PATHOGENICITY CHARACTERISTICS OF STOCKED AND FRESH YEASTS STRAINS

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ABSTRACT

In order to evaluate the pathogenicity of yeasts of medical importance, 15 strains stocked in mineral oil at the URM Culture Collection of the Department of Mycology, Biological Sciences Centre, Federal University of Pernambuco and 15 fresh strains isolated from AIDS patients interned at the Clinical Hospital, Centre of Health Sciences, Federal University of Pernambuco, were selected. As pathogenicity characteristics, the ability to grow at 37°C and production of phospholipase and proteinase were tested. All samples grew at 37°C and exhibited proteinase activity. However, when tested on solid medium, the proteinase activity was negative. Among 15 stocked samples, 13 (86.66%) were phospholipase positive and two (13.34%) were phospholipase negative. From the 15 samples isolated from AIDS patients, four (26.66%) were phospholipase positive and 11 (73.34%) were phospholipase negative.

Key words: yeasts, pathogenicity, temperature, phospholipase, proteinase.

INTRODUCTION

Candidosis is an infection caused by Candida species. It can be acute or chronic, superficial or deep and its clinical spectrum can be so variable that a more specific definition can not be given (16).

The principal etiological agent of candidosis is Candida albicans. The majority of the studies show that this species constitute at least 60% of Candida species isolated from clinical samples. Since this yeast is a part of the normal human microbiota, candidosis can be considered an opportunistic infection (16,17,29).

However, some considerations have to be taken because another Candida species have been frequently reported in literature as etiological agents of candidosis, like for example: C. tropicalis, C. parapsilosis, C. krusei, C. guilliermondii, C. glabrata, C. kefir, C. lusitaniae, C. viswanathii e C. famata. All these species were already isolated from clinical samples (7,23).

Trichosporon species can cause many human infections similar to candidosis. The disseminated form, denominated thricosporonosis (acute or chronic), can be life-threatening in patient with malign diseases, mainly imunossupressed patients (11,14).

The ability of some fungi to grow up at 37°C, and also to produce enzymes like phospholipases and proteinases permit the establishment of a relation with pathogenicity (13,16,20, 22,25,33).

The purposes of the present study were: a) To confirm the identification of 15 samples of stocked yeasts at the URM Culture Collection, through biochemical and physiological assays; b) To determinate pathogenicity characteristics in vitro, of these stocked samples and also of yeasts isolated from AIDS patients including growth, at 37°C, and enzymatic (phospholipase and proteinase) activity.

MATERIALS AND METHODS

Strains

Thirty yeasts strains isolated from clinical samples were selected: 15 stocked in mineral oil at the URM Culture Collection of

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Department of Mycology, Centre of Biological Sciences, Federal University of Pernambuco and 15 fresh samples isolated from AIDS patients interned at the Clinical Hospital, Centre of Health Sciences, Federal University of Pernambuco (Tables 1 and 2).

Sample Reactivation

The stocked samples preserved in mineral oil were reactivated by growth in glycoside broth and then transferred to slants containing Sabouraud agar plus yeast extract (SAB+YE).

Culture Media

For confirmation of the identification: Bovine bile water medium, C and N basic medium and sugar fermentation medium (17). For characterization of enzymatic activity: Semi-quantitative phospholipase determination medium (28) modified, substituting 20 g of sterilized egg yolk (Difco) by two natural egg yolk, and qualitative proteinase determination medium (17,18). For species confirmation, the classical methods of Lodder (19), Kreger van Rij (15) and Barnett et al. (1) were utilized.

Pathogenicity characteristics detection

Growth at 37ºC

The yeasts samples were smeared in duplicate into SAB+YE slants, the first slant kept at room temperature (28ºC ± 1ºC) and the other one incubated at 37ºC. They were monitored for 72 h, with the purpose of comparing the cultures growth.

Enzymatic Activity

Semi-quantitative phospholipase detection (28): yeasts, grown for 72 h were inoculated on the referred culture medium and incubated at room temperature. Cultures were observed for 10 to 15 days for formation of an opaque zone of precipitation, measured in centimeters. Phospholipase Zone (PZ) was calculated as the ratio between the diameter of the colony and the diameter of colony plus the diameter of zone of precipitation. When PZ was = 1.0, the samples tested were considered phospholipase negative and when PZ was < 1.0, they were considered phospholipase positive.

