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Molecular heterogeneity of *Malassezia pachydermatis* through RAPD-PCR

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ABSTRACT. Several methodologies in molecular biology have been used in the investigation of *Malassezia pachydermatis* and its differentiation into subtypes. Recent molecular research of this species includes the use of samples isolated from canine otitis externa and dermatitis, as well as from healthy animals, having in view an epidemiological study of the yeast. The aim of this study was to identify molecular differences in *M. pachydermatis* samples isolated from dogs with otitis externa. The *M. pachydermatis* strains were analyzed by means of the Random Amplification Primer DNA - Polimerase Chain Reaction (RAPD–PCR) for molecular heterogeneity research. DNA extraction was carried out with phenol-chloroform and the RAPD technique using the AGAATCCGCC primer. A variation was observed in the number and arrangement of the bands among the 49 studied isolates, grouped into nine patterns. Isolate groupings were not found to be related to animal breed, age or sex. It was concluded that *M. pachydermatis* has differences in its molecular profile, as shown by the molecular technique (RAPD – PCR), which allows isolates to be classified into nine subtypes.

Key words: *Malassezia pachydermatis*, molecular biology, otitis externa, dogs.

RESUMO. Heterogeneidade molecular da *Malassezia pachydermatis* através de RAPD-PCR. Várias metodologias em biologia molecular têm sido aplicadas para estudar a *M. pachydermatis* diferenciando-a em subgrupos. Recentemente utiliza-se a investigação molecular desta espécie isolada de otite externa e dermatite, e também de isolados da mesma de animais hígidos, para um estudo epidemiológico da levedura. O objetivo deste trabalho foi identificar diferenças moleculares entre isolados de *M. pachydermatis* obtidos de casos de otite externa canina. Para isto, amostras da levedura provenientes de cães com esta enfermidade foram estudadas através da técnica de Polimorfismo de DNA Amplificado ao Acaso - Reação da Polimerase em Cadeia (RAPD–PCR) para pesquisa de heterogeneidade molecular. A extração de DNA foi realizada no processo fenol-cloroformio e a técnica de RAPD foi estudada com o primer AGAATCCGCC. Pode-se observar com esta metodologia, variação no número e posição das bandas entre os 49 isolados estudados, podendo-se agrupá-los em nove padrões. Os agrupamentos formados pelos isolados não apresentaram relação com raça, idade ou sexo do animal. Concluindo-se que a *M. pachydermatis* apresenta diferenças em seu perfil molecular, observado pela técnica molecular (RAPD-PCR) que permite classificar os isolados desta espécie em até nove subtipos.

Palavas-chave: *Malassezia pachydermatis*, biologia molecular, otite externa, cães.

Introduction

Otitis externa is a common acoustic meatus illness diagnosed in veterinary clinics (HARVEY et al., 2004), and *M. pachydermatis* is a yeast closely related to this pathology. Its prevalence has been often investigated in such cases (DUFFAIT, 1983; HUANG, 1994; NOBRE et al., 2001; NASCENTE et al., 2004; 2005; PRADO et al., 2008). Molecular research has become a new tool for the study of the epidemiology and diagnosis of diseases (ANTHONY et al., 1994; AIZAWA et al., 1999; 2001; CASTELLÁ et al., 2005).

Recent developments in molecular biology have enabled the use of the genetic material structure as a tool for subspecies typing. Several methods have been used in the classification of *M. pachydermatis* into subgroups, and some epidemiological studies have performed molecular investigation of otitis externa and dermatitis isolates, as well as isolates from healthy animals, of this yeast (AIZAWA et al.,

1999; 2001). Polymerase Chain Reaction (PCR) is based on the fact that oligonucleotides (primers) hybridize specifically with a DNA tape mold. Amplified nucleic acid can then be examined (size, amount, string) or used in subsequent experimental protocols (e.g. cloning) (LIMA et al., 1998).

