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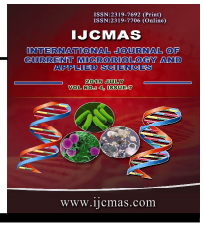

Original Research Article

Virulence Factors among *Trichopsoron* Species from Clinical Isolates in a Tertiary Care Centre

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A B S T R A C T

Trichosporon sp are medically important genus, which colonize and proliferate in different parts of the body. They generally cause opportunistic infections. Virulence factors of this genus are major contributors for their pathogenicity. In our study we have analyzed the presence of virulence factors among 45 *Trichopsoron* species isolated from various clinical samples by phenotypic methods. The tests used were as follows: biofilm production (modified Christensen et al tube method), phospholipase production (by Egg yolk agar method of Samaranayake et al), proteinase activity (Modified Staib method), esterase (Tween-80 opacity medium) and hemolytic activity (SDA with 7% sheep blood agar). About 18 (40%) of the isolates produced biofilm, 27 (60%) showed phospholipase activity and 43 (95.5%) had esterase activity. None of the 45 isolates tested produced proteinase and or had haemolytic activity. Virulence of *Trichosporon* sp is not attributed by a single factor but a combination of multiple factors. This study can be used as reference for further extensive studies on virulence factors of *Trichopsoron* spp.

Introduction

Trichosporon species are basidiomycetes yeast like organisms (Basidiomycota, Hymenomycetes, Tremelloidae, *Trichosporon*ales) which are ubiquitous in nature. They are colonizers in skin, respiratory tract, gastrointestinal system, and vagina. *Trichosporon* sp may cause superficial, deep-seated or mucosa-associated infections (Arnaldo *et al.*, 2011). The extent and severity of infections depends on the immunity of the host.

Trichosporon sp have found to be one of the common causes of non- candidal yeast infections predominantly in patients with underlying risk factors like haematological malignancies, solid tumour, AIDS, burns etc (Pfaller *et al.*,2004). Recently, the incidences of disseminated infections caused by *Trichosporon* sp have increased among the general population (Rastogi *et al.*, 2007). Disseminated infections with *Trichosporon* sp are characterized by high mortality rate,

since it is resistant to most of the antifungal agents (Serena *et al.*,2006 and Zhikuan Xia *et al.*, 2012) Hence, exploring the virulence factors which are primary contributors of pathogenicity and infections caused by *Trichopsoron* sp is important.

Biofilm formation plays an important role in the pathogenicity of *Trichosporon* species especially with the use of catheters and catheter-related devices. This ability to form biofilms on implanted devices accounts for invasive Trichosporonosis and it can also promote the resistance to antifungal drugs and evasion of host immune response (Arnaldo *et al.,* 2011). Secretion of enzymes by the organism for scavenging nutrients from the environment also serves as an important virulence factor.

The most common enzymes produced by the organism, proteases and phospholipases, increases the pathogenicity by breaking up proteins and disrupting host cell membranes (Chaves *et al.,* 2003). This extracellular proteolytic activity plays an important role in its pathogenicity by facilitating invasion through degradation of keratin and collagen (Arnaldo *et al.,* 2011).

Microorganisms invading the host cells can penetrate and damage the outer cell envelope. Phospholipids and proteins represent the major chemical constituents of the host cell envelope. The enzymes which are capable of hydrolyzing these chemical constituents, such as phospholipases and proteinases, are probably involved in the membrane disruption activity that occurs during host cell invasion (Hube *et al.,* 2001 and Ibrahim *et al.,* 1995). Furthermore, the survival and the capacity of the yeast to cause infections in human beings are mainly related to its ability to produce elemental iron through hemolysin production.

Materials and Methods

This study was done at Sri Ramachandra Medical College and Research Institute, Chennai which is a tertiary care centre. It is a retrospective hospital based descriptive study which was initiated after getting approval from Institutional Ethics Committee (CSP/13/JUN/29/113).

About 45 genotypically confirmed and archived strains of *Trichosporon* species isolated from various clinical samples were used for the study. The isolates were tested for various virulence factors like biofilm production, haemolytic activity and production of enzymes likes phospholipase, proteinase, and esterase.

