

DR. JEKYLL AND MR. HYDE: THE DUAL NATURE OF *MALASSEZIA PACHYDERMATIS*

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Abstract

Malassezia pachydermatis is a part of the physiological biota of the skin and mucous membranes of most mammals and birds. This opportunistic species causes mainly surface skin infections. Since *M. pachydermatis* is isolated almost equally from diseased animals and those without clinical symptoms of the disease, accurate diagnosis of infections caused by this yeast is a problem of great importance in veterinary and medicine. Therefore, the isolation of these fungi from patients is not equivalent to the identification of the etiologic agent of infection. According to the current state of our knowledge, the problem of diseases associated with *M. pachydermatis* is correlated with the metabolic, hormonal and immunological state of the host. However, in the light of a review of the literature, a significant question arises, whether the pathogenicity of *M. pachydermatis* is solely conditioned by host-dependent factors, or maybe the pathogenic strains have independent virulence mechanisms while the other factors only trigger their expression.

Key words: yeast, pathogen, commensal, pathogenicity markers

Introduction

The most common disease caused by *Malassezia pachydermatis* diagnosed in dogs is *otitis externa* (inflammation of the external auditory canal; Patterson and Frank 2002). However, the etiology factors predisposing to this disease and the role of mentioned microorganisms in the pathogenesis of the disease are not fully explained. Precise and early diagnosis determining the causes of the disease is very important for proper and effective treatment. According to the literature data, *M. pachydermatis* is isolated with almost the same frequency from sick and healthy animals (Dworecka-Kaszak and Adamski 2005, Girao *et al.* 2006, Prado *et al.* 2008, Campbell *et al.* 2010). This suggests that the development of disease symptoms depends on the immune as well as the metabolic and hormonal status of the host (Patterson and Frank 2002). On the other hand, it may indicate the presence of certain features of fungi, by which they regulate their impact on the host organism, which results in inhibition or stimulation of the immune response. Therefore, *M. pachydermatis* is referred to as the immune paradox (Dworecka-Kaszak and Adamski 2005), and the issue of a closer understanding

of their biology and mutual relationship with the host organism is still valid and current.

Although fungi of the *M. pachydermatis* species are commonly found in the microbiota of animals, especially in dogs and cats (Prado *et al.* 2008, Duarte *et al.* 2009), the methods of their influence on the host organism are still only partly explained. Most authors suggest that *M. pachydermatis* plays the role of commensal and opportunist (Cafarchia *et al.* 2008, Bond 2010, Shokri *et al.* 2010, Saunders *et al.* 2012). Nevertheless, there are data on the comparison of the characteristics of *M. pachydermatis* strains originating from animals without disease symptoms and suffering from *otitis externa*, which indicate some substantial differences between the strains representing these two groups (Kobayashi *et al.* 2011, Czyżewska *et al.* 2016, Czyżewska *et al.* 2018, Czyżewska *et al.* 2019). The existence of a correlation between the properties of strains and their potential virulence leads to the question – is it possible that different strains of *M. pachydermatis* have a distinct nature: commensal or pathogenic? In the case of a positive answer, we should also search for the discriminatory features characteristic of each particular ecological property. Consequently, there are two possibilities: either *M. pachydermatis* is (i) a typical opportunist and the infection develops as a result of host homeostasis disorders, or (ii) typically commensal strains that have specific characteristics which, in combination with conditions related to the physiological condition of the host, allow them to induce infection.

Genetic findings and properties

If we assume that certain biological features of the fungus affect its virulence, we should expect that potentially pathogenic strains should be more closely related to other virulent isolates, not to commensal strains. Data obtained by the nucleotide sequencing of the intergenic transcribed spacer (ITS1) fragment allow us to trace and reconstruct the phylogeny of the studied microorganisms. Moreover, the lack of natural selection effects on these fragments means that all their variability reflects phylogenetic relationships and the population structure of *M. pachydermatis*.