Several molecular methods are currently being used for identification and typing of the *Malassezia* genus, including kariotyping (ANTHONY et al., 1994), RAPD (Random Amplified Polymorphism DNA) (BOEKHOUT et al., 1998; AIZAWA et al., 1999; CASTELLÁ et al., 2005), RFLP (Restriction Fragments Loci Analysis) (THELLEN et al., 2001; MIRHENDI et al., 2005), sequence analysis (KANO et al., 1999; AIZAWA et al., 2001), among others (AFFES et al., 2009). Gupta et al. (2000) have differentiated seven *Malassezia* species through molecular testing based on PCR reactions and restriction endonucleases. The RAPD-PCR technique has already been employed to test clinical isolates of *M. pachydermatis*, dividing them into several molecular subtypes (AIZAWA et al., 1999; 2001). The results obtained have resulted in the classification of the *M. pachydermatis* species into dermatitis and otitis externa pathology or healthy animal subdivisions. This study aimed to identify molecular differences among 49 *M. pachydermatis* yeast isolates of clinical cases of external canine otitis.

Material and methods

M. pachydermatis samples from external canine otitis were investigated through the RAPD technique - PCR for the determination of molecular heterogeneity. Forty-nine samples with the AGAATCCGCC primer were evaluated.

Dogs with otitis externa symptoms referred to veterinary clinics in Pelotas, Rio Grando do Sul State – Brazil were included in this study. During clinical examination the presence of dark cerumen, characteristic of otic malasseziosis, was found. Sample collection was done with a sterile swab moistened in saline solution, which was then rubbed on the external acoustic meatus area. All swabs with the collected material, after being identified with the record number of the animal under investigation, were forwarded to the Laboratory of Infectious Diseases – Mycology Sector of the Federal University of Pelotas.

The material was sown in agar Sabouraud dextrose plus chloramphenicol and cycloheximide[1] and incubated at 37°C for up to ten days. The characteristics of the macroscopic colonies were observed daily and the cellular morphology was determined by the Gram staining method and biochemical tests.

For DNA extraction of *M. pachydermatis* isolates, a sample of the yeast with up to 48h, previously suspended in 600 μL of SE (EDTA/NaCl/H_2O) and treated with proteinase K (3 μL) 20 μg mL^{-1} 10' at 56°C, was used. Extraction was done through the phenol/chloroform process.

In order to perform this study, reactions containing 25 µL com 2.5 U of Taq polymerase (5 U μL^{-1}), 0.8 mM DNTP, 40 pmol primer, buffer (200 mM/tris/HCL/(pH 8.4) 500 mM); $MgCl_2$ KCL, and 2.5 target DNA were done. DNA amplification was done in a Perkim Elmer thermocycler (480), with two cycles: 94°C for 5 minutes for denaturing, 33°C for 5 minutes to ringing plus 72°C for 5 minutes for extension. Next, 35 cycles at 94°C for 90 seconds, 33°C for 90 seconds and 72°C for 90 seconds, with a final extension at 72°C for 5 minutes were done. PCR product reading was carried out in agarose gel (1.5%) stained with ethidium bromide and visualized under UV light in electrophoresis (LIMA et al., 1998).

Results and discussion

The results obtained with the 49 *M. pachydermatis* isolates using the AGAATCCGCC primer showed a variation in the number and arrangement of the bands and the sample grouping into nine subtypes (Figure 1). Within each pattern, dogs from which *M. pachydermatis* samples were isolated included both males and females of different breeds and ages (Table 1).

According to Schiottfeldt et al. (2002), species differentiation based on molecular characteristics can be performed by a number of tests, among which PFGE (Pulse-Field Gel Electrophoresis) and RAPD are the most used. However, these two techniques are used for different purposes, for while PFGE is very useful for species identification, RAPD provides information about molecular changes within the species, identifying subtypes. The RAPD technique used in this study enabled the detection of differences in *M. pachydermatis* isolate molecular weight patterns (Table 2), though these differences were found to be unrelated to breed, age or sex of the animals.

[1]Mycobiotic - Merck

Table 1. A description of investigated dogs with otitis externa, according to breed, sex, age and band arrangement as observed by the RAPD technique with the AGAATCCGCC primer-PCR.