Test for virulence factors

Bio film formation

Biofilm production of the *Trichosporon* isolates was identified by modification of the standard method by Christensen *et al.* 1982. A loopful of the *Trichosporon* isolate from overnight culture plates was inoculated in two milliliters of Sabouraud dextrose broth in 12 x 75 mm borosilicate test tubes and incubated at 37°C for 48 hours. The contents were then decanted from the tubes, washed with phosphate buffered solution (PBS) at pH 7.3, and left to dry at room temperature. The tubes were then stained by adding 4% crystal violet solution to the tubes and were rotated to ensure uniform staining. The contents were gently decanted, the tubes were placed upside down to drain and then observed for biofilm formation. Development of a visible film which lined the sides and bottom of the tube was considered as positive. However formation of ring at the liquid interface was not considered as biofilm formation. The scoring was performed as done by Mathur *et*

al. (2006) (Absent - 0, Weak - 1, Moderate - 2, Strong - 3) by visual observation.

Enzyme production

Preparation of *Trichosporon* suspension

A twenty four to forty eight hour old culture of *Trichosporon* from Sabouraud dextrose agar was suspended in sterile PBS and the turbidity was adjusted to 0.5 Mc Farland standard. The suspension prepared was used to evaluate the phospholipase, esterase, proteinase and hemolytic activity (Staib *et al.*, 1965 and Vinitha Mohan Das *et al.*, 2008)

Determination of phospholipase activity by using egg yolk agar

To determine phospholipase activity, the egg yolk agar method of Price *et al.* (1982) which was modified by Samaranayake *et al.* (2005) was employed. The culture medium contained 1 M NaCl, 0.005 M $CaCl_2$, and 10% sterile egg yolk in 1L of SDA. Ten micro liters of previously prepared *Trichosporon* suspension was inoculated onto plates; and then incubated at 37 °C for 5 days (Vinitha Mohan das *et al.*, 2008; Melek Inci *et al.*, 2012). Formation of a zone of precipitation around the *Trichosporon* colonies indicated the presence of enzymatic activity. Phospholipase activity (Pz) was calculated by dividing the diameter of the colony by the diameter of the colony plus precipitation zone. If Pz = 1, negative phospholipase activity; Pz = 0.64–0.99, positive phospholipase activity; Pz ≤ 0.63, very strong phospholipase activity.

Determination of proteinase activity by using bovine serum albumin agar

Proteinase activity was detected by using the Modified Staib method. Medium containing 2% dextrose, 0.1% KH_2PO_4, 0.05% $MgSO_4$ and 2% agar was prepared. After cooling to 50˚C, 1% bovine serum albumin solution was added. Ten micro liters of *Trichosporon* suspension was inoculated into the wells punctured onto the surface of the medium. The plates were incubated at 37˚C for 10 days. After incubation, the plates were stained with amidoblack. Opaqueness of the media around the wells that could not be stained with amidoblack, corresponds to a zone of proteolysis which indicated the degradation of protein (Vinitha Mohan Das *et al.,* 2008). The diameter of unstained zone around the well was measured as the degree of proteinase production. The proteinase activity (Pz) = diameter of the well / diameter of the proteolytic unstained zone (Kemker *et al.,* 1991).

Determination of hemolytic activity by using SDA with 7% sheep blood

To determine hemolytic activity, SDA containing sheep blood 7% and glucose 3%, with final pH adjusted to 5.6 was used. Ten microliters of previously prepared *Trichosporon* suspension was inoculated onto plates; these were then incubated at 37°C for 48 hrs (Melek Inci *et al.,* 2012 and Manns *et al.,* 1994). A transparent/semitransparent zone around the site of inoculation was considered as positive hemolytic activity (Manns *et al.,* 1994).

Determination of esterase activity by using tween-80 opacity test medium:

To determine esterase activity, Tween-80 opacity test medium was used. The test medium consisted of 1% peptone, 0.5% NaCl, 0.01% $CaCl_2$, and 1.5% agar with pH adjusted to 6.8. After cooling the medium (50°C), 0.5% of Tween-80 was added. Ten micro liters of previously prepared *Trichoporon* suspension of each isolate was

inoculated onto the Tween-80 opacity test medium, this was then incubated at 37 °C for 10 days (Melek Inci *et al.,* 2012). Presence of a halo pervious to light around the inoculation site was considered as positive for esterase activity (Slifkin *et al.,* 2000).

All strains were tested 3 times in the enzymatic evaluation and interpreted by two independent observers.

Results and Discussion

The results of the ezymatic activity, biofilm production and haemolytic activity of all the 45 *Trichosporon* isolates tested is given in table 1.

