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COMPARATIVE ANALYSIS OF PROTEIN PROFILES OF *MALASSEZIA PACHYDERMATIS* STRAINS ISOLATED FROM HEALTHY DOGS AND DOGS WITH OTITIS EXTERNA

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Abstract

The study included 40 strains of *Malassezia pachydermatis* obtained in pure culture from external ear canal of dogs and the reference strain. Protein fractions were received by mechanical disruption of the fungal cells. Denaturing protein electrophoresis was performed according to Laemmli method. More than 90% of the all tested strains were characterised by the presence of the following protein fractions: 83.0; 77.0; 59.0; 55.0; 48.0; 38.0; 35.0; 28.0, and 27.0 kDa. In other regions of electrophoregrams, a relatively high differentiation was observed. The strains isolated from animals with otitis externa were characterised by the presence of the following protein fractions: 75.0; 61.0; 52.0; 36.0; 19.5; 16.0; 13.8, and 13.0 kDa. These fractions were absent in the commensal strains. The use of statistical analysis confirmed the obtained results and allowed to generate dendrogram grouping potentially pathogenic and commensal strains on two different branches. Such results may indicate significant differences between protein profiles of strains of *M. pachydermatis* isolated from healthy and diseased animals.

Key words: dogs, otitis externa, *Malassezia pachydermatis*, protein profile.

Malassezia sp. yeasts are the part of the normal biota of human and animal skin and mucous membranes (2, 6) but simultaneously can be associated with various types of skin infections (pityriasis versicolor, seborrhoeic dermatitis, atopic dermatitis, and folliculitis in humans, and dermatitis and otitis externa in animals (3, 18) and, much more rarely, with systemic mycoses or even fungemias (21).

Malassezia pachydermatis is a lipophilic yeast and the only lipid-independent species, most commonly isolated from animals, mainly from dogs (12). As a result of weakening of host defence mechanisms, or due to the changes in cutaneous microenvironment, the microorganism may undergo conversion into a pathogenic form (10). *M. pachydermatis* is responsible for about 30%-80% of cases of otitis externa and is associated with 30% of cases of seborrhoeic or atopic dermatitis in dogs (19). In humans, it can cause fungemias, especially in newborns in intensive therapy units (5).

Studies of the antigenic structure of *Malassezia* sp. and its interactions with the immune system, even carried out on a large scale, encounter serious difficulties. This fact is connected with the multi-effects of *Malassezia* cells on the elements of the immune system, involving activation of classical and alternative complement pathways and stimulation of humoral and cellular immune responses, affecting the secretion of cytokines (4). Moreover, eukaryotic fungal cells have a complex and variable structure, depending on the gene expression in different stages of their life. In yeast of the genus *Malassezia*, in contrast to dermatophytes (13), no correlation was found between the antigenic profile and the morphological form of the fungus: both mycelial hyphae and blastospores had a very similar structure of antigens (24).

Spectrum of the obtained antigens is associated with the phase of fungal growth (15), metabolites produced, conditions for fungal growth (13), and with type of the strain and method of antigen extraction (16, 17). The aim of most of these studies was to determine and identify protein antigens and to evaluate their impact on the host's immune system. They resulted in identification of allergens associated with atopic *Malassezia* infections or *Malassezia* overgrowth in humans and animals.

Considering the fact that the pool of antigens present in *Malassezia* cells contains not only allergens but also includes proteins which induce cell-mediated responses, to determine the protein profile in terms of the source of strain (healthy and affected animals) could be clinically important. Such an analysis would allow to demonstrate the degree of antigenic variation, particularly regarding the pathogenicity of the tested

strain. So far, only rare studies have been conducted in this area, which concern a very limited number of strains (11), therefore the aim of the study was to determine the protein profiles of *M. pachydermatis* strains, with focus on comparative analysis of fractions obtained from strains isolated from healthy dogs and from dogs with otitis externa.

