Original Article

Evaluating the Typing Power of Six Isoenzymatic Systems for Differentiation of Clinical and Standard Isolates of *Candida* Species

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Abstract

Background: Due to the increasing prevalence of candidiasis, early detection of the causative agents may pave the way for the management of this infection. The present study aimed to assess the discriminative power of the six isoenzymatic systems for differentiating the *Candida* species.

Materials and Methods: Sixteen standard *Candida albicans* and *Candida dubliniensis* strains and 30 fluconazole-sensitive and fluconazole-resistant clinical strains of *Candida albicans* were analyzed using a Multilocus Enzyme Electrophoresis (MLEE) method, including six enzymatic systems consisting of malate dehydrogenase (MDH), phosphoglucomutase (PGM), glucose-phosphate isomerase (GPI), glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGD), and malic enzyme (ME).

Results: Among the six enzymatic systems, ME showed no diagnostic activity, whereas MDH provided the best species-specific pattern for species discrimination. In addition, the MDH and G6PD systems provided a discriminatory pattern for differentiating *C. dubliniensis* from *C. albicans* isolates. The same isoenzymatic activity was detected in all 36 standard and clinical isolates. Moreover, the results showed no correlation between the isoenzymatic profiles and drug resistance.

Conclusion: Among the investigated MLEE systems, MDH was able to differentiate between *Candida albicans* and *Candida dubliniensis*. Although no association was detected between isoenzyme patterns and fluconazole resistance in this investigation, isoenzyme patterns are likely correlated with virulence factors between species and even within species. To answer these questions, additional studies should be done on more strains.

Keywords: *Candida*, electrophoresis, isoenzymes, mycological typing techniques

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Introduction

Candidiasis is a vast spectrum fungal infection caused by *Candida* opportunistic yeasts species, which are from normal microflora.[1-3] Following some predisposing factors, such as immunosuppression, the microflora population of yeasts switches to infection.[4] The most prevalent etiological agents are *Candida albicans*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. krusei*, and *C. dubliniensis*.[5-8] Moreover, *Candida* species are the most frequent fungi isolated from cancer patients, the

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second causative agent of catheter-associated urinary tract infections, and the third pathogenic organism responsible for catheter-associated and pediatric sepsis.[9-12] There are several methods to diagnose *Candida* species in addition to conventional (direct examination and culture), biochemical, and molecular methods.[13,14] One of these methods is isoenzyme (isozyme) isotyping.

The term "isoenzyme" describes an enzyme that performs the same tasks despite the different molecular weights. It is possible to separate these molecules based on their different molecular weights and electric charges using electrophoretic systems. Each enzyme may have two or more isozymes. Zymodeme refers to a population of an organism with the same electrophoretic patterns of enzymatic systems.[15] *Candida* species exhibit different electrophoretic patterns (different zymodemes). Assessment of several isoenzymes has been suggested for typing an organism.[16-18] Recently, researchers have been developing different comparative methods for isoenzyme biotypes to discriminate different types of microorganisms.[19] Based on their molecular weights and electric charges, nondenaturized enzymes can be seen moving physically in the chemical background of substances like cellulose acetate, polyacrylamide, and agarose using the multilocus enzyme electrophoresis (MLEE) approach.

The current study aims to evaluate the diagnostic efficacy of the MLEE tool composed of six isoenzyme systems for the differentiation of medically significant *Candida* species: malate dehydrogenase (MDH), phosphoglucomutase (PGM), glucose-phosphate isomerase (GPI), glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGD), and malic enzyme (ME). In addition, the isozyme pattern of fluconazole-sensitive and resistant strains of *C. albicans* was studied and compared.

Materials and Methods

Yeast isolates

A total of 16 American type culture collection (ATCC) (www.atcc.org) and the centralbureau voor schimmelcultures (CBS) (www.wi.knaw.nl) strains of *Candida,* including *C. albicans* (ATCC 10261, ATCC 4356, CBS 5982, CBS 2730, CBS 1949, CBS 1912, CBS 1905, and CBS 562), *C. tropicalis* (ATCC 750), *C. krusei* (ATCC 6258), *C. glabrata* (ATCC 90030), *C. parapsilosis* (ATCC 4344), and *C. dubliniensis* (CBS 8500, CBS7501, CBS7277, and CBS 7277) were cultured in Sabouraud Dextrose Agar (SDA) (37°C, 48 h). In addition, 11 clinical strains of *C. dubliniensis* and 17 clinical strains of *Candida albicans* identified previously by PCR-RFLP method,[20] including fluconazole-sensitive and fluconazole-resistant strains, were used in this study.[21]