Pattern	Breed	Sex	Age
1	Boxer	F	2y
2	O. E. Sheep Dog	M	3y
2	Rottweiler	F	10y
2	English Cocker S.	F	10y
2	English Cocker S.	F	9y
2	English Cocker S.	M	14y
2	English Cocker S.	M	14y
2	Poodle	M	9y
2	Poodle	F	9y
2	Rottweiler	M	NI
2	Mixed-breed	M	10m
2	Mixed-breed	M	8y
3	Mixed-breed	F	3y
3	Mixed-breed	M	NI
4	English Cocker S.	F	1y
4	Mixed-breed	F	2y
4	Mixed-breed	M	NI
5	Poodle	M	1M
5	Poodle	M	1M
5	Poodle	M	4y
5	English Cocker S.	F	3y
6	Poodle	M	3y
6	Poodle	F	11y
6	Mixed-breed	F	13y
6	Daschund	M	11y
6	Daschund	M	9y
6	Poodle	M	4y
6	Labrador R.	F	3y
6	Labrador R.	M	8y
6	Shar Pei	M	2y
6	Mixed-breed	M	NI
6	Mixed-breed	M	5y
6	Mixed-breed	M	10y
6	Mixed-breed	F	2y
6	Mixed-breed	F	8y
6	Siberian Husky	F	NI
6	Mixed-breed	F	9y
7	Shar Pei	M	7m
7	Mixed-breed	M	2y
7	Mixed-breed	F	NI
8	Mixed-breed	F	NI
8	Poodle	F	2y
8	Mixed-breed	F	NI
9	Mixed-breed	M	NI
9	English Cocker S.	F	2y
9	Chow Chow	F	4y
9	Mixed-breed	M	NI
9	English Cocker S.	F	6y
9	Poodle	M	4y

y-years; m-months; NI-unidentified; F-female; M-male

The molecular analysis performed by Cabañes et al. (2005) with the alleged new species of *Malassezia* genus (*M. nana*, *M. dermatis* and *M. equi*) found identical genes of these isolates when compared to the well-known *M. sympodialis*. Guého and Guillot (1999) believe that these findings suggesting new species are in fact *M. pachydermatis* variants with phenotypic differences, such as those observed by Bond and Anthony (1995). According to literature review, the *Malassezia* genus, when first identified, would be physiologically divided into two clear-cut entities: the *M. pachydermatis* species that may grow in cultivation media used in laboratory routine and in the mycology laboratory, and the lipodependent species, which depends on lipid supplement in its cultivation medium for its development. In this study, all species identified showed non-lipodependent species characteristics. However, when studied through molecular (RAPD technique - PCR), nine subtypes of the same species, differentiated in number and band weight, were found.

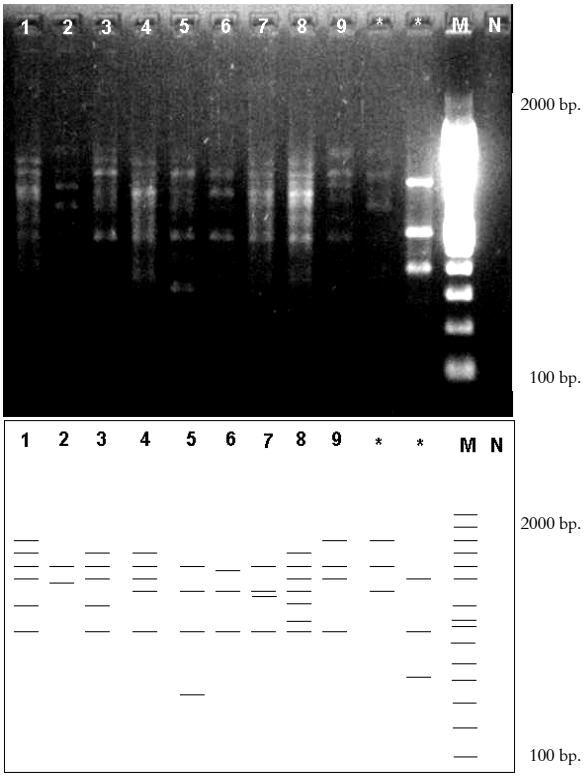

Figure 1. Nine subtypes of yeast *M. pachydermatis* from canine otitis externa observed through RAPD technique – PCR – photo and schema statement. *Two strains of *Candida* sp.: positive control. (-) Negative control; m-molecular weight marker; BP-base pairs.

Table 2. Distribution of 49 isolates of *M. pachydermatis* as observed through the Random Amplified DNA Polymorphism-PCR technique with AGAATCCGCC primer in accordance with the number of samples and bands in each of the nine subtypes.

