Comparison of Cytology and Cultural Examination and Intradermal Test Results in Atopic Dogs with Evidence of *Malassezia Pachydermatis*

Senka Babić[1], Amir Zahirović[2], Šemso Pašić[1]
[1]*Department of Microbiology and Infectious Diseases, Veterinary Faculty, University of Sarajevo, Sarajevo, Bosnia and Herzegovina*
[2]*Department of Internal Diseases, Veterinary Faculty, University of Sarajevo, Sarajevo, Bosnia and Herzegovina*

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Abstract. *The purpose of this study was to determine association between cytological and/or cultural examination in atopic dogs with evidence of Malassezia pachydermatis; association regarding the results of examination and age, sex, breed, onset of symptoms of atopic dermatitis, intradermal test (IDT) results to common allergens and to M. pachydermatis allergen was also analyzed. Thirty-seven atopic dogs with Malassezia evidence were evaluated. Malassezia yeast was detected in 9 of 37 (24.32%) dogs by cytology (Group I); 12 of 37 (32.43%) dogs were culturally positive to M. pachydermatis (Group II), and M. pachydermatis was evidenced by cytology and culture in 16 of 37 (43.24%) dogs ($P > 0.05$). Purebred dogs were in the greater number in Groups II and III. The hypersensitivity to the house dust and house dust allergen group was considered as most common in all three groups of dogs, 8 (88.88%), 12 (100%) and 13 (81.25%), respectively. In Group II and III, the greater number of dogs were with pruritus than without it ($P < 0.05$). In Group I, the greater number of dogs were with a positive IDT to M. pachydermatis allergen and with pruritus (margin of significant difference; $P = 0.056$). Dogs with a positive IDT to M. pachydermatis allergen were in the greater number (7 of 14) positive by culture, while dogs with a negative IDT to M. pachydermatis allergen were in the greater number (11 of 23) positive by cytology and culture; there was no statistically significant difference found. It is important to control presence of Malassezia yeast in dogs with atopic dermatitis to minimize the risk of sensitization to M. pachydermatis allergens, since the low number of yeast cells may cause hypersensitivity reactions in dogs predisposed to development of atopic dermatitis.*

Introduction

Malassezia pachydermatis yeast is considered as part of normal cutaneous microflora of most warm-blooded animals (Guillot and Bond, 1999; Bond et al., 2020; Di Tomaso et al., 2021). Also, this yeast can act as an opportunistic pathogen, whenever alteration of skin surface microclimatic conditions or host defense occurs (Guillot and Bond, 1999; Negre et al., 2009; Oldenhoff et al., 2014; Bond et al., 2020). Some conditions may predispose dogs to *M. pachydermatis* overgrowth; atopic dermatitis (AD) is one of those (Kim et al., 2007; Bond et al., 2020). In hypersensitivity diseases, such as atopy, the proliferation of yeasts is suspected to be promoted by excessive sebum production or disruption of the epidermal barrier (Bond et al., 1996). Analysis of skin swab samples from healthy, naturally affected allergic, and experimentally sensitized atopic dogs by using next generation sequencing (NGS) and quantitative real-time PCR (qPCR) methods has shown that *M. pachydermatis* was more abundant on naturally affected allergic skin (by next generation sequencing-NGS) and on allergen induced skin lesions (by quantitative real-time PCR-qPCR) (Meason-Smith et al., 2019).

A cytology evidence of *Malassezia* overgrowth is a common finding in dogs with AD (Kim et al., 2007), while routine cultures provide primarily qualitative data on presence or absence of yeast (Bond et al., 2020). Using the culture technique has been shown to be more sensitive than both the cytological tape and the dry swab staining method in identifying the presence of *Malassezia* on the skin (Omodo-Eluk et al., 2003). Therefore, in case of negative cytology results, a culture examination of samples should be performed to rule out the suspicion of *Malassezia* infections in animals with otitis or dermatitis (Cafarchia et al., 2005). Furthermore, molecular techniques are pivotal in the accurate identification of many currently recognized *Malassezia* species, with the usual exception of *M. pachydermatis* (Bond et al., 2020).

