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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 phospolipase 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 specie 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 37ºC, 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 37ºC, and enzymatic (phospholipase and proteinase) activity.

MATHERIALS 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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co d agar plus yeast extract (SAB+YE).

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on of the identification: Bovine bile water m asic medium and sugar fermentation medium (1 terization of enzymatic activity: Semi-qu holipase termination medium (28) modified, su of ste ed egg yolk (Difco) by two natural eg ualita proteinase determination medium (1 ecies firmation, the classical methods of Lo ger j (15) and Barnett *et al*. (1) were utilized.

Pa eristics detection
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s es were smeared in duplicate into SAB+YE sla rs 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.

Spe	Registration number (URM)	Storage period (years)	Origin
Cand *albi*	720	44	Bronchoalveolar secretion

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 10^6/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 thawn

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 spectophotometer). 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 *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 37ºC

All strains were able to grow at 37ºC. Twenty-eight *Candida* and two *Trichosporon pullulans* strains grew similarly at room temperature and 37ºC.

The ability of some fungi to grow at 37ºC is cited in the literature as a pathogenicity factor (16,17,22,29,35). *Histoplasma capsulatum* and *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 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 *C. albicans*, three (23.08%) *C. parapsilosis*, two (15.39%) *C. tropicalis*, one (7.69%) *C. glabrata* and one (7.69%) *C. krusei* (Fig. 1).

The Phospholipase Zone (PZ) for the 15 stocked strains ranged from 0.179 to 0.310. One strain presented a PZ band 0.179, 11 strains bands between 0.204 and 0.270 and one strain presented a PZ band 0.310 (Table 3).

From the 15 fresh strains isolated from AIDS patients, four (26.66%) were phospholipase positive and 11 (73.34%) were negative. From the four positive strains, three (75%) were *C. albicans* and one (25%) *C. parapsilosis* (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 *C. albicans*, other *Candida* species were also able to produce phospholipase. Only two strains did not produce phospholipase: one *C. parapsilosis* and one *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.

Species	URM Registration number	Phospholipase activity (PZ)	Proteinase activity
Candida albicans	720	0.310	1.066 U/mL
C. albicans	743	0.217	1.386 U/mL
C. albicans	2224	0.270	0.673 U/mL
C. albicans	2252	0.259	1.053 U/mL
C. albicans	2253	0.260	0.733 U/mL
C. albicans	3622	0.223	0.726 U/mL
C. glabrata	943	0.204	1.353 U/mL
C. krusei	934	0.261	0.803 U/mL
C. parapsilosis	729	-	0.973 U/mL
C. parapsilosis	3621	0.179	0.686 U/mL
C. parapsilosis	3624	0.240	0.700 U/mL
C. parapsilosis	3627	0.254	0.686 U/mL
C. tropicalis	1150	0.269	0.673 U/mL
C. tropicalis	916	0.267	0.860 U/mL
T. pullulans	1323	-	0.830 U/mL

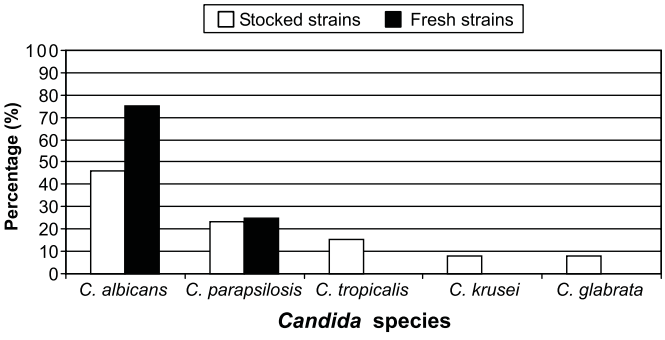

Figure 1. Phospholipase activity percentage in *Candida* strains.

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

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

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. pulullans* 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 from 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, phosplipase 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 Ghannoum (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 -20ºC and - 80ºC 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 melittus patients. It is likely that the samples of our study have decreased their ability to produce phospholipase as consequence of antifungal tratment 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 0.886 $U.mL^{-1}$ 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*) 84 (*C. albicans*) 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

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 phagocitic cells, showed that *C. parapsilosis* did not produced acid proteinase *in vivo*.

C. albicans is able to produce proteinases *in vitro* in high quantities (3, 6, 8, 12, 24, 30). Chakabrati *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 C*andida* 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 Biiológicas, 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 37ºC, bem como apresentarem atividade de fosfolipase e protease. As 30 amostras cresceram a 37ºC 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 positiva e duas (13,34%) atividade fosfolipásica negativa e das 15 amostras isoladas de pacientes com AIDS, quatro (26,66%) apresentaram atividade de fosfolipase positiva e 11 (73,34%) apresentaram atividade fosfolipásica negativa.

