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Candida Lusitaniae. ISOLATION, IDENTIFICATION AND CLINICAL RELEVANCE

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ABSTRACT

Five isolates of *Candida* sp; three from blood cultures and two from food were studied and compared with reference strains. The studies included macroscopic and microscopic morphology, automated biochemical tests, susceptibility to itraconazole, as well as cytoplasmic protein profile analysis. The five isolates were identified as *Candida lusitaniae*. The isolates formed abundant pseudohyphae with lateral blastoconidia in short chains, they did not form a germinative tube and chlamyconidia. Isolates from blood cultures were highly susceptible to itraconazole (minimum inhibitory concentration 0.022 µg / mL). The analysis of cytoplasmic proteins revealed an intra-species similarity coefficient of 91 to 100% and separation coefficients with *Candida albicans*, and *Candida parapsilosis* of 63 and 73%, respectively. The morphological characteristics together with the specific biochemical tests are resources with high reliability to identify this opportunistic pathogen. The data show that *Candida lusitaniae*, *Candida albicans* and *Candida parapsilosis* are three species that have a clear taxonomic separation. The procedure followed in this work is accessible to basic mycology laboratories.

KEYWORDS: *Candida lusitaniae*, *Candida parapsilosis*, *Cytoplasmic proteins*, Itraconazole.

1. INTRODUCTION

Candida lusitaniae is an opportunistic levaduriform fungus emerging, difficult to identify, that is related to nosocomial infections and severe systemic infections in immune-compromised patients.[1] In some cases, therapeutic failure occurs due to acquired resistance, mainly to amphotericin B[2], as well as to imidazoles and 5-fluorocytokine.[3] The conventional tests used in the identification of the fungus include the morphological analysis of the colonies, the study of their reproductive proccesses[3,4] and the use of manual and automated biochemical tests.[5-10] Sometimes the identification can be wrong as has happened with *Candida tropicalis*, *Candida parapsilosis*, *Candida albicans* and *Saccharomyses cerevisiae*.[3] Some crops have been identified as *Candida tropicalis* varieties, due to the great similarity of their physiological properties.

The frequency of *Candida lusitaniae* in blood cultures is lower compared with other species including *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis* and *Candida glabrata*, considered of medical importance and that have been isolated in cases of fungemia. However, *Candida lusitaniae* has been isolated from various clinical samples, varying its frequency in respiratory mucosa, oral mucosa, peripheral blood, urine, feces, expectoration, vaginal exudates, chronic ulcers and catheters.[3,11,12]

In this work we report on the isolation and identification of *Candida lusitaniae* from blood and food samples. The identification was made by conventional and automated methods. The sensitivity to itraconazole, and its electrophoretic protein profiles were compared with those of *Candida albicans* and *Candida parapsilosis*.

2. MATERIALS AND METHODS

2.1. Biological Material

In 2012, three strains referred to as *Candida* sp (Cl1, Cl2, Cl3) obtained from blood cultures of pediatric patients were received, two strains were sent by the State Public Health Laboratory of Puebla, Mexico and one by the General Children's Hospital of Morelia, Mexico, as well as a strain of *Candida* sp (Cl4) isolated in a fortuitous way, when trying to isolate *Cryptococcus neoformans* from grapefruit and a strain of *Candida* sp isolated from a

ham sandwich (Cl5), sent by the State Laboratory of Public Health of Aguascalientes, Mexico.

The cultures were studied using conventional methods that included analysis of their morphological characteristics, germ tube formation, production of chlamyconidia and sporulation characteristics by leaf cultures[13], identification by the automated Vitek system[14], susceptibility tests to itraconazole by a modification to the agar dilution method[15-18] and analysis of its protein electrophoretic profiles.[19-22]

2.2. Reference strains

Candida parapsilosis ATCC 22019

Candida albicans ATCC 10231

Candida albicans ATCC 90028

2.3. Experimental procedure

2.3.1. Germinative tube formation

In order to observe the development of the germ tube, a sample of each strain was resuspended in 0.5 mL of commercial horse serum. The suspension was homogenized and incubated at 37 °C for two hours.

2.3.2. Production of chlamyconidia

Strains were seeded in corn flour agar, added tween 80 and trypan blue. Each culture was striated in parallel lines and separated approximately 1 cm, a coverslip was placed on the striae and the observations were made between 48 and 96 hours. In this medium the formation of pseudohyphae, hyphae and blastoconidia was observed.