It is known that the presence of *Malassezia* yeasts on the skin, both in normal and excessive numbers, activates the skin immune system in dogs and cats (Grice and Dawson, 2017). *Malassezia* antigens can stimulate innate, antibody and cell mediated immune responses, as well as trigger hypersensitivity reactions

Correspondence to Senka Babić, Department of Microbiology and Infectious Disease, Veterinary Faculty, University of Sarajevo, Zmaja od Bosne 90, Sarajevo, Bosnia and Herzegovina.
Email: senka.babic@vfs.unsa.ba

(Bond et al., 2010). Furthermore, continuous interactions with the host immune system will maintain low numbers of the yeast without generating a clinically appreciable inflammatory response (Bond et al., 2020; Guillot and Bond, 2020). Thus, the commensal presence of *M. pachydermatis* or cutaneous disease caused by this yeast is not just a consequence of a particular number or density of yeast cells within the stratum corneum, but also involves complex interactions between yeast and host (Bond et al., 2020).

The suggested mechanism for sensitization of atopic dogs to *Malassezia* yeast is epicutaneous contact with allergens produced by yeast which induces atopic cascade (Farver et al., 2005). In dogs with *Malassezia* dermatitis, immunological hyper-responsiveness can be present as none, immediate, delayed and contact (Bond et al., 2020). Serological and intradermal tests are tests for immediate hypersensitivity (Marsella et al., 2012; Bond et al., 2020; Di Tomaso et al., 2021). A greater chance for sensitization is expected in dogs with an increased number of yeasts on the skin (Farver et al., 2005). However, this is not always the rule, as dogs with no evidence of *Malassezia* on the skin can react to an IDT (Farver et al., 2005). Due to the fact that serological and cutaneous test reactivity can occur in some unaffected dogs, these immunological tests must be assessed in the context of clinical and cytological data, and should not be used as individual diagnostic tests (Bond et al., 2020). It should also be mentioned that in dogs with hypersensitivity to *Malassezia* allergens few yeasts may elicit pruritus and associated skin lesions (Hensel et al., 2015).

The purpose of this study was to determine whether there is an association between cytological and/or cultural presence of the yeast and age, sex, breed, onset of symptoms of AD, and intradermal test (IDT) results to common allergens and to *M. pachydermatis* allergen in atopic dogs with *Malassezia* evidence.

Material and methods

Thirty-seven atopic dogs (20 female and 17 male dogs) with cytological or/and cultural evidence of *Malassezia* yeast were included in this study. The average age was 4.3 years (16 dogs up to 3 years and 21 dogs over 3 years of age). Twenty-nine dogs were purebred and eight dogs were crossbreed. The diagnosis of AD was based on history, clinical signs, exclusion of food allergy, ectoparasites and other pruritic diseases, and presence of positive intradermal tests (IDT) (Prelaud et al., 1998; Hensel et al., 2015). The seasonality of the first onset of signs was recorded. Pruritus intensity was assessed by the owners as no pruritus, mild, moderate and severe (Rybnicek et al., 2008). Intradermal tests were performed on all included dogs with 15 allergens (Greer, USA), as well as positive (histamine-0.0275 mg/mL) and negative (diluent) controls according to the manufacturer's instructions. For data analysis, allergens were grouped as follows: 1 – house dust and dust mite; 2 – tree pollen (pine mix; eastern seven tree mix); 3 – grass and weed pollen (plantain-sorrel; seven grass mix; ragweed mix); 4 – fungi (*Trichophyton mentagrophytes*; mold mix); 5 – insects (house fly; flea antigen; *Culicoides*); 6 – epithelia and feathers (cat epithelia allergen; feather allergens) and 7 – *Malassezia pachydermatis*.

For the detection of *Malassezia* yeasts, samples from ear external canals (n = 72) and skin (including legs, paws, back, head, neck, tail, chest, abdomen, hips, groin, axillae, inguinal and genital region) (n = 70) were collected from 37 dogs, using sterile cotton swabs. The skin samples were collected from different areas and in a different number for each dog. All samples were examined by cytology and culture. Gram's stained slide smears were used for cytology examination. Five random fields were examined under an oil immersion objective (x 1000 high power field). Yeast cells were characterized according to their morphology compatible to *Malassezia* yeast. Evaluation of cytology examination was done according to Nascente et al. (2015); absence of yeast cells per field was considered negative, while presence of one and more cells per field was considered positive. Since *M. pachydermatis* is the most common *Malassezia* species isolated from the skin and ear canal in dogs (Matousek and Campbell, 2002; Bond et al., 2020), evaluation of cultural examination was performed based on its growth on *Sabouraud's Dextrose Agar* with *Chloramphenicol*. *M. pachydermatis* was identified by macro- and micro morphology characteristics and ability to hydrolyze *Christensen's* urea medium (Warren and Shadomy, 1991; Guillot and Bond, 1999; Girao et al., 2006; Čonkova et al., 2011). Dogs in which the presence of yeast from at least one sampling site was detected by cytological and/or cultural examination are designated as dogs with evidence of *Malassezia* yeast.

