

Protein profiles of *Malassezia pachydermatis* isolated from dogs

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Abstract

The protein fractions of 30 *Malassezia pachydermatis* isolates from dogs (15 with otitis and 15 with dermatitis) were investigated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoresis runs resulted in bands ranging from 15.0–145.7 kDa in the ear canal secretion isolates. The values for the skin isolates varied between 16.0–141.1 kDa. The presence of protein bands occurred most frequently within the 30–40 kDa bands (66.7%), 40–50 kDa (70.0%), 50–60 kDa (73.3%) and 80–90 kDa (83.3%). No statistical differences were determined, considering the behavior of *M. pachydermatis* isolates from ear and skin in each band of molecular weight values, nor in the number of bands obtained.

Key words: *Malassezia pachydermatis*, dermatomycosis, otomycosis, dogs, electrophoresis, proteins

Introduction

Malassezia pachydermatis is a commensal yeast from mammalian skin and mucosa that, under certain circumstances, shows exacerbated development leading to subsequent clinical disease [1]. In a veterinarian's pet practice, *M. pachydermatis* is highlighted as an agent of dermatitis and otitis externa, particularly in dogs [2, 3].

Although malasseziasis might represent a major disease in such animal species, the characterization of the pathogen is mostly restricted to morphology and dependence or non-dependence on lipids for its development. Such facts lead to scarce information about the biochemical profile of *M. pachydermatis*. It is not known whether samples originating in the two major parasitism sites in the dog, namely the ear canal and skin, behave alike.

In the last few years studies on *M. furfur* have been carried out, proving that serum of atopic patients presenting antibodies IgE reacts with certain protein bands, obtained from the separation of proteins through electrophoresis in polyacrylamide gel by using the crude extract of yeast [4, 5].

Bruneau & Guinet [6] studied *M. pachydermatis* samples through quantitative immunoelectrophoresis, verifying its total protein content. Notwithstanding,

they did not study the most frequent bands of molecular weights within which protein decomposes, an essential piece of information for checking the antigens presented by *M. pachydermatis*.

Therefore, this study investigated the cell wall protein profile of *M. pachydermatis*, isolated from canine ear canals and skin, to determine whether there were any differences in behavior among the samples related to the parasitized sites.

Material and methods

Malassezia pachydermatis isolates

Thirty canine isolates were studied as follows: 15 cultures isolated from otic secretion of animals with otitis numbered from o1 to o15; 15 cultures obtained from hair and skin scraped from animals with dermatitis numbered from p1 to p15.

Obtaining crude extracts from the cell walls of *Malassezia pachydermatis*

Malassezia pachydermatis was cultured in Roux bottles containing 130 ml of Fava Netto's agar medium [7] for 7 days at 37 °C, when large yeast masses were obtained. Growth was blocked by adding 30 ml

thimerosal (1 : 5000), centrifuging the suspension for 15 min at 3000 rpm and discarding the supernatant. The cellular mass was held at 37 °C for drying over a 7-day period. The dried mass was then weighted and resuspended in 5% Coca's solution [8]. For slow extraction of proteins from the cellular walls [9], the suspensions were kept for 7 days at 4 °C, again centrifuging the material and collecting the supernatant, then introduced in dialysis tubing (Dialysis tubing-cellulose membrane. Sigma), in order to promote concentration through preevaporation. The concentrated extracts were sterilized by filtration through 22 µm membranes (STL Millex 25 MM-0.22 Millipore), in sterile flasks and freeze-dried at 4 °C until used.

Study of protein fraction

By using crude extracts, the total content of proteins was determined [10], estimating 60–70 µg as the concentration in each well (2 µg/µl). The protein fractions were studied by electrophoresis in polyacrylamide gels with sodium dodecyl-sulphate (SDS-PAGE), as described by Nichols et al. [11], in order to separate the protein sub-units. The run was carried out in a Mini-Protein Dual Slab Cell (Bio-Rad), through discontinuous electrophoresis (5% and 12% gels). The techniques for preparing the gels, the samples and the electrophoretic run were as employed by Portocarrero [12]. A low molecular weight standard (Prestained SDS-PAGE Standard-Low Range. Bio-Rad) was then applied for calibration purposes, along with the samples to be tested in gel; carrying the electrophoresis out under a direct current of 30 mA and voltage of 70 V for 2 h. The gels were then stained with Coomassie blue R-250 at 0.05% overnight and later destained until bands became distinct [11]. The approximate molecular weight calculation was carried out by a computer, using the DNA-Star software, at a 3% error margin.