2.3.3. Identification by the Vitek system, YST card

The cultures were striated on Sabouraud dextrose agar and incubated at 35 °C for 48 hours. Several isolated colonies were selected to prepare an inoculum in 0.5% saline solution, with reading in the range of 1.8 to 2.0 in the densiCHEK plus McF. The cells were filled with the cell suspensions of each strain, sealed and incubated at 35 °C for 24 hours. The cards with the biochemical patterns were examined and the results stored for analysis. The data included crop number, time, date, specific species and percentage of reliability.

2.3.4. Preparation of plates and dilution of antifungal

The culture medium used was Yeast morphology agar, using a buffer solution of 3- (N-Morpholino) propanesulfonic acid (MOPS, Sigma Cat. M-9381), 0.165 M, pH 7.0.[16] Sterilized at 121 °C for 15 minutes, cooled to a temperature close to 45 °C and added the antifungal at different concentrations. The medium was homogenized and distributed in plastic Petri dishes (Falcon 351012) in 25 mL volumes. The control included plaques without drug (Control). The culture media was subjected to a sterility test for 24 hours at 37 °C.

The concentration interval of itraconazole used was 0.032-0.5 µg / mL, which was dissolved in dimethylsulfoxide and sterilized by filtration according to the recommendations of the National Committee for Clinical Laboratory Standard[17], using the reference strain *Candida parapsilosis* ATCC 22019

2.3.4.1. Inoculum preparation

Strains were seeded by cross striae on Sabouraud dextrose agar and incubated at 35 °C for 48 hours. Several colonies were then randomly selected and suspended in 12 x 75 mm tubes with 3 mL of 0.5 % saline, vortexed to obtain a uniform suspension. The suspensions were adjusted to an optical density of 0.5 in the densiCHEK plus McF.[14] A final dilution of 1:10 was made from this suspension.

2.3.4.2. Inoculation of the medium

Subsequently, an inoculum of 5 µL of each cell suspension was deposited in the culture medium with and without antifungal, equivalent to a final concentration between 500 and 2500 colony forming units according to the recommendations of the European Society of Clinical Microbiology and Infectious Diseases.[23] The boxes were left to stand for 10 minutes to allow the inoculum to be absorbed, then inverted and incubated at 35 °C for 48 hours.

2.3.4.3. Reading the test

For the test to be valid, the base culture medium plate must show a free growth button on all samples. In the plates with the different concentrations, the presence of growth at a certain concentration and subsequently its lack of development at the next concentration corresponds to the minimum inhibitory concentration of that sample, under these conditions. A sensitive strain will be one that does not present development at a certain concentration, while a resistant strain will be one that shows growth similar to that found in the base culture

medium. The buttons with incipient growth or in points are considered negative and the cultures are considered sensitive to the antifungal.

2.3.5. Protein pattern

The strains were striated on Sabouraud dextrose agar and incubated at 35 °C for 48 hours. From each strain was prepared a suspension in saline solution approximately to tube 6 of McFarland, 1.5 cm of sterile glass beads with a diameter of 425 to 600 µm (tube of 12x75 mm) were added. The suspensions were shaken in a vortex during cycles of 30 seconds in agitation and 30 seconds in ice, for a total time of 60 minutes.

The suspensions were centrifuged, the supernatants were separated and filtered with 0.45 nm pore. To each mL of supernatant was added 5 µL of the following protease inhibitors: aprotinin, phenyl methyl sulfonyl fluoride and ethylene diamine tetraacetic acid. The supernatants were stored at -20 °C and each protein concentration was determined by the Lowry method.[19]

Protein separation of each cell extract was performed with a Mini-PROTEAN 3 Electrophoresis Cell (Bio-Rad Laboratories), using 11% polyacrylamide gels under reducing conditions (SDS-PAGE 11%), at a concentration of 20 µg of protein per lane and 100 volts during the entire shift and subsequent staining with comassie brilliant blue.[20,21] To document the banding profiles, the image was acquired by means of a CCD camera (Eagleeye, Stratagene) and with the help of the computer program RFLPscan Plus V3.0 (Scanalytics), the molecular weights were calculated with respect to reference standards (BRMWM Cat V8491, Promega Co.).

