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Article

Virulence potential of filamentous fungi isolated from poultry barns in Cascavel, Paraná, Brazil

Cleison Ledesma Taira[1], Nadir Rodrigues Marcondes[2], Valdeci Aparecido Mota[1], Terezinha Inez Estivalet Svidzinski*[1]

Laboratory of Medical Mycology, Department of Clinical Analysis, State University of Maringá,
[2]Laboratory of Microbiology, Pharmacy Course, State University of Western Paraná

Opportunistic fungi are those that normally would not cause diseases in otherwise healthy people, but are able to cause problems under some circumstances, and for this they need to possess a certain virulence potential. The objective of this study was to identify samples of filamentous fungi isolated from poultry barns in Cascavel, Paraná, and also to evaluate their virulence potential by assessing proteinase production, hemolytic activity, urease production, and growth rate at 37 ºC. We have evaluated the following samples: *Acremonium hyalinulum* (1 sample), *Aspergillus* sp. (12), *Beauveria bassiana* (1), *Curvularia brachyspora* (1), *Paecilomyces variotti* (1), and *Penicillium* sp. (2). Out of the 18 samples analyzed, 44.4% showed proteolytic activity using albumin as the substrate versus 66.7% when using casein; 66.7% showed hemolytic activity, 83.3% were positive for urea, and 88.9% grew at a temperature of 37 ºC. The results demonstrated that the majority of the isolates expressed virulence factors. Therefore, these isolates have the potential to harm human hosts, such as those working at poultry barns, especially predisposed or susceptible individuals.

Uniterms: Filamentous fungi/identification. Filamentous fungi/virulence potential. Filamentous fungi/enzymatic activity. Filamentous fungi/hemolytic activity.

Fungos oportunistas são aqueles que normalmente não causariam doenças em pessoas saudáveis, mas eles são capazes de causar problemas sob certas circunstâncias e, para isso, eles necessitam possuir algum potencial de virulência. O objetivo deste trabalho foi identificar amostras de fungos filamentosos isolados de granjas de aves em Cascavel, Paraná, e também avaliar o seu potencial de virulência, verificando a produção de proteinase, atividade hemolítica, produção de urease e crescimento a 37 ºC. Foram avaliados *Acremonium hyalinulum* (01), *Aspergillus* sp (12), *Beauveria bassiana* (01), *Curvularia brachyspora* (01), *Paecylomices variotti* (01) e *Penicillium* sp (02). Das 18 amostras, 44,4% apresentaram atividade proteolítica usando como substrato a albumina e 66,7% com caseína; 66,7% demonstraram atividade hemolítica, 83,3% foram uréia positivas e 88,9% cresceram em temperatura de 37 ºC. Os resultados demonstram que a maioria dos isolados expressaram fatores de virulência e, portanto, têm potencial para causar danos a hospedeiros humanos como os trabalhadores dos aviários, sobretudo em indivíduos predispostos ou suscetíveis.

Unitermos: Fungos filamentosos/identificação. Fungos filamentosos/potencial de virulência. Fungos filamentosos/atividade enzimática. Fungos filamentosos/atividade hemolítica.

INTRODUCTION

Fungi are heterotrophic organisms, and can act as saprobionts, parasites, and symbionts. Under certain conditions, saprobionts can become pathogenic, and in this case are called opportunistic fungi. Immunosuppressed individuals are susceptible to infections caused by these types of fungi, which can be found in the human body or carried by the air or feed. In their natural habitat, fungi grow and reproduce in the presence of appropriate nutrients and environmental conditions, such as temperature and humidity.

***Correspondence:** T. I. E. Svidzinski. Laboratório de Micologia Médica, Departamento de Análises Clinicas, Universidade Estadual de Maringá. Av. Colombo 5790, Bloco J90, Sala 11, Zona Sete - 87020-900 - Maringá - PR, Brazil. E-mail: tiesvidzinski@uem.br

The dispersal efficiency of fungi is closely related to the high production of the dissemination elements, in this case spores. In addition to spores, fragments of vegetative mycelium or other fungal structures can also act as dissemination elements. Through the efficient dispersal processes of the fungi, these microorganisms are found in high concentrations in the environment, and are transported by the air. When they encounter an appropriate substrate and environmental conditions for their growth, these propagules will germinate, grow, and colonize the surfaces where they were inserted. Fungi are characterized by their great enzymatic variability. Consequently, they can colonize several types of substrates, breaking down materials such as wood, glass, paints, paper, rubber, clothing, foods, skins, and others (Clarice *et al.,* 1998).

