12.

The isoenzymatic system that presented the most diversity was MDH, followed by SDH. The ADH, GDH and ASD systems presented minor quantity of distinct band (two bands each). Among the enzymatic systems investigated, peroxidase was the only one that did not present activity in any strain analysed.

The band combinations available in the five enzymatic systems revealed 27 electrophoretic types (ET-1 to ET-27) in the 28 isolates of *C. albicans* analysed (Table 1). ET-5 was the unique electrophoretic type for two isolates (10.4 and 13.6) that were indistinguishable.

The genetic interpretation of the MLEE pattern (Table 2) in this population demonstrated that the loci were polymorphic for two or three alleles (two alleles for *Adh*, *Gdh*, *Asd*, *Sdh-1*, *Sdh-2* and *Mdh-3*, and three alleles for *Mdh-1*). Only one enzymatic locus (*Mdh-2*) was monomorphic. The combination of the alleles at the eight isoenzymatic loci yielded 27 ETs (96.42% of the isolates). Isolates 10.4 and 13.6 presented the same pattern of alleles. These results confirm the findings by the numerical interpretation of the MLEE patterns.

Generally, heterozygotes produced two or three enzymatic bands (two bands for Adh, Gdh, Asd, Sdh-1 and Sdh-2, and three bands for Mdh-1). In the homozygotes, we observed one allele at the Sdh-1, Mdh-2 and Mdh-3 loci and two alleles at the Gdh, Asd and Mdh-1 loci. A Hardy–Weinberg test showed significant differences ($P < 0.01$) between the observed and expected genotypic frequencies at three of the eight enzymatic loci analysed (*Adh*, *Gdh* and *Sdh-2*), which may indicate that these loci are not in Hardy–Weinberg equilibrium. The average heterozygosity observed (0.195) was lower than the average heterozygosity expected (0.344); this result may be related to high

Table 1 Numerical interpretation of electromorphic profiles of *Candida albicans* isolated from women with vaginal candidiasis.

		Electromorphs of five enzymes																
ET	No. isolates	Adh		Gdh		Asd		Sdh				Mdh						
1	1	1	0	1	1	0	1	0	0	1	0	0	1	0	0	0	0	0
2	1	0	1	0	1	0	1	0	1	0	0	0	1	1	0	0	0	0
3	1	0	1	0	1	0	1	0	0	1	1	0	0	1	0	0	0	0
4	1	0	1	0	1	0	1	0	0	1	0	0	1	0	0	0	0	0
5	2	0	1	0	1	0	1	0	0	1	1	0	0	1	0	0	0	1
6	1	1	1	0	1	1	0	0	0	1	0	0	0	0	0	1	1	1
7	1	0	1	0	1	1	0	0	0	1	0	1	0	1	0	0	0	0
8	1	1	0	0	1	1	1	0	0	1	0	1	0	1	0	0	0	0
9	1	0	1	0	1	1	1	0	0	0	1	1	0	1	0	0	0	0
10	1	0	1	1	0	0	1	0	1	0	0	0	0	1	1	0	0	0
11	1	1	1	0	1	0	1	0	0	0	1	0	0	0	1	0	0	0
12	1	0	1	0	1	1	0	0	1	0	0	0	0	0	1	0	0	0
13	1	0	1	1	1	0	1	0	0	1	0	1	0	1	0	0	0	0
14	1	0	1	1	1	1	0	0	1	0	0	0	0	1	1	0	0	0
15	1	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	0
16	1	0	1	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0
17	1	0	1	1	0	0	1	0	0	0	1	0	0	1	0	0	0	1
18	1	0	1	0	1	0	1	0	0	1	0	0	0	1	1	0	0	0
19	1	1	0	0	1	0	1	1	1	0	0	1	0	1	0	0	0	0
20	1	0	1	0	1	0	1	0	0	0	1	1	0	1	0	0	0	0
21	1	0	1	0	1	0	1	0	0	0	1	0	0	1	0	0	0	0
22	1	1	0	1	0	1	1	0	0	0	1	1	0	1	0	0	0	0
23	1	0	1	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0
24	1	1	1	0	1	1	1	0	1	0	0	0	0	1	0	0	0	0
25	1	0	1	0	1	0	1	0	0	0	1	0	0	1	0	0	0	1
26	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0	0	0	1
27	1	0	1	0	1	1	1	0	0	1	0	0	0	1	0	0	0	0