2.3.6. Statistic analysis.

To determine the presence of yeasts foreign to the isolates and reference strains, the matrix n x m was transformed by the Jaccard metric to a triangular matrix n x n of similarity, where n are the isolates or reference strains. The UPGMA linkage algorithm (intergroup linkage) was established on this matrix, which establishes the rules by which the isolated strains or reference strains will join the groups based on their similarities among the n elements[22], using the program of computation NTSYSPC V2.02j.

3. RESULTS

3.1. Morphological characteristics

The five strains of *Candida* sp did not form a germ tube or chlamycoconidia. The sporulation in corn flour showed small variations. Abundant pseudohyphae were observed, lateral blastoconidia on both sides, small, ovoid to spherical in short chains with tendency to branch. The two ATCC strains of *Candida albicans* formed a germ tube and chlamycoconidia. There are a large number of hyphae, pseudohyphae and blastoconidia, the latter arranged laterally in clusters and spherical in shape. The ATCC strain of *Candida parapsilopsis* did not form a germinative tube or chlamyconidia, a large number of pseudohyphae, hyphae and blastoconidia were observed, whose arrangement is symmetrical, observing small groups of ovoid to spherical shape. The morphological characteristics are shown in Figure 1.

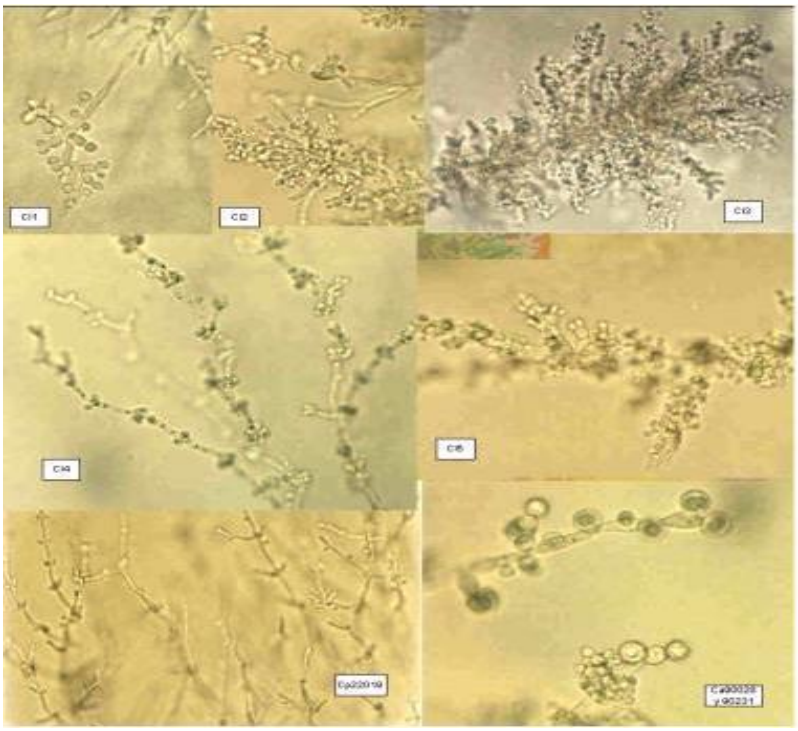

Figure 1: Microscopic morphological characteristics of *Candida lusitaniae* (Cl1-Cl5), *Candida. albicans* (10231,90028) and *Candida parapsilosis* (22019). (120 X).

3.2. Biochemical Characteristics

The five strains of *Candida* sp were identified by the Vitek system as *Candida luisitaniae*, which was confirmed with the reference strains. There are biochemical differences that clearly separate the three species (Table 1). The use of cellobiose and melicitosa seems to be constant in *Candida lusitaniae*, while the two strains of *Candida albicans* and that of *Candida parapsilopsis* did not hydrolyze these sugars. Another determining substrate was

methyl-D-glucoside, which is not hydrolyzed by *Candida lusitaniae*, whereas the reference strains *Candida albicans* and *Candida parapsilosis* do hydrolyze it. Other substrates useful in the separation of these were galactose, arabinose, glycerol and susceptibility to cycloheximide. Of these, arabinose separates *Candida parapsilopsis* from *Candida albicans* and *Candida lusitaniae*. Finally, *Candida lusitaniae* (5/5) and *Candida parapsilosis* are susceptible to cycloheximide while the two reference strains of *Candida albicans* were resistant.