Statistical analysis. Fisher's exact test and χ^2 were used for analysis of correlation between cytological and/or cultural examination results regarding age, sex, breed, onset of symptoms of atopic dermatitis, and intradermal test (IDT) results to common allergens and to *M. pachydermatis* allergen. A probability value of $\leqslant 0.05$ was considered statistically significant.

Results

Among 37 included dogs, the greatest number (29 dogs) was purebred ($P < 0.05$). According to data obtained from the owners, the intensity of pruritus was evaluated as follows: mild in 2 dogs, moderate in 12 dogs, severe in 11 dogs, while 7 dogs showed no signs of pruritus. The owners could not determine intensity of pruritus in 5 dogs. The onset of signs was noted in 18 dogs in summer-spring, in 7 dogs in autumn-winter, and non-seasonally in 12 dogs.

According to cytology and cultural examination results, the dogs were arranged into three groups.

Group I comprised dogs with positive cytology and negative cultural examination to *Malassezia* yeast. Group II consisted of dogs with positive cultural and negative cytology examination to *M. pachydermatis*. Group III included dogs with positive cytology and cultural examination to *M. pachydermatis* (Table 1). Furthermore, Table 1 shows the results of cytological and cultural examination of atopic dogs with evidence of *M. pachydermatis* regarding the compared parameters.

In this study, 9 of 37 (24.32%) dogs had cytological presence of typical *Malassezia* yeast (Group I); *M. pachydermatis* were isolated from 12 of 37 dogs (32.43%) (Group II); and 16 of 37 (43.24%) of the examined dogs were positive both cytological and cultural (Group III) ($P > 0.05$). Among 37 dogs with evidence of *Malassezia* yeast, 14 (37.8%) dogs were IDT positive and 23 (62.2%) were IDT negative to *M. pachydermatis* allergen ($P > 0.05$).

In Group I, the greatest number of dogs had a positive IDT to the house dust and house dust mite group of allergens than to other tested groups of allergens ($P < 0.05$). A greater number of dogs was noted with a negative IDT to *M. pachydermatis* allergen, as well as with pruritus (margin of the significant difference; $P = 0.056$).

In Group II, a greater number of dogs were characterized as follows: dogs were purebred, had spring-summer onset of signs rather than autumn-winter, had a positive IDT to the house dust and house dust mite group of allergens rather than to other tested allergen groups, and to the grass and weed pollen allergen group rather than to epithelia and feather allergen group, had a positive IDT to the grass and weed pollen allergen group and to *M. pachydermatis* allergen than to the epithelia and feather group of allergens, and had pruritus ($P < 0.05$).

In Group III, a greater number of dogs were purebred ($P < 0.05$). The dogs were IDT positive in a greater number to the house dust and house dust mite allergen group than to other tested allergen groups (except to grass and weed pollen and tree pollens allergen group). Also, more dogs were with pruritus than without it ($P < 0.05$).

Age and sex predisposition were not found in any group of dogs ($P > 0.05$). Dogs with a positive IDT to *M. pachydermatis* allergen were in a higher number (7 of 14 dogs) positive by culture, while dogs with a negative IDT to *M. pachydermatis* allergen were positive in a higher number (11 of 23 dogs) by cytology and cultural, but no significant difference was found.

Table 1. Comparison of atopic dogs with evidence of *Malassezia* yeast

Parameter (number of dogs)		Group I (n = 9)	Group II (n = 12)	Group III (n = 16)
Breed	Purebred (n = 29)	4	12	13
	Crossbred (n = 8)	5	0	3
Sex	Male (n = 17)	5	6	6
	Female (n = 20)	4	6	10
Age	Up to 3 years of age (n = 16)	4	4	7
	Over 3 years of age (n = 21)	5	8	9
Seasonality	Spring-summer (n = 18)	4	7	7
	Autumn-winter (n = 7)	3	1	2
	Non-seasonally (n = 12)	2	4	6
Pruritus	With pruritus (n = 30)	7	11	12
	Without pruritus (n = 7)	2	1	4
Intradermal test (IDT)	House dust and house dust mite (n = 32)	8	12	13
	Grass and weed pollen (n = 17)	4	7	6
	Tree pollen (n = 15)	3	6	6
	Fungi (n = 9)	2	2	5
	Insects (n = 11)	1	3	7
	Epithelia and feathers (n = 7)	2	1	4
IDT to *M. pachydermatis*	Positive (n = 14)	2	7	5
	Negative (n = 23)	7	5	11

Group I – positive cytology and negative cultural examination to *Malassezia* yeast;
Group II – positive cultural and negative cytology examination to *M. pachydermatis*;
Group III – positive cytology and cultural examination to *M. pachydermatis*.