Patterns	Number of bands	Number of samples
1	6	1 (2%)
2	2	11 (22.4%)
3	5	2 (4.1%)
4	5	3 (6.1%)
5	4	4 (8.2%)
6	3	16 (32.7%)
7	4	3 (6.1%)
8	7	3 (6.1%)
9	4	6 (12.2%)
Total		49 (100%)

All patterns showed 1400 base pair (bp) molecular weight bands and with the exception of two, all others showed a 670 bp band weight, which was present in most patterns. The other bands differed in weight and arrangement, as shown in Table 3.

Table 3. Distribution of molecular weights of each of the nine subtypes detected in 49 *M. pachydermatis* isolates through.

bp	Patterns								
	1	2	3	4	5	6	7	8	9
	1700								1700
	1560		1560	1560				1560	
	1400	1400	1400	1400	1400	1400	1400	1400	1400
	1320	1320	1320	1320				1320	1320
				1100	1100	1100	1100	1100	
							1000		
	800		800					800	
								710	
	670		670	670	670	670	670	670	670
					310				

In this study, two repetitions of the test with each of the 49 samples were done in order to reproduce the results with the same primer. After this analysis, a copy of each pattern was selected for control and comparison to those found in previous studies. The test with samples of the nine subtypes can be seen in Figure 1. Two species of *Candida*, apart from the molecular weight marker and the negative control whose reaction was not carried out without DNA presence, were added.

Aizawa et al. (1999) have investigated 16 strains of *M. pachydermatis* isolated from dogs in Japan which were submitted to the same method (RAPD) and Chitin Promoter 2 (CHS2) gene frequency analysis; three out of 16 strains were genetically differentiated by RAPD. Aizawa et al. (2001) assessed 110 samples from dogs and cats with malasseziosis by RAPD and CHS2 to investigate the epidemiology of *Malassezia* yeast infection in small animals, and differences were also found. Before, Guillot et al. (1996) had shown the phylogenetic ribossomal RNA phylogenetic tree of *M. pachydermatis* suggesting that this species should be divided into seven distinct sequential types. Kano et al. (1999) also verified by RAPD and analysis of clinical isolates that CHS2 *M. pachydermatis* can be divided into four subtypes (a, b, c and d).

Castellá et al. (2005) also differentiated *M. pachydermatis* isolates and subdivided them into four genetic types. Type A appeared in all animal groups, which included both healthy and diseased cats and dogs. Typse B and D originated from sick dogs and type C from healthy dogs. In the present study, patterns 2 and 6, despite having a higher representation, were unrelated to animal age, breed or sex, once all submitted patterns were representative of different breeds and ages (Table 2). In accordance with Leśniak and Dworecka-Kaszak (2006), there is a high heterogeneity degree in the *M. pachydermatis* DNA investigated. This could be attributed to either pathogen resistance factors or sensitivity against antifungals, including previous otitis externa treatments. Because this etiological agent can perpetuate the disease, the drugs used to fight bacteria can also act against *M. pachydermatis*, but without causing the death of the yeast. Thus, not only animal species, breed and/or age, but also previous yeast treatments, would be factors that affect the molecular differences found.

In 1994, Anthony et al. (1994) used the molecular karyotyping, RFLP and Southern blotting techniques to study *M. pachydermatis*. The Southern blotting method showed considerable variation in *M. pachydermatis* isolates; also, five isolates differed from the other 99 for having seven rather than six bands. These six bands observed in the yeast under study were described by Kiuchi et al. (1992). Bond and Anthoni (1995) observed through RFLP techniques that 30 out of 244 *M. pachydermatis* samples showed differences which were later related to lipodependence features. In this study no sample presented lipodependence, confirming an *M. pachydermatis* species characteristic.

The identification of *Malassezia* genus yeasts is performed by morphological, physiological and biochemical tests in most cases. The procedures for species classification may conceal an array of fundamental differences, such as pathogenicity and molecular techniques could be used for checking them. Differences observed by molecular typing methods do not correspond to those observed in biochemical tests and morphology; these methods not only identify species differences in this genus, but also identify differences within the same species. Gueho and Guillot (1999) and Cabañes et al. (2005) stated that both physiological and genetic differences can be observed in a known species.

Conclusion

M. pachydermatis yeast samples from canine otitis externa have shown a molecular profile observed by the molecular (RAPD technique – PCR), which enables their classification into nine subtypes; there is no relationship between these differences and animal age, breed or sex, suggesting that other intrinsic factors, including *M. pachydermatis*, can influence these molecular differences.

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