DOI: 10.5433/1679-0359.2016v37n5p3173

RAPD-based genotyping of *Malassezia pachydermatis* from Domestic and wild animals

Genotipificação de *Malassezia pachydermatis* através da técnica de RAPD

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Abstract

Malassezia pachydermatis (*M. pachydermatis*) is a fungus of importance in human and veterinary medicine. Although a part of the normal microbiota, it can sometimes be present in its pathogenic form, particularly causing otitis and dermatitis in animals. Among human beings, it mainly affects immuno compromised patients and newborns, causing simple pustulosis, seborrheic dermatitis, tinea versicolor or fungemia. This study aimed to analyze the genomic polymorphism in *M. pachydermatis* samples isolated from *Canis familiaris* (domestic dog), *Felis catus* (domestic cat), and *Myrmecophaga tridactyla* (giant anteater). Two hundred and fourteen samples were collected and cultured in Sabouraud agar with chloranphenicol (100mg L^{-1}) and incubated at 37 °C for a period of 7 to 10 days. One hundred and sixty six samples that appeared morphologically comparable to yeast cultures were processed for DNA extraction and PCR was performed for a specific region in the Internal Transcribed Spacer (ITS) of *M. pachydermatis*. Among these, seven (4.21%) were negative and 159 (95.79%) were positive. Of the 159 positive samples, 102 (64.15%) were from animals with clinical signs and 57 (35.85%) without clinical signs. Fifty-seven samples were selected at random for RAPD-PCR based genotyping and distributed into four genetic groups. Types I and II were more frequent in animals with clinical signs while type III was frequent in healthy animals. Type IV occurred evenly across animals with or without clinical signs. These results indicate differences in pathogenicity of the fungus based on the genotype.
Key words: *Malassezia pachydermatis.* PCR. RAPD.

Resumo

A levedura *Malassezia pachydermatis* é de importância na medicina humana e veterinária por se apresentar de forma comensal e por vezes sob a forma patogênica. Em animais, causa principalmente otites e dermatites e em humanos acomete principalmente pacientes imunocomprometidos e neonatos, causando desde pustulose simples, dermatite seborréica, pitiríase versicolor até fungemia. Este trabalho

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Received: Oct. 02, 2015 - Approved: May 18, 2016

teve como objetivo analisar o polimorfismo genômico de amostras de *M. pachydermatis* nas 208 amostras das espécies *Canis familiaris* (cão doméstico), 03 amostras de *Felis catus* (gato doméstico) e 03 amostras de *Myrmecophaga tridactyla* (tamanduá bandeira). As 214 amostras coletadas foram cultivadas em agar Sabouraud acrescido de cloranfenicol (100mg/l) e incubados a 37°C, por um período de sete à dez dias. Os 166 isolados morfologicamente compatíveis com a levedura foram processados para extração do ácido desoxirribonucleico (DNA) e realização da Reação em cadeia pela polimerase (PCR) com oligonucleotídeos específicos para região *ITS* (*Internal Trancribed Spacer*) da levedura *M. pachydermatis.* Quando submetidos à PCR, 07 (4.21%) foram negativas e 159 (95. 79%) tiveram identificação positivas. Das 159 amostras positivas, 102 (64.15%) eram oriundas de animais com sinais clínicos e 57 (35,85%) sem sinais clínicos. Destes, 57 isolados com e sem sinais clínicos, confirmados na PCR foram submetidos a técnica de RAPD-PCR, sendo distribuídos em 4 padrões genéticos. A maioria dos animais doentes foi classificada nos tipos I e II, enquanto os saudáveis no tipo III; no tipo IV houve equivalência entre os isolados, sugerindo diferenças na patogenicidade dos isolados.
Palavras-chave: *Malassezia pachydermatis*. PCR. RAPD.