Discussion

M. pachydermatis is most frequently isolated *Malassezia* species from healthy dogs and those with disease (Matousek and Campbell, 2002; Cafarchia et al., 2005; Bond et al., 2020). It is a complicating factor in many dermatological diseases (Matousek and Campbell, 2002) which plays an important role in developing AD in dogs (Sihelska et al., 2017). In dogs with clinical signs consistent with *Malassezia* dermatitis, both cytological evaluation and tests for hypersensitivity may be useful (Oldenhoff et al., 2014).

In the present study, in a greater number of dogs, 16 (43.24%), *M. pachydermatis* was detected by both methods, but a significant difference was not found compared with the number of cytology positive (9; 24.32%) and cultural positive (12; 32.43%) dogs with AD. This confirms the importance of cytology and cultural examination for the diagnosis of *Malassezia* infections (Cafarchia et al., 2005). Certainly, it should be remembered that in the case of a negative cytological examination, a cultural examination should be performed to rule out suspicion of infection with *Malassezia* species (Cafarchia et al., 2005). This is consistent with data from the current study, where 12 cytology negative dogs were positive by cultural examination.

Some atopic dogs show immediate reactivity to intradermal injections of *Malassezia* antigens, suggesting that hypersensitivity to yeast antigens may exacerbate the clinical signs in those individuals (Morris et al., 1998; Guillot and Bond, 1999; Bond et al., 2002a). Determinations of immediate type hypersensitivity to *M. pachydermatis* are often made through IDT (Oldenhoff et al., 2014). An intradermal test indirectly measures reactivity of the cutaneous mast cell due to the presence of IgE (Marsella et al., 2012), so intradermal testing with *M. pachydermatis* allergen can be helpful to assess skin immunity to yeast (Bond et al., 2002b). A positive hypersensitivity test to *Malassezia* may lead the clinician to consider *M. pachydermatis* overgrowth and cytological evaluation, if previously neglected, to perform it (Oldenhoff et al., 2014). On the other hand, cytological definitions of *Malassezia* overgrowth may be insufficiently sensitive in defining the number of yeasts required for sensitization (Farver et al., 2005). It should be kept in mind that diagnosis based on the assessment of yeast numbers does not take into account that some yeast could possess unusually potent virulence factors, or hosts could be unusually sensitive to these yeasts; and signs of *Malassezia* dermatitis would likely develop in the presence of low numbers of yeasts in these cases (Negre et al., 2009).

This study shows a lower percentage of IDT positive dogs to *M. pachydermatis* allergen than research by Farver et al. (2005), where 93% of dogs with *Malassezia* dermatitis (based on cytology results) were reactive to *M. pachydermatis*. In our study, totally 14 (37.8%) dogs showed a positive IDT to *M. pachydermatis* allergen. There was no significant difference compared with IDT negative dogs. Among 9 dogs with cytology evidence of the yeast, 2 (22.22%) dogs were IDT positive, and among 12 dogs with cultural evidence of *M. pachydermatis*, 7 (58.33%) were IDT positive to *M. pachydermatis* allergen. Also, our results showed that, among 16 dogs positive by cultural and cytology examination, 5 (31.25%) were IDT positive to *M. pachydermatis* allergen. In the group of cytology positive dogs, on the margin of the significant difference, it was noted that a greater number of dogs were IDT negative to *M. pachydermatis* allergen ($P = 0.056$). Also, our results are not in accordance with the study of Morris et al. (1998), where reactivity was higher in atopic dogs with cytology evidence of *Malassezia* dermatitis compared with those without. In this study, the dogs with positive IDT to *M. pachydermatis* allergen in a greater number (7 of 14 dogs) were positive by culture and negative by cytology; while the dogs with negative IDT to *M. pachydermatis* allergen were positive in a greater number (11 of 23 dogs) by cytology and cultural; there was no significant difference.

