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Full Length Research Paper

Enzymatic characterization of *Malassezia pachydermatis* isolates from dogs

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The yeast *Malassezia pachydermatis* is part of the normal cutaneous microflora of most homoeothermic vertebrates. However, under certain conditions as high humidity, seborrheic skin, corticotherapy and immunodeficiency, it can become pathogenic and cause dermatopathies. The pathogenic role of the genus *Malassezia* seems to be related to physical, chemical and immunological disturbances and to the production of enzymes, including lipases, phospholipases and hydrolases. The Api-Zym® system is a semiquantitative method designed for the study of 19 enzymatic activities of tissues, cell suspensions, biological fluids, microorganisms, soil and others. Herein, the use of the Api-Zym system in 30 *M. pachydermatis* isolates detected the presence of the phosphohydrolases: acid phosphatase and naphtol-AS-BI-phosphohydrolase among all isolates, independently of the clinical sign associated to the animal. The knowledge of the enzymatic profile of *M. pachydermatis* aims to contribute to the comprehension of the role of each enzyme in the pathogeny of this yeast.

Key words: *Malassezia pachydermatis*, enzymes, Api-Zym®, enzymatic profile, dogs.

INTRODUCTION

The genus *Malassezia* consists of lipophilic yeasts that are found in the skin microflora of humans and animals. Its isolation from the environment is exceptional because its survival is related to the presence of a lipid source, with the exception of *Malassezia pachydermatis*, which does not exhibit an absolute requirement for a lipid (Guého et al., 1996; Guillot and Bond, 1999a; Sidrim and Moreira, 1999).

M. pachydermatis is the species that has adapted the most to animals. It is frequently isolated from the ear canal and skin of dogs, cats and other wild and domestic animals. In canines, *M. pachydermatis* has been often associated with external otitis and dermatitis, with intense proliferation in cases of local or systemic imbalance (Guillot and Bond, 1999a; Leite et al., 2003; Machado et al., 2003). Once colonization takes place, *M. pachydermatis* is thought to release enzymes (proteases, lipases, phosphatases and ureases) that alter the sebum quality and disrupt the epidermal surface (Mathieson et al., 1998).

Most of the methods used in clinical microbiology laboratories for the identification of microorganisms require growing organisms and detecting the end result of their complex metabolic activities. An alternative method is to use a system for detecting esterases, lipases, peptidases and other enzymes described by Buissiere et al. (1967); it has been modified and made commercially available as the Api-Zym® system (Humble et al., 1977). From this system, the aim of this work was the characterization of the *M. pachydermatis* isolates through your enzymatic profile.

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MATERIALS AND METHODS

Thirty *M. pachydermatis* isolates were used. Twenty-four genotyped

samples of subtype A1 were obtained from dogs that were referred to Hospital de Clínicas Veterinárias. The samples were processed at Setor de Micologia of the Universidade Federal do Rio Grande do Sul (UFRGS), state of Rio Grande do Sul. The other six samples were obtained from dogs from the state of Mato Grosso and processed by Laboratório de Pesquisas Micológicas (LAPEMI) of the Universidade Federal de Santa Maria (UFSM), state of Rio Grande do Sul.

The extracellular production of 19 enzymes was assayed by the semi-quantitative method Api-Zym® (BioMérieux, São Paulo, Brazil).

The following enzymes were researched: alkaline phosphatase, esterase (C_4), esterase lipase (C_8), lipase (C_{14}), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, quimotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidade, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase.

The samples were cultivated into Dixon's modified medium, incubated at 37°C in CO_2 atmosphere during 48 h, for posterior quantification through spectrophotometry. The inoculum suspensions were prepared in 5 ml of 0.085% sterile saline and read spectrophotometrically at wavelength 530 nm to match approximately 85 of transmittance.

Each well of the Api-Zym® tray was inoculated with 65 µl of the solution and incubated for 4 h at 37 °C. Following the recommended protocol, the color developed by the reaction was revealed with the Zym A and Zym B reagents. An arbitrary scale ranging from 1 to 5 was used to score the intensity of the color reaction for each tested enzyme. The results were determined according to the intensity of the reaction in nanomoles (nmol) of the hydrolysed substrate (that is that is., 1 = 5 nmol, 2 = 10 nmol, 3 = 20 nmol, 4 = 30 nmol and 5 = > 40 nmol).