1 and 0 correspond to the presence and absence of bands (electromorphs), respectively.
Adh, alcohol dehydrogenase; Gdh, glucose dehydrogenase; Asd, aspartate dehydrogenase; Sdh, sorbitol dehydrogenase; Mdh, malate dehydrogenase; ET, electrophoretic type.

frequency of null alleles encountered at the *Sdh*-1, *Sdh*-2, *Mdh*-1, *Mdh*-2 and *Mdh*-3 loci.

The genetic diversity of the *C. albicans* isolates was determined based on the values in the genetic distance matrix, d_{ij}.[26] From this, seven groups were distinguished (Fig. 1). Figure 1 summarises graphically the relationship between strains determined by the UPGMA method for matrix generation; 57.15% (16) of the isolates were considered highly related ($0.19 > d_{ij} > 0$) or indistinguishable ($d_{ij} = 0$). The average relatedness of individuals in each cluster was 2.28; 12 isolates (42.85%) were considered moderately related or unrelated (isolates 4.2, 40.1, 8.3, 32.1, 73V3, 74e2, 39.3, 38.1, 47.1, 73e2, 35.1 and 15.2.) The cluster with the highest number of isolates was cluster IV, with six strains. Two isolates (10.4 and 13.6 from group III) were considered indistinguishable.

Analysis using the criteria described by Tenover *et al.* [27] led to similar results: 25 individuals were highly related (two or three different bands), and two individuals were indistinguishable (equal numbers and sizes of bands) and can be considered, epidemiologically, as representatives of the same strain. Finally, just one strain was considered possibly related to the others; it presented between four and six bands that were different from the other samples.

Discussion

Yeast identification and extracellular enzyme production

Our results show that the use of phenotypic and molecular (snPCR) parameters yielded similar identifications. The use of various identification methods is important for minimising the possibility of confusion between *C. albicans* and the closely related species *Candida dubliniensis*.

The production of hydrolytic enzymes by *Candida* species is frequently studied with the intention of comparing

Table 2 Genetic interpretation: allelic profiles of 27 electrophoretic types of *Candida albicans* isolated from women with vaginal candidiasis.

		Alleles of eight enzyme loci*							
ET	No. isolates	*Adh*	*Gdh*	*Asd*	*Sdh-1*	*Sdh-2*	*Mdh-1*	*Mdh-2*	*Mdh-3*
1	1	bb	ab	aa	–	bb	aa	–	–
2	1	aa	aa	aa	aa	–	ab	–	–
3	1	aa	aa	aa	–	ab	aa	–	–
4	1	aa	aa	aa	–	bb	bb	–	–
5	2	aa	aa	aa	–	ab	aa	–	aa
6	1	ab	aa	bb	–	bb	–	–	ac
7	1	aa	aa	bb	–	bb	ac	–	–
8	1	bb	aa	ab	–	bb	ac	–	–
9	1	aa	aa	ab	–	aa	ac	–	–
10	1	aa	bb	aa	aa	–	aa	aa	–
11	1	ab	aa	aa	–	aa	–	aa	–
12	1	aa	aa	bb	aa	–	–	aa	–
13	1	aa	ab	aa	–	bb	ac	–	–
14	1	aa	ab	bb	aa	–	aa	aa	–
15	1	aa	aa	aa	aa	–	ac	–	–
16	1	aa	aa	aa	aa	–	cc	–	–
17	1	aa	bb	aa	–	aa	aa	–	aa
18	1	aa	aa	aa	–	bb	aa	aa	–
19	1	bb	aa	aa	ab	–	ac	–	–
20	1	aa	aa	aa	–	aa	ac	–	–
21	1	aa	aa	aa	–	aa	aa	–	–
22	1	bb	bb	ab	–	aa	ac	–	–
23	1	aa	aa	aa	–	aa	ac	–	–
24	1	ab	aa	ab	aa	–	aa	–	–
25	1	aa	aa	aa	–	aa	aa	–	aa
26	1	ab	ab	ab	ab	bb	ab	–	aa
27	1	aa	aa	ab	–	bb	ac	–	–

Adh, alcohol dehydrogenase; Gdh, glucose dehydrogenase; Asd, aspartate dehydrogenase; Sdh, sorbitol dehydrogenase; Mdh, malate dehydrogenase; ET, electrophoretic type.