Statistical method

In order to evaluate the results, variance analysis tests by Friedman's posts, of multiple comparisons and of Mann-Whitney [13, 14] were applied. The rejection level of the nullity hypothesis was fixed at 0.05 or 5% ($\alpha \leq 0.05$).

Results

The electrophoretic runs, carried out by using protein extracts from the cell walls of *M. pachydermatis*, gave

bands ranging from 15.0–145.7 kDa for the otic secretion cultures and from 16.0–141.1 kDa for the skin isolates (Figures 1–6).

For better visualization, otic secretion and skin data were gathered, discriminating the percentage of samples showing protein bands throughout the different molecular weight strips (Table 1). The presence of protein bands for *M. pachydermatis* occurred at a higher frequency within the 30–60 and 80–90 kDa bands.

Statistical analysis comparing skin and otic secretion samples proved that there were no meaningful differences concerning either the total number of bands shown by both groups (mean values of 5 and 6, respectively) or each of the molecular weight strips.

Discussion

Since no literature with regard to obtaining *M. pachydermatis* protein extracts was found, a pilot experiment for evaluating growth time and extraction liquid was carried out. The 7-day period and the Coca's solution were adapted to the extraction of proteins from the cell walls of *M. pachydermatis*.

The SDS-PAGE technique is widely employed nowadays, since it is very sensitive for the purification of allergens by fractionating them into molecular weight bands [8]. The technique employed in our study allowed the detection of numerous protein bands ranging from 15.0–145.7 kDa for *M. pachydermatis*. Due to the several cultures submitted to electrophoresis (30) and to the multiplicity of bands, the results were shown on molecular weight strips once; in that manner, the data were more easily interpreted. Therefore in the *M. pachydermatis* isolates originating in otic secretions and in skin, most samples showed bands within molecular strips of 30–40, 40–50, 50–60 and 80–90 kDa (Table 1), without detecting meaningful differences in behavior among the isolates originating in either parasitized sites.

Regarding *M. furfur*, the literature investigated also reported an elevated number of protein fractions, with bands ranging from 14–120 kDa [4, 5, 15]. Given the vast amount of bands verified by the researchers, the major ones were selected based on how frequently they appeared or were recognized by the antibodies [4, 5].

The protein profile found for *M. pachydermatis* has no parallel, since no other report of such a nature was found in the literature. Nevertheless, acknowl-

CONTROLS	o1	o2	o3	p1	p2
<u>112.0</u> ¹				<u>114.2</u>	<u>141.1</u>
<u>84.0</u>	<u>95.1</u>	<u>80.9</u>	<u>95.1</u>	<u>80.9</u>	<u>80.9</u>
	<u>80.9</u>		<u>69.9</u>		
		<u>61.2</u>			
<u>53.2</u>	<u>54.0</u>			<u>48.1</u>	<u>54.0</u>
	<u>43.1</u>	<u>43.1</u>	<u>43.1</u>		
		<u>38.8</u>			
<u>34.9</u>	<u>35.1</u>			<u>31.8</u>	<u>35.1</u>
<u>28.7</u>		<u>29.0</u>			
			<u>26.4</u>		
<u>20.5</u>					

1. Molecular weights in Kilodaltons

Figure 1. Molecular weights of protein bands, obtained from SDS-PAGE, by using extracts of *Malassezia pachydermatis* cell walls from otic secretions (o1, o2 and o3) and skin (p1 and p2).