Modification of cultures

Since the presence of amino acids in culture media may inhibit the identification of isoenzymes,[22] fresh yeast colonies were transferred to 50 mL of Yeast Nitrogen Broth (YNB) (Merck-Germany) as the amino acid-free medium. The flasks were incubated at room temperature and rotated at 100 rpm for 24 h.

Protein extraction

Following 5 min at 1000 rpm, the supernatant was removed, cell suspensions were washed in Tris hydrochloride buffer (0.1 M, pH 8.0), and they were centrifuged at 2000 rpm for 10 min (3–4 times). The washed yeasts were transferred to 1.5 mL tubes filled with 0.5 mL glass beads (0.45–0.50 mm diameter) and tris hydrochloride buffer. The tubes were agitated vigorously and placed in an ice bath (−20°C) to avoid the destruction of proteins by high temperatures. This procedure was repeated four times. The supernatant was collected after centrifugation (14,000 rpm, 2°C, 2 min), and the extracts were kept in tubes at −20°C for further assays.[23]

Determination of protein concentration

The protein content of the extracts was measured by the Bradford–Lowry method.[24,25] The yeast proteins were extracted, as explained previously.[26] Bradford solution and bovine serum albumin were used as standards, and stock solutions were prepared and stored in dark tubes. The quality of the protein concentrations was evaluated by drawing standard curves with an albumin solution. Serial dilutions were prepared for the standard albumin solution, and each dilution's optical absorption or OD was recorded. Finally, the Bradford curves were drawn for each sample [Figure 1].

Isoenzyme electrophoresis

The protein concentration of the extracts was diluted in Tris hydrochloride buffer (0.1 M, pH = 8.0) to achieve 0.5–1.5 mg/mL of protein per sample. Then, 10 mL of each sample was loaded on a discontinuous polyacrylamide gel electrophoresis (PAGE) tank (1.5-mm-thick slab). Electrophoresis was performed using 4% stacking gel, 7.0% separating gel, and a tank buffer of tris-HCl (pH = 8.3), which ran under 2 mA/well for 150 min. Six enzymatic systems (PGM, ME, 6PGD, GPI, MDH, and G6PD) were used to isolate and differentiate *Candida* species. Each enzymatic system contained particular

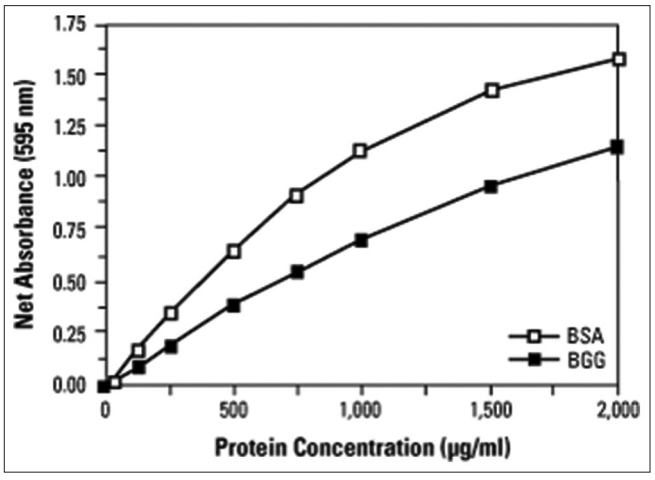

Figure 1: The standard Bradford curves

substrates, buffers, coenzymes, and color catalysts [Table 1]. Accordingly, 3–5 µg of the protein concentration was used for the ME system, 0.5–1 µg for MDH, and 1.2-1.5 µg for 6PGD, GPI, G6PD, and PGM. The enzymatic systems used $MgCl_2$ as the biochemical reaction enhancer.[26-28]

Numerical interpretation of isoenzyme patterns

The relative migration or relative factor (RF) value of each band was measured separately. The RF value has been defined as the ratio of the distance moved by the electrophoretic band to the distance moved by the bromophenol blue color marker. Here, RF was indicated by the d/D equation (d is the distance between the beginning of the gel and the band formation site, and D is the distance between the front of the gel and the bromophenol blue color marker). Electrophoretic bands were interpreted according to the previous reports.[26,29-32]

Hamming distance matrix

To better understand the results of the MDH MLEE assays, the distance matrix was calculated based on Hamming distance method. This matrix displayed the pairwise distances between all of the banding patterns obtained. The corresponding dendrogram was drawn using the average-linkage method.