*Heterozygotes are shown as ab and ac. [--] null allele.

the activity of these enzymes in isolates from symptomatic or asymptomatic patients or in isolates from different anatomical sites or with the intention of biotyping, as suggested by Williamson *et al.* [28]. According to these authors, only the presence or absence of enzymatic activity is a precise criterion for the biotyping of *C. albicans*. In this work, we consider the proposal by Williamson *et al.* [28]. In the present study, the frequency of phospholipase-positive isolates (75.7%) was more significant than proteinase-positive isolates (42.4% of isolates). However, the averages of the coefficients (*Pz*) of phospholipase and proteinase production were similarly high for both enzymes.

Phospholipase and proteinase activities are considered to play important roles in the pathogenesis of opportunistic fungi. The roles of these two hydrolytic enzymes in *C. albicans* and other yeast appear to be associated with the invasion of the host mucosal epithelia.[1,29] However, researchers have already mentioned that enzyme production determines the potential not only for pathogenesis but also for commensal colonisation by yeast.[29] Oksuz *et al.* [30] verified a high production of these enzymes in different *Candida* species isolated from anatomically distinct sites of healthy adults.

In contrast with our results (a higher frequency of phospholipase-positive isolates), previous investigation with *Candida* strains from vaginitis cases showed proteolytic activity in a majority of strains.[1] This difference may be associated with factors such as the great phenotypic variability.

Numerical and genetic interpretation of the enzymatic patterns

It is known that the band patterns revealed by enzymatic activity can establish the genetic intraspecific relationship of clinically important organisms isolated from the same or different sites.[16–18,31–33] Thus, many works in medical mycology have utilised MLEE patterns to support the understanding of *Candida* spp.