Qualitative proteinase detection (17)

The strains were inoculated at the same conditions of phospholipase detection assay. When a transparent zone of precipitation occurred, the result was considered positive, regardless the diameter.

Quantitative proteinase detection (18)

The strains were inoculated into 250 mL flasks containing 50mL of Sabouraud broth. The flasks were incubated at room temperature, 120 r.p.m., for 24 h and the number of cells were adjusted to 10⁹/mL using a Neubauer chamber. The strains were inoculated into 250 mL flasks containing 50 mL of casein enzymatic broth. The flasks were incubated at room temperature, 120 r.p.m., for 48 h, and 5 mL of each sample were frozen at 0ºC until enzymatic dosage was done. The frozen samples were thawed

<table>
<thead>
<tr>
<th>Species</th>
<th>Registration number (URM)</th>
<th>Storage period (years)</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida albicans</td>
<td>720</td>
<td>44</td>
<td>Bronchoalveolar secretion</td>
</tr>
</tbody>
</table>
Pathogenicity of Yeasts Strains

at room temperature for 1 h, centrifuged for 15 min. at 2,000 xg and the supernatants submitted to proteinase activity determination. The assay mixture (0.25 mL of azocasein 1% w/v on Tris-HCl buffer, pH = 7.6, and 0.15 mL of the medium containing proteinase) was incubated for one hour at room temperature, and then 1.2 mL of trichloroacetic acid (TCA) 10% w/v was added. The samples were centrifuged for 10 min. at 8,000 xg, at 4ºC. Then 0.8 mL of the supernatants were transferred to test tubes containing 1.4 mL of sodium hydroxide 1M. Each assay was performed twice. Negative controls comprised by solutions without the enzyme were included in the tests. The absorbancy was read at 440 nm (Shimatzu UV-visible spectrophotometer). The proteinase unit, expressed as U.mL⁻¹, was defined as the amount of enzyme required to produce a variation of absorbancy equal to 1.

RESULTS AND DISCUSSION

Strains Viability

The stocked yeasts strains at the URM Culture Collection (15) were viable regardless the time of preservation, presenting growth when transferred from glycoside broth to SAB+YE. Confirmation of the identification: All stocked yeasts strains (15) showed patterns of sugar assimilation and fermentation that corresponded to the specie previously identified. However, the \textit{C. albicans} samples lost their ability to produce clamidospore on bovine bile water, suggesting physiological modifications caused by genetic instability due to the storage for long periods on mineral oil (36).

Pathogenicity Characteristics

Growth at 37°C

All strains were able to grow at 37°C. Twenty-eight \textit{Candida} and two \textit{Trichosporon pullulans} strains grew similarly at room temperature and 37°C.

The ability of some fungi to grow at 37°C is cited in the literature as a pathogenicity factor (16,17,22,29,35). \textit{Histoplasma capsulatum} and \textit{Criptococcus neoformans}, frequently isolated from animals without causing disease, are human pathogens, probably because this animals higher body temperature in than humans (35).

Phospholipase Activity

From the 15 fresh strains stocked at the URM Culture Collection, 13 (86.66%) were phospholipase positive and two (13.34%) were phospholipase negative. From the 13 phospholipase positive strains, six (46.15%) were \textit{C. albicans}, three (23.08%) \textit{C. parapsilosis}, two (15.39%) \textit{C. tropicalis}, one (7.69%) \textit{C. glabrata} and one (7.69%) \textit{C. krusei} (Fig. 1).

The Phospholipase Zone (PZ) for the 15 fresh samples ranged from 0.276 to 0.482, with one sample presenting PZ band 0.276, one 0.319, one 0.459 and one 0.482 (Table 4).

Among the stocked strains, a higher percentage for phospholipase activity was observed. In addition to \textit{C. albicans}, other \textit{Candida} species were also able to produce phospholipase. Only two strains did not produce phospholipase: one \textit{C. parapsilosis} and one \textit{Trichosporon pullulans}. In general, the strains presented low PZ values, indicating high phospholipase activity.