Diagnosis of *Malassezia* dermatitis is made on the basis of clinical signs and proliferation of yeast, but the number of *Malassezia* yeast could be insignificant, because some very virulent strains of yeast may be present and/or some sensitive individuals could exhibit signs with low numbers of yeast (Negre et al., 2005). According to Oldenhoff et al. (2014), clinical signs of *Malassezia* dermatitis, the cytology finding of yeast and demonstration of potential *Malassezia* hypersensitivity (by serological or IDT methods) are three separated, but often related concepts, and may or may not occur at the same time in a given case (Oldenhoff et al., 2014). These authors pay attention to the fact that those three concepts are independent elements of disease evaluation and that diagnosis should not rest on any single element (Oldenhoff et al., 2014). Failure to find yeast organisms on cytology does not rule out the possible contribution of yeast to clinical signs, as well as a negative *Malassezia* IgE test does not rule out a pathological role for this organism in an atopic dog (Oldenhoff et al., 2014). We found that 7 dogs IDT positive to *M. pachydermatis* allergen with a negative cytology test were cultural positive. It is in accordance with Farver et al.'s (2005) observation that if cultural examination had been performed instead of the cytological tape analysis, it is possible that malassezia dermatitis negative but IDT positive dogs would have been assigned to the malassezia dermatitis positive group (Farver et al., 2005).

In this study, the absence of a positive IDT test for the *M. pachydermatis* allergen can be explained by the fact that this yeast is part of the normal skin microflora of dogs, because continuous interactions with the host immune system will maintain low numbers of the yeast without generating a clinically appreciable

inflammatory response (Bond et al., 2020; Guillot and Bond 2020); or there may be an insufficient number of yeasts to cause a hypersensitivity reaction (Farver et al., 2005); or due to the small number of dogs included in the analysis. However, the pathological role of the yeast and its possible contribution to clinical signs should not be ruled out. Once again, it is important to remember that allergy tests (both IDT and allergen specific IgE serology) are not recommended as screening tests. They should only be used to confirm the clinical diagnosis of canine AD, and they are useful to identify the offending allergens in order to formulate an allergen-specific immunotherapy (Hensel et al., 2015).

Furthermore, in research by Bond et al. (2002b), only two dogs showed immediate skin test reactivity; on the other hand, nearly all dogs with *Malassezia* dermatitis developed delayed type reactions to *M. pachydermatis*. The subject of our study was not delayed type of hypersensitivity; therefore, it is possible that dogs with malassezia evidence and a negative IDT may have this type hypersensitivity.

In Group II and Group III of dogs, we noted a significantly greater number of purebred dogs. Polysensitization was observed in all included dogs; the most common positive reaction was noted to the house dust and house dust mite allergen group ($P < 0.05$). It is in accordance with previous studies that reported house dust and house dust mite as the most common allergens in dogs with AD (Zur et al., 2002, Di Tomaso et al., 2021), and that purebred dogs are more susceptible to *M. pachydermatis* than crossbred dogs (Marin et al., 2018). We did not find influence of sex and age in all three groups of dogs, which is in accordance with other research (Čonkova et al., 2011; Sihelska et al., 2017).

Certain research suggests an association of seasons and related temperature differences, humidity, and allergy seasons with the development of malassezia infection (Patterson and Frank, 2002; Čonkova et al., 2011). In this study, a seasonal onset of AD signs had no influence on cytology examination, but in a cultural examination it was found that a greater number of dogs had a spring-summer rather than autumn-winter onset of AD signs.

Our results showed that in all groups of dogs there were greater numbers of dogs with pruritus than without it. This is consistent with previous publications that, in animals with overgrowth of yeast or in individuals predisposed to allergic sensitization, the consequent inflammatory response can lead to clinical signs such as dermatitis and pruritus (Bond et al., 2020; Guillot and Bond, 2020). Furthermore, inflammation and pruritus caused by pathogenic mechanisms of *M. pachydermatis* lead to favorable microenvironment for yeast overgrowth (Patterson and Frank, 2002).

Conclusion

The results of this study confirmed that cytology and cultural examination are important for detecting presence of *M. pachydermatis* in dogs with AD. This study demonstrated intradermal reactivity to *M. pachydermatis* allergen in atopic dogs with *Malassezia* evidence, suggesting that hypersensitivity to it should be suspected. It is important to control presence of *Malassezia* yeast in dogs with AD to minimize the risk of sensitization to *M. pachydermatis* allergens, since the low number of yeast cells may cause hypersensitivity reactions in dogs predisposed to AD development. Although not statistically significant, the dogs with a positive IDT to *M. pachydermatis* allergen were positive in a greater number by culture examination; while dogs with a negative IDT to *M. pachydermatis* allergen were positive in a greater number by cytology and cultural examination.

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