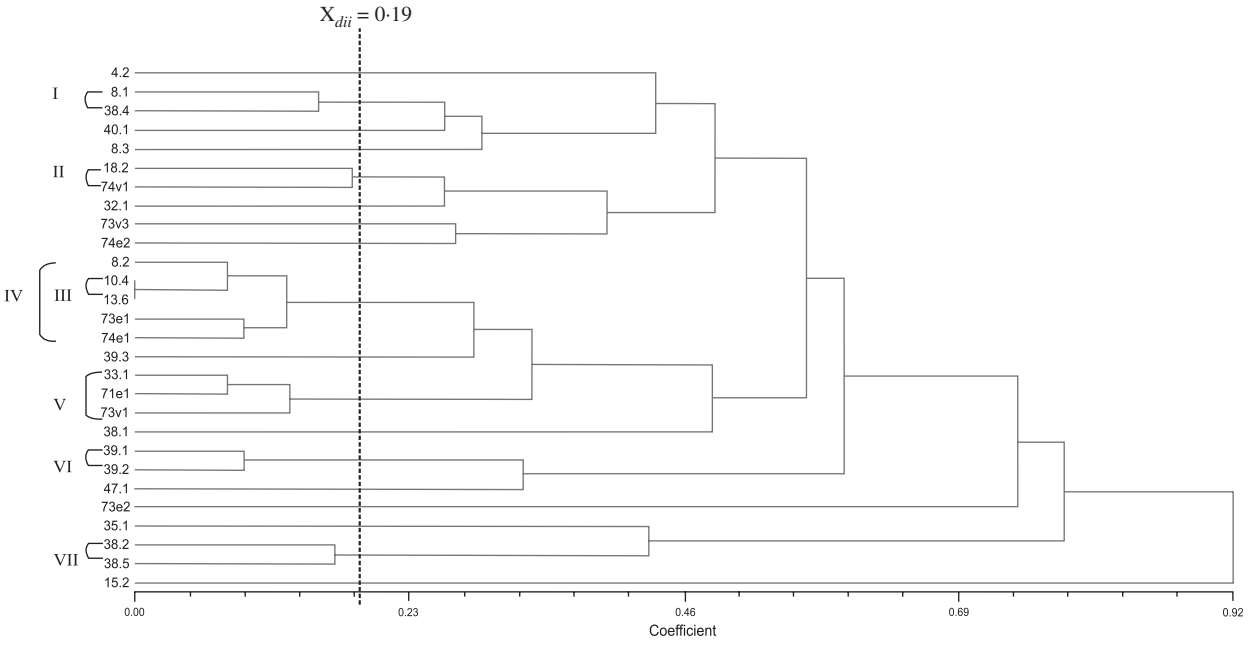

Figure 1 Genetic diversity among 28 samples of *Candida albicans* isolated from women with vaginal candidiasis. Dendrogram of genetic distances obtained by analysis of unweighted pair-group method using an arithmetic average (UPGMA) starting from the d_{ij} coefficient (Nei, 1972).[26] The genetic diversity of the *C. albicans* isolates was determined based on the values in the genetic distance matrix, d_{ij}. The perpendicular line represents the average genetic distance, X_{dij}. Isolates from groups that are formed to the right of this line are considered moderately related or unrelated, whereas isolates from groups formed to the left of this line are considered closely related.

epidemiology and ecology. However, according to Rosa *et al.* [34] MLEE patterns are only efficient for intraspecific characterisations for systematic or epidemiological purposes. Vanhee *et al.* [35] concluded that MLEE patterns are equivalent to RAPD, SSDP (sequence-specific DNA polymorphism), MSP (microsatellite polymorphism) and STR (short tandem repeat) in a comparative study.

The MLEE patterns can be used to measure the intraspecific genetic diversity, by allelic and genic frequency, polymorphic loci perceptual index, heterozygosity, average number of alleles and average number of alleles per polymorphic locus.

In our study, the percentage of polymorphic loci (structural genic loci at which the frequency of the most common allele was lower than 0.99) found was 87.5% (7 of 8 isoenzymatic loci). This result indicates that the number of analysed loci was sufficient to discriminate between the isolates in the studied population. According to the criteria of Botstein *et al.* [36], the polymorphic loci analysed in this study can be considered very informative because the percentage of the more common allele was above 50%.

The numbers of loci related to MDH and SDH (3 and 2) verified herein are in accordance with the findings of other authors.[17,18,26,37] Numerical interpretation allows the detection of isoenzymatic diversity by the presence or absence of bands for that enzyme. The number of ETs identified by genetic (allelic frequency) and numerical interpretation was the same, presenting a concordance of 100%. These results support and validate the numerical interpretation and agree with Boriolo *et al.* [17], who said that discriminatory power of the numerical interpretation of enzymatic profiles is around 97%.

Discordance between observed and expected genotypic frequencies of the *Adh*, *Gdh* and *Sdh*-2 loci may be related to high frequencies of the null alleles.

In general, the observed heterozygosity average observed in the total population of *C. albicans* (0.195) was less than the expected frequency (0.344). This result indicates an excess of homozygotes that, in the population structure of some organisms, can be interpreted as a group of interconnected strains.[38] The H values revealing a low degree of heterozygosity (<50%) were similar to those found by Caugant *et al.* [31] (H = 0.13) and Boriollo [39] (0.19). Some authors say that the electrophoresis conditions used, the fact that all isolates were from the same site and the number of isoenzymatic systems studied[39] can explain the low

values of H. We do not think these facts interfere with our results because, as mentioned, the number of isoenzymatic systems used allowed the distinction of three alleles and five genotypes. Moreover, of the 28 isolates analysed, it was possible to differentiate 27 strains.

However, the low heterozygosity values can be attributed to the high frequency of null alleles. According to Alfenas [25], null alleles are defined as alleles that control the alloenzymes, and these alleles do not express activity during the gel colouration process. The null alleles can, however, represent either a defective enzyme or one that is unstable during the extraction, storage and electrophoresis processes. Null alleles that persist in the genome are those that are balanced either by alternative alleles in heterozygotes or by isoenzymes controlled by another locus. These types of balancing may explain the high frequency of null alleles in this study.