Table 3. Phospholipase and proteinase activity of the yeasts strains stocked in The URM Cultures Collection.

<table>
<thead>
<tr>
<th>Species</th>
<th>URM Registration number</th>
<th>Phospholipase activity (PZ)</th>
<th>Proteinase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Candida albicans}</td>
<td>720</td>
<td>0.310</td>
<td>1.066 U/mL</td>
</tr>
<tr>
<td>\textit{C. albicans}</td>
<td>743</td>
<td>0.217</td>
<td>1.386 U/mL</td>
</tr>
<tr>
<td>\textit{C. albicans}</td>
<td>2224</td>
<td>0.270</td>
<td>0.673 U/mL</td>
</tr>
<tr>
<td>\textit{C. albicans}</td>
<td>2252</td>
<td>0.259</td>
<td>1.053 U/mL</td>
</tr>
<tr>
<td>\textit{C. albicans}</td>
<td>2253</td>
<td>0.260</td>
<td>0.733 U/mL</td>
</tr>
<tr>
<td>\textit{C. albicans}</td>
<td>3622</td>
<td>0.223</td>
<td>0.726 U/mL</td>
</tr>
<tr>
<td>\textit{C. glabrata}</td>
<td>943</td>
<td>0.204</td>
<td>1.535 U/mL</td>
</tr>
<tr>
<td>\textit{C. krusei}</td>
<td>934</td>
<td>0.261</td>
<td>0.803 U/mL</td>
</tr>
<tr>
<td>\textit{C. parapsilosis}</td>
<td>729</td>
<td>-</td>
<td>0.973 U/mL</td>
</tr>
<tr>
<td>\textit{C. parapsilosis}</td>
<td>3621</td>
<td>0.179</td>
<td>0.686 U/mL</td>
</tr>
<tr>
<td>\textit{C. parapsilosis}</td>
<td>3624</td>
<td>0.240</td>
<td>0.700 U/mL</td>
</tr>
<tr>
<td>\textit{C. parapsilosis}</td>
<td>3627</td>
<td>0.254</td>
<td>0.686 U/mL</td>
</tr>
<tr>
<td>\textit{C. tropicalis}</td>
<td>1150</td>
<td>0.269</td>
<td>0.673 U/mL</td>
</tr>
<tr>
<td>\textit{C. tropicalis}</td>
<td>916</td>
<td>0.267</td>
<td>0.860 U/mL</td>
</tr>
<tr>
<td>\textit{T. pullulans}</td>
<td>1323</td>
<td>-</td>
<td>0.830 U/mL</td>
</tr>
</tbody>
</table>

Figure 1. Phospholipase activity percentage in \textit{Candida} strains.
Among the fresh strains, there was a lower number of phospholipase positive samples. In addition, only one strain of *C. parapsilosis* was able to present phospholipase activity. It was also observed that one *C. albicans* and one of *T. pullulans* strain were phospholipase negative.

Saramanayake *et al.* (32), Mayser *et al.* (21) and Candido *et al.* (5), studying *Candida* strains isolated from oral cavity of patients presenting or not lesions, only detected phospholipase activity only in *C. albicans*, and observed that *C. glabrata*, *C. parapsilosis* and *C. tropicalis* did not produce phospholipase. However, Shimizu *et al.* (34), testing yeasts strains isolated from clinical samples, detected phospholipase activity in other species of *Candida*, such *C. parapsilosis*, *C. tropicalis*, *C. guilliermondii* and *C. krusei*. However these strains produced lower quantity of enzyme than *C. albicans*.

Oliveira *et al.* (26), investigating yeasts samples isolated from oral cavity of cancer patients, observed that all isolates (24 *C. albicans* and one *C. krusei*) were able to produce phospholipases, but showed a large PZ variation (0.12 to 0.61). In our study, phospholipase activity was detected in all *Candida* species tested, contributing with the authors which have found the same results.

Although some authors such as Vidotto *et al.* (37) and Ghannoun (9) have indicated that *Trichosporon* is able to produce phospholipase, Mayser *et al.* (21) did not observe phospholipase activity in *T. cutaneum*, *T. capitatum* and *T. inkin*. The literature does not refer *T. pullulans* showing positive phospholipase activity.