Introduction

Malassezia pachydermatis (*M. pachydermatis*) is an opportunistic yeast of importance in both human and veterinary medicine. In animals, it causes ear infections, dermatitis, and eye infections (LEDBETTER et al., 2015; LEDBETTER; STARR, 2015; NOBRE et al., 1998), while in human beings it affects mainly mmunocompromised patients and newborns, causing simple pustulosis, seborrheic dermatitis, tinea versicolor or fungemia (GLATZ et al., 2015; JAGIELSKI et al., 2014; ZAITZ et al., 2000).

The genus was first described by Baillonin (1889), and currently includes a total of 14 species. These lipid-dependent species constitute the lipophilic microbiota, which occurs in the skin of horses and several ruminants. They have been isolated from most domestic as well as free or captive wild animals (CABAÑES, 2014).

Previously, *Malassezia* species were identified and classified by biochemical tests (catalase, urease, esculin hydrolysis and Tween 20%, 40%, 60%, and 80% testing), but these were often inconclusive or provided false positive/negative results, apart from being time-consuming (MIRHENDI et al., 2005). Currently, molecular techniques, such as PCR are used for their classification (SUGITA et al., 2001).

The genetic diversity of *M. pachydermatis* has been identified by several molecular techniques such as the Amplified Fragment Length Polymorphisms(AFLP), Random Amplified Polymorphic DNA (RAPD) (CASTELLÁ et al., 2005; THEELEN et al., 2001) and analysis of sequences of the Internal Transcribed Spacers1 (ITS 1) (MAKIMURA et al., 2000), which suggested that some of the genotypes are pathogenic (CASTELLÁ et al., 2005).

The objective of this study was to analyze the genetic variability of *M. pachydermatis* isolates from domestic and wild animals, with or without clinical signs, using the RAPD technique.

Materials and Methods

Sample collection

This study was conducted in the city of Cuiabá-MT, Brazil and the samples were collected between 2010 and 2012. Swabs were collected from the ear canal and/or scraped from the fur of pets treated at the veterinary hospital or in private veterinary clinics in the city. After collection, samples were sent to the Veterinary Microbiology Laboratory for culture and isolation. A total of 214 samples were collected. Of these, 208 were from dogs, three from cats and three from giant anteaters.

DNA Isolation and PCR

The samples were isolated in Sabouraud Dextrose Agar (SDA) plus chloramphenicol (100mg L^{-1}) and incubated at 37°C, for a period ranging between seven and ten days. The colonies presenting macro and micromorphological characteristics of *M. pachydermatis* yeast culture as per Quinn et al. (1994), were re-seeded in SDA plus 20 µl chloranphenicol (100 mg L^{-1}) and sterile olive oil to optimize their growth, and incubated at 37°C for three days.

After proliferation, yeasts were cultured in *Sabouraud* medium at 37 °C for seven days. DNA extraction was performed following the protocol of Mseddi et al. (2011) with some adaptations. A volume of 2mL of culture was centrifuged at 4,000 g for 5 minutes. The precipitate was resuspended in a buffer containing lyticase enzyme (5 $U\mu l^{-1}$; Sigma-Aldrich), and incubated at 37 °C for 2 hours. The precipitate was washed, resuspended in lysis buffer (100mMNaCl, 10 mMTris pH-8.0, 25mM EDTA, 0.5% SDS, 0.1$mgmL^{-1}$ proteinase K), incubated for 18 hat 65°C and then extracted with phenol:chloroform. DNA was precipitated using sodium acetate (0.3M) and isopropanol, collected by centrifugation at 12,000g for 10 min, rinsed with 70% ethanol and resuspended in 30 µl ultrapure water.

M. pachydermatis samples were confirmed by PCR technique, using primers specific for the ITS region of the yeast, namely M.pa-F (5'CTGCCATACGGATGCGCAAG3') and 5.8S-R (5'TTCGCTGCGTTCTTCATCGA3'), as described by Sugita et al. (2001), which amplify a 220 bp fragment. Previously sequenced *M. pachydermatis* DNA was used as positive control and ultrapure water as negative control. The amplification products were analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide (10 μgml^{-1}), for 1 h at 10Vcm^{-1} and observed in an UV transilluminator with a 100 bp DNA Ladder marker.