Results

Protein extracts from different species of *Candida* were subjected to one-dimensional electrophoresis, which revealed that only the ME system among the investigated systems lacked any enzymatic activity. The remaining enzymes provided electrophoretic bands, as shown in Figures 2 and 3. Accordingly, the MDH indicated the best species-specific pattern to discriminate all the examined species. Other enzymatic systems such as MDH, GPI, and G6PD provided different patterns for differentiating *C. dubliniensis* from *C. albicans.* The enzymatic pattern of GPI has been depicted in Figure 2.b. Accordingly, all the species exhibited one allele for this enzyme with two RF ratios (0.6 and 0.5), except for *C. dubliniensis,* which presented two alleles (RFs: 0.5 and 0.52). As shown in Figure 2.c, all species revealed similar bands for 6PGD (RF = 0.25), except for *C. krusei* and *C. glabrata*. Although PGM revealed slightly weaker bands than the other enzymes, it discriminated the examined species, except for *C. krusei* and *C. tropicalis.* However, the enzymatic activity of this enzyme was much higher in *C. krusei* than in *C. tropicalis* [Figure 2.d].

In the MDH system, four patterns were found within the examined species [Figure 2.e]. Among *Candid*a species, the gene that corresponded to this enzyme had one allele in *C. parapsilosis* and *C. krusei* (RF = 0.5), two alleles in *C. glabrata* and *C. tropicalis* (RFs = 0.5 and 0.52) and *C. dubliniensis* (RFs = 0.25 and 0.75), and three alleles in *C. albicans* (RFs = 0.37, 0.67, and 0.7). All studied *Candid*a species had one allele for G6PD with an RF ratio of 0.75 for *C. krusei*, *C. tropicalis,* and *C. glabrata*, 0.5 for *C. parapsilosis*, 0.45 for *C. dubliniensis*, and 0.55 for *C. albicans* [Figure 2.f]. Seven distinct isoenzyme profiles were detected based on the banding patterns resulting from the MDH MLEE assays in the 46 standard and clinically isolated strains of *C. albicans* and *C. dubliniensis*. These profiles and the number of strains in each group have been presented in Table 2. The Hamming distance matrix of the obtained profiles

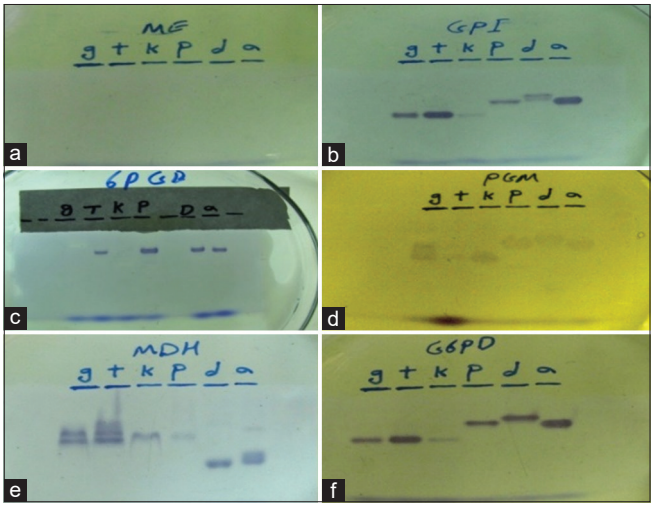

Figure 2: The isoenzyme profiles of Candida species (g: *C. glabrata*, t: *C. tropicalis*, k: *C. krusei*, p: *C. parapsilosis*, d: *C. dubliniensis*, and a: *C. albicans*). a. ME: Malic enzyme b. GP: Glucose-phosphate isomerase, c. 6PGD, 6-phosphogluconate dehydrogenase, d. PGM: Phosphoglucomutase, e. MDH: Malate dehydrogenase, f. G6PD: Glucose-6-phosphate dehydrogenase

Table 1: Isoenzymatic systems and their compositions.