RESULTS

Of the 30 *M. pachydermatis* isolates, 27 were obtained from a great variety of pathologies including the following: autoimmune diseases such as atopy (15) and pemphigus (1); dermatopathies such as flea allergy dermatitis (2), demodicosis (2) and seborrheic dermatitis (1); and otitis (6). The remaining three samples were obtained from healthy dogs.

The enzymes leucine arylamidase and naphthol-AS-BI-phosphohydrolase were detected in 100% of the samples. Individually, the presence of the enzymes among the isolates was the following, in decreasing order: acid phosphatase (96.7%), esterase (93.3%), esterase lipase (90%), β-glucosidase (46.7%) and alkaline phosphatase (43.3%) (Table 1).

The extracellular enzymes with an intermediate presence were reported to be valine arylamidase (40%), cystine arylamidase (30%), lipase, α-quimotrypsin and α-glucosidase (26.7%), trypsin (23.3%) and N-acetyl-β-glucosaminidase (20%). Lastly, α-galactosidase, β-galactosidase, β-glucuronidase, α-mannosidase and α-fucosidase were detected in only 10% of the *M. pachydermatis* isolates.

The mean enzymatic activities were the following, in decreasing order: phosphatase acid (30 nmol), naphthol-AS-BI-phosphohydrolase (24 nmol), esterase (12 nmol), leucine arylamidase (11 nmol), esterase lipase (8 nmol), β-glucosidase (2 nmol), valine arylamidase (1 nmol), alkaline phosphatase (1 nmol), cystine arylamidase and α-glucosidase (1 nmol), lipase and α-quimotrypsin (0.8 nmol), trypsin (0.7 nmol), N-acetyl-β-glucosaminidase (0.4 nmol) and α-galactosidase, β-galactosidase, β-glucuronidase, α-mannosidase and α-fucosidase (0.2 nmol) (Figure 1).

The samples 47 CM, 84 CM and 11 4AS isolated, respectively, from dogs with atopy, pemphigus and without clinical signs, showed the most production of different enzymes, totalizing 19 enzymes.

DISCUSSION

Malassezia produces many enzymes (including lipases and proteases) that can contribute to cutaneous inflammation through proteolysis, lipolysis (which alters the lipidic cutaneous film), changes of cutaneous pH, eicosanoid release and complement activation (Mason, 1993). The pathogenic mechanisms of *M. pachydermatis* cause inflammation and pruritus, which promote the formation of a favorable microenvironment for yeast overgrowth (Patterson and Frank, 2002).

With the exception of *M. pachydermatis*, all other known *Malassezia* species require an external lipid source for growth (Brunke and Hube, 2006). Lipid-dependent species of this yeast produce a wide range of enzymes. Lipase activity was demonstrated within the fungus cell wall and on in vitro membrane sites, using a histochemical techniques and electronic microscopy. This suggests that the enzyme is synthesized intracellularly and transported to the cell surface (Catterall et al., 1978). Several researches about the biochemical profile of *M. pachydermatis* also showed *in vitro* production of various enzymes including the following: acid and alkaline phosphatase, chondroitin-sulphatase, esterase, esterase lipase, galactosidase, glucosidase, hyaluronidase, leucine arylamidase, lipase, lecithinase, peroxidase, phosphoamidase, phospholipase, phosphohydrolase, protease and urease (Mathieson et al., 1998; Guillot and Bond, 1999a; Coutinho and Paula, 2000; Bond, 2002).

Proteases are believed to be the mediator of pruritus at free nerve endings in the skin; therefore, proteases released by *Malassezia* organisms can also contribute to pruritus. *Malassezia* organisms also produce lipases, which alter sebum production and produce free fatty acids on the skin surface. Released lipids can be used by yeasts for nutrition, and free fatty acids can provide protection by inhibiting other organisms (Chen and Hill, 2005). As a consequence, lipolytic enzymes such as lipase, esterase, phospholipase and lysophospholipase are considered to be closely associated with the virulence of *Malassezia* spp. (Juntachai et al., 2009).

The results of our study are similar to those of Mancianti et al. (2000), who analyzed 20 *M. pachydermatis* isolates using the Api-Zym® system and

Table 1. *M. pachydermatis* isolates classificated by source, disease of the host and extracellular enzymes analyzed through the Api - Zym® test.