The results of the comparison of the nucleotide sequence of 65 strains of *M. pachydermatis* (Czyżewska *et al.* 2018) enabled us to identify 11 different genotypes that could be grouped into 3 major clades (Figure 1). The distinction of 3 separate genotypic groups is consistent with other available data in the literature in which *M. pachydermatis* strains originating from the skin and ears of dogs in Korea were analyzed (Han *et al.* 2013). What is more, a few authors have pointed out the relationship between the genetic profile of the studied fungi and their geographical origin (Machado *et al.* 2010), while others did not see any correlation at the genetic level, but noted relationships at the phenotypic level regarding the presence and activity of selected enzymes (Teramoto *et al.* 2015). Nevertheless, further research contributing to a deeper understanding of *M. pachydermatis* biology will help us to understand better the existing relationships between host and pathogen or commensal. Nevertheless, data from

various sources clearly indicate that we are dealing with a dualistic microorganism, and the genesis of *M. pachydermatis*-associated skin infections are at least partly dependent on the fungus properties.

The diversity of the nucleotide sequence of the ITS1 fragment indicates that the virulence of *M. pachydermatis* isolates may be correlated with their phylogenesis (Czyżewska *et al.* 2018). It should be noted that phylogenetic clade II (Figure 1) is composed of about 80% of the isolates from clinical cases. What is more, they represent two slightly different genotypes. Despite the definite dominance of potentially virulent strains in this clade, about 1/5 of the strains were isolated from animals without any signs of infection. On the other hand, in clades I and III, we observed the domination of isolates obtained from healthy dogs, while strains from *otitis externa* cases were constituting 35% and 31%, respectively. These observations are consistent with previous data on the great importance of the host animal's condition for the risk of disease development (Chang *et al.* 1998, Masuda *et al.* 2000, Tragiannidis *et al.* 2010, Gaitanis *et al.* 2012, Al-Sweih *et al.* 2014). Nevertheless, the close kinship of strains derived from sick animals supports the supposition of the participation of specific properties of the fungus in the development of infection, which may indicate the validity of the hypothesis about the occurrence of strains displaying both a pathogenic and commensal nature within the studied species. In addition, some premises from the literature also indicate the importance of features (which correlates with the genetic profile) of the fungus in the genesis of inflammation in animals. For example, it has been observed that specific subtypes of *M. pachydermatis*, established on the basis of the sequencing of the intergenic segment (IGS-1) correlate with excessive development of the fungal population and the synthesis of phospholipases that are a factor of its virulence (Kobayashi *et al.* 2011). Also Han *et al.* (2013), analyzing the IGS-1, ITS-1 fragments and the 26S rRNA gene found a different frequency of isolates representing different genotypes in healthy and sick animals. Kobayashi *et al.* (2011) noted the fact that strains grouped from sick animals regardless of strains isolated from healthy animals. However, there is no unambiguous data to answer the question whether the clear separation of strains involved in clinical cases is not the result of a speciation process associated with, for example, lack of gene flow (isolation) and natural selection. On the basis of present knowledge, there is no clear evidence of speciation (of virulent strains) among *M. pachydermatis*.

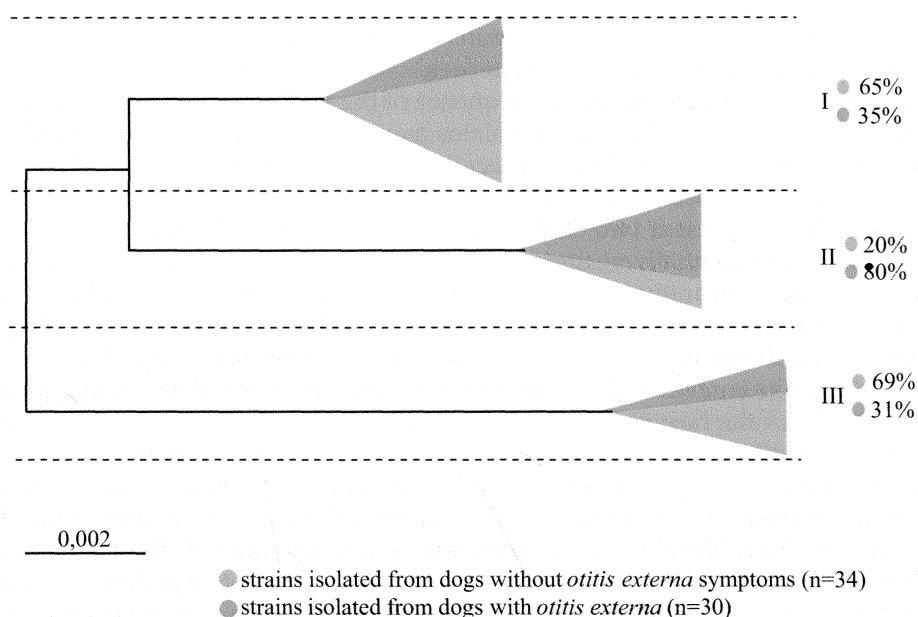


Figure 1. Dendrogram depicting the genetic relationships between *M. pachydermatis* isolates from healthy dogs and dogs with *otitis externa* (Czyżewska *et al.* 2018).