The genetic diversity shown in the dendrograms based on Nei's coefficient confirmed that the ADH, GDH and ASD loci present minor discriminatory power, producing few clusters with higher numbers of isolates per cluster (data not shown). This information supports our idea that the high discrimination produced by the combination of all of the enzymes occurs because of the SDH and MDH band profiles, and these are mostly responsible for the distinction between isolates. This information also supports the individual, system-by-system interpretation of results and does not support the conclusion of Lehmann *et al.* [40] that the variability pattern of SDH is relevant only to *C. albicans* strain distinction. According to our results, it is possible to make an intra-specific distinction.

Analyses of highly related, moderately related and unrelated individuals by the criteria of Nei [26] and Tenover *et al.* [27] showed that they have a similar discriminatory power and can be used for this kind of analysis. The clusters identified in the dendrogram show the existence of highly and moderately related isolates of *C. albicans*; however, we did not observe a correlation with the results of phenotypic tests (phospholipase and proteinase activity). Similarly, Boerlin *et al.* [32], Guennec *et al.* [41] and Pujol *et al.* [7], using MLEE and cluster analysis in isolates of *C. albicans* from healthy and immunocompromised patients (in Montpellier, France; Lausanne, Switzerland and Abidjan, Costa do Marfim), found highly related clusters without any correlation with clinical or phenotypic characteristics. In other studies the use of different methods of typing for *C. albicans* also did not find a relationship between phospholipase activity and genotypes.[42–44] This could be related to different ecosystems with their specific characteristics and many factors that influence the expression of these enzymes, and having as a consequence, various degrees of enzymatic activity.[43]

The analysis by MLEE patterns revealed that *C. albicans* isolates could be classified into seven groups by the criteria of Nei [26] and Tenover *et al.* [27]. Furthermore, the allelic frequency and heterozygosity analysis point to high genetic diversity.

In summary, we can conclude that our results revealed distinct *C. albicans* genotypes, isolated from the same anatomic site and involved in the same kind of infection, without correlation with phospholipases and proteinase production. This can be used to demonstrate the importance of the knowledge of the prevalent strains characteristics from a determined place and to valorise molecular and phenotypic characterisation as a precious tool for understanding the medical importance of microorganisms.

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References

1 De Bernardis FL, Agatensi IK, Ross GW *et al.* Evidence for a role for secreted aspartate proteinase of *Candida albicans* in vulvovaginal candidiasis. *J Infect Dis* 1990; **161**: 1276–83.
2 Lipsky MS, Waters T, Sharp LK. Impact of vaginal antifungal products on utilization of health care services: evidence from physician visits. *J Am Board Fam Pract* 2000; **13**: 178–82.
3 Sobel JD, Faro S, Force RW *et al.* Vulvovaginal candidiasis: epidemiologic, diagnostic, and therapeutic considerations. *Am J Obstet Gynecol* 1998; **178**: 203–11.
4 Sobel JD. Genital candidiasis. *Medicine* 2005; **33**: 62–65.
5 Gil-Lamaignere C, Roilides E, Hacker J, Müller F-MC. Molecular typing for fungi—a critical review of the possibilities and limitations of currently and future methods. *Clin Microbiol Infect* 2003; **9**: 172–85.
6 Holmberg K, Feroze F. Evaluation of an optimized system for random amplified polymorphism DNA (RAPD)-analysis for genotypic mapping of *Candida albicans* strains. *J Clin Lab Anal* 1996; **10**: 59–69.