Isolates	Source	Disease	ALP	E	EL	L	LA	VA	CA	T	αQ	AP	N	αGA	βGA	βGL	αG	βG	NA	αM	αF
38AM	UFRGS	Atopy	+	+	+	+	+	-	-	-	-	+	+	-	-	-	-	+	+	-	-
64AM	UFRGS	Atopy	-	+	+	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-
70AM	UFRGS	Atopy	-	+	+	-	+	-	-	-	+	+	+	-	-	-	-	+	-	-	-
91AM	UFRGS	Atopy	-	+	+	-	+	+	-	-	-	+	+	-	-	-	-	+	-	-	-
98AM	UFRGS	Atopy	-	+	+	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-
13BM	UFRGS	Atopy	+	-	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-
19BM	UFRGS	Atopy	-	+	+	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-
39BM	UFRGS	Atopy	-	+	+	-	+	-	-	-	-	+	+	-	-	-	-	+	-	-	-
95BM	UFRGS	Atopy	-	+	+	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-
97BM	UFRGS	Atopy	-	+	+	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-
102BM	UFRGS	Atopy	-	-	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-
22CM	UFRGS	Atopy	+	+	+	-	+	-	-	-	-	+	+	-	-	-	-	+	-	-	-
47CM	UFRGS	Atopy	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
63CM	UFRGS	Atopy	-	+	+	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
113CM	UFRGS	Atopy	+	+	+	+	+	-	-	-	-	+	+	-	-	-	-	+	-	-	-
189M	Cuiabá	Otitis	-	+	+	-	+	-	-	-	-	+	+	-	-	-	+	-	-	-	-
414M	Cuiabá	Otitis	+	+	+	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-
528M	Cuiabá	Otitis	+	+	+	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
564M	Cuiabá	Otitis	-	+	+	-	+	+	-	-	-	-	+	-	-	-	+	-	-	-	-
570M	Cuiabá	Otitis	-	+	+	-	+	+	-	-	-	+	+	-	-	-	+	-	-	-	-
589M	Cuiabá	Otitis	-	+	+	-	+	+	-	-	-	+	+	-	-	-	+	-	-	-	-
12AM	UFRGS	FAD*	-	+	+	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
115CM	UFRGS	FAD*	+	+	+	+	+	-	-	-	-	+	+	-	-	-	-	+	-	-	-
49AM	UFRGS	D*	-	+	+	-	+	-	+	-	-	+	+	-	-	-	-	+	-	-	-
86AM	UFRGS	D*	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	-
99AS	UFRGS	Absent	+	+	+	+	+	+	+	-	-	+	+	-	-	-	-	-	+	-	-
114AS	UFRGS	Absent	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
175CS	UFRGS	Absent	-	+	+	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-
37BM	UFRGS	SD*	-	+	+	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-
84CM	UFRGS	P*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

*FAD= Flea Allergy Dermatitis - *D= Demodicosis - *SD= Seborrheic dermatitis - *P= Pemphigus- ALP (Alkaline phosphatase) - E (Esterase) - EL (Esterase lipase) - L (Lipase) - LA (Leucine arylamidase) - VA (Valine arylamidase) - CA (Cystine arylamidase) - T (Trypsin) - αQ (α-Quimotrypsin) - AP (Acid phosphatase) - N (Naphthol-AS-BI-phosphohydrolase) - αGA (α-Galactosidase) - βGA (β-Galactosidase) - βGL (β-Glucuronidase) - αG (α-Glucosidase) - βG (β-Glucosidase) - NA (N-acetyl-β-glucosaminidase) - αM (α-Mannosidase) - αF (α-Fucosidase).

found 15 enzymatic activities that highlight the presence of esterase lipase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. These data corroborate the importance of such enzymes in the biochemical profile of *M. pachydermatis*, although there are no studies that prove the