Material and Methods

***M. pachydermatis* isolates.** The study included 40 strains of *M. pachydermatis,* freshly isolated from the external ear canal of dogs. Twenty strains were obtained from clinically healthy animals, while the remaining strains were received from dogs with clinical symptoms *Malassezia* otitis (*Malassezia* overgrowth, redness, itching, the presence of characteristic brownish exudate in the ear canal). The samples were cultured on Sabouraud agar at 37°C for 72 h. The selected strains were isolated from pure culture (microscopic examination and culture samples indicated no presence of other bacterial, fungal, or parasitological biota). The study also included *M. pachydermatis* reference strain CBS7925, isolated from healthy dog (Centraalbureau voor Schimmelcultures, the Netherlands). After phenotypic and genotypic identification (14), the strains were freeze-stored (-70°C) as a cell suspension at a concentration of $2x10^9$ cfu ml^{-1} in sterile saline with an addition of 20% glycerol, until protein extraction.

Extraction of proteins. All the tested strains were defrosted at the same time and suspensions of individual strains in the amount of 0.5 ml were incubated on solid Sabouraud medium at 37˚C for 3 d. In order to obtain the full panel of proteins, the homogenisation of cells was carried out according to the previously standardised procedure (26). Briefly, *Malassezia* cultures were suspended in sterile distilled water with 0.02% merthiolate (Merck Schuchardt) at a ratio of 10 ml of solution per plate. The cells were disintegrated in a homogeniser (Braun MSK) at 4˚C in 1-min cycle (min. 95% cell disintegration). Homogenates were concentrated (Millipore 0.22 µm) to a volume of 200 µl and collected. The total amount of protein was measured, by the use of the Total Protein 60 kit (Cormay).

Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE).
Denaturing protein electrophoresis (SDS-PAGE) was performed according to Laemmli method (20) using a mini-protean system (Bio-Rad). Electrophoretic separation of the samples was repeated three times at various intervals. The individual electrophoregrams were analysed and documented using Gel-Doc 2000 (Bio-Rad).

Statistical analysis. Statistical analysis was performed using NTSYS-pc 2.02 and FENAL 1.0 β-software. The similarity of the tested isolates was expressed in percentage of similarity coefficients, SM (simple matching), while to construct of the dendrogram the UPGMA method (unweighted pair group method with arithmetic mean) was used. In the statistical analysis, the reference strain was included as a commensal isolate.

Results

Preliminary evaluation of protein content in individual samples showed a great diversity. The values obtained ranged from 0.8 g/dl to 15.8 g/dl with a mean value of 6.3 g/dl (with standard deviation σ = 3.1). Average protein content in samples of strains obtained from diseased animals was higher ($P<0.05$) when compared to commensal strains (including the reference strain). These values were 7.3 g/dl (σ=2.5) and 5.2 g/dl (σ = 3.4), respectively.

The electrophoretic separation of the protein extracts revealed a high level of diversity reflecting their very complex structure (Figs 1A, B, C).

Total number of protein fractions ranged from 3 to even 42, with a mean value of 29.1 (σ=9.5). Similarly to the protein concentration in samples from the strains isolated from diseased animals, the number of fractions was significantly higher ($P<0.05$) compared to the commensal strains and was 31.7 (σ=10) and 26.7 (σ=8.6), respectively, for both groups of the tested strains. The molecular weights of individual protein fractions were within the range of 13.0-259 kDa and their distribution was irregular.

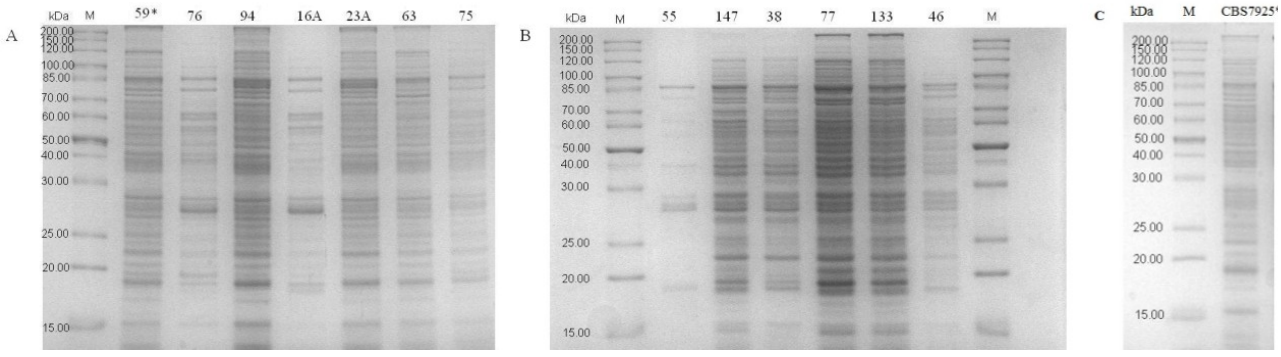


Fig. 1. Electrophoregrams (SDS-PAGE) of selected *M. pachydermatis* isolates: **A –** strains isolated from healthy dogs, **B** – strains isolated from dogs with otitis externa, **C** - *M. pachydermatis* reference strain CBS7925. * No of strain, **M** – protein marker

