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EVALUATION OF CRITERIA FOR DIAGNOSIS OF ATOPIC DERMATITIS AND DETECTION OF ALLERGEN SPECIFIC IgE ANTIBODIES IN DOGS ALLERGIC TO *AMBROSIA ARTEMISIIFOLIA* POLLEN

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Common ragweed (Ambrosia atremisiifolia) is one of the most frequent causes of pollen-induced allergic reactions both in humans and dogs. It has not been defined yet, what is the major allergen(s) to which most dogs allergic to ragweed show a positive result on intradermal skin test (IDST). In the present study sensitization to Ambrosia artemisiifolia pollen allergens in dogs with atopic dermatitis was examined with both in vivo and in vitro tests, including IDST and serum allergen specific IgE test. Detection of specific-IgE antibodies against ragweed allergens by immunoblotting in the sera of allergic dogs was optimized, as well. Dogs that were positive, as judged by IDST reactions to ragweed pollen allergens, also had alergen specific IgE antibodies in their sera. Results indicate that major allergens of A. artemisifolia pollen in dogs are Amb a 1 and Amb a 2. Further characterization of ragweed allergens is needed before they could potentially be used in intradermal testing or allergen immunotherapy in affected dogs. Also, we evaluated new Favrots diagnostic criteria for canine atopic dermatitis in dogs allergic to Ambrosia atremisiifolia pollen. It might be concluded that proposed criteria are of great assistance for seting up suspected diagnosis of canine atopic dermatitis, after ruling out other pruritic dermatoses.

Key words: Ambrosia artemisiifolia, canine atopic dermatitis, immunoglobulin E (IgE), ragweed pollen

INTRODUCTON

Canine atopic dermatitis is one of the most common skin diseases, and it is defined as a genetically predisposed inflammatory and pruritic allergic skin disease with characteristic clinical features associated with IgE antibodies most commonly directed against environmental allergens (Halliwell, 2006). The diagnosis of this condition in dogs is difficult, because none of these typical signs are pathognomonic. It relies upon a careful evaluation of the owner's history,

presence of appropriate clinical signs and exclusion of other resembling or coexisting pruritic skin diseases (De Boer and Hillier, 2001). Recently, new sets of diagnostic criteria for canine atopic dermatitis were proposed (Favrot *et al*., 2010), and one of the aims of this study was to evaluate them in dogs allergic to *Ambrosia atremisiifolia* pollen.

Common ragweed (*Ambrosia artemisiifolia L*.) is the most widespread plant of the genus *Ambrosia* in North America. It was imported to Europe as ballast weed at the beginning of the last century and was initially limited to Hungary. In the 1990s, the plant migrated rapidly into new areas of central and southern Europe (DžAmato *et al*., 2007). Recent phytogeographical studies that analyzed the distribution and abundance of ragweed populations and aerobiological studies that examined the characteristics of airborne ragweed pollen, have shown that Serbia is severely infected by ragweed plants and threatened by ragweed airborne pollen (Janjic *et al*., 2007; Sikoparija *et al*., 2009).

Common ragweed emerges in the late spring and sets seed in late summer or fall. Pollen of this plant is involved in the development of hay fever in humans with the symptoms of rhinitis, conjunctivitis, and/or allergic asthma, an exacerbating factor in atopic dermatitis. The sensitization rate of subjects allergic to ragweed pollen in the European population is quite high and still increasing (Jager, 2000; Asero *et al*., 2006). Data from new pan-European study shows that the prevalence for ragweed sensitization in humans was above 2.5% in all European countries, highest in Hungary (50%), Netherlands (15.2%) and Germany (14.2%) (Burbach *et al*., 2009).

In veterinary medicine, there are little published data concerning the sensitization of dogs to this allergen, but authors agree that ragweed is the most significant weed pollen allergen (Reedy *et al.*, 1997; Scott *et al.*, 2001; Hillier et DeBoer, 2001). Results from Serbia indicate that allergens from *Ambrosia artemisifolia* are of great importance in the etiopathogenesis of atopic dermatitis in dogs and they are the second most common allergen to yield positive intradermal test reactions just after houst dust mites (Milcic Matic *et al*., 2010). Consequently, there is increasing demand for improving diagnostic tools, and treatment of dogs suffering from this allergy. Despite these facts until now there are no studies related to dog allergy induced by *A. artemisiifolia* pollen. Allergens from *A. artemisifolia* pollen, inducing allergic reactions in dogs, have not yet been defined in veterinary medicine. Characterization of these allergens could enable intradermal testing with the specific allergen (or group of allergens), and hence increase the diagnostic value of intradermal tests in terms of increasing its sensitivity and specificity, and minimizing the number of false positive and false negative reactions. This will significantly improve the efficiency and safety of immunotherapy by application of specific allergen to which the dog reacted.