Enzyme	EC No.	Buffer	Substrate	Coenzyme	Salt	Dye catalyzer
G6PD	1.1.1.49.	0.3 M tris hydrochloride pH=7.4	G6pD (1 mL)	NADP, agarose	$MgCl_2$ (250 µ)	MTT, PMS (0.5 mL)
GPI	5.3.1.9.	0.3 M tris hydrochloride pH=8 (6.7 mL)	Dp6p (1 mL), G6PD	NADH, agarose	$MgCl_2$ (0.2 mL)	MTT, PMS (0.5 mL)
MDH	1.1.1.37.	0.3 M tris hydrochloride pH=7.1	Malic acid (750 µ)	NAD, agarose		MTT, PMS (0.5 mL)
ME	1.1.1.39.	1 M tris hydrochloride pH=7.1 (10 mL)	Malic acid (0.6 mL)	NADP, agarose	$MgCl_2$	MTT (1.2 mg) PMS (3 mg)
6PGD	1.1.1.43.	0.3 M tris hydrochloride pH=7.4 (6 mL)	6-phosphogluconic acid (1 mL)	NADP, agarose	$MgCl_2$ (250 µ)	MTT, PMS (0.5 mL)
PGM	5.4.2.2.	0.3 M tris hydrochloride pH=8 (6 mL)	Glucose phosphate (20 mg/1 mLDW)	NADP, DW, agarose	$MgCl_2$ (0.2 mL)	MTT, PMS (0.5 mL)

and the corresponding dendrogram has been displayed in Figure 4. The results revealed no significant relationships between the isoenzymatic patterns and the susceptibility or resistance of *Candida* species.

In what follows, it is suggested that further research be conducted on additional isoenzyme systems, *Candida* species, and strains, and the potential relationship between these isoenzymes and geographic characteristics, the severity of pathogenicity in the host, treatment response, biofilm formation, and other yeast characteristics.

Discussion

The present study tested the typing potential of six enzymatic systems for *Candida* yeasts. The PGM system provided the best species-specific pattern for discriminating between *Candida* species. The MDH system provided two distinct patterns for differentiating *C. albicans* from *C. dubliniensis*. The results revealed no significant relationship between the isoenzymatic patterns and the susceptibility or resistance of *Candida* species. Yet, these enzymatic systems showed the same activity in all standard and clinical isolates.

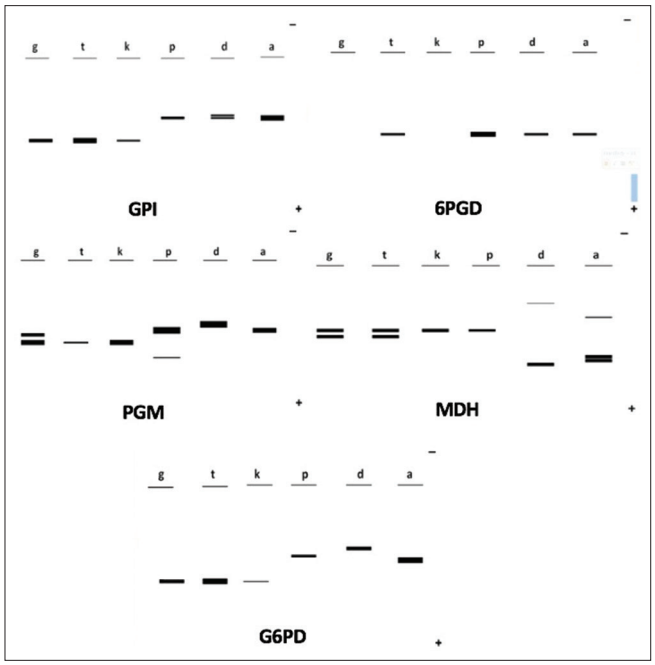

Figure 3: Schematic diagrams of the isoenzyme profiles of *Candida* species (g: *C. glabrata*, t: *C. tropicalis*, k: *C. krusei*, p: *C. parapsilosis*, d: *C. dubliniensis*, and a: *C. albicans*). GPI, glucose-phosphate isomerase; 6PGD, 6 phosphogluconate dehydrogenase; PGM, phosphoglucomutase; MDH, malate dehydrogenase; G6PD, glucose-6-phosphate dehydrogenase