Comparison of lipid profiles

Data obtained from genetic analyses indicate the high diversity of *M. pachydermatis* strains. As mentioned above, a relationship between the origin of the fungus (from a sick or healthy animal) and its genetic profile was found. Hence, the natural consequence is the search for potential, genetically conditioned features of the fungus enabling it to effectively overcome the host's defense barriers.

Lipid profiles are used as an easy, fast and highly selective method that allows the identification of fungal pathogens such as *H. capsulatum* (Zarnowski *et al.* 2007), *C. albicans* and *Candida dubliniensis* (Mahmoudabadi and Drucker 2006), *Cryptococcus neoformans*, as well as also non-pathogenic fungus, *S. cerevisiae* (El Menyawi *et al.* 2000). In 2012, the lipid profile of *M. pachydermatis* was also compared with profiles established for *C. albicans* and *S. cerevisiae*; however, the research concerned only the reference strain (Tylicki *et al.* 2012). In 2016, lipid profiles of *M. pachydermatis* strains derived from dogs were determined (Czyżewska *et al.* 2016). These studies indicated that strains derived from dogs without *otitis externa* symptoms contain much more fatty acids than those isolated from sick dogs. This difference is maintained regardless of culture conditions (medium supplemented with lipids and medium without the addition of lipids). Differences between both groups of tested strains were also observed in

the content of particular fatty acids. Thin layer chromatography also provided interesting information on the differences in lipid profiles between the strains studied. Strains isolated from dogs with *otitis externa* showed significantly lower levels of triacylglycerols and ergosterol esters. These results show that the strains from the two studied groups are different, which may be a basis for determining whether a given strain is potentially pathogenic.

If we assume that *M. pachydermatis* is a typical opportunist, it is possible that strains derived from animals without disease symptoms are in a dormant phase (Brand 2012). *M. pachydermatis*, in addition to the ability to synthesize myristic acid, also has the ability to obtain lipids from the environment. Lower lipid content in strains derived from dogs with *otitis externa* may be due to the rapid proliferation of cells correlated with the appearance of disease symptoms. This process requires large amounts of energy, which could be also provided by lipids previously accumulated in the cells. Therefore, it can be assumed that the ability to quickly activate lipid reserves is necessary to recover *M. pachydermatis* cells from dormancy.

However, looking at the problem from a different perspective, the question arises whether the differences observed in lipid profiles may indicate the pathogenic nature of *M. pachydermatis* strains? Triacylglycerols are the primary source and storage form of energy in fungal cells, as well as a source of fatty acids necessary for the biosynthesis of membrane phospholipids (Rajakumari *et al.* 2010). Triacylglycerols are hydrolyzed to diacylglycerols and fatty acid residues, which can then be used in the β -oxidation process to release energy. Diacylglycerols, resulting from this hydrolysis, can serve as a scaffold for the biosynthesis of phosphatidylcholine or phosphatidylethanolamine in the Kennedy cycle (Czabany *et al.* 2007, Rajakumari *et al.* 2008). Sterols, which are released from sterol esters, can be introduced into the membrane, where they modulate the properties of the protein-lipid bilayer (Czabany *et al.* 2007). It was also observed that some nutrient components stimulate *in vitro* mycelium formation by *Malassezia* sp. Porro *et al.* (1977) obtained the growth of the mycelium of *Malassezia* sp. by adding cholesterol and its esters to the medium. Perhaps potentially pathogenic strains of *M. pachydermatis*, having a lower concentration of triacylglycerols and ergosterol esters, must compensate the deficiencies of these compounds using the resources of the host organism. This strategy combines potentially pathogenic strains with causing clinical manifestations of the disease.