Healthy dogs have a high level of total IgE (1-41 μg/mL of serum), being hundred times higher than in humans (Ledin *et al*., 2006). This difference is caused by the presence of intestinal parasites (Meeusen, 1999), and high IgE level is retained even in the serum of dogs regularly treated with anitiparasite drugs (Pullola *et al*., 2006). There is no significant difference in the level of total IgE in atopic dogs and nonallergic dogs (Halliwell, 1990; Halliwell and Gorman, 1989;

Reedy *et al*., 1997; Scott *et al*., 2001; DeBoer and Hill, 1999; Hill *et al*., 1995). The reason for this is that in atopic dogs there is a very low proportion of allergen-specific out of the total IgE antibodies, which is sufficient to induce allergic reaction, but does not increase significantly total IgE level (DeBoer, 2004). As a result, the detection of very low concentrations of specific IgE antibodies to allergens is very complex and difficult in terms of methodology. Therefore, there are very few publications describing dogs' allergens in general, and there are no data about allergens from *A. artemisifolia* that induce the allergic reaction in dogs. To support diagnostic clinical data in this study we have optimized the detection of specific-IgE antibodies against ragweed allergens by immunoblotting.

MATERIAL AND METHODS

Population study

Dogs with atopic dermatitis (AD) were recruited from the Small Animals' Clinic at the Faculty of Veterinary Medicine in Belgrade, Serbia. Diagnosis was based on a combination of a compatible history and clinical signs, and exclusion of other pruritic dermatoses. Coat brushings, skin scrapings and test therapy were used to eliminate ectoparasites. All dogs underwent a 6-week, commercial hypoallergenic diet trial in order to eliminate possibility of adverse food reactions. Seborrhea, S*taphylococcal* pyoderma and *Malassezia* infection were managed appropriately. No anti-inflammatory medication was given for at least 3 weeks prior to intradermal skin testing.

Intradermal skin test (IDST)

Dogs were sedated with 0.15 mg/kg xylazine IM. Hair was clipped from the lateral flank and 0.05 mL of *Ambrosia artemisiifolia* allergen extract (1000 PNU/mL), 1/100,000 w/v histamine (positive control) and diluent (negative control) were injected intradermally. The diameter of wheals was measured 15 min after the injections. The sizes of the wheals were scored from 0 to 4 compared to the controls. Reactions 2 or greater were considered positive.

Sample collection

Blood samples from dogs with a positive reaction to *A. artemisiifolia* allergen extract (n=50) were collected by jugular venepuncture. Blood samples from healthy dogs (n=10) were collected by cardiac puncture immediately after euthanasia. Serum was separated by centrifugation and stored at -80^{o}C.

Control samples were taken from healthy dogs presented for euthanasia at the University of Veterinary Medicine in Belgrade, with no history and clinical signs of pruritus or conditions likely to alter immune function.

Allergenic extracts

Ambrosia artemisiifolia pollen samples, obtained from the Institute of Virology, vaccines and sera - Torlak (Belgrade, Serbia), were collected in the Belgrade surrounding area. Pollen grains (5 g) were defatted by ethyl ether, suspended in 50 mL of distilled water with 2% polyvinylpoly-pyrrolidone (PVPP)

and shaken at 4°C for 4 h. Suspension was centrifuged at 10 000 × g for 30 min. The supernatant was dialyzed against destilled water (cut off 3 kDa). Protein content in the pollen extracts was determined by the Bradford method (Bradford, 1976).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) and Western blot

SDS PAGE was carried out according to Laemmli using a 14% Tris–glycine polyacrylamide resolving gel and a 4% stacking gel in a discontinuous buffer system. *A. artemisiifolia* extract was mixed with four volumes of either reducing (60 mM Tris–HCl, 2% SDS, 25% glycerol, 0.1% bromophenol blue and 14.4 mM 2-mercaptoethanol) or non-reducing (without 2-mercaptoethanol) sample buffer and heated at 95°C for 5 min. The extract was then loaded into a single broad well with the molecular weight marker alongside and electrophoresis was run using a Hoefer Scientific Instruments apparatus (Amersham Biosciences). Separated proteins were transferred during 1h to nitrocellulose membrane (0.45 mm pore size; Millipore, Billerica, MA, USA) by a semi-dry electro blotter system (Serva, Germany). Finally, the membrane was cut into strips, so that each strip has an identical set of pollen proteins separated by molecular weight.

