Serological identification of house dust mite allergens in dogs with atopic dermatitis[1]

Victor E.S. Cunha[2]*, Maria Helena Silva[3] and João Luiz H. Faccini[2]

ABSTRACT.- Cunha V.E.S., Silva M.H. & Faccini J.L.H. 2012. **Serological identification of house dust mite allergens in dogs with atopic dermatitis.** *Pesquisa Veterinária Brasileira 32(9):917-921.* Departamento de Parasitologia Veterinária, Universidade Federal Rural do Rio de Janeiro, BR 465 Km 7, Seropédica, RJ 23890-000, Brazil. E-mail: mv.victor@uol.com.br

House dust mite antigens have been used for decades to diagnose allergic diseases in humans and animals. The objective of this study was to identify allergens in commercial *Dermatophagoides farinae* and *Blomia tropicalis* extracts by immunoblotting using sera from allergic dogs and anti-dog IgE conjugate. The analysis of antigens present in the *D. farinae* extract (FDA Allergenic) using sera from 10 dogs allergic to *D. farinae* showed that eight sera recognized a band of approximately 102 kDa, eight recognized two bands of 52 to 76 kDa, five recognized one band of approximately 76 kDa, four recognized one band of 31 to 38 kDa, and two recognized one band of 12 to 17 kDa. Immunoblot assays of the *B. tropicalis* extract (FDA Allergenic) using sera from 10 animals allergic to *B. tropicalis* showed that five sera recognized two bands of 52 to 76 kDa. These results demonstrate the importance of the two house dust mite species for the pathogenesis of canine atopic dermatitis in Brazil. In addition, the results indicate which allergens should be present in allergenic extracts used for diagnosis and allergen-specific immunotherapy.

INDEX TERMS: Atopic dermatitis, allergens, dogs, *Dermatophagoides farinae*, *Blomia tropicalis*.

RESUMO.- [**Identificação sorológica de alérgenos de ácaros da poeira domiciliar em cães com dermatite atópica.**] Antígenos de ácaros da poeira domiciliar são utilizados por décadas para diagnóstico de doenças alérgicas em seres humanos e animais. O objetivo do presente trabalho foi identificar proteínas alergênicas presentes em extratos de *Dermatophagoides farinae* e *Blomia tropicalis* através de "immunoblotting" utilizando-se soros de animais alérgicos e conjugado anti-IgE canina. A análise por "immunoblotting" dos antígenos presentes no extrato de *D. farinae* (FDA Allergenic), utilizando soros de dez animais alérgicos, mostrou que oito soros reconhecem uma banda com peso molecular de aproximadamente 102 kDa; oito soros duas bandas entre 52 e 76 kDa; cinco soros uma banda com aproximadamente 76 kDa; quatro soros uma banda entre 31 e 38 kDa; e dois soros uma banda entre 12 e 17 kDa. A análise por "immunoblotting" dos antígenos do extrato de *B. tropicalis* (FDA Allergenic) mostrou que cinco soros reconhecem duas bandas com pesos moleculares entre 52 e 76 kDa. Esses resultados demonstram a importância dessas duas espécies de ácaros da poeira domiciliar na patogênese da dermatite atópica canina no Brasil, assim como indicam alérgenos que devem estar presentes nos extratos alergênicos utilizados para diagnóstico e imunoterapia alérgeno-específica.

TERMOS DE INDEXAÇÃO: Dermatite atópica, alérgenos, cães, *Dermatophagoides farinae*, *Blomia tropicalis*.

INTRODUCTION

Canine atopic dermatitis (CAD) is a common inflammatory pruriginous dermatitis, which is characterized by the excessive production of specific IgE immunoglobulins against environmental allergens (Halliwell 2006). The main environmental allergens involved in the pathogenesis of CAD are house dust and storage mite antigens; pollens from grasses, trees and weeds; mould spores; epidermal antigens, and insect antigens (Hill & DeBoer 2001). House dust mites, particularly *Dermatophagoides farinae* (DEF) and *D.*

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[2] Departamento de Parasitologia Veterinária, Universidade Federal Rural do Rio de Janeiro (UFRRJ), BR 465 Km 7, Seropédica, RJ 23890-000, Brazil. *Corresponding author: mv.victor@uol.com.br

[3] Instituto de Microbiologia, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro (UFRJ), Cidade Universitária, Ilha do Fundão, Rio de Janeiro, RJ 21944-970, Brazil.

pteronyssinus (DEP) (family Pyroglyphidae), are the main allergens responsible for immediate hypersensitivity in dogs, affecting 30 to 100% of dogs with CAD (Randall et al. 2003). Mites of the family Glycyphagidae, particularly *Blomia tropicalis* (BLO), are frequently found in homes of tropical and subtropical areas and are often the predominant house dust mite species (Binotti et al. 2001). Some antigens of this species are recognized as an important cause of allergic reactions in humans, but their role in CAD is still unknown.

Allergens are designated according to the accepted taxonomic name of their source as follows: the first three letters of the genus, a space, the first letter of the species name, a space, and an Arabic number. The numbers are attributed in the order of identification of the allergen and the same number is generally used for homologous allergens of related species (King et al. 1995). For example, Der f 1 and Der p 1 belong to group 1 allergens of DEF and DEP, respectively.