Among 45 *Trichosporon* isolates tested for biofilm production using tube method, 18 were weakly positive (Fig. 1A) and others showed a negative result (Fig. 1B). Among the 45 *Trichosporon* isolates tested for esterase activity 43 (95.5%) were esterase positive (Fig. 2A) and the rest of the two isolates were negative (Fig. 2B). Among all the 45 *Trichosporon* isolates tested for phospholipase activity, 27 (60%) expressed phospholipase activity (Fig. 3). Pz value ≤ 0.63 indicates very strong positive result (Gueho *et al.,* 1992). All the 27 isolates which expressed phospholipase activity had their Pz value between 0.3-0.5 which indicated a very strong positive result. Proteinase activity was found to be negative in all the tested strains by using modified Staib method (Fig. 4). All the 45 isolates showed no hemolytic activity (Fig. 5) in our study. About 21 (47%) *Trichosporon* isolates produced both phospholipase and esterase. Only two isolates were negative for both the enzymes. Among the 18 (40%) weakly positive biofilm producers, 15 (33%) of them also produced the enzymes esterase and phospholipase.

Trichosporon sp have been documented as an opportunistic agent causing rising, invasive infections all over the world, though most of them isolated from clinical laboratories are colonizers or cause superficial infections. Invasive Trichosporonosis has been documented in critically ill patients, patients with malignancies and those who are exposed to multiple invasive medical procedures.

Various factors like the presence of glucuronoxylomannan (GXM) in the cell wall of *Trichosporon* spp, their ability to produce enzymes like phospholipases, proteinases, lipases and production of slime, are likely to be associated with the virulence of this genus. These virulence factors may lead to the progression of invasive Trichosporonosis by escaping host immune defences, Even though *Trichosporon* species are the second most common cause of non candida yeast infections in high risk patients causing invasive disease, only very few studies have been done addressing the virulence factors of this genus.

In the present study about 60% of *Trichosporon* isolates i.e. 27/45 were phospholipase producers and 96% of them i.e (43/45) produced esterase. In a previous study on production of virulence factors from 50 *T. ashaii* strains by Dag and Cerikcioglu (2006), proteinase and phospholipase activity could not be detected in any of the isolates although all were esterase positive. The result of esterase activity in our study is almost the same as the previous study (Dag and Cerikçioglu, 2006). In another study conducted by Cafarchia and his collegues from pigeon cloacae and droppings, of the 14 *T. beigelii* isolates tested, 6 were positive for phospholipase production (Cafarchia *et al.,* 2008).

Table.1 Virulence factors of *Trichosporon* species

Virulence factors	No. of clinical isolates(45)
Biofilm formation	18(40%)
Hemolytic activity	0
Phospholipase activity	27(60%) (Mean Pz = 0.23)
Proteinase activity	0
Esterase activity	43(95.5%)