Immunoblot

The membrane strips with transferred proteins were first blocked with 1% (w/v) solution of bovine serum albumin (BSA; Fluka, Sigma–Aldrich) in Tris-buffered saline (pH 7.4) with 0.1% Tween-20 (TTBS) for 1h at 37°C before being probed with the dog sera samples. After blocking the membrane, strips were incubated with diluted sera during 12h at 4°C. After membrane washing with TTBS bound IgE was detected using biotinylated anti-canine IgE antibody (Sigma-Aldrich, Germany) diluted with 0.1% BSA followed by streptavidin labeled alkaline phosphatase (streptavidin – AP) diluted with 0.1% BSA. Visualisation of IgE binding was carried out with a substrate solution consisting of 1.5 mg BCIP (5-bromo-4-chloro- 3-indolyl phosphate; Sigma–Aldrich) and 3 mg NBT (nitroblue tetrazolium; Sigma-Aldrich) in 10 mL of 100 mM Tris buffer, containing 150 mM NaCl, and 5 mM $MgCl_2$, pH 9.6.

RESULTS

One of the goals of this study was to determine the validity of Favrot's diagnostic criteria. The study included 50 dogs, that had a positive reaction on *Ambrosia artemisiifolia* allergen extract on intradermal skin test and manifested clinical symptoms of allergic dermatitis. Based on the proposed Favrot's diagnostic criteria for canine atopic dermatitis, it was necessary to define the onset of clinical signs, what was the first symptom that appeared and was pruritus responsive to glucocorticosteroid treatment. Also, it was necessary to record how many hours dogs spend at home. During the physical examination, special attention was directed towards the four skin sites, including: front feet, ear pinnae,

ear margins and the dorso-lumbar area. Results of our and Favrot's study are presented in Table 1.

Table1. Frequency of clinical symptoms occurrence in canine atopic dermatitis: comparative review with Favrot's results

Diagnostic criteria	Our results	Favrot's results
Age at onset <3 years	88%	78.8%
Mostly indoor	96%	84%
Corticosteroid-responsive pruritus	84%	78%
Pruritus *sine materia* at onset	78%	61%
Affected front feet	80%	79%
Affected ear pinnae	56%	58%
Non-affected ear margins	96%	92%

Dogs included in Favrot's research had chronic pruritus (more than 2 months) or recurrent (more than two episodes) pruritus. It was suggested that patients should have at least 5 out of 8 of the proposed criteria, to be set by suspected diagnosis of atopic dermatitis. Ninety percent (90%) of the dogs included in our examination had five or more of Favrot's proposed criteria.

Optimization of methodology to detect IgE binding proteins on Ambrosia artemisiifolia immunoblots

In attempt to detect allergen-specific IgE antibodies, comprising only a very small proportion of total serum IgE antibodies, immunoblot with pool of sera from dogs allergic and nonallergic to *A. artemisifolia* pollen was performed. Also, in order to examine non-specific binding of anti-canine IgE antibodies, as well as streptavidin-AP, to *A. artemisifolia* proteins, strips were incubated with 1% BSA instead of dog's sera. Immunoblot optimization was performed by using different concentrations of anti-canine IgE antibodies and streptavidin-AP.

In Figure 1, intensive binding of IgE from the sera of allergic, as well as non-allergic dogs can be observed. However, bands are noticeable also on the strips incubated without serum (Figure 1, strips 9 and 10). It appears that anti-canine IgE antibodies or streptavidin-AP binds nonspecifically on transferred ragweed pollen proteins. Streptavidin binds to biotin (biotinylated anti-canine IgE antibodies) in 1:4 ratio. If anti-canine IgE antibodies binds nonspecifically, bands should be less visible (Figure 1, strips 5-8 compared to 1-4) with dilution (no matter the concentration of streptavidin). However, bands intensity on the set of samples with a dilution of 1:500 (Figure 1, strips 1-4) are almost identical to the band intensity on the set of samples with a dilution of 1:1000 (Figure 1, strips 5-8). To minimize non-specific binding we used 1:1000 dilutions of anti-canine IgE antibodies in the further work. On the other hand, dilution of streptavidin resulted in increasingly less visible bands, indicating that the concentration of streptavidin dictates the intensity of the color, with or without serum, supporting the

assumption of direct binding of streptavidin to the ragweed pollen proteins. In order to minimize nonspecific binding of streptavidin we have used higher dilution of streptavidin (1:100,000).