Price *et al.* (28), comparing the plate method and biochemical assays, verified that the easy method using a solid medium is very useful to investigate the variability of phospholipase activity of different clinical samples. In addition, these authors also verified that some isolates frozen for long periods at -20°C and -80°C presented a decrease in phospholipase activity. These data do not agree with the results obtained in the present study, since strains preserved in mineral oil maintained the enzymatic activity. The high phospholipase activity was already expected, because all samples in the present study were isolated from patients. However, a lower phospholipase activity was observed among the fresh strains isolated from AIDS patients.

Willis *et al.* (38) analyzed the effect of fluconazole and nystatin on phospholipase production and observed that fluconazole decreased phospholipase activity in clinical samples isolated from oral cavity of diabetes mellitus patients. It is likely that the samples of our study have decreased their ability to produce phospholipase as consequence of antifungal treatment to which the patients interned at The Clinical Hospital have been submitted (azoles and nystatin).

### Proteinase Activity

Qualitative proteinase determination: None of the strains, including the stocked and the fresh strains produced proteinase in the casein medium, suggesting that this solid medium is not an suitable alternative for yeasts proteinase activity detection.

Quantitative proteinase determination: This test indicated that all stocked strains (15) were able to produce detectable levels of proteinase: three strains produced from 0.673 to 0.686 U.mL^-1^; three samples from 0.700 to 0.733 U.mL^-1^; four samples from 0.803 to 0.860 U.mL^-1^; one sample produced 0.973 U.mL^-1^ and four samples from 1.053 to 1.386 U.mL^-1^.

The samples 1150 (*C. parapsilosis*) and 2224 (*C. albicans*) was the best proteinase producer (0.673 U.mL^-1^). The strain 743 (*C.albicans*) produced more proteinase (1.386 U.mL^-1^) (Table 3).

All the fresh samples isolated from AIDS patients (15) also produced detectable levels of proteinase. It was observed that seven samples produced from 0.706 to 0.780 U.mL^-1^; three samples from 0.840 to 0.866 U.mL^-1^ and five samples from 0.933 to 0.993 U.mL^-1^.

The samples 23 (*C. albicans*) and 97 (*C. parapsilosis*) produced less proteinase (0.706 U.mL^-1^). The samples 16 (*C. albicans*) produced 0.973 U.mL^-1^ and 58 (*C. krusei*) were the best proteinase producers (0.993 U.mL^-1^) (Table 4).

Wu *et al.* (39), analyzing clinical samples of *C. albicans*, *C. tropicalis* and *C. parapsilosis* from oropharyngeal secretion, investigated SAPs genes expression on a proteinase inductor medium (YCB-BSA) and observed that *C. albicans* samples were more proteolytic than other species of the same genus.

Bistoni *et al.* (2) and Rüchel *et al.* (31) demonstrated that *Candida parapsilosis* samples virulence was low or absent even in immunossupressed animals. De Bernardis *et al.* (8) however, observed elevated in *vitro* production of proteinase but one of the yeasts samples did not produce acid proteinase in *vivo*. This same authors, comparing the acid proteinase

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### Table 4. Phospholipase and proteinase activity of the yeasts isolated from AIDS patients interned at The Clinical Hospital, Federal University of Pernambuco.