Activity of extracellular phospholipases

M. pachydermatis is capable of extracellular secretion of many enzymes, including phospholipases. These enzymes are considered to be one of the factors leading to skin lesions (Juntachai *et al.* 2009). Phospholipases are active against the cell membrane composed of phospholipids and proteins. This naturally leads to the formation of pores and disruption of membrane function. In this situation, the pathogen has easier access to the cell, which further results in tissue invasion and the use of host resources to maintain fungal metabolic

transformations (Coutinho and Paula 2000). Inflammatory processes of the host skin are also associated with the release of arachidonic acid from cells, under the influence of fungal phospholipases (Buommino *et al.* 2016). In their research, Cafarchia and Otranto (2008), Vlachos *et al.* (2013) and Teramoto *et al.* (2015) found that *M. pachydermatis* strains isolated from dogs suffering from *otitis externa* have higher overall phospholipase activity compared to strains from dogs without clinical signs of disease. However, the method for determining the overall phospholipase activity is not completely specific and is considered pre-screening (Pini and Faggi 2011). In 2015, Teramoto *et al.* determined phospholipase C activity. It turned out to be higher in strains derived from sick dogs. The increased activity of phospholipases in strains obtained from dogs with *otitis externa* in comparison with strains isolated from healthy animals indicates that these enzymes play an important role in the pathogenesis of diseases caused by *M. pachydermatis*. Due to the significant differences in phospholipase activity between strains from different sources (infected and healthy animals), phospholipase activity can be used as a marker for distinguishing between typically commensal and potentially pathogenic strains. By using such an additional criterion, it would be possible to improve the effectiveness of diagnostics of diseases caused by *Malassezia* fungi.

Differences in protein profiles

One of the tasks of proteomic research is to look for differences that can be used to improve and facilitate clinical diagnosis, choose an appropriate therapy, and constantly monitor the course of the disease (Kossowska *et al.* 2009). Currently, proteomic tests can be used in clinical diagnostics. They make it possible to indicate possible differences between the protein profiles of sick and healthy people, because the cause or consequence of a given disease may be the presence of a specific protein or proteins (Nahaczewska *et al.* 2014). Nowakiewicz and Ziółkowska (2013) indicated significant differences in the presence or concentrations of 8 proteins produced by *M. pachydermatis* by comparing isolates from infected and unaffected dogs. There are also reports in the literature that strains of *M. pachydermatis* from sick dogs have proteins that induce a strong immune response in animals (Bond and Lloyd 2002, Chen *et al.* 2002, Kim *et al.* 2010). In 2019, Czyżewska *et al.* (2019) compared the protein profiles of *M. pachydermatis* strains isolated from dogs suffering from *otitis externa* and from dogs without signs of disease. The performed analyses were based on electrophoretic separation of proteins by two-dimensional electrophoresis, followed by their identification by liquid chromatography coupled with tandem mass spectrometry (LC-MS / MS). As a result of spectroscopic studies, the authors found the presence of some proteins that occur only in one of the groups of strains studied and other proteins that differ between both groups in terms of quantity. The obtained results indicate differences in protein profiles between *M. pachydermatis* strains of different origins (Table 1). Naturally, we should assess if the identified proteins could be considered as a marker of pathogenicity.

In this context, the presence of NADP-dependent mannitol dehydrogenase protein in dog strains is of greatest interest. This enzyme is involved in the mannitol cycle, catalyzing the oxidation of D-mannitol to D-fructose, which is involved in the regeneration of NADPH in the cells of many fungi. This enzyme has also been shown to exist as a single isoform. Characterization of this protein in *Aspergillus parasiticus* was done by Niehaus and Dilts in 1982. This protein may be associated with the pathogenicity of microorganisms. Simon-Nobbe *et al.* (2006) showed that this protein is the main allergen of *Cladosporium herbarum*, which is considered to be the cause of allergic diseases in almost all climate zones. They found that NADP-dependent mannitol dehydrogenase is recognized by IgE antibodies in approximately 57% of all patients suffering from allergies associated with *C. herbarum*.

It is also interesting that the presence of ketol-acid reductoisomerase was also found in strains from clinical cases of *otitis externa*, but not in strains obtained from uninfected dogs. This enzyme is involved in the branched-chain amino acid biosynthesis pathway that occurs in various organisms from bacteria to fungi and higher plants, except for animals. Liu *et al.* (2014) characterized the homologue of *S. cerevisiae* ketol-acid reductoisomerase from *Fusarium graminearum*, which is an optional parasite of many plant species from cereals to tropical crops. In their studies using mutants with a deletion of the gene encoding this protein, they showed that this enzyme is necessary for the complete development of infections caused by *F. graminearum*. Therefore, there are premises to conclude that ketol-acid reductoisomerase may be of significant importance in the process of infections caused by *M. pachydermatis*.