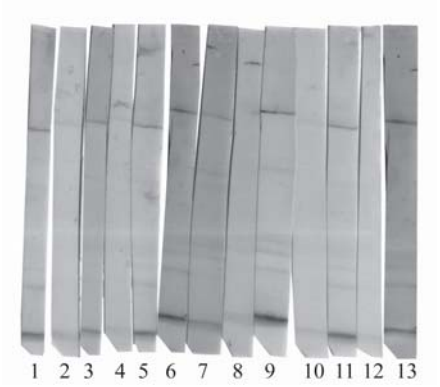

Figure 1. IgE immunoblot of dogžs sera nonallergic and allergic to *A. Artemisiifolia* pollen (BSA as blocking agent). 1-8, 13- pooled sera of allergic dogs (1:10 dilution); 11, 12 – pooled sera of non-allergic dogs (1:10 dilution); 9,10 - 0.1% BSA. Different dilutions of anti-canine IgE: 1-4, 9, 11 (1:500); 5-8, 10, 12, 13 (1:1000). Different dilutions of streptavidin-AP: 1, 5, 9, 11 (1:20.000); 2, 6 (1:50.000); 3, 7 (1:100.000); 4, 8, 10, 12, 13 (1: 200.000)

In the next set of analyses we attempted to solve the problem of nonspecific binding of protein by using skimmed milk proteins, instead of BSA, as a blocking agent at a concentration of 5%, and as an antibody stabilizer at a concentration of 0.5%. Each pair of antigen-antibody has unique properties and there is no ideal

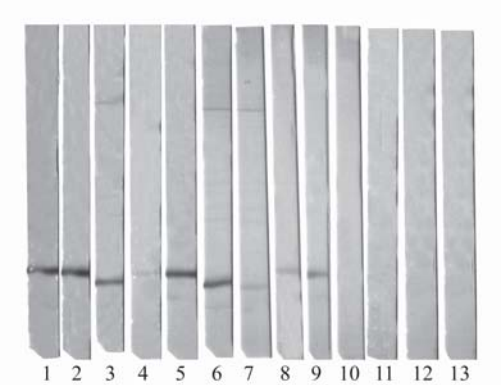

Figure 2. IgE Immunoblot of dog's sera nonallergic and allergic to *A. artemisiifolia* pollen (milk proteins as blocking agent). 1,3,5,7,9,12, 13- pooled sera of allergic dogs; 2,4,6,8,10- pooled sera of non-allergic dogs; 11- 0.5% milk proteins. Different dilutions of dogs sera: 1,2 (1:10); 3,4 (1:20); 5,6 (1:40); 7,8 (1:80); 9,10 (1:160). Different dilutions of anti-canine IgE: 1-11, 13 (1:1000), 12- 0.5% milk proteins. Different dilutions of streptavidin-AP: 1-12 (1:100.000); 13- 0.5% milk proteins

blocking agent. The aim was to find the best blocking agent giving a maximum ratio of signal/background and achieving maximum ratio of specific/non-specific binding. We tested also different dilutions of sera, in order to obtain an optimum, at which there is minimal nonspecific binding with sufficient sensitivity. Also, we included a sample without the addition of anti-canine IgE antibodies to determine whether there is non-specific binding of sterptavidin to IgE antibodies.

With milk as a blocking/stabilizing agent, serum IgE from both allergic and non-allergic dogs recognizes ragweed protein (Figure 2). Low serum dilution leads to nonspecific binding of IgE antibodies, which has low affinity, but is present in high concentrations. When the dog serum is diluted sufficiently, non-specific IgE cannot be detected because of the low affinity, but specific IgE can be detected due to high affinity (Figure 2, strip 9 compared to 10). Immunoblot results show that skimmed milk as the blocking agent and a dilution of streptavidin-AP 1:100,000 eliminates its non-specific binding, and that milk proteins and anti-canine IgE dilution of 1:1,000 eliminate non-specific binding of anti-canine IgE. Although we have improved the specificity of the test, its sensitivity was still low, as only one reactive band was observed in the tested pooled serum sample. Thereby we have tested separation of pollen proteins in different conditions and using different blocking agent for probing.