<table>
<thead>
<tr>
<th>Species</th>
<th>Registration number</th>
<th>Phospholipase activity (PZ)</th>
<th>Proteinase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Candida albicans</em></td>
<td>16</td>
<td>0.993 U.mL</td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>84</td>
<td>0.319 0.993 U.mL</td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>14</td>
<td>0.482 0.946 U.mL</td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>18</td>
<td>0.276 0.780 U.mL</td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>23</td>
<td>0.706 U.mL</td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>97</td>
<td>0.706 U.mL</td>
<td></td>
</tr>
<tr>
<td><em>C. glabrata</em></td>
<td>64</td>
<td>0.714 U.mL</td>
<td></td>
</tr>
<tr>
<td><em>C. krusei</em></td>
<td>58</td>
<td>0.993 U.mL</td>
<td></td>
</tr>
<tr>
<td><em>C. parapsilosis</em></td>
<td>33</td>
<td>0.459 0.933 U.mL</td>
<td></td>
</tr>
<tr>
<td><em>C. parapsilosis</em></td>
<td>35</td>
<td>- 0.840 U.mL</td>
<td></td>
</tr>
<tr>
<td><em>C. parapsilosis</em></td>
<td>69</td>
<td>- 0.860 U.mL</td>
<td></td>
</tr>
<tr>
<td><em>C. parapsilosis</em></td>
<td>44</td>
<td>- 0.886 U.mL</td>
<td></td>
</tr>
<tr>
<td><em>C. trophi</em></td>
<td>08</td>
<td>- 0.726 U.mL</td>
<td></td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>34</td>
<td>- 0.710 U.mL</td>
<td></td>
</tr>
<tr>
<td><em>T. pullulans</em></td>
<td>86</td>
<td>- 0.726 U.mL</td>
<td></td>
</tr>
</tbody>
</table>

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G.M.Chaves *et al.*
production by *C. albicans* and *C. parapsilosis* isolated from vaginal secretion, observed a very similar enzyme production kinetics. Nevertheless, Rüchel et al., (30), developing an infection model with phagocytic cells, showed that *C. parapsilosis* did not produce acid proteinase in vivo.

*C. albicans* is able to produce proteinases *in vitro* in high quantities (3, 6, 8, 12, 24, 30). Chakrabarti et al. (6) call the attention for the proteinase activity detection by *C. tropicalis*, *C. parapsilosis* and *C. glabrata* isolated from clinical samples like anal mucous secretion, sputum, oropharyngeal secretion and urine, where the proteinase production was never investigated. According to those authors, this test is important for the definition of the microorganism as etiological agent or colonizer, in sites where they are part of the normal microbiota.

In the present study, it was observed that stocked strains presented a higher proteinase activity than the fresh samples recovered from AIDS patients.

The literature have recently pointed out that anti-retroviral agents utilized in AIDS patients may cause proteinase inhibition in *Candida* species, like *C. albicans*, *C. parapsilosis*, *C. tropicalis* and *C. lusitaniae* (10,27). The aspartic proteinase secreted by *Candida* have similarities to the HIV aspartic proteinases (9). It is likely that in the present study proteinase activity of the fresh strains have been inhibited, once the patients were submitted to anti-retroviral therapy.

RESUMO

**Características de patogenicidade de amostras de leveduras preservadas e recém-isoladas**

Com o objetivo de avaliar a patogenicidade de leveduras de interesse médico, foram selecionadas 15 amostras preservadas em óleo mineral na Micoteca URM, Centro de Ciências Biológicas, Universidade Federal de Pernambuco e 15 isolados recentes, provenientes de amostras clínicas de pacientes com AIDS, internados no Hospital das Clínicas, Centro de Ciências da Saúde, Universidade Federal de Pernambuco. Com relação as características de patogenicidade, foram avaliados a capacidade desses microorganismos crescerem a 37°C, bem como apresentarem atividade de fosfolipase e protease. As 30 amostras cresceram a 37°C e apresentaram atividade quantitativa de protease, entretanto não apresentaram essa atividade quando testadas em meio sólido. Das 15 amostras preservadas, 13 (86,66%) apresentaram atividade fosfolipase e protease. As 30 amostras cresceram a 37°C e apresentaram atividade quantitativa de protease, entretanto não apresentaram essa atividade quando testadas em meio sólido. Das 15 amostras preservadas, 13 (86,66%) apresentaram atividade fosfolipase e protease. Das 15 amostras isoladas de pacientes com AIDS, quatro (26,66%) apresentaram atividade de fosfolipase e protease. As 15 amostras isoladas de pacientes com AIDS, quatro (26,66%) apresentaram atividade de fosfolipase e protease.

**Palavras-chave:** leveduras, patogenicidade, temperatura, fosfolipase, protease.
36. Smith; Onions The preservation and maintenance of living fungi. 1ª ed., USA, Commonwealth Mycological Institute, 1983, 51p.