Figure.1 Biofilm formation

A. weak positive B. Negative

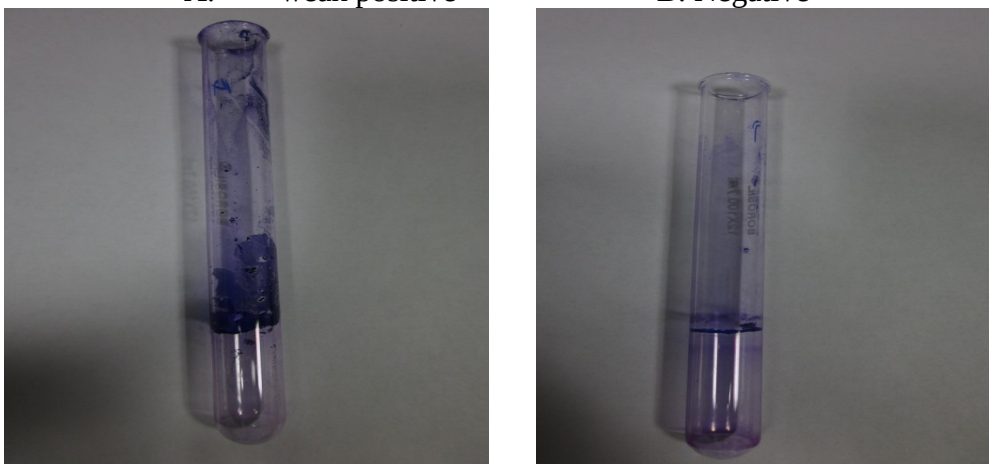

Fig.2 Esterase activity

A. Esterase positive B. Esterase negative

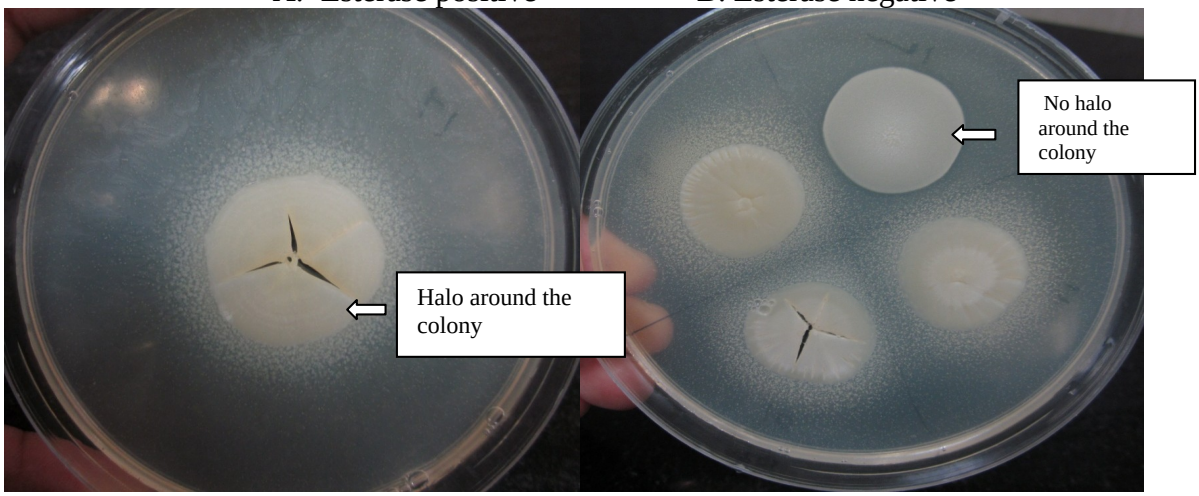

Fig.3 Phospholipase activity

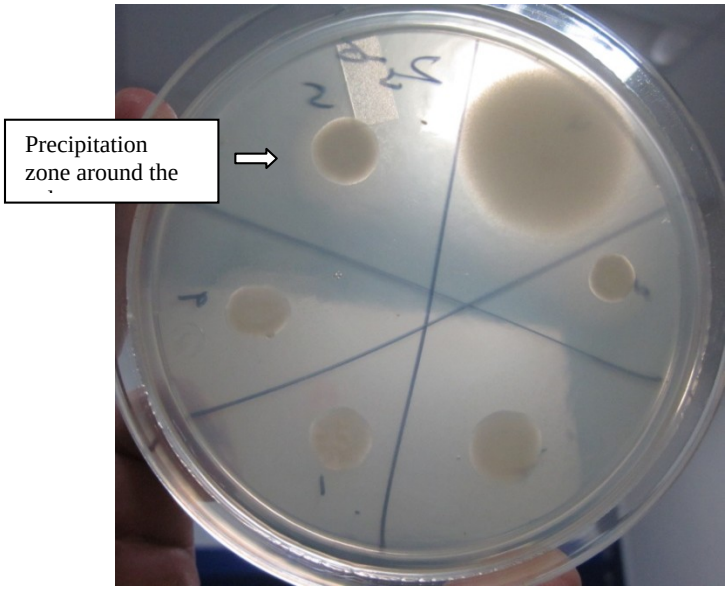

Fig.4 Proteinase activity

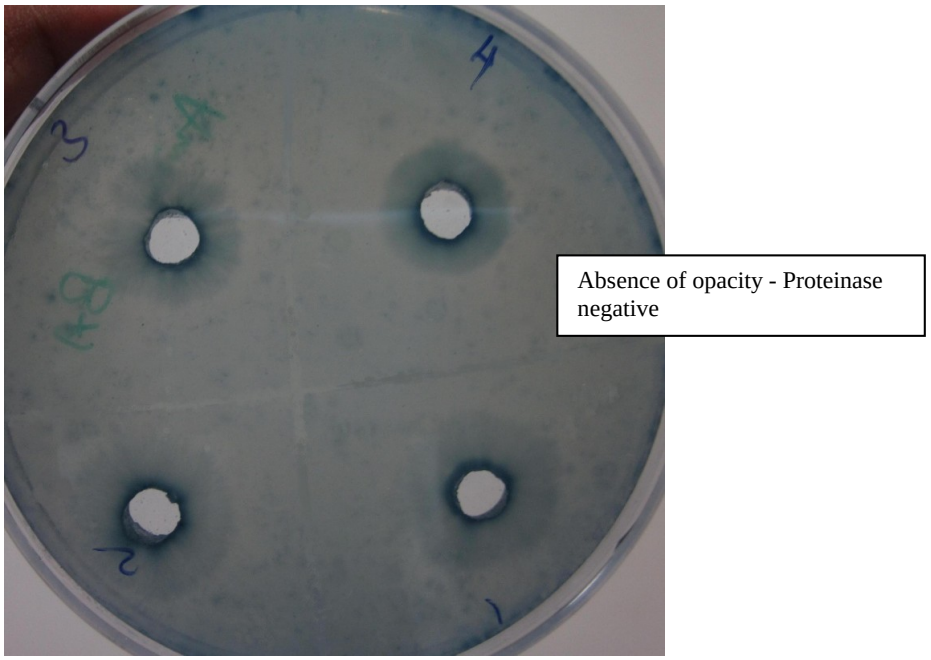

Fig.5 Hemolytic activity

The present study detected higher phospholipase activity 27/45 (60%) when compared to the study by Cafarchia *et al.* (2008) and also there was no phospholipase activity detected by Dag and Cerikçioglu (2006) in his study. To increase the yield of the phospholipase activity particularly in poor phospholipase producing strains, it is necessary that it has to be confirmed with specific radiometric or colorimetric assays and the use of concentrated culture filtrate.

In the present study biofilm production was weakly positive in about 40% (18/45) of the *Trichosporon* spp. In a study by Dag and Cerikçioglu (2006), out of 50 *T. ashaii* strains 10 were moderate slime producers, 18 strains were weak slime producers and 20 strains were slime non producers. In this study detection of biofilm production was less compared to the previous ones. Hence a better method using microtitre plates for detection of slime production could be adopted to increase the percentage of positivity.

In this study none of the isolates produced the enzyme proteinase which is similar to the results of study done by Dag and Cerikçioglu (2006). In a study on proteinase production in yeasts isolated from bovine milk at different temperature, it was found that 16·81% of the strains produced proteinase at 37°C and 4·09% under refrigeration (Melville *et al.*, 2011). In the present study proteinase was detected using routinely used Bovine Serum Albumin agar method. However it is a relatively insensitive method which cannot detect very low levels of proteinase activity (Naglik *et al.*, 2003). A solution based Bovine Serum Albumin hydrolysis assay is a better method which can overcome this drawback.