In response to allergens, immune system produces IgE antibodies against sequential and conformational epitopes of allergens. After SDS PAGE in reducing conditions, fully unfolded proteins are transferred to a membrane where they can partially refold. However they do not refold to the native conformation due to cleavage of disulfide bridges, by which most proteins are directed to refold in native conformation. Thus, SDS PAGE in reducing conditions abolished conformational epitopes, leaving mostly the sequential epitopes that IgE antibodies can bind to. In previous analyzes allergen-specific IgE antibodies were not detected, because of difficulties with nonspecific binding, which often occurs when dealing with dogs sera. We assumed that we could detect specific IgE

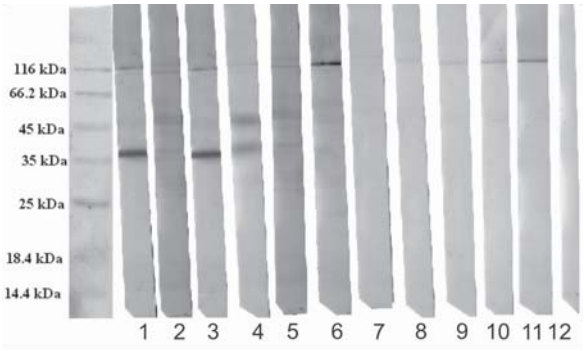

Figure 3. IgE immunoblot of individual sera of dogs nonallergic and allergic to *A. Artemisiifolia* pollen (reducing conditions). 1-6 - individual sera of allergic dogs (1:2 dilution); 7-9 - individual sera of non-allergic dogs (1:2); 10 - 0.1% gelatin. Anti-canine IgE: 1-10, 12 (1:1000 dilution), 11- 0.1% gelatin. Streptavidin-AP: 1-11 (1:100.000 dilution); 12- 0.1% gelatin

antibodies to conformational epitopes, which are probably dominant in dogs and present in higher concentrations. Therefore, pollen proteins were separated by SDS PAGE in non-reducing conditions. To gain insight into individual differences in the recognition of allergens, individual sera were tested instead of pooled sera. In order to further improve elimination of non-specific binding, in the next step a gelatin was used as the blocking/stabilizing agent instead of milk protein. The advantage of gelatin is that it effectively blocks the membrane surface, although it can mask the surface of bounded proteins and thereby can reduce the sensitivity of the essay. Therefore, we used low dilutions of individual sera (1:2), assuming that we would get less nonspecific binding of IgE antibodies, due to reduced availability of complete pollen protein sequence to non-specific IgE antibodies.

The results shown in Figure 3 confirm that the non-reducing conditions almost completely eliminate non-specific IgE binding (Figure 3, strips 7-12). However, it may be noted, that all sera from allergic dogs, more or less extensively, recognize the dominant band with a molecular weight of approximately 35-40 kDa and a band of 45-50 kDa, although certain allergens were not recognized in all of the tested patients.

DISCUSSION

Validity of Favrot's diagnostic criteria

The International Task Force on Canine Atopic Dermatitis supports the recommendation of using these diagnostic criteria in two situations: as a help for veterinarians general practitioners, in setting up a suspected diagnosis and for increasing the homogeneity of future clinical studies (Olivry, 2010).

The majority of examined dogs (88%) had the first clinical symptoms until the age of 3 years. These findings are entirely consistent with previously published data (Scott *et al*., 2001; Nødtvedt *et al*., 2006; Favrot *et al*., 2010). Clinical symptoms during the first year of life are usually very weak, the owner usually does not notice them and they do not require any treatment. The appearance of the first symptoms of the disease in very young dogs, up to the age of 6 months is mainly observed when both parents had symptoms of atopy and breed in the area with a high concentration of allergens (Scott *et al*., 2001). After three years of age, the onset of symptoms of this disease is less common; usually in dogs that change the living environment (Scott *et al*., 2001; Reedy *et al*., 1997). However, food allergy often occurs in the very young and elderly dogs (Chesney, 2002; Verlinden *et al*., 2006), which can be used for differential diagnostic purposes.