Table 1: Biochemical characteristics of *Candida lusitaniae* (Cl-1-Cl-5), *Candida albicans* (90028, 10231) and *Candida parapsilosis* (22019).

	Cl-1	Cl-2	Cl-3	Cl-4	Cl-5	Ca 90028	Ca 10231	Cp 22019
Galactose	-	-	-	-	+	+	+	+
Lactose	-	-	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	-	-	-
Methyl-D-Glucoside	-	-	-	-	-	+	+	+
Xylose	+	-	-	+	+	+	+	+
Arabinose	-	-	-	-	-	-	-	+
Trehalose	+	+	+	+	+	+	+	+
Melecitosa	+	+	+	+	+	-	-	+
Rafinose	-	-	-	-	-	-	-	-
N-Acetyl-D-Glucosamine	+	+	+	+	+	+	+	+
Xylitol	+	-	-	+	-	+	+	+
Dulcitol	-	-	-	-	-	-	-	-
Adonitol	+	-	-	+	-	+	+	+
Palatinosa	+	+	+	+	+	+	+	+
Glycerol	+	-	-	+	-	+	+	+
Sorbitol	+	+	+	+	+	+	+	+
Erythritol	-	-	-	-	-	-	-	-
Melobiosa	-	-	-	-	-	-	-	-
Cycloheximide	-	-	-	-	-	+	+	-
Glucose	+	+	+	+	+	+	+	+
Inositol	-	-	-	-	-	-	-	-
Nitrates	-	-	-	-	-	-	-	-
2-Keto-D-Gluconate	+	+	+	+	+	+	+	+
Urea	-	-	-	-	-	-	-	-

3.3. *In vitro* tests for sensitivity to itraconazole

The three strains of *Candida lusitaniae* isolated from blood cultures showed high sensitivity to itraconazole. The minimum inhibitory concentration was below 0.032 µg / mL. The two strains of *Candida lusitaniae* isolated from food presented lower susceptibility (0.5 µg / mL). The strains used as controls; *Candida parapsilosis* ATCC 22019, *Candida albicans* ATCC

90231 and *Candida albicans* ATCC 90028 were highly sensitive to the action of itraconazole (0.032 to 1 µg / mL).

Table 2: Presence-absence matrix of the banding profile by Coomassie brilliant blue staining. Cl-1 to Cl-5 isolates *Candida lusitaniae*, Ca-1 *Candida albicans* 90028 ATCC, Cp-1 *Candida parapsilosis* 22019 ATCC, Ca-2 *Candida albicans* 10231 ATCC. (Full box, presence, Empty box, absence).

PM	Cl-1	Cl-2	CL-3	Cl-4	Cl-5	Ca-1	Cp-1	Ca-2
110.3	0	0	0	0	0	0	1	0
108.3	1	1	1	1	1	0	0	0
103.0	1	1	1	1	1	1	0	1
96.7	0	0	0	0	0	0	1	0
92.0	1	1	1	1	1	1	0	1
90.0	0	0	0	0	0	0	1	0
87.8	1	1	1	1	1	1	0	1
83.8	0	0	0	0	0	0	1	0
74.3	0	0	0	0	0	1	1	1
72.4	1	1	1	1	1	0	0	0
71.5	0	0	0	0	0	1	0	1
68.8	0	0	0	0	0	1	0	1
64.1	0	0	0	0	0	1	0	1
62.4	0	0	0	0	0	0	1	0
60.7	1	1	1	1	1	1	0	1
58.4	0	0	0	0	0	0	1	0
55.1	1	1	1	1	1	1	1	1
52.0	0	0	0	0	0	1	1	1
49.5	0	0	0	0	0	1	1	1
47.1	0	0	0	0	0	1	1	1
44.1	1	1	1	1	1	1	1	1
41.7	1	1	1	1	1	1	0	1
39.6	1	1	1	1	1	1	1	1
37.1	1	1	1	1	1	0	1	0
35.1	1	1	1	1	1	1	0	1
33.6	0	0	0	0	0	0	1	0
32.8	1	1	1	1	1	0	0	0
30.5	1	1	1	1	1	1	1	1
26.7	1	1	1	1	1	1	1	1
26.2	0	0	0	0	0	0	1	0
25.7	0	0	0	0	0	1	0	1
24.8	1	1	1	1	1	1	1	1
24.3	0	0	0	0	0	0	1	0
23.3	1	1	1	1	1	0	1	0
22.6	0	0	0	0	0	1	0	1
21.2	1	1	1	1	1	0	0	0

20.7	0	0	0	0	0	1	1	1
19.5	1	1	1	1	1	0	0	0
18.4	0	0	0	0	0	1	0	1
16.4	1	1	1	1	1	1	1	1
15.1	1	1	1	1	1	1	0	1

3.4. Protein electrophoretic profiles

A rectangular matrix nxm of presence or absence of proteins was constructed, where n are the electrophoretic patterns of each strain m, including a range of 15.1 to 110.3 KDa (Table 2).