The MLLE was initially used to classify bacteria such as *Mycobacterium*, *Pseudomonas,* and *Escherichia coli*. It has been successfully applied for fungal biotyping and taxonomy, especially *Candida* yeasts.[16,31,33] Generally, three main electrophoresis systems are used to determine enzyme zymodemes: focusing, discontinuous, and continuous electrophoresis.[34] In the focusing isoelectric technique, isoenzymes (proteins) are separated based on their electric charges.[34,35] Each band represents the expression of the enzyme's gene and protein. Therefore, in this method, the genetic background of the organism can be indirectly traced via the evaluation of the presented band patterns.[34,35] The results of these assays are so accurate that they are called fingerprints. Hence, this method can be considered an important genetic marker for diagnostic purposes. In recent years, the MLEE has been used as the standard method for analyzing eukaryotic populations. Moreover, medical mycologists have been using MLEE as a modified technique in the genetic taxonomy and epidemiology of fungi. As the first study in the field, Lehmann *et al.*[23] developed the polyacrylamide gel electrophoresis method for analyzing the isoenzyme profiles of *Candida* species.[27] In addition, Doebbeling *et al.*[36] evaluated several isoenzyme profiles of *C. tropicalis* isolates from an outbreak of sternal wound infections. Guennec *et al.*[37] also assayed *C. albicans* strain diversity in four AIDS patients with recurrent oropharyngeal candidiasis who showed resistance to fluconazole and itraconazole using MLEE and *in vitro* susceptibility testing via the broth microdilution method. Badoc *et al.*[38] investigated the possible differences among *C. dubliniensis*, *C. albicans*, and atypical *C. albicans* using phenotypic and MLEE methods.

Moreover, Rosa *et al.*[28] assessed the diversity of five common *Candida* species isolated from the oral cavity using MLEE and

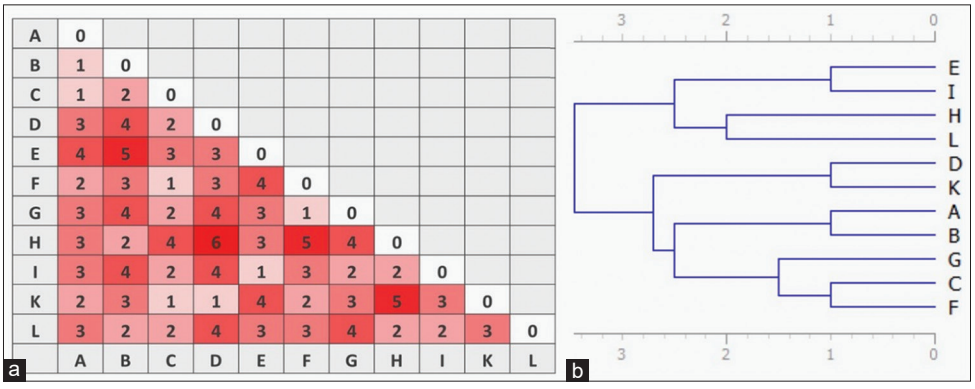

Figure 4: The Hamming distance matrix of seven MDH MLEE banding profiles (a) and the corresponding dendrogram (b)

Table 2: The electrophoretic patterns of isoenzymatic systems for typing of clinical isolates of *C. albicans* and *C. dubliniensis*.

MDH isozymes (RF)	MDH MLEE banding patterns										
	A	B	C	D	E	F	G	H	I	K	L
MDH1 (0.23)	+	+	-	-	-	-	-	+	-	-	-
MDH2 (0.27)	-	-	-	+	-	-	-	-	-	+	-
MDH3 (0.34)	-	+	-	-	-	-	-	+	-	-	+
MDH4 (0.40)	-	-	-	+	+	-	-	-	-	-	-
MDH5 (0.53)	-	-	-	-	+	-	-	+	+	-	+
MDH6 (0.60)	+	+	+	+	-	+	-	-	-	+	+
MDH7 (0.69)	-	-	-	-	-	+	+	-	-	-	-
C. albicans (25 strains)	5	1	4	1	1	1	8	0	0	1	2
C. dubliniensis (15 strains)	0	0	1	0	0	0	7	1	2	4	0