CONTROLS	o4	o5	p3	p4	p5
<u>112.0</u> ¹		<u>114.5</u>	<u>114.5</u>		
<u>84.0</u>	<u>100.5</u>	<u>89.3</u>	<u>100.5</u>	<u>89.3</u>	<u>89.3</u>
			<u>80.1</u>		
	<u>66.0</u>				
<u>53.2</u>	<u>55.6</u>	<u>60.4</u>	<u>60.4</u>	<u>60.4</u>	<u>60.4</u>
		<u>47.6</u>		<u>55.6</u>	
	<u>44.3</u>			<u>44.3</u>	<u>44.3</u>
<u>34.9</u>					
	<u>32.1</u>	<u>32.1</u>			
<u>28.7</u>					
	<u>23.0</u>				
<u>20.5</u>					

1. Molecular weights in Kilodaltons

Figure 2. Molecular weights of protein bands, obtained from SDS-PAGE, by using extracts of *Malassezia pachydermatis* cell walls from otic secretions (o4 and o5) and skin (p3 and p5).

CONTROLS	o6	o7	o8	p6	p7
<u>112.0</u> ¹	<u>126.0</u>	<u>126.0</u>	<u>114.1</u>	<u>104.0</u>	<u>114.1</u>
<u>84.0</u>	<u>87.7</u>	<u>81.1</u>	<u>81.1</u>	<u>81.1</u>	<u>81.1</u>
			<u>61.1</u>	<u>61.1</u>	
<u>53.2</u>	<u>53.8</u>	<u>53.8</u>	<u>47.7</u>	<u>50.6</u>	<u>53.8</u>
		<u>47.7</u>	<u>47.7</u>		<u>47.7</u>
<u>34.9</u>	<u>38.0</u>	<u>38.0</u>	<u>36.0</u>		
	<u>36.0</u>	<u>36.0</u>			
<u>28.7</u>				<u>26.5</u>	
<u>20.5</u>					<u>20.6</u>

1. Molecular weights in Kilodaltons

Figure 3. Molecular weights of protein bands, obtained from SDS-PAGE, by using extracts of *Malassezia pachydermatis* cell walls from otic secretions (o6, o7 and o8) and skin (p6 and p7).

CONTROLS	o9	o10	p8	p9	p10
<u>112.0</u> ¹	<u>128.7</u>	<u>145.7</u>	<u>115.1</u>	<u>115.1</u>	<u>128.7</u>
<u>84.0</u>	<u>86.9</u>	<u>86.9</u>	<u>80.2</u>	<u>86.9</u>	<u>86.9</u>
	<u>69.2</u>	<u>74.3</u>			
			<u>60.8</u>		
<u>53.2</u>	<u>51.0</u>	<u>54.0</u>	<u>51.0</u>		<u>57.2</u>
		<u>48.4</u>	<u>46.0</u>	<u>48.4</u>	<u>51.0</u>
				<u>46.0</u>	
<u>34.9</u>	<u>39.8</u>	<u>39.8</u>		<u>38.1</u>	<u>39.8</u>
		<u>38.1</u>			
<u>28.7</u>			<u>29.9</u>		
<u>20.5</u>					

1. Molecular weights in Kilodaltons

Figure 4. Molecular weights of protein bands, obtained from SDS-PAGE, by using extracts of *Malassezia pachydermatis* cell walls from otic secretions (o9 and o10) and skin (p8, p9 and p10).

CONTROLS	o11	o12	o13	p11	p12
<u>112.0</u> ¹	<u>137.5</u>	<u>137.5</u>	<u>137.5</u>	<u>111.0</u>	<u>137.5</u>
<u>84.0</u>	<u>92.5</u>	<u>85.3</u>	<u>92.5</u>	<u>85.3</u>	<u>85.3</u>
	<u>78.9</u>	<u>73.4</u>	<u>85.3</u>	<u>73.4</u>	<u>73.4</u>
	<u>60.3</u>	<u>60.3</u>	<u>73.4</u>	<u>64.2</u>	
<u>53.2</u>	<u>56.8</u>	<u>56.8</u>	<u>53.6</u>	<u>56.8</u>	<u>53.6</u>
		<u>50.7</u>	<u>48.1</u>	<u>50.7</u>	<u>48.1</u>
	<u>39.4</u>	<u>39.4</u>	<u>39.4</u>	<u>39.4</u>	
<u>34.9</u>		<u>37.7</u>	<u>37.7</u>	<u>37.7</u>	<u>37.7</u>
<u>28.7</u>					
<u>20.5</u>					
			<u>16.6</u>		<u>16.0</u>

1. Molecular weights in Kilodaltons

Figure 5. Molecular weights of protein bands, obtained from SDS-PAGE, by using extracts of *Malassezia pachydermatis* cell walls from otic secretions (o11, o12 and o13) and skin (p11 and p12).