Table 1
Protein fractions of *M. pachydermatis* reference strain
and isolates obtained from healthy dogs and dogs with otitis externa

Molecular mass (kDa)	Number of strains showing determined protein fractions		
	Strains obtained from animals:		The reference strain CBS7925
	healthy	with otitis externa	
75.0	0 (0%)	2 (10%)	-*
61.0	0 (0%)	4 (20%)	-
52.0	0 (0%)	16 (80%)	-
36.0	0 (0%)	7 (35%)	-
19.5	0 (0%)	6 (30%)	-
16.0	0 (0%)	4 (20%)	-
13.8	0 (0%)	4 (20%)	-
13.0	0 (0%)	2 (10%)	-
98.0	8 (40%)	15 (75%)	-
72.0	11 (55%)	18 (90%)	+
40.0	7 (35%)	18 (90%)	+
32.0	7 (35%)	18 (90%)	+
27.3	7 (35%)	16 (80%)	+
20.5	9 (45%)	15 (75%)	+
17.0	8 (40%)	15 (75%)	+
24.5	7 (35%)	0 (0%)	-
19.0	18 (90%)	6 (30%)	-

*+or - indicates the presence or absence of a fraction of the reference strain protein profile

A significant increase in the number of bands was observed in the regions of 85.0-35 kDa; 29.0-27.0 kDa, and 22.0-19.0 kDa (Figs 1A, B, and C). In these regions, the fractions present in more than 90% of the all examined strains (including reference strain) were found. These selected fractions were characterised by the following molecular weights: 83.0(95.1%); 77.0 (97.5%); 59.0 (92.6%); 55.0 (92.6%); 48.0 (95.1%); 38.0 (92.6%); 35.0 (92.6%); 28.0 (97.5%), and 27.0 kDa (92.6%). Other areas were characterised by the great diversity in the presence of individual bands. The absence or presence of certain bands was associated with a particular strain, especially with its origin. The strains isolated from animals with otitis externa were characterised by the presence of the following protein fractions: 75.0, 61.0, 52.0, 36.0, 19.5, 16.0, 13.8, and 13.0 kDa; these fractions were absent in commensal strains. The isolation frequency of the individual bands was variable: fractions of 52 kDa (80% of strains), 36.0 kDa (35%), 19.5 kDa (30%), 61.0, 16.0, and 13.8 kDa (20%), and 75.0 and 13.0 kDa (10%) were the most frequently detected (Table 1). Protein analysis also showed that the fractions: 98.0, 72.0, 40.0, 32.0, 27.3, 20.5, and 17.0 kDa were present almost two times more commonly in pathogenic strains when compared to commensal strains (Table 1). A characteristic feature of the strains isolated from healthy animals was the presence of the 24.5 kDa fraction (35% of strains); this band was absent in the pathogenic strains. The band with a molecular weight of 19.0 kDa was present in 90% of the commensal strains but only in 30% of the pathogenic strains.

Due to the very complex protein profiles of the tested strains and the presence of other bands in similar percentage in strains from healthy and diseased dogs, the comparative statistical analysis of the strains was performed using Nt-Sys software. The programme generated a dendrogram focusing individually commensal and potentially pathogenic strains (Fig. 2). The relationships among individual strains specified on the basis of their protein profiles were represented by the distribution of the strains on individual branches.

The strains isolated from cases with otitis externa were clustered, in general, in one group, (with the exception of two strains - 46, 55), whereas the commensal strains formed a clearly separate group (degree of similarity between these two groups was about 62%). The reference strain was found in the group of isolates originating from diseased animals.

Large differences between protein profiles of individual strains resulted in the formation of very small clusters. The resultant clusters included no more than five strains each (Fig. 2) and in most cases clusters contained only two strains.