7 Pujol C, Joly S, Lockhart SR, Noel S, Tibayrenc M, Soll DR. Parity among the randomly amplified polymorphic DNA method, multilocus enzyme electrophoresis, and Southern blot hybridization with the moderately repetitive DNA probe Ca3 for fingerprinting *Candida albicans*. *J Clin Microbiol* 1997; **35**: 2348–58.
8 Tietz HJ, Kussner A, Thanos M, De Andrade MP, Presber W, Schonian G. Phenotypic and genotypic characterization of unusual vaginal isolates of *Candida albicans* from Africa. *J Clin Microbiol* 1995; **33**: 2462–5.
9 Clemons KV, Feroze F, Holmberg K, Stevens DA. Comparative analysis of genetic variability among *Candida albicans* isolates from different geographic locales by three genotypic methods. *J Clin Microbiol* 1997; **35**: 1332–6.
10 Chong PP, Hadi SRA, Lee YL *et al.* Genotyping and drug resistance profile of *Candida* spp. In recurrent and one-off vaginitis, and high association of non-albicans species with non-pregnant status. *Infect Genet Evol* 2007; **7**: 449–56.
11 Abbes S, Sellami H, Sellami A *et al.* Variability of *Candida albicans* strains in ICU in Tunisia hospital. *J Mycologie Méd* 2008; **18**: 10–15.
12 Dunyach C, Bertout S, Phelipeau C. Detection and identification of *Candida* spp. in human serum by LightCycler real-time polymerase chain reaction. *Diag Microbiol Infect Dis* 2008; **60**: 263–71.
13 Metwally L, Hogg G, Coyle PV. Rapid differentiation between fluconazole-sensitive and -resistant species of *Candida* directly from positive blood culture bottles by real-time PCR. *J Med Microbiol* 2007; **56**(Pt 7): 964–70.
14 Walczak E, Czaplińska A, Barszczewski W, Wilgosz M, Wojtatowicz M, Robak M. RAPD with microsatellite as a tool for differentiation of *Candida* genus yeasts isolated in brewing. *Food Microbiol* 2007; **24**: 305–12.
15 Botterel F, Desterke C, Costa C, Bretagne S. Analysis of microsatellite markers of *Candida albicans* used for rapid typing. *J Clin Microbiol* 2001; **39**: 4076–81.
16 Rosa EAR, Rosa RT, Boriollo MFG, Bernardo WLC., Hofling JF. Oral *Candida albicans* and *Candida dubliniensis* differentiation by multilocus enzyme electrophoresis and sodium dodecyl sulphate-polyacrylamide gel electrophoresis. *Rev Argent Microbiol* 2003; **35**: 24–28.
17 Boriollo MFG, Rosa EAR, Bernardo WLC, Spolidorio DMP, Goncalves RB, Hofling JF. Multilocus enzyme electrophoresis typing of *Candida albicans* populations isolated from healthy children according to socioeconomic background. *Rev Bras Epidemiol* 2005; **8**: 1–16.
18 Boriollo MFG, Rosa EAR, Rosa RT, Hofling JF. Parity among interpretation methods of MLEE patterns and disparity among clustering methods in epidemiological typing of *Candida albicans*. *J Microbiol Methods* 2006; **64**: 346–65.
19 Kurtzmam CP, Feel JW. *The Yeasts, Taxonomic Study*. Amsterdan, The Netherlands: Elsevier Science, 2001.
20 Milde K, Kostkaa V, Kaletaa EF, Willems HC, Jager C. Multiplex-PCR-based differentiation and characterization of *Candida*-isolates derived from tortoises (Testudinidae). *Vet Microbiol* 2001; **76**: 395–402.
21 Ahmad S, Khan Z, Mustafa SA, Khan UZ. Seminested PCR for diagnosis of candidemia: comparison with culture, antigen detection and biochemical methods for species identification. *J Clin Microbiol* 2002; **40**: 2483–9.
22 Price MF, Wilkinson ID, Gentry LO. Plate method for detection of phospholipase activity in *Candida albicans*. *Sabouraudia* 1982; **20**: 7–10.
23 Samaranayake P, Raeside JM, Macfarlane TW. Factors affecting the phospholipase activity of *Candida* species *in vitro*. *Sabouraudia* 1984; **22**: 201–7.
24 Mohan V, Ballal M. Proteinase and phospholipase activity as virulence factors in *Candida* species isolated from blood. *Rev Iberoam Micol* 2008; **25**: 208–10.
25 Alfenas AC *Eletroforese e marcadores bioquímicos em plantas e microrganismos*. Viçosa: Ed. UFV, 2006: Vol. **627**: 216–89.
26 Nei M. Genetic distances between populations. *Am Naturalist* 1972; **106**: 283–92.
27 Tenover Fc, Arbeit Rd, Goering Rv, Mickelsen PA. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* 1995; **33**: 2233–9.
28 Williamson MI, Samaranayake LP, MacFarlane TW. Phospholipase activity as a criterion for biotyping *Candida albicans*. *J Med Vet Mycol* 1986; **24**: 415–7.
29 Niewerth M, Korting HC. Phospholipases of *Candida albicans* phospholipases von *Candida albicans*. *Mycoses* 2001; **44**: 361–7.
30 Oksuz S, Sahin I, Yildirim M. Phospholipase and proteinase activities in different *Candida* species isolated from anatomically distinct sites of healthy adults. *J Inf Dis* 2007; **60**: 280–3.
31 Caugant DA, Sandven P. Epidemiological analysis of *Candida albicans* strains by multilocus enzyme electrophoresis. *J Clin Microbiol* 1993; **31**: 215–20.
32 Boerlin P. Applications of multilocus enzyme electrophoresis in medical microbiology. *J Microbiol Methods* 1997; **28**: 221–31.
33 Rosa EAR, Rosa RT, Pereira CV, Hofling JF. Inter and intra-specific genetic variability of oral *Candida* species. *Rev Iberoam Micol* 2001; **18**: 60–64.
34 Rosa EAR, Rosa RT, Pereira CV, Hofling JF. Grouping oral *Candida* species by multilocus enzyme electrophoresis. *Int J Syst Evol Microbiol* 2000; **23**: 1343–9.
35 Vanhee LME, Symoens F, Jacobsen MD, Nelis HJ, Coenye T. Comparison of multiple typing methods for *Aspergillus fumigatus*. *Clin Microb Infection* 2009; **15**: 643–50.
36 Botstein D, White RL, Skolmick H *et al.* Construction of a genetic linkage map in man using restriction fragment length polymorphism. *Am J Human Genetics* 1980; **32**: 314–31.
37 Rosa EAR, Rosa RT, Pereira CV, Boriollo MFG, Hofling JF. Analysis of parity between protein-based electrophoretic