RAPD technique

For the RAPD technique, the OPT-20 (5'-GACCAATGCC-3') primer described by Amoah et al. (1995) was used. The reaction was performed in a final volume of 25 µl containing 1x PCR buffer, 3mM $MgCl_2$, 200 µM each dNTP, 2.5 U Taq DNA polymerase and 1.44 µM OPT-20 oligonucleotide. Initial denaturation of 5 minutes at 95 ºC was followed by 30 cycles of 1 min at 95 ºC for denaturation, 1 min at 31 ºC for annealing, and 30 sat 72 ºC for extension, followed by a final extension of 6 min at 72 ºC. The amplification products were analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide (10 μgml^{-1}), for 3 h at 12 $Vcm^{-1}$and observed in an UV transilluminator with a 100 bp DNA Ladder marker. The samples were classified into four genetic patterns, type I, II, III and IV, as described by Castellá et al. (2005).

Results and Discussion

Of the 214 samples collected, 166 (77.57%) presented cultures that were morphologically similar to *M. pachydermatis.* This frequency is comparable to that described by Han et al. (2013), who collected 228 samples, of which 141 were *M. pachydermatis* isolates (61.8%).

Amongst these, 95.78% (159/166) were positive for *M. pachydermatis*by PCR, with102 (64.15%) being from animals that presented clinical signs, and 57 (35.85%) from animals without any clinical signs. The site with the highest frequency of isolation was the ear, both in clinically ill and healthy animals.

The isolation of microorganisms, especially from the ear canal, does not necessarily mean that these are pathogenic, since the external ear of dogs is inhabited by various potentially pathogenic microbiota (OLIVEIRA et al., 2008; PRADO et al., 2008). However, the role of this yeast as a perpetuating factor for ear infections is highly probable.

Currently the pathological occurrence of *M. pachydermatis* is associated with predisposing host factors, such as changes in skin microclimate, dysfunction of the epidermal barrier, pendulous "pinnae", increased production of cerumen, lack of cleanliness of the ear canal, as well as concomitant hypersensitivity reactions, cornification disorders, endocrine disorders and the use of corticosteroids for long periods of time (GIRÃO et al., 2006).

Wurfel et al. (2009) reported that the occurrence of *M. pachydermatis* in dogs is not directly associated with the development of skin, ear canal and oral mucosa diseases that are caused by yeasts that often colonize the body surface of animals. On the other hand, Nardoni et al. (2007) reported a statistical correlation between *M. pachydermatis* detection and skin changes.

The lowest frequency of isolates in cats (1.8%) can be justified by their clean habits and housing characteristics, since these animals usually have less access to the street without the supervision of their owners, which decreases contact with other animals (AHMAN; BERGSTRÖM, 2009).

Regarding giant anteaters held in captivity, there are only a few studies focusing on their biology and habits. Moreover, data reporting the frequency of *M. pachydermatis* infection in giant anteaters are scarce. In our study, an occurrence of 100% (3) was found in the animals analyzed, which was higher when compared to the incidence observed by Bentudo et al. (2006). In their study, the frequency of isolation was 11.1% amongthe 13 giant anteaters (*Tamandua tetradactyla)* evaluated. The high incidence found in our study is probably because only three animals were evaluated, all of which presented a certain level of stress; one was a road-killed victim and the other two came from a zoo.