Specific bands were observed for the three species studied (Figure 2). The group of strains of *Candida lusitaniae* presented five specific bands, absent in the reference strains (19.5, 21.2, 32.8, 72.4, 108.3 KDa). The reference strains of *Candida albicans* had six specific bands (18.4, 22.6, 25.7, 64.1, 68.8 and 71.5) absent in the reference strain of *Candida parapsilopsis* and in the strains of *Candida lusitaniae*. *Candida parapsilopsis* presented 8 bands (24.3, 26.2, 33.6, 58.4, 62.4, 90, 96.7, 110.3), which were not observed in the strains of *Candida lusitaniae* and in the two reference strains of *Candida albicans*. The rest of the bands shared common characteristics among the three species. There are **seven** bands that share the five strains of *Candida lusitaniae* with the two reference strains of *Candida albicans* (15.1, 35.1, 60.7, 74.3, 87.8, 92 and 103), while there are only two bands (23.3, 37.1) that share the strains of *Candida lusitaniae* with the reference strain of *Candida parapsilopsis*.

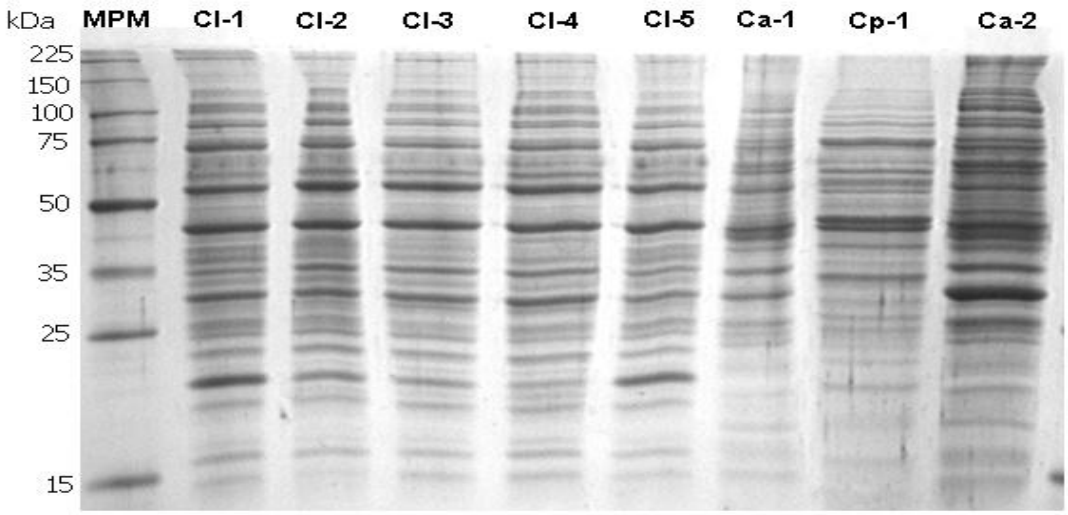

Figure 2. Protein profile. 11% SDS-PAGE, 10% glycerol total protein, stained with Coomassie Brilliant Blue. Cl-1 to Cl-5 strains of *Candida lusitaniae*, Ca-1 *Candida albicans* ATCC 90028, Cp-1 *Candida parapsilosis* ATCC 22019, Ca-2 *Candida albicans* ATCC 10231 and MPM molecular weight markers.

There is great similarity between the electrophoretic patterns of the strains of *Candida lusitaniae*. The two strains of *Candida albicans* also show a similar pattern of proteins. By submitting the banding map to the conglomerate analysis technique for group formation, the formation of two clearly separated groups can be seen; in one, isolated strains of *Candida lusitaniae* (C4-1, Cl-2, Cl-1, Cl-3 and Cl-5) are grouped with an important similarity. On the other hand *Candida albicans* and *Candida parapsilosis* form another group; however, *Candida albicans* (Ca 90231 and Ca 90028) are grouped differently from *Candida parapsilosis* (Cp 22019). Figure 3 shows a relationship dendogram of the three species, based on biochemical characteristics and protein banding profiles.