In 78% of dogs pruritus was the first sign that the owners noticed. As already mentioned, the majority of dogs with atopic dermatitis have no visible primary lesions, while in some cases erythema may occur (Scott *et al*., 2001). Most authors report that in the initial stage of the disease, pruritus is not intensive and appears in the region of head and mouth (Nødtvedt *et al*., 2006; Chanthick *et al*., 2008), which is consistent with our results. In most cases, the owner does not pay attention to those first signs of scratching and often forgets to mention them. Occurrence of pruritus *sine materia*, besides in atopic dermatitis occurs in other

allergic dermatoses, and in numerous ectoparasitic infestations, and therefore is not of great importance in differential diagnostic purposes.

The usual treatment prescribed by veterinarians for reducing scratching is topical and systemic application of glucocorticosteroids. Their anti-inflammatory effect is based on the fact that they change macrophage function (phagocytosis and reduced response to cytokines), lymphocytes (decreased proliferation and antigen processing), reduce vascular permeability, reduce the release of vasoactive amines, inhibit the synthesis of prostaglandins and leukotrienes, reduce the concentration of complement levels and stabilize the cell plasma membrane (Scott, 1995, Scott *et al*., 2001; Adcock and Ito, 2000). Positive effects of these drugs in reducing pruritus and skin lesions were noted in 50% to 80% of patients (Reme *et al*., 2005; Steffan *et al*., 2003; Olivry *et al*., 2002). It is believed that the poor response to therapy is a result of most commonly present secondary staphylococcal or malassezia infections of the skin (Hillier, 2002). In our trial favorable effects of glucocorticosteroids were recorded in 84% of dogs. These dogs generally received prednisolone orally, and the initial daily dose was 0.5-1.0 mg/kg body weight. Glucocorticosteroids also show good effects in other allergic skin conditions. However, in nonallergic pruritic dermatoses, especially in the infestation of the skin with parasitic mites, their effect is weaker and does not lead to symptoms withdrawal. Failure to respond to systemic corticosteroid therapy in an adequate dose, in most cases excludes atopic dermatitis as a cause of scratching.

Changes on the front feet were observed in 80% of dogs. Licking of the paws and consequent changes on the feet are considered characteristic for atopic dermatitis, and there is a hypothesis that feet are predilection sites for percutaneous absorption of airborne allergens (Chalmers and Medleau 1994; Frank and McEntee, 1995). Also, the highest numbers of mast cells are present in the interdigital skin and in the ear lobe (9.2 to 11.3). Some authors believe that this is the reason why pedal and pinnal pruritus is often present in canine atopic dermatitis (Auxilia and Hill, 2000). Changes on the feet in the form of erythema, reddish discoloration of hair, papules and pustules, alopecia and edema of the skin, were the result of licking and biting the paws skin.

Erythema and reddish discoloration of the hair was mostly observed in West Highland white terriers and golden retrievers. This change of hair color is a result of saliva porphyrin (Griffin, 1996). Bacterial pododermatitis in the form of papules and pustules was noted in a high percentage in Bulldog dogs. These symptoms usually occur in dogs with chronic disease (symptoms present for more than a year), and the changes were more intense in the wet period of the year (spring and autumn). As stated in the literature, recurrent staphylococcal skin infections often occur in dogs with atopic dermatitis (DeBoer and Marsella, 2001). The skin of these dogs has a larger number of bacteria in relation to the skin of healthy dogs (increased colonization) and they are mainly found in the superficial layers of the epidermis (Mason and Lloyd, 1989). Adherence of *Staphylococcus pseudointermedius* to keratinocytes of atopic dogs, is also increased, both in inflamed and non-inflamed skin (McEwan, 2000, 2003). Besides infection, cocci can cause an allergic reaction. Protein A (cell wall antigen that is found on many

strains of *Staphylococcus pseudointermedius*) may non-specifically bind to IgE on the mast cells and provoke their degranulation (Mason and Lloyd, 1989). This increases the permeability of the epidermis for staphylococcal antigens and leads to further degranulation of the mast cells.

More than a half of the dogs (56%) had skin lesions on the ear pinnae in the form of erythema, hyperpigmentation, lichenification and alopecia. This clinical symptom is considered as one of the most characteristic for atopic dermatitis and occurs in a similar percentage as in Favrotžs examination (Favrot *et al*., 2010). Besides inflammatory response, changes on the skin of ear pinnae result from self trauma in terms of frequent scratching and rubbing this area.