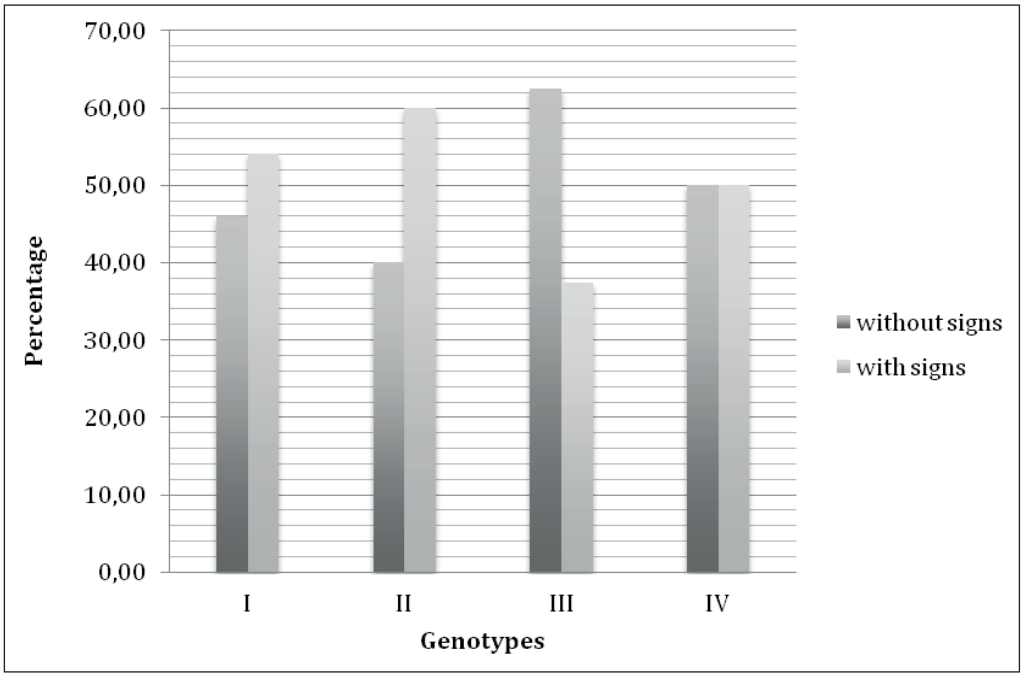
To evaluate the genetic polymorphism, 57 isolates were selected at random for RAPD PCR; 32 (56.14%) with clinical signs and 25 (43.86%) without clinical signs. They were divided into four categories: 37 (64.91%) isolates belonged to type I, 5 (8.77%) to type II, 13 (22.8%) to type III and 2 (3.5%) to type IV (Table 1).

Table 1. The distribution of *M. pachydermatis* genotypes in isolates from domestic and wild animals with or without clinical signs, from 2010 to 2012, in the city of Cuiabá-MT.

Clinical sign	Genotype			
	Type I	**Type II**	**Type III**	**Type IV**
Present	20	3	5	1
Absent	17	2	8	1

Type I (n=37) was the most predominant in our study, followed by genotypes III (n=13), II (n=5) and IV (n=2) (Table1). The distribution of the genotypes according to the presence of clinical signs is shown in Figure 1. Genotypes I and II were detected more frequently in isolates from animals with clinical signs; genotype III was detected mostly in healthy animals; and genotype IV was distributed equally among animals with or without clinical signs (Figure 1).

Figure 1. The percentage distribution of *M. pachydermatis* genotypes in isolates from domestic and wild animals with or without clinical signs, from 2010 to 2012, in the city of Cuiabá-MT.



In our investigation, the distribution profile of the genotypes is similar to that described by Castellá et al. (2005), although in their study genotype III was found only in healthy animals. This is probably because the isolates used by Castellá et al. (2005) were from only 11 healthy or sick animals (5 dogs, 3 cats, 1 horse, 1 goat and 1 pig).

Similar results were also observed by Kobayashi et al. (2011), who described differences in the occurrence of *M. pachydermatis* genotypes based on the nucleotide sequence of the IGS region. Two subtypes (IB and 3D) were prevalent in sick animals, but at a lower frequency in healthy animals.

Conclusions

RAPD-PCR of *M. pachydermatis* isolates from animal shelped to identify correlation between genotype and the presence of clinical signs. Some genotypes were more frequent in healthy animals (type III) and others in animals with clinical signs (type I and II). Thus, genotyping can assist in identifying clinical isolates with greater pathogenic potential.

Acknowledgements

We are greatful to Capes for support.

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