Brazil is a major producer of chickens and currently ranked as the third-largest producer and the second-largest exporter of poultry in the world. This growth has encouraged management practices that increase the number of birds on the same platform (Chernki-leffer *et al.,* 2002). This kind of practice allows the accumulation of organic matter such as feces, urine, feathers, and remains of feed, providing optimal conditions for the proliferation of diverse microorganisms, including fungi. Normally, fungi are carried by air and dust that are rich in organic matter, which can be subsequently inhaled by humans. increasing the health risks for workers in these environments.

Although the epidemiology of fungal infections is not yet clear or well established, the mycoses caused by pathogenic agents such as *Paracoccidioies brasiliensis*, *Candida albicans*, *Cryptococcus neoformans*, and also *Aspergillus fumigatus* have been well documented. However, a number of species of environmental fungi previously characterized as nonpathogenic have emerged as human infection agents (Ponton *et al.,* 2000). Mycoses caused by this group of fungi are designated as opportunistic, because normally they are considered saprobiontic agents, incapable of causing disease in immunocompetent individuals. On the other hand, these microorganisms in immunocompromised individuals are responsible for serious infections. Investigation of these microorganisms is therefore justified.

Virulence factors are products or attributes of a fungus that increases its capacity to attack the host. Many virulence factors are obvious and important, such as growth ability at 37 ºC and physiological pH, which is essential for the pathogenicity of a dimorphic fungus (Hogan *et al.*, 1996). Several studies have shown that samples of *C. albicans* and other species of this genus produce proteinases and phospholipases, providing compelling evidence that these enzymes are virulence determinants, independently from the anatomical site or clinical conditions from which they were isolated (Ruiz *et al.,* 2005).

Despite great progress in environmental fungus knowledge and also on opportunistic mycosis, little it is known about virulence factors of these microorganisms. The objective of this study was to identify and evaluate the virulence potential of ambient filamentous fungi isolated from poultry farms.

MATERIAL AND METHODS

The fungi were isolated from the litter platforms of poultry barns on farms in Cascavel, Paraná, Brazil according to Marcondes *et al.* (2007). Identification of the fungal isolates was based upon macroscopic and microscopic morphology in V8 agar, according to De Hoog *et al.* (2001).

C. albicans ICB 12A, maintained in stock cultures (peptone, beef extract, sodium chloride, yeast extract, distilled water, glycerol) at - 20 º C, was reactivated on Sabouraud dextrose broth (Difco – Becton Dickinson, Sparks, MD, USA) for 24 h at 25 ºC. Subsequently, a loopful of this young culture was streaked on Sabouraud dextrose agar (SDA) and incubated for 18 h at 25 ºC. Inoculum of this culture was used as control for all experiments.

The inoculum of the ambient filamentous fungi, isolated from poultry farms to investigate proteinase and hemolytic activity, was prepared from a suspension of the fungus in sterile physiological saline (SPS). A small fragment of the colony was placed into a tube containing 2 mL of SPS, pulverized with a microbiological loop, and vigorously stirred in a Vortex mixer (Phoenix AP56) for 30s. The inoculum size was adjusted to 1.0×10^6 and 5.0×10^6 spores/mL, by microscopic counting using a cell-counting hematocytometer (Neubauer chamber; Merck, S.A., Madrid, Spain). To test, a spot with 1.0 µL was placed on agar surfaces with the respective substrate.

Proteinase production was evaluated in Petri dishes containing agar and supplemented with two other substrates, as follows: bovine albumin (BSA) and casein, according to the methodology proposed by Ruchel *et al.* (1982). The presence of the enzymes was detected by the formation of a translucent degradation halo of the respective proteins around each colony. Enzyme activity (Pz) was measured based on the proportion between the colony diameter and the degradation zone. Enzyme activity was interpreted as follows: Pz=1 absence of activity; $0.64<Pz<1$ positive enzyme activity; and $Pz<0.65$ strongly positive activity (Price *et al.,* 1982). The dishes were incubated at 25 ºC for up to eight days, and readings were taken at the beginning of the fourth day (96 hours).

Hemolytic activity was observed in SDA medium supplemented with 5% glucose and 5% sheep blood (Bonassoli *et al*., 2005). The dishes were incubated at 25 ºC for up to seven days, and hemolysis was classified as absent, partial, or total.

The urease test was conducted using Christensen's urea agar medium or urea-agar, in tubes incubated for three days at 25 ºC. A red color in the medium indicated a positive result, evidencing raised pH (alkalinisation) due to urea breakdown and subsequent medium color change.