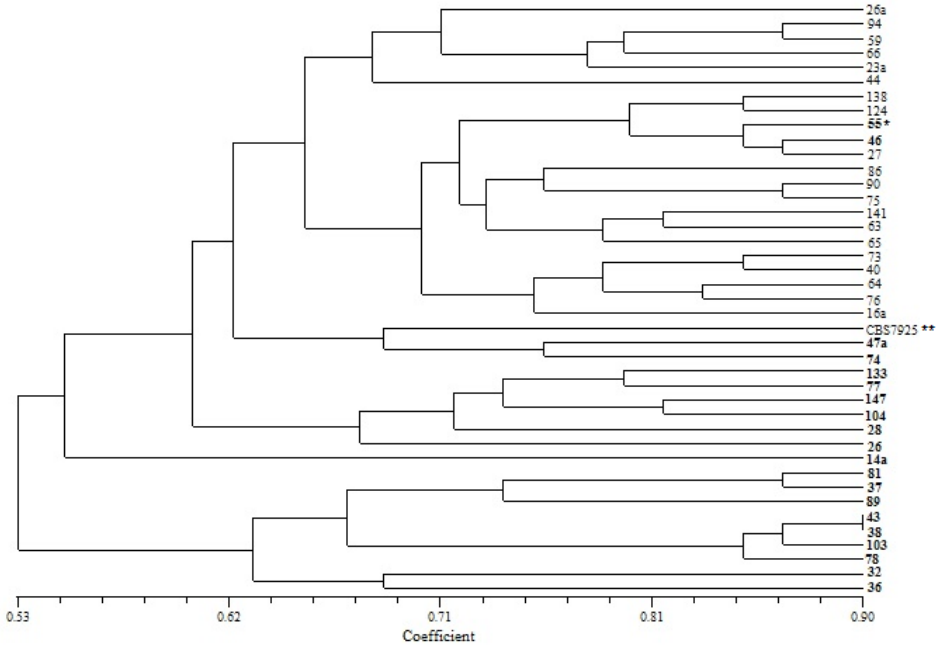


Fig. 2. Dendrogram of *Malassezia pachydermatis* strains
* strains isolated from diseased dogs (bold type), ** the reference strain

Discussion

The results of the study demonstrated that antigenic structure of *M. pachydermatis* yeasts is very complex and it has a high degree of heterogeneity. Due to the fact that most antigens stimulating the host's immune system are poorly associated with the fungal cell wall, they can be very easily lost during the extraction process (1). To obtain a protein profile containing the complete pool of antigens, total homogenisation of cells was carried out. Moreover, expression of antigens is affected by many factors (11), so it is important to optimise the conditions for obtaining representative and reproducible pool of antigens. Habibah *et al.* (15) showed that the incubation period and phase of fungal growth significantly affect the antigenic profile and the most effective yield was given at 2-d incubation. *M. pachydermatis* strains incubated for longer than 4 d showed a significantly lower immunoreactivity, which could indicate the loss of some antigens. Similar observations were obtained by Zargari *et al.* (25) for lipid-dependent *Malassezia* species. However, not all strains of *M. pachydermatis* reached an appropriate cell mass after 2 d, and this fact could significantly reduce the amount of antigen (26). Therefore, as concerns the research undertaken, it was decided to extend the incubation for up to 3 d. During this period, cells of *M. pachydermatis* displayed an intensive growth, which resulted in a rich and diverse set of antigens with determinable content (high cell mass). However, the pool of tested strains included five isolates (124, 27, 46, 55, and 138), which, despite the extended incubation period, revealed very weak growth, a low protein content in the samples, and poor antigenic profile, as reflected by the placement of these strains on a common branch of the dendrogram (Fig. 2).

As confirmed by other authors, the absence of protease inhibitors did not change the electrophoretic pattern observed for *M. pachydermatis* strains (7). The analysis of the protein profiles of the individual strains of *M. pachydermatis* based on SDS-PAGE electrophoregrams revealed a rich and diverse set of fractions within the mass range of 259.0 to 13.0 kDa. Protein profiles contained fractions present in more than 90% of the tested strains and, presumably, these could be antigen fractions characteristic for the species *M. pachydermatis* in particular. These nine proteins (83.0, 77.0, 59.0, 55.0, 48.0, 38.0, 35.0, 28.0 and 27.0 kDa) were also observed in the profile of the reference strain.