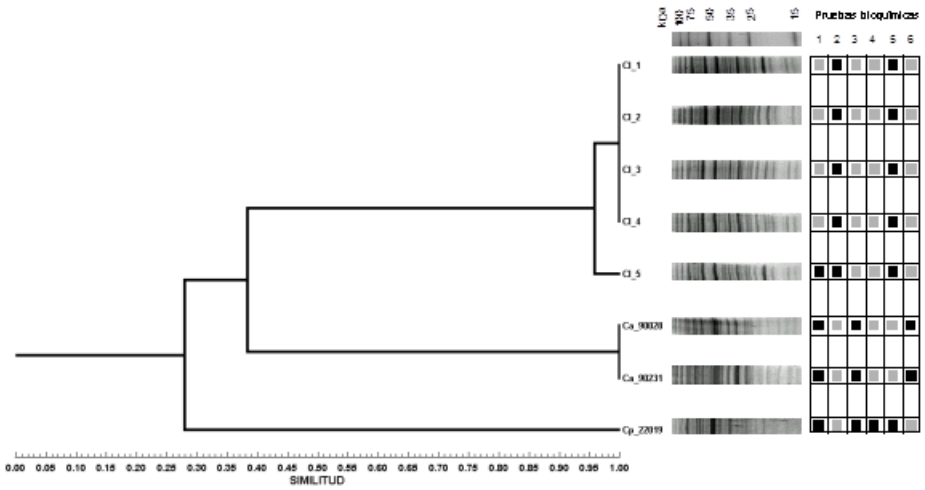

Figure 3: Dendrogram that relates electrophoretic profiles and biochemical tests in the formation of conglomerates of *Candida lusitaniae* (Cl-1, Cl-2, Cl-3, Cl-4, Cl-5), *Candida albicans* 10231, *Candida albicans* 90028 and *Candida parapsilosis* 22019. 1.Galactose, 2.Cellobiose, 3. Arabinose, 4. Methyl-D-glucoside, 5. Melecitosa, 6. Cyclohexamide. *Candida lusitaniae* (19.5, 21.2, 32.8, 72.4, 108.3), *Candida albicans* 10231 and 90028 (18.4, 22.6, 25.7, 64.1, 68.8, 71.5) and *Candida parapsilosis* 22019 (24.3, 26.2, 33.6, 58.4, 62.4, 90.0, 96.7, 110.3).

4. DISCUSSION

Candida albicans continues to be the most important opportunistic species in infections by the genus *Candida*. The other species; *Candida parapsilosis*, *Candida glabrata*, *Candida tropicalis* and *Candida guilliermondii* have increased their importance, accentuating regional differences in their frequency.[24] In 1995, *Candida lusitaniae* was considered an emerging opportunistic pathogen.[1] Since then, this yeast has gained greater medical importance due to

its high natural and acquired resistance to amphotericin B during treatment.[25] The fact that this yeast was found in five hospitalized patients suggests that *Candida lusitaniae* can be acquired directly in hospitals.[26] However, if the incidence of colonization is low and infections are infrequent, the isolation of *Candida lusitaniae* from the hospital environment constitutes a potential risk of transmission for immunocompromised patients.

In the last decades, systemic candidosis has increased due to non-albicans species. In Mexico, only two cases have been reported by *Candida lusitaniae*, one case of candidemia and the other, an infection of the lower respiratory tract.[27] Most laboratories still use manual biochemical tests to identify important species of the genus *Candida*, but it is not a reliable resource to identify this opportunistic pathogen. Automated testing (Vitek System) has proved to be a reliable, simple and effective method for the identification of the main species of the *Candida* genus, including rare species such as *Candida lusitaniae*.[28, 29] The Vitek system used in this study allowed the identification of the five strains of *Candida* sp, such as *Candida lusitaniae*, 24 hours later. Of the 28 substrates studied, 7 tests are determinants (cellobiose, melicitose, methyl-D-glucoside, arabinose, raffinose, galactose and cycloheximide) in the differentiation of *Candida lusitaniae* with *Candida parapsilosis* and *Candida albicans*.