The growth assay at 37 ºC was carried out in tubes containing SDA medium. For each fungus isolated, 10 µL of suspension in SPS was inoculated into two different tubes. One tube was incubated at 25 ºC and the other at 37 ºC, in order to evaluate the capacity of the species to develop in above-ambient temperature.

RESULTS AND DISCUSSION

A total of 19 samples of filamentous fungi were isolated from poultry farms, identified by genus or species level. All samples were assumed to be saprobiontic fungi, except for *Beauveria bassiana*, which is an entomopathogenic fungus used as a biological control for insect control on farms.

One isolate of *Acremonium hyalinulum*, 1 *Alternaria tenuissima*, 12 *Aspergillus* sp., 1 *Beauveria bassiana*, 1 *Curvularia brachyspora,* 1 *Paecilomyces variotti,* and 2 *Penicillium* sp, were identified. Several virulence factors were evaluated in 18 samples. The *Alternaria tenuissima* sample proved to be fastidious, since it was difficult to manipulate and its behaviors against albumin, casein, and urea tests were inconsistent. Therefore, this isolate was not compared with the other isolates which, in contrast to this particular sample, developed easily on the substrates used and yielded reliable readings.

A total of nine samples (50.0%) degraded albumin, whereas 12 degraded casein (66.7%). In addition, 12 samples showed hemolytic activity (66.7%) while 15 were urease-positive (83.3%), and 16 grew at a temperature of 37 ºC (88.9%), as seen in Table 1. Only 7 samples were able to degrade both proteins (albumin and casein), and 5 of these showed no specificity for either of the two substrates.

Virulence factors are properties that increase the survival, growth, and propagation of fungi in animal tissue (De Hoog *et al.*, 2001). Some factors are well known, such as the ability of the organism to grow at 37 ºC in neutral pH, to excrete proteinase and phospholipase, and the capacity for adhesion (De Hoog *et al*., 2001). Other factors are not yet well studied and detailed, such as the production of urease (Cox *et al.,* 2000) and hemolytic activity (Luo *et al.,* 2001).

All fungi found in the poultry barns were considered saprobionts, but depending on the situation they might have the potential to become opportunistic. Some of these have been reported as causing human infections, such as fungemia by *Acremonium* sp. (Mattei *et al*., 2003), subcutaneous mycosis by *Alternaria* sp. (Vieira *et al.,* 1998), whereas *Penicillium chrysogenum* has been reported as a cause of infection of the central nervous system (Kantar-

TABLE I - Virulence potential of filamentous fungi isolated from poultry barns

Fungi	N	Albumin	Casein	Hemolysis	Urease	37 ºC
Acremonium hyalinulum	1	Abs	Abs	Hp	-	+
Aspergillus alliaceus	1	Abs	Abs	Hp	+	+
Aspergillus caesiellus	1	Pos	Pos	Hp	+	+
Aspergillus deflectus	1	Abs	Pos	Abs	+	+
Aspergillus hollandicus	3	Abs	Abs	Abs	+	+
Aspergillus janus	1	Abs	Pos	Abs	+	+
Aspergillus niveus	2	Pos	Pos	Hp	+	+
Aspergillus terreus	3	Pos	Pos	Hp	+	+
Beauveria bassiana	1	Strong	Pos	Hp	-	-
Curvularia brachyspora	1	Abs	Pos	Hp	+	+
Paecylomices variotti	1	Pos	Abs	Hp	-	+
Penicillium chrysogenum	1	Pos	Pos	Abs	+	+
Penicillium expansum	1	Abs	Pos	Hp	+	-

N = number of isolated strains; Albumin and Casein: Abs = absence of activity; Pos = positive; Strong = strongly positive; Hemolysis: Abs = Absence of activity; Hp = Partial hemolysis; Positive for urease = +; Positive for growth at 37 ºC = ; - = Negative cioglu *et al.,* 2004) and intestinal invasion followed by dissemination (Barcus *et al*., 2005). According to Thomas (2003), species from *Aspergillus* and *Paecilomyces* genera*,* including *A. terreus* and *P. variotti,* which were isolated in this study, could cause ophthalmic infections.

Dematiaceous fungi such as *Curvularia,* despite their ubiquitous presence in the environment, may cause infections in humans. *Curvularia* was originally thought to be a pathogen of plants, but has been described as a pathogen in humans and animals over the last half-century, that causes respiratory tract, cutaneous, and corneal infections. Carter and Boudreaux (2004) reported a fatal case of cerebral *Curvularia* infection in a patient with no apparent medical history involving an immunocompromised individual or evidence of prior respiratory tract or sinus infection.