Changes at the ear margins were observed in 2 dogs (4%). Flakes and crusts on the ear margins with intense pruritus are characteristic of infestation with *Sarcoptes scabiei var.canis*. Atopic dermatitis and scabies in dogs in many cases shows same clinical symptoms, intense pruritus is usually only a primary symptom and it is difficult to differentiate. For this reason, changes at the top of the ear pinnae are used for differential diagnostic purposes in order to exclude mange infestation.

Chewing or scratching the lower part of the back is one of the main symptoms of allergy to flea saliva. In these dogs, secondary changes occur in the form of alopecia, erythema, hyperpigmentation, and in chronic cases bacterial infections and lichenification are frequent. In our study changes in the dorso-lumbar part of the body were observed in 16% of dogs that is a slightly lower percentage compared to Favrot's criteria.

As mentioned before, diagnosis of atopic dermatitis is one of the greatest challenges in human and veterinary medicine. There is no pathognomonic symptom, test, laboratory procedure, or response to therapy, as well as histopathological examination of skin specimens that can confirm the diagnosis of atopic dermatitis. For this reason, intensive work has been done in establishing certain diagnostic principles in order facilitate the diagnosis of this disease and uniformity of next clinical studies. International Task Force of Canine Atopic Dermatitis, which consists of leading experts in the field of veterinary allergology, recommended the use of Favrot's diagnostic criteria (Olivry, 2010).

As can be seen in Table 1, we noticed slightly higher percentage of positive criteria compared to Favrot's study. In our research a high number of participating dogs spends most of their time indoors. The owners of these dogs can give more precise answers to the questions from the list of medical history, and observe changes in the skin at an early stage. Also, our attention was focused only on 8 parameters defined by Favrot's. Medical history and physical examination were conducted only by 2 veterinary dermatologists, unlike Favrot's, where 34 veterinary dermatologists from 15 different countries took part.

By definition, the "specificity test" is the number of non-hypersensitive individuals that will be identified as negative, and the "sensitivity" refers to the number of hypersensitive individuals who will have a positive test reaction (DeBoer and Hillier, 2001). According to the authors, Favrot's criteria have a sensitivity of 85% and a specificity of 79%. In our research, negative controls were not included, so we were not able to determine specifity, just the sensitivity of this

test, and it was 90%. Therefore, it can be concluded that the proposed Favrot's criteria are of great assistance to setup suspect diagnosis of canine atopic dermatitis. This set of criteria is not intended to replace a complete clinical examination, it is important to exclude ectoparasites, bacterial and fungal infections, and to perform an elimination diet trial test.

Immunochemical analysis Ambrosia atremisiifolia allergens in dogs

For valid diagnosis and efficient therapy it is necessary to characterize components of *A. atremisiifolia* pollen in terms of defining major and minor allergens inducing clinically manifested allergic reaction in dogs. Regarding difficulties in the detection of allergen specific IgE antibodies our aim was to optimize conditions for their detection in immunoblot. Chen and colleagues (Chen *et al*., 2002) have tested several systems for detection of IgE antibodies in dogs specific for *Malassezia pachydermatis*. They noticed that visualization by chromogen substrates (diaminobenzidine, BCIP/NBT) has low sensitivity, while only hemiluminescence has satisfactory sensitivity. These authors pointed at several difficulties with cross-reactivity observing non-specific binding of goat and bovine anti - canine IgG, as well as binding of streptavidin-peroxydase to components of *M. pachydermatis* extract. Due to low sensitivity of immunoassays with chromogen substrates, in the most of studies dealing with allergens in dogs ELISA assays were performed with fluorescent substrates (Nuttall *et al*., 2001; Sakaguchi *et al*., 2001; Hou *et al*., 2005). In this study, detection was based on chromogen substrate and streptavidin, in order to find out conditions for the assay that will have sufficient sensitivity and minimal non-specific binding.