Table 1. Proteins identified in selected spots in *Malassezia pachydermatis* strains (Czyżewska *et al.* 2019).

Proteins	The name of the protein
characteristic for strains isolated from dogs with <i>otitis externa</i>	<ul style="list-style-type: none"> – NADP– dependent mannitol dehydrogenase – cytochrome–b5 reductase – atpase v1 a1 complex subunit e – ketol–acid reductoisomerase – phosphoglycerate kinase
common for strains isolated from dogs	<ul style="list-style-type: none"> – cytochrome c – hypothetical protein Malapachy_0291 – aldo keto reductase – fructose–bisphosphate aldolase – ubiquinol–cytochrome c reductase complex core protein 2 – NAD–malate dehydrogenase – glyceraldehyde–3–phosphate dehydrogenase
characteristic for strains isolated from dogs without <i>otitis externa</i> symptoms	<ul style="list-style-type: none"> – guanine nucleotide binding protein beta subunit – ATP synthase f1 gamma – polyadenylate–binding protein – glycyl–tRNA synthetase

Conclusion

In summary, we should try to evaluate if all the mentioned facts are significant enough to be used as criteria for differentiation between pathogen and commensal. It is not easy to provide a clear answer. However, taking into account the presented research results, it cannot be completely excluded that *M. pachydermatis* comprises two, partly independent groups with different ecological properties. Furthermore, there are some pieces of evidence that support this hypothesis at the level of lipid profiles, phospholipase activity, and the protein profiles of *M. pachydermatis* strains isolated from dogs. These studies have shown that the lipid profiles of individual strains differ depending on the source of their origin. It is worth noting that some differences (reduced content of ergosterol esters, reduced total fatty acid content, presence of nervonic acid in strains isolated from sick dogs) are conservative and observed despite any changes in culture conditions. In addition, strains isolated from *otitis externa* cases showed significantly higher phospholipase C activity. These strains also showed the presence of characteristic proteins, the most important of which seem to be NADP-dependent mannitol dehydrogenase and ketol-acid reductoisomerase.

The situation observed in the case of *M. pachydermatis* appears to be similar to a recent finding in the field of bacteriology. For example, the *Bacillus cereus* sensu lato group of strains is also formed by strains with quite similar genetic properties and a common evolutionary history confirmed by multi-locus sequence typing and 16S rRNA gene sequencing (Bartoszewicz and Czyżewska, 2017a; Bartoszewicz and Czyżewska, 2017b; Bartoszewicz and Marjanska, 2017). Surprisingly, even among representatives of one species, there are often distinct virulence-associated strains with the ability to synthesize toxins. Thus, an approach that distinguishes different ecotypes (eg. pathotypes, thermotypes) in the entire species has recently become widely accepted and favored. Consequently, in the entire *M. pachydermatis* species, slight genetic differences result in small dissimilarities in the ecological properties of the fungus.

Considering the information presented above, it can be concluded that the results of all these studies can be used in practice to more accurately diagnose and determine whether we are dealing with a potentially pathogenic strain. If the host has no symptoms of the disease and is a carrier of *M. pachydermatis*, the lipid profile of this strain can be determined and, based on its analysis, it can be assessed whether the carrier is at risk of developing the disease. Therefore, preventative treatment can be given before the symptoms of the disease are revealed. To more accurately determine the risk of disease, a combination of several characteristics, eg. determination of the phospholipase activity or a search for characteristic proteins can be specified. This combination of traits may be the most effective marker of pathogenicity among clinically isolated strains.

In order to finally confirm this hypothesis, besides the necessary clinical experiments on animals, a description of the entire proteome of *M. pachydermatis* strains of different origins should also be carried out. Expression analysis of genes coding identified proteins can also be performed by the use of real-time RT-PCR in order to determine the level of expression of marker proteins

in clinical isolates. Summing up these facts, we strongly believe that *M. pachydermatis* consists of both ecotypes, pathogenic and commensal isolates, but still, host-associated factors are important for the development of the infection.

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