The fact that few *Trichosporon* isolates were positive for both the enzymes (Phospholipase, esterase) and biofilm production, and all the isolates tested negative for the other enzymes (proteinase) and hemolytic activity, emphasizes the fact that various other virulence mechanisms are

also involved in the pathogenesis of the genus *Trichosporon.*

The virulence of *Trichosporon* species is attributed to a combination of various factors like production of proteinase, phospholipase, biofilm production and other unknown enzymes which has not been described. With growing incidence of disseminated *Trichosporon* infections, it is essential to conduct further studies regarding their virulence mechanisms in a larger scale by improvising the techniques for the detection of virulence factors.

Very few studies have been done pertaining to the virulence factors of *Trichosporon* species, considering this as a pilot study and further studies can be performed comparing the virulence factors production of pathogenic and environmental strains.

References

Arnaldo, L., Colombo, Ana Carolina, B., Padovan, Guilherme M. Chaves, 2011. Current Knowledge of *Trichosporon* spp. and Trichosporonosis. *Clin. Microbiol. Rev.*, 24(4): 682. doi: 10.1128/CMR.00003-11.

Cafarchia C., Romito D., Coccioli C., Camarda A., Otranto D. 2008. Phospholipase activity of yeasts from wild birds and possible implications for human disease. *Med. Mycol.*, 46: 429–434

Chaves, G.M., Cavalcanti, M.A.Q., Porto, A.L.F. 2003. Pathogenicity characteristics of stocked and fresh yeasts strains. *Braz. J. Microbiol.*, 34: 197–202.

Christensen, G.D., Simpson, W.A., Bisno, A.L., Beachy, E.H. 1982. Adherence of biofilm producing strains of *Staphylococcus epidermidis* to smooth surfaces. *Infect. Immun.*, 37: 318–326.

Dag, A., Cerikçioglu, N. 2006. Investigation of some virulence factors of *Trichosporon asahii* strains isolated from the clinical samples of hospitalized patients. *Microbiol. Bul.*, 40: 225–235

Gueho, E., de Hoog, G.S., Smith, M.T. 1992. Neotypification of the genus *Trichosporon*. *Antonie Van Leeywenhoek.*, 61: 285–288.