In the process of immunoblot optimization three blocking agents were tested, 1% BSA, 5% milk proteins and 1% gelatin. Gelatin exereted the best effect due to efficient membrane blocking, which resulted in minimizing non-specific binding. By using different anti-canine IgE dilutions we demonstrated that there was no non-specific binding of this antibody to pollen proteins. However, non-specific binding of streptavidin-AP was observed. In fact, streptavidin poses the tripeptide sequence Arg-Tyr-Asp (RYD) mimicking Arg-Gly-Asp (RGD)-binding sequence of fibronectin, thus leading to non-specific binding difficulties in many assays based on streptavidin (Alon *et al*., 1990). Our hypothesis was that by RYD sequence streptavidin binds to short sequence in pollen proteins which is similar to the intergin sequence. Proving this hypothesis in further studies would contribute to the explanation of mechanisms of allergen uptake: binding of integrin-like sequence in pollen proteins to RGD sequence of fibronectin would improve retaining of allergen in respiratory pathways. By streptavidin-AP dilution of 1:100, 000 its non-specific binding was completely eliminated. Non-specific binding of IgE was minimized by choosing of gelatin as blocking agent, by Western blot of proteins separated under reducing conditions, as well as by high dilution of anti-canine IgE and streptavidin-AP. Assay sensitivity was improved by low sera dilution (1:2).

In this study pooled sera from allergic dogs, as well as individual sera, were examined. Although individual differences in the recognition of pollen allergens were noticed, similar to human population, there are common allergens

recognized by most dog sera with molecular mass around 40 kDa. Amb a 1 is the most aboudant protein of *A. atremisifolia* pollen comprising 6% of total pollen proteins. This protein with molecular mass of 38 kDa reacts with 97% of sera from allergic human patients (Wopfner *et al*., 2005; Oberhuber *et al*., 2008). Amb a 2 is a protein of 38 kDa with 70-80% sequence homology with Amb a 2, and it is recognized by sera of 88 % allergic patients (Wopfner *et al*., 2005). The results of this study indicate that, as within the human population, major allergens of *A. artemisifolia* in dogs are most probabaly Amb a 1 and Amb a 2.

In conclusion, this study confirmed that Favrots criteria can be used as a screening test, following exclusion of ectoparasites, bacterial and fungal infections and elimination diet trial test. The dogs showed positive IDST reactions to *A. Artemisifolia* pollen allergens and demonstrated specific IgE to major allergens Amb a 1 and Amb a 2. However, further characterization of these allergens is needed before they could potentially be used in intradermal testing or allergen immunotherapy in affected dogs.

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EVALUACIJA KRITERIJUMA ZA DIJAGNOZU ATOPIJSKOG DERMATITISA I DETEKCIJA ALERGEN SPECIFIČNIH IgE ANTITELA KOD PASA ALERGIČNIH NA POLEN BILJKE *AMBROSIA ARTEMISIIFOLIA*

MILČIĆ-MATIĆ NATALIJA, OGNJENOVIĆ JANA, BURAZER LIDIJA, BLAGOJEVIĆ G, POPOVIĆ N, LAZAREVIĆ M i STANIĆ-VUČINIĆ DRAGANA

SADRŽAJ

Kratka ambrozija (*Ambrosia artemisiifolia*) je jedan od najčešćih uzročnika alergijskih reakcija izazvanih polenom kod ljudi i pasa. Još uvek nije definisano koji je glavni alergen (i), na koji, većina pasa alergičnih na polen ambrozije, ispoljava pozitivnu reakciju na intradermalnom testu. U ovoj studiji je ispitana senzibilizacija na polen ove biljke kod pasa sa simptomima atopijskog dermatitisa *in vivo* i

in vitro testovima, uključujući intradermalni test i dokazivanje prisustva alergen specifičnih IgE antitela u serumu. Optimizovani su uslovi za detekciju IgE specifičnih antitela iz seruma pasa alergičnih na polen ambrozije imunoblot tehnikom. Psi koji su imali pozitivnu reakciju na polen ove biljke na intradermalnom testu, takođe su imali specifična IgE antitela u serumu. Dobijeni rezultati ukazuju da su glavni alergeni *Ambrosia artemisiifolia* kod pasa Amb a 1 i Amb a 2. Neophodna je dalja karakterizacija alergena ambrozije kako bi se oni mogli primeniti pri rutinskom intradermalnom testiranju ili u alergen specifičnoj imunoterapiji obolelih pasa. Takođe je razmatrana i validnost Favrotovih dijagnostičkih kriterijuma kod pasa alergičnih na polen ambrozije. Može se zaključiti da su predloženi kriterijumi od velike pomoći u postavljanju suspektne dijagnoze atopijskog dermatitisa pasa, nakon isključenja drugih pruritičnih dermatoza.