The preliminary assessment showed that the strains isolated from dogs with clinical signs of otitis externa had a richer and more strongly expressed protein set than the commensal strains. Moreover, characteristic bands were observed only for the pathogenic strains (75.0, 61.0, 52.0, 36.0, 19.5, 16.0, 13.8 and 13.0 kDa) or some bands were found in significantly higher percentage in those strains (98.0, 72.0, 40.0, 32.0, 27.3, 20.5 and 17.0 kDa). Due to the large heterogeneity and complex structure of the protein profiles of *M. pachydermatis* strains, it should not be concluded that these bands are unique only to strains that cause clinical signs of otitis externa. However, it may suggest a high probability that the expression of these antigens may depend on the clinical status of the host (healthy or diseased animal) and be connected with the pathogenic properties of the strain (23). Studies in this area are rare (11) and mostly concern the detection and identification of antigens involved in the humoral immune response, particularly in atopic patients with clinical signs of otitis or dermatitis. Chen *et al.* (8, 9) observed proteins at 45-63 kDa and 25kDa, that reacted with sera of more than 50% dogs with atopic dermatitis (major allergens), while

the sera of healthy dogs were much less reactive to the same proteins. Similary, Bond and Lloyd (7), determined protein fractions with high molecular weights (219.0, 110.0, 71.0 and 45.0 kDa) reacting with sera of dogs with clinical signs of dermatitis. Similar reactivity of the sera of atopic dogs in relation to the protein mass of 98 kDa was observed by Kim *et al.* (17). In the study, the selected proteins of similar molecular weight could also correspond to antigens isolated by other authors: proteins characteristic only for strains derived from dogs with signs of otitis externa (61, 52, 36 kDa) can be the equivalent of some allergens identified by Chen and Hill (10) and Bond and Lloyd (7), and proteins detected in more than 90% of tested strains (55 and 48 kDa) can reproduce allergens recognised by sera of both healthy and atopic dogs (7). Profiles of protein antigens from *M. pachydermatis* strains obtained by different research groups are quite similar, despite small differences in the molecular weight of individual allergens, especially when considering the differences in the accuracy of the various methods of determination of molecular weights of proteins, different periods of culture, or different host immune system activity (7, 10, 15, 25). Recent studies have shown that some of extracted proteins can possibly correspond to molecular weights of allergens (61, 52, 36 kDa); however, *M. pachydermatis* can also produce antigens undetectable by immunoblotting methods, as well as by stimulating cell-mediated response (11, 22). Another obstacle is to determine the status of the tested strain. In the study, the strains were divided into two groups: isolated from clinically healthy animals and from animals with signs of otitis externa. However, there was no possibility to determine whether healthy animals had not previously had ear or skin infections due to *Malassezia*, which could significantly change the protein expression profile of the tested strain. In addition, *M. pachydermatis* is a component of commensal biota, constantly in contact with the host immune system and it is not possible to determine accurately, which antigens are expressed during infection. All of these factors could affect such a large discrepancy in the percentage of occurrence of fractions separated only from strains isolated from otitis externa (from 10% to 80%). Similarly, demonstration of 24.5 kDa fraction in 35% of the strains isolated from healthy animals cannot be interpreted as a typical fraction only for commensalic strains. Simultaneously, the use of the statistical analysis to interpret the obtained electrophoregrams allowed to demonstrate certain differences between the groups of “pathogenic” and “commensal” strains. The protein profiles of both groups formed two separate branches of the dendrogram, which showed a high degree of similarity. The dendrogram contained clusters grouping maximum five strains each, which could indicate either a high heterogeneity of the strains or that in the study none of the examined pool of strains was large enough. The reference strain was among the strains isolated from diseased animals, although it originated from a healthy animal (Fig. 2). The protein profile of the isolate was characterised by the lack of proteins that are specific to isolates derived from diseased animals, but also the majority (six out of seven) of the proteins were present in an amount almost twice as high as in the profiles of "pathogenic" strains. Simultaneously, the reference strains did not show the proteins observed in healthy animals (24.5 and 19 kDa) in their profile. Probably these factors contributed to such a position of strain on the dendrogram and a relatively low degree of similarity (approximately 69%) from the nearest neighbouring strain.

In summary, the differences in protein profiles of strains of *M. pachydermatis* isolated from healthy and diseased animals were demonstrated. However, at this stage of the study, it is not possible to determine which protein is responsible for the pathogenicity of the strain, and therefore a further analysis is required for the determination of biological activity of individual fractions and their potential role in the pathogenesis of *Malassezia* infection.

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