Hube, B., Naglik, J. 2001. *Candida albicans* proteinases: resolving the mystery of a gene family. *Microbiology*, 147: 1997–2005.

Ibrahim, A.S., Mirbod, F., Filler, S.G., Banno, Y., Cole, G.T., Kitajima, Y., Edwards, Jr. J.E., Nozawa, Y., Ghannoum, M.A. 1995. Evidence implicating phospholipse as a virulence factor of *Candida albicans. Infect. Immun.*, 63: 1993–1998.

Kemker, B.J., Lehmann, P.F., Lee, J.W., Walsh, T.J. 1991. Distinction of deep versus superficial clinical and nonclinical isolates of *Trichosporon beigelii* by isoenzymes and restriction fragment length polymorphisms of rDNA generated by polymerase chain reaction. *J. Clin. Microbiol.*, 29:1677–1683.

Manns, J.M., Mosser, D.M., Buckley, H.R.1994. Production of a hemolytic factor by *Candida albicans*. *Infect. Immun.*, 62: 5154–6.

Mathur, T., Singhal, S., Khan, S., Upadhyay, D.J., Fatma, T., Rattan, A. 2006. Detection of biofilm formation among the clinical isolates of staphylococci: an evaluation of three different screening methods. *Indian J. Med. Microbiol.*, 24: 25–29.

Melek Inci, Mustafa Altay Atalay,Ayse Nedret Koc, Erkan Yula, Omer Evirgen, Syleyman Durmaz. 2012.

Investigating virulence factors of clinical isolates in relation to atmospheric conditions and genotype. *Turk. J. Med. Sci.,* 42(sup): 1476–1483.

Melville, P.A., Benites, N.R., Ruz-Peres, M., Yokoya, E. 2011. Proteinase and phospholipase activities and development at different temperatures of yeasts isolated from bovine milk. *J. Dairy Res.,* 78(4): 385–90. doi: 10.1017/S0022029911000434. Epub 2011 Jul 27.

Naglik, J.R., Rodgers, C.A., Shirlaw, P.J., Dobbie, J.L., Fernandes-Naglik, L.L., Greenspan, D., Agabian, N., Challacombe, S.J. 2003. Differential expression of *Candida albicans* secreted aspartyl proteinase and phospholipase B genes in humans correlates with active oral and vaginal infections. *J. Infect. Dis.,* 188: 465–475.

Pfaller, M.A., Diekema, D.J. 2004. Rare and emerging opportunistic fungal pathogens: concern for resistance beyond *Candida albicans* and *Aspergillus fumigatus*. *J. Clin. Microbiol.,* 42(10): 4419–31.

Price, M.F., Wilkinson, I.D., Gentry, L.O. 1982. Plate method for detection of phospholipase activity in *Candida albicans*. *Sabouraudia,* 20: 7–14.

Rastogi, V.L., Nirwan, P.S. 2007. Invasive Trichosporonosis due to *Trichosporon asahii* in a non-immunocompromised host. A rare case report. *Ind. J. Med. Microbiol.,* 25(1): 59–61.

Samaranayake, Y.H., Dassanayake, R.S., Jayatilake, J.A.M.S., Cheung, B.P.K., Yay, J.Y.Y. 2005. Phospholipase B enzyme expression is not associated with other virulence attributes in *Candida albicans* isolates from patients with human immunodeficiency virus infection. *J. Med. Microbiol.,* 54: 583–593.

Serena, C., Gilgado, F., Marine, M., Pastor, F.J., Guarro, J. 2006. Efficacy of voriconazole in a guinea pig model of invasive Trichosporonosis. *Antimicrob. Agents Chemother.,* 50: 2240–2243.

Slifkin M. 2003. Tween 80 opacity test responses of various *Candida* species. *J. Clin. Microbiol.,* 38: 4626–8.

Staib, F. 1965. Serum-proteins as nitrogen source for yeastlike fungi. *Sabouraudia.,* 4: 187–193.

Vinitha Mohan Das, Mamatha Ballal, 2008. Proteinase and phospholipase activity as virulence factors in Candida species isolated from blood. *Rev. Iberoam Micol.,* 25: 208–210.

Zhikuan Xia, Rongya Yang, Wenling Vang, Ling Cong, 2012. Genotyping and antifungal drug susceptibility of *Trichosporon asahii* isolated from Chinese patients. *Mycopatholgia.,* 173(2-3): 127–133