The use of cellobiose by *Candida lusitaniae* is differential. In unpublished data, using strains from the library of the Institute of Epidemiological Diagnosis and Reference (InDRE), 63 strains of *Candida albicans* and 50 strains of *Candida parapsilosis* did not hydrolyze this carbohydrate. On the other hand, while *Candida lusitaniae* does not hydrolyze methyl-D-glucoside, 63 strains of *Candida albicans* use this substrate as a carbon source, as well as 40 of 50 strains of *Candida parapsilosis*. Arabinose is another carbohydrate that is also not used by *Candida lusitaniae* but is a substrate for 42 of 50 strains of *Candida parapsilosis*.

The susceptibility to cycloheximide also has a differential character; *Candida lusitaniae* (5/5) and *Candida parapsilosis* (50/50) are susceptible in 100%, whereas in *Candida albicans* most are resistant (48/63). These biochemical tests are within the reach of basic mycology laboratories and, together with the morphological characteristics described, can be a reliable resource in the identification of *Candida lusitaniae*.

The susceptibility to itraconazole is of interest, the three strains isolated from peripheral blood were highly sensitive, so this drug can be an alternative when it comes to strains

resistant to amphotericin B. The two strains isolated from food behaved differently, the minimum inhibitory concentration obtained was 0.5 µg / mL, which according to the guidelines proposed by Espinel-Ingroff and Pfaller (30) are considered dose-dependent susceptible.

The use of reference strains is very useful, *Candida parapsilosis* 22019 behaved as a sensitive strain according to that established in the literature[17, 23], the minimum inhibitory concentration was 0.032 µg / mL. The strains of *Candida albicans* have not been used consistently in quality control programs, and there are no data on their behavior to itraconazole.[31, 32] Strain 10231 was highly sensitive, while strain 90018 was resistant to the range of 0.032 to 1 µg / mL. The method of dilution in agar used in this work can be an alternative in basic laboratories. The results obtained with reference strains are reproducible with standardized methods.[23] Regarding the standard inoculum established by the Clinical Laboratory Standard Institute and the European Society of Clinical Microbiology and Infectious Diseases, we used the latter, with dilution 1:10 of the inoculum prepared to the 0.5 tube of Mcfarland, which allowed to establish a volume of 5 µl, accessible to recommended Petri dishes.

The electrophoretic profiles show clear differential characteristics for the three *Candida* species. The coefficient of similarity between *Candida albicans* and *Candida lusitaniae* is 37%, while between *Candida parapsilosis* and *Candida lusitaniae* it is 27%. Four strains of *Candida lusitaniae* have a similarity coefficient very close to each other (99%) and the Cl-5 culture isolated from the ham sandwich has a similarity of 91%, which seems to indicate that strains of the species *Candida lusitaniae* tend to to behave in a homogeneous way.

Recent studies[33] with molecular techniques found a clear separation between the isolates of *Candida albicans* and *Candida parapsilosis*. The reference strains used in this study; *Candida albicans* 10231, *Candida albicans* 90028 and *Candida parapsilosis* 22019, also exhibited a clear separation in the electrophoretic profiles. Other authors have reported similar results by electrophoresis in DNA fragments.[34] *Candida albicans* constitutes a more homogeneous group, while *Candida parapsilosis* behaves heterogeneously, but with a clear separation between these two species.

5. CONCLUSIONS

In conclusion, this study provides experimental evidence for the correct identification of *Candida lusitaniae*. With the Vitek system five strains were identified with an excellence level of 99%. Electrophoresis of cytoplasmic proteins revealed that *Candida. lusitaniae*, *Candida albicans* and *Candida parapsilopsis* present specific proteins of each species. The five bands that *Candida lusitaniae* are absent in *Candida albicans* and *Candida parapsilopsis*. The proteins of *Candida lusitaniae* of the five strains revealed an intra-species similarity coefficient of 91 to 100% and a separation coefficient with *Candida albicans,* and with *Candida parapsilosis* of 63 and 73%, respectively.

Isolates from blood cultures were highly susceptible to itraconazole, whereas food isolates were susceptible in a dose-dependent manner. The agar dilution method for susceptibility tests to itraconazole is a simple and practical resource with reproducible results by the standardized methods by the Clinical Laboratory Standard Institute and the European Society of Clinical Microbiology and Infectious Diseases.

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