Palavras-chave: leveduras, patogenicidade, temperatura, fosfolipase, protease.

REFERENCES

1. Barnett, J.A.; Payne R.W.; Yarrow D. *Yeasts: characteristics and identification*. 4 ed. Cambridge, Cambridge: University Press, 1990, 1002p.
2. Bistoni, F.; Vecchiiarelli, A.; Cenci, A.; Sbaraglia, G.; Perito, S.; Cassone, A. A comparision of experimental pathogenicity of *Candida* species in cyclophophamide-immunodepressed mice. *Sabouraudia*, 22:409-418, 1984.
3. Borg-von, M.; Grünes, V. Characterization of two monoclonal antibodies against secretory proteinase of *Candida tropicalis* DSM 4238. *J. Med. Vet. Mycol.*, 21:1-15, 1993.
4. Borg-von, M.; Meyer, I.; Thomssen, R.; Wurzner R.; Sanglard D.; Monod HIV-Protease inhibitors reduce cell adherence of Candida albicans by inhibition of yeast secreted aspartic proteases. *J. Invest. Dermat.*, 113(5):747-751, 1999.
5. Candido, C.C.; Azevedo, R.V.P.; Komesu, M.C. Enzimotipagem de espécies do gênero *Candida* isoladas da cavidade bucal. *Rev. Soc. Bras. Med. Trop.*, 33(5):437-442, 2000.
6. Chakrabarti, A.; Nayak, N.; Talwar, P. *In vitro* proteinase production by *Candida* species. *Mycopathologia*, 141:163-168, 1991.
7. Coleman, D.C.; Rinaldi, M.G.; Haynes, K.A; Rex, J.H.; Summerbell, R.C.; Anaisse, E.J.; Li, A.; Sullivan, D.J. Importance of *Candida* species other than *Candida albicans* as opportunistic pathogens. *Med Mycol*, 36:156-165, 1998.
8. De Bernardis, F.; Morelli, L.; Ceddia, T.; LorenzenI, R.; Cassone, A. Experimental pathogenicity and acid proteinase secretion of vaginal isolates of *Candida parapsilosis*. *J. Med. Vet. Mycol.*, 28:125-137, 1990.
9. Ghannoum, M.A. Potential role of phospholipases in virulence and fungal pathogenicity. *Clin. Microb. Ver.*, 13(1):122-143, 2000.
10. Gruber, A.; Berlit, J.; Speth, C.; Lass-Florl; Kofler, G.; Nagl, M.; Borg Von Zepelin, M.; Dierich, M.P.; Wurzner R. Dissimilar attenuation of *Candida albicans* virulence properties by human immunodeficiency virus type 1 protease inhibitors. *Immunobiology*, 201(1):133-144, 1999.
11. Hsu, C.F.; Wang, C.G.; Hung, C.S.; Cheng, S.N., Chen, Y.H.; Chu, M.L. *Trichosporon beigelli* causing oral mucosit and fungaemia: report of one case. *Chung Hua Min Kuo Hsiao Erh Ko I Hsueh Hui Tsa Chih*, 39(3):191-194, 1998.
12. Hube, B.; Turver, C.J.; Odds, F.C.; Eiffert, H.; Boulnois, G.J.; Köchel; H.; RücheL, R. Sequence of the *Candida albicans* gene enconding the secretory aspartate proteinase. *J. Med. Vet. Mycol.*, 29:129-132, 1991.
13. Khozel, T.R. Virulence factors of *Cyptococcus neoformans*. *Trends Microbiol.*, 3:295-299, 1995.
14. Krcmery, V.Jr.; Mateicka, F.; Kunova, A. Spanik, S.; Giarfas, J.; Sycova, Z.; Trupl, J. Hematogenous trichosporonosis in cancer patients: report of 12 cases including 5 during prophylaxis with itraconazol. *Support Care Cancer*, 7(1):39-43, 1999.
15. Kreger-Van RIJ, N.J.W. *The yeast: a taxonomic study*. 3.ed. Elsevier Sci. Publication: Amsterdan, 1984.
16. Kwon Chung; Bennet. *Med. Mycol.*, 615. 1992.
17. Lacaz, C.S.; Porto, C.; Martins, J.E.C. *Micologia Médica: fungos, actinomycetes e algas de interesse médico*. 8ª ed., São Paulo: Sarver-EDUSP, 1991. 695p.
18. Leighton, T.J.; Doi, R.H. The relationship of serine proetease activity to RNA polymerase modification and sporulation in *Bacillus subtillis*. *J. Molec. Biol.*, 76:103-122, 1973.
19. Lodder, J. *The Yeast: a taxonomic study*. Oxford: North Holland Publishing Company, 1970. 1385p.
20. Mago, K. Subcellular localisation of enzymes of phospholipase metabolism in *Candida albicans*. *J. Med. Vet. Mycol.*, 28:355-362, 1990.