methods for characterization of oral *Candida* species. *Mem Inst Oswaldo Cruz* 2000; **95**: 801–6.
38 Hartl DL, Clark AG. *Principles of Population Genetics*. Massachusetts: Sinauer Associates, Inc. Publishers Suderland, 1997.
39 Boriollo MFG. *Análise da diversidade genética de amostras de Candida albicans isoladas da cavidade bucal de crianças saudáveis por eletroforese de enzima multiloco*. Piracicaba, São Paulo, Brazil: Tese apresentada à Faculdade de odontologia de Piracicaba, da Universidade Estadual de Campinas, 2004, 202 p.
40 Lehmann PF, Kemker BJ, Hsiao CB, Dev S. Isoenzyme biotypes of *Candida* species. *J Clin Microbiol* 1989; **27**: 2514–21.
41 Guennec RL, Reynes J, Mallie M, Pujol C, Janbon F, Bastide J-M. Fluconazole- and itraconazole-resistant *Candida albicans* strains from AIDS patients: multilocus enzyme electrophoresis analysis and antifungal susceptibilities. *J Clin Microbiol* 1995; **33**: 2732–7.
42 Vidotto V, Koga-Ito CY, Milano R, Fianchino B, Poto J. Correlation between germ tube production, phospholipase activity and serotype distribution in *Candida albicans*. *Rev Iberoam Micol* 1999; **16**: 208–10.
43 Mardegan RC, Klein MI, Golvea MB, Rodrigues JAO, Gonçalves RB, Hofling JF. Biotyping and genotypic diversity among oral *Candida albicans* strains from caries-free and caries-active healthy children. *Braz J Microbiol* 2006; **37**: 26–32.
44 Barros LM, Boriollo MFG, Alves ACBA, Klein MI, Gonçalves RB, Höfling JF. Genetic diversity and exoenzyme activities of *Candida albicans* and *Candida dubliniensis* isolated from the oral cavity of Brazilian periodontal patients. *Arch Oral Biol* 2008; **53**: 1172–8.