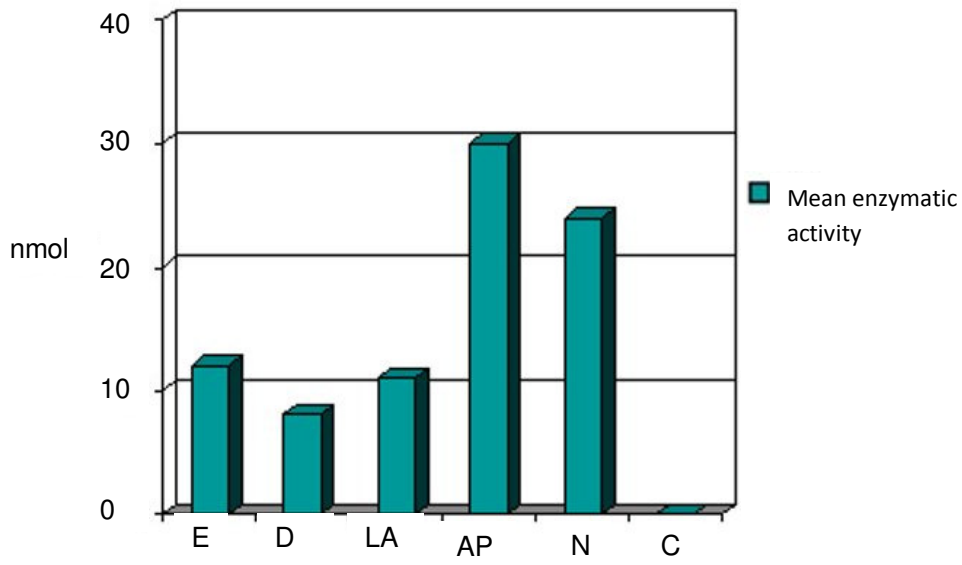

Figure 1. Prevalent enzymatic activity of analysed M. Pachydematic isolate. E (Esterase) EL (Esterase Lipase) -LA (Leucine Arylamidase) – AP (Acid Phosphate) – N (Naphthol-AS-BI-Phos phohydrolase) – C(Control).

relation of these enzymes with the pathogenicity of this commensal yeast.

Studying the hydrolytic properties of dermatophytes, Nowicki (1995) observed high activity of the enzymes acid phosphatase, alkaline phosphatase and naphthol-AS-BI-phosphohydrolase. Kurnatowska (1998) also observed statistical differences among the enzymatic activities of esterase, leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-glucosidase and N-acetyl-β-glucosaminidase in your study about hydrolytic properties of *Candida albicans*.

The samples 47 CM, 84 CM and 11 4AS showed the most production of different enzymes, a total of 19 enzymatic activities. These samples were obtained from dogs with atopy, pemphigus and without clinical signs, respectively. This could suggest that there is no correlation between greater or minor enzymatic activity of yeast and specific dermatopathys, although a great number of isolates is necessary to support this theory.

Coutinho and Paula (2000) analyzed four enzymes: chondroitin sulphatase, hyaluronidase, phospholipase and protease, investigated in 30 strains of *M. pachydermatis* isolated from dogs with otitis or dermatitis. When analyzing the four enzymes produced by the otic secretion and skin isolates, similar results were found. All 30 strains studied showed proteinase and chondroitin-sulphatase activity and 29 showed activities of phosphor-lipase and hyaluronidase.

Although the study of Coutinho and Paula (2000) did not aim to determine the degree of influence of these enzymes in determining the virulence of *M. pachydermatis* infections, the data obtained suggest the potential value of such studies. By the other side, according to Bond (2002), greater activity of esterase (C_4) has been detected in cellular suspensions of *M. pachydermatis* strains obtained from health dogs when compared to those with dermatitis.

Notably, age, sex and sampling period are not often considered predisposing factor to malasseziosis in dogs (Carlotti and Taillieu-Le Roy, 1997; Crespo et al., 2002). Thus, these data are not included in our study.

The biggest frequencies and population sizes of *Malassezia* spp. reported in animals with otitis, when compared to the healthy animals of our study, indicate that the yeast grows at infection sites and develops a function in the pathogenesis of otitis externa. Factors related to the transition from a commensal lifestyle to the exhibition of pathogenic behaviors are not completely clear, but they probably reflect disturbances of the physi-cal, chemical and immunological mechanisms that restrict the colonization of skin by microorganisms (Scott and Miller, 1995; Guillot and Bond, 1999b; Morris, 1999). Moreover, overgrowth of the yeast at infection sites has been shown to be an important factor of disease induction, as well as phospholipase production (Cafarchia and Otranto, 2004).

The presence of the phosphohydrolases acid phosphatase and naphtol-AS-BI-phosphohydrolase were detected among all of the isolates, independent of the clinical signs associated with the animal. This data also had been found in similar studies, demonstrating the importance of these enzymes in the biochemical process of *M. pachydermatis*, although a wide range of researches is necessary to determinate the specific role of such enzymes and its correlation with the pathogenic

process of the yeast.

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