21. Mayser, P.; Laabs, S.; Heuer, K.; Gründer, K. Detection of extracellular phospholipase activity in *Candida albicans* and *Rhodotorula rubra*. *Mycopathologia*, 135:149-155, 1996.
22. Mcdonald, F.; Odds, F.C. Virulence for mice of a proteinase secreting strain of *Candida albicans* and proteinase deficient mutant. *J. Gen. Microbiol.*, 129:431-438, 1983.
23. Milan, E.P.; Malheiros, E.S.; Fischman, O.; Colombo, A.L. Evaluation of the auxacolor system for the identification of clinical yeasts isolates. *Mycopathologia*, 137:153-157, 1997.
24. MilenwskI, S.; MigninI, F.; CovellI, I.; Borowsky, E. Specific inhibition of acid proteinase secretion in *Candida albicans* by LYS-NVA-FMDP. *J. Med. Vet. Mycol.*, 32:1-11, 1994.
25. Neder, R.N. *Microbiologia: Manual de Laboratório*. São Paulo, ed. Nobel, 98p, 1992.
26. Oliveira, E.E.; Silva, S.C.; Soares, A.J.; Attux, C.; Cruvinel, B.; Silva, M.R.R. Toxinas killer e produção de enzimas por *Candida albicans* isoladas da mucosa bucal de pacientes com câncer. *Rev. Bras. Med. Trop.*, 31:523-527, 1998.
27. Pichova, I; Pavlickova, L; Dostal, J; Dolejsi, E; Hruskova-Heidingsfeldova, O; Weber, J; Ruml, T.; Soucek, M. Secreted aspartic proteases of *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis* and *Candida lusitaniae*. Inhibition with peptidomimetic inhibitors**.** *Eur. J. Biochem.*, 268(9):2669-2677, 2001.
28. Price, M.F.; Wilkinson, I.D.; Gentry, L.O. Plate Method for detection of phospholipase activity in *Candida albicans*. *Saboraudia*, 20:7-14, 1982.
29. Rippon, J.W. Medical Mycology: Hongos y Actinomicetos Patógenos. 3ª ed., México: Copyright, 1990, 855p.
30. RücheL, R. A variety of *Candida* proteinases and their possible targets of proteolytic attack in the host. *Zbl. Bakt. Mikrob. Hyg.*, 257(2):266-274, 1984.
31. RucheL, R.; Boning, B.; Borg, M. Characterisation of a secretory proteinase of *Candida parapsilosis* and evidence for the absence of enzyme during infection in vitro. *Infect. Immun.*, 56:411-419, 1986.
32. Samaranayake, L.P.; Raeside, J.M.; Macfarlane, T.W. Factors affecting the phospholipase activity of *Candida* species in vitro. *J. Med. Vet. Mycol.*, 22:201-207, 1984.
33. Samuels, K.D.Z.; Heale, J.B.; Lewellyn, M. Characteristics relating to the patogenicity of *Metarhizium anisopliae*, toward *Nilaparvata lugens*. *J. Invertebr. Pathol.*, 53:25-31, 1989.
34. Shimizu, M.T. Fosfolipase em espécies de *Candida*. *Rev. Microbiol.*, 20:338-341, 1989.
35. Sidrim, J.J.C; Moreira, J.L.B. *Fundamentos Clínicos e Laboratoriais da Micologia Médica*. 1ª ed., Rio de Janeiro: Guanabara Koogan, 1999, 287p.
36. Smith; Onions *The preservation and maintenance of living fungi.* 1ª ed., USA, Commonwealth Mycological Institute, 1983, 51p.
37. Vidotto, V.; Sinnico, A; Di Fraia, D.; Cardaropoli, S.; Aoki, S.; Ito-Kuwa, S. Phospholipase activity in *Criptococcus neoformans*. *Mycopathologia*, 126:119-123, 1996.
38. Willis, A.M.; Coulter, W.A.; Fulton, C.R.; Hayes, J.R.; Bell, P.M.; Lamey, P.J. The influence of antifungal drugs on virulence properties of *Candida albicans* in patients with diabete mellitus. *Oral Surg.*, 91(3):317-321, 2001.
39. Wu, T; Saramanayake, L.P. The expression of secreted aspartyl proteinases of *Candida* species in human whole saliva. *J. Med. Microbiol.*, (8):711-720, 1999.