Particular attention should be paid to *Aspergillus*, because 12 out of our 19 isolated samples belong to this genus, which is commonly associated with sinusitis (Monteiro *et al.,* 2002), keratitis (Hofling-Lima *et al.,* 2005) and respiratory allergies (Menezes *et al.,* 2004; Mezzari *et al.,* 2003). Recently, Corry and Kheradmand (2009) showed a major environmental link between asthma and exposure to environmental proteinases, especially airway infection by proteinase-producing organisms, such as fungi from *Aspergillus* spp. Pending verification in human cases, these findings suggest entirely new therapeutic interventions for asthma, including restricted use of anti-inflammatory therapy and administration of universal anti-fungal agents. In addition, other species from this same genus can attack immunocompromised patients, a situation associated with high rates of morbidity and mortality (Baddley *et al.,* 2003).

Six of the 12 *Aspergillus* isolates showed the presence of all analyzed virulence factors, and all samples from this genus were capable of growing at 37 ºC. In fact, *Aspergillus* spp. are capable of secreting a variety of proteinases, many of them used to degrade dead animals and plants as a nutritional substrate, or to degrade structural barriers of a living host, facilitating tissue invasion (Hogan *et al.,* 1996). According to Rhodes (2006), germination rate, efficient growth at 37 ºC, and nutritional versatility, together with high sensitivity and rapid response to a nitrogen source, are important properties for a fungus to become an opportunist pathogen. All the species of *Aspergillus* isolated in this study exhibited rapid growth at 37 ºC, and developed in a medium containing bovine albumin (BSA) as the only nitrogen source.

Proteinase production is a well-studied parameter in isolates of *Candida albicans* (Kantarcioglu *et al.,* 2002, Oliveira *et al.,* 1998; Penha *et al.,* 2000). Louie *et al.* (1994) demonstrated a positive correlation *in vivo* between proteinase activity and virulence in *C. albicans* (Louie *et al.,* 1994). The majority of the 75 yeasts (98.7%) had strongly positive enzyme activity (Ruiz *et al.,* 2005). In relation to true pathogenic fungi, Assis *et al.* (1999) demonstrated that 100% of 20 *P. brasiliensis* isolates were strong proteinase producers in dishes containing base medium and bovine albumin, characteristics associated to pathogenicity.

Hemolytic activity was observed in 66.7% of the isolates. This test was used by Luo *et al.* (2001) with different species of *Candida*, and by Bonassoli *et al.* (2005) with *Candida parapsilosis*.

The capacity to hydrolyze urea is recognized as an important factor for pathogenicity of bacteria such as *Helicobacter pylori* and *Proteus mirabilis*. Many human pathogenic fungi produce urease, including *C. neoformans, Coccidioides immitis, Histoplasma capsulatum, Sporothrix schenckii,* and species of *Trichosporon* and *Aspergillus* (Cox *et al.,* 2000). Cox *et al.* (2000) tested a urease-negative mutant of *C. neoformans* in experimental cryptococcosis using three animal models. The authors noted that urease activity was one of the pathogenicity factors of this genus. In the present study, 15 out of 18 samples (88.9%) were urease-positive, corroborating the virulence potential of these ambient fungi.

Except for *B. bassiana* and *P. expansum,* all the samples tested had the ability to grow at 37 ºC. This result differs from the observations of Silva *et al.* (1999), who achieved growth in only a few saprobiotic fungi. This difference may be explained by the fact that poultry barns are heated to 37ºC, and therefore the fungi isolated in this study may have been selected over time, which would have potentiated their capacity to survive in human tissue. The thermotolerance in filamentous fungi is commonly associated to virulence potential increase (De Crecy *et al.*, 2009).

B. bassiana was strongly positive for enzyme activity, for degrading albumin, and causing partial haemolysis. This fungus is entomopathogenic and generally used as a biological control on farms. However, it is important to point out that this agent is also capable of causing keratomycosis in humans (Sonoyama *et al.* 2008). Furthermore, Westwood *et al.* (2006) identified four presumed allergens from *B. bassiana*, confirming its potential for causing allergic reactions in humans.

CONCLUSION

This study has demonstrated the presence of virulence factors in fungi considered nonpathogenic, suggesting that these microorganisms might have some ability to afflict humans. Given the fact that farming has a huge world impact and involves a large workforce, it is important to establish the virulence potential and mechanisms of these fungi, since farm workers are constantly exposed when performing their activities. It would be useful to evaluate possible prophylactic measures, particularly to avoid inhalation of these microorganisms which exhibit high potential for causing disorders in humans when there is predisposition in the host.

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