CONTROLS	o14	o15	p13	p14	p15
<u>112.0</u> ¹	<u>134.9</u>	<u>134.9</u>		<u>134.9</u>	<u>122.8</u>
<u>84.0</u>	<u>89.0</u>	<u>82.9</u>	<u>89.0</u>	<u>95.8</u>	<u>95.8</u>
		<u>72.6</u>	<u>77.5</u>	<u>77.5</u>	<u>72.6</u>
<u>53.2</u>	<u>57.3</u>	<u>57.3</u>	<u>54.2</u>	<u>54.2</u>	<u>54.2</u>
	<u>51.4</u>	<u>51.4</u>		<u>48.8</u>	<u>48.8</u>
	<u>40.0</u>	<u>40.0</u>	<u>40.0</u>	<u>38.2</u>	<u>38.2</u>
<u>34.9</u>					
<u>28.7</u>					
<u>20.5</u>	<u>23.6</u>	<u>22.7</u>			
	<u>15.0</u>	<u>15.0</u>			

1. Molecular weights in Kilodaltons

Figure 6. Molecular weights of protein bands, obtained from SDS-PAGE, by using extracts of *Malassezia pachydermatis* cell walls from otic secretions (o14 and o15) and skin (p13, p14 and p15).

Table 1. Isolates of *Malassezia pachydermatis* from otic secretions and skin grouped according to the presence of protein bands of different molecular weights.

Isolates	Molecular weight bands (kDa) ^a															
	10-20	20-30	30-40	40-50	50-60	60-70	70-80	80-90	90-100	100-110	110-120	120-130	130-140	140-150		
Otic secretion (15)	Absolute number	3	5	12	11	11	8	5	12	4	1	2	3	5	1	
	% ^b	20.0	33.33	80.0	73.3	73.3	53.3	33.3	80.0	26.7	6.7	13.3	20.0	33.3	6.7	
Skin (15)	Absolute number	1	3	8	10	11	6	5	13	2	2	6	2	2	1	
	% ^b	6.7	20.0	53.3	66.7	73.3	40.0	33.3	86.7	13.3	13.3	40.0	13.3	13.3	6.7	
Total (30)	Absolute number	4	8	20	21	22	14	10	25	6	3	8	5	7	2	
	% ^b	13.3	26.7	66.7	70.0	73.3	46.7	33.3	83.3	20.0	10.0	26.7	16.7	23.3	6.7	

^akDa = kilodaltons.^bValues > s 100% once the same sample may present several protein bands.

edging the main protein fractions are the basis for studies within the field of atopic or therapeutic dermatitis among others, where the mapping of such allergenic epitopes not only will contribute to extract standardizing but also as a diagnostic aid.

In that manner, Bond & Lloyd [16], concluded that the proteins from *M. pachydermatis* wall are important for the adherence of the yeast cells to corneocytes and that such proteins remain to be well defined, so that inhibitors that could be employed in a prophylactic or therapeutic way may be investigated.

In dogs, subcutaneously inoculated with live or inactive *M. pachydermatis*, local itching and eosinophilia reactions were noted, thus suggesting the allergenic properties of this microorganism [17]. This experiment nevertheless proved attenuation of symptoms and even recovery of sick animals. It was not determined, however, which fractions were responsible for the remission of the medical diagnosis, leaving the question unanswered, since the protein fractions generally form the main fungal antigen [8].

Both for these cases and as a principle for the study of *M. pachydermatis* allergens, protein fractions are recommended for use, based on those most frequently found in this experiment.

The technique we adopted was appropriate for the study of the protein profile of *M. pachydermatis*, without taking into account the differences in behavior among the isolates from the skin and ear canals. These preliminary studies are to be looked into in forthcoming studies, aimed at applicability for diagnosis and therapy.

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