numerical taxonomic methods, but they could not separate the strains. Another study surveyed different enzyme profiles of *C. albicans* in non-neutropenic patients typed by MLEE in different care units. The results demonstrated a noticeable difference among the strains.[39] Boriollo *et al.*[40] analyzed the hydrolytic enzyme activity and genetic diversity profiles of *C. albicans* strains isolated from the oral sites of patients with diabetes and their nondiabetic peers using Secreted Aspartyl Proteinases (SAPs) and Phospholipases (PLs) systems. They applied MLEE, Electrophoretic Karyotyping (EK), and microsatellite markers to the 75 oral isolates and compared their discriminatory power and ability to differentiate and group *C. albicans* isolates. They also evaluated the similarity of each set's fingerprinting method.[29] Santos *et al.*[41] clustered different strains of *C. albicans* obtained from women with vaginal candidiasis using the MLEE method. In addition, the genetic diversity of the isolates was measured via allelic and genomic frequencies.

Lehmann *et al*.[23] separated two species by distinct isoenzyme patterns. Within each species, variations were found for several isoenzymes. These results allowed the development of new methods for biotyping yeasts. Moreover, they analyzed the isoenzyme profiles of multiple strains of two commonly used *C. albicans* reference cultures. Some strains showed variations in their G6PDH isoenzyme system.[27] In comparison with the present study, the G6PDH system showed higher discriminatory power in the research carried out by Lehmann *et al*.[27] This difference in the activity of G6PDH was also evident in the results of the studies conducted by Doebbeling *et al.*[36] and Rosa *et al.*[28] In the study by Doebbeling *et al*.,[36] the outbreak isolates and their isoenzymes were readily identified, which were quite different from those of the control isolates.

The electrophoretic karyotypes and their CHEF-RFA types were established, as well. The genetic relationships of the isolates were previously established via restriction fragment analysis. In the study by Badoc *et al*.,[38] five of the 17 enzyme systems (chiefly G6PDH) presented no discriminatory roles. Similar results were obtained by Briollo *et al*.,[40] which revealed heterozygosity in MDH and G6PDH systems with low efficacy for *Candida* yeasts grouping. The MDH system's ability to differentiate *Candida* yeasts was also assessed by Lehmann *et al*.,[27] and their results were repeated in our findings. However, contradictory results were achieved by Badoc *et al.*[38] On the other hand, Santos *et al.*[41] reported the high diversity, activity, and frequent alleles of the *MDH* gene loci. Rosa *et al.*[28] applied the ME system and presented the high discriminatory power of *Candida* yeasts, contrary to the current study results. In addition, the ME system showed no enzymatic activity, which was consistent with the previous studies that disclosed the inactivity of some of the tested systems.[23,30] This finding may be attributed to the numerous subcultures that lead to mutations in different gene loci of fungi. Arnavielhe *et al.*[39] assayed the G6PDH, GPI, PGM, ME, and MDH systems and indicated that the ME system had a monomorphic band in the gel electrophoresis run. They also established the heterozygosity of the MDH gene loci in all tested *Candida* species. Boriollo *et al*.[40] indicated that all three methods (MLEE, SSR, and EK) were powerful tools for typing *C. albicans* strains and were thus valuable for further epidemiological studies. In that study, isoenzyme typing was performed using 11 enzyme systems: ADH, SDH, M1P, MDH, IDH, GDH, G6PDH, ASD, CAT, PO, and LAP. The results revealed the discriminatory power of the G6PDH system.

On the other hand, four out of the six yeast species showed almost similar enzymatic patterns in the 6-PG. Therefore, this system was not suitable for differentiating *Candida* species. These findings were not in agreement with those of the research by Arnavielhe *et al*.[39] Overall, isoenzyme systems have shown several intraspecies variations resulting from genetic mutations.[30,42]

Conclusion

Candidiasis is a widespread infection that has recently been listed among "priority pathogens" by the World Health Organization. Therefore, the methods for identifying species and strains of this yeast are essential. Among the MLEE systems studied, MDH provided the best pattern for *Candida* species differentiation. Furthermore, the analyzed isozyme patterns were similar between strains of the examined species,

and no significant correlation was observed between the investigated isozyme patterns and azole drug resistance.

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Ethics approval and consent to participate

Not applicable for this study

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Conflicts of interest

There are no conflicts of interest.

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