Table 1. Major mite allergens identified in humans and deposited in the database of the International Union of Immunological Societies (IUIS, www.allergen.org)

Mite species	Allergen	Molecular weight (Da)	Identity
Blomia tropicalis	Blo t 1	25,126	Cysteine peptidase
	Blo t 2	13,528	Lipid-binding protein
	Blo t 3	23,824	Trypsin
	Blo t 4	55,055	Alpha-amylase
	Blo t 5	13,497	Structural protein
	Blo t 6	24,743	Chymotrypsin
	Blo t 8	27,630	Glutathione S-transferase
	Blo t 9	23,598	Collagenase
	Blo t 10	33,003	Tropomyosin
	Blo t 11	102,028	Paramyosin
	Blo t 12	14,337	Chitin-binding protein
	Blo t 13	14,8	Fatty acid-binding protein
	Blo t 14	39,353	Vitellogenin
	Blo t 18	49,231	Chitinase
	Blo t 19	7,226	Antimicrobial peptide
	Blo t 21	14,944	Structural protein
Dermatophagoides	Der p 1	25,394	Cysteine peptidase
	Der f 1	25,148	Cysteine peptidase
	Der p 2	14,121	Lipid-binding protein
	Der f 2	14,081	Lipid-binding protein
	Der p 3	24,987	Trypsin
	Der f 3	24,954	Trypsin
	Der p 4	57,15	Alpha-amylase
	Der p 5	13,587	Structural protein
	Der f 5	13,614	Structural protein
	Der p 6	24,885	Chymotrypsin
	Der f 6	25,034	Chymotrypsin
	Der p 7	22,179	Unknown function
	Der f 7	21,863	Unknown function
	Der p 8	25,668	Glutathione S-transferase
	Der p 9	23,756	Collagenase
	Der p 10	32,901	Tropomyosin
	Der f 10	32,955	Tropomyosin
	Der p 11	102,417	Paramyosin
	Der f 11	81,372	Paramyosin
	Der f 13	14,980	Fatty acid-binding protein
	Der f 14	39,668	Vitellogenin
	Der p 15	61,413	Chitinase
	Der f 15	61,111	Chitinase
	Der f 16	55,131	Gelsolin
	Der p 18	49,227	Chitinase
	Der f 18	49,468	Chitinase
	Der p 20	40,477	Arginine kinase

By convention, "major" allergens are defined as allergens that are recognized by more than 50% of sera from atopic patients, whereas "minor" allergens are those recognized by less than 50% of patient sera (Thomas et al. 1998). Table 1 lists the major allergens of DEF, DEP and BLO so far identified in humans and deposited in the database of the Allergen Nomenclature Subcommittee (www.allergen.org) that operates under the auspices of the International Union of Immunological Societies (IUIS) and World Health Organization (WHO).

Using immunoassays, some DEF and DEP allergens have been identified in sera of dogs with CAD (Noli et al. 1996, McCall et al. 2001, Weber et al. 2003) (Table 2). No such studies have so far been conducted in Brazil and since the pattern of antigen recognition may vary between dog populations due to genetic and environmental differences, the identification of the antigen recognition pattern in Brazilian dogs is of major importance. Since the major allergens described for humans may differ from those of dogs (McCall et al. 2001), the objective of the present study was to identify important allergens in dogs by immunoenzymatic assays using commercial DEF and BLO allergenic extracts.

Table 2. Mite allergens identified in dogs

Mite species	Allergen	Molecular weight (kDa)	Identity
Dermatophagoides	Der p 1 Der f 1	25	Cysteine proteinase
	Der p 2 Der f 2	14-20	Epididymal protein
	Der f 10	37	Tropomyosin
	Der f 15	98	Chitinase
	Der f 18	60	Chitinase

Major allergens are highlighted in bold.

MATERIALS AND METHODS

The presence of IgE antibodies in sera of allergic animals and controls was evaluated by immunoblotting using DEF and BLO allergenic extracts provided by FDA Allergenic Ltda.

Dog sera

Blood samples were collected by venipuncture (cephalic or jugular vein) from 26 dogs with CAD and from five dogs without clinical signs of the disease. After collection, the blood samples were kept at room temperature for 30 min to allow clotting. Next, serum was separated by centrifugation in an analytical centrifuge at 3000 rpm. The sera were divided into 500-µL aliquots and stored at -20ºC until the time for use.

The diagnosis of CAD was made according to the criteria established by Willense (1986) and modified by Prélaud et al. (1998). In addition, all dogs with CAD participating in the study had a positive skin test to the DEF (FDA Allergenic) and/or BLO (FDA Allergenic) extract. The accuracy of the skin tests performed with these allergenic extracts has been demonstrated by Cunha et al (2007).

Only 10 of the 26 sera from dogs with CAD presenting high allergen-specific IgE titers (optical density: 0.436 to 1.919) measured by enzyme-linked immunosorbent assay (ELISA) were used for immunoblotting.

Allergenic extracts for in vitro assays

The DEF (batch 09PF01276) and BLO (batch 10BB00055) allergenic extracts used for immunoblotting were produced and provided by FDA Allergenic Ltda. The extracts were filtered and concentrated through an Amicon YM 10 centrifugal filter device (Millipore) equipped with a nitrocellulose membrane that separates proteins with a molecular weight > 10 kDa. Next, protein concentration was determined by the method of Bradford (1976), with slight modifications, using bovine serum albumin (Sigma Chemical Co., USA) as standard.

The extracts were submitted to electrophoresis on polyacrylamide gel under denaturing conditions (SDS-PAGE) for the analysis of their protein composition (Laemmli 1970). Briefly, stacking and separating gels containing 4% and 12% polyacrylamide, respectively, were prepared. Next, the gels were mounted in vertical units and samples of the extracts were prepared in sample buffer containing 2% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol. The samples were boiled for 10 min and then applied to the stacking gel (10 µg protein/well). For the determination of molecular weights, 10 µl of a protein standard (Precision Plus Protein Unstained Standards, Bio-Rad) was applied. The gels were run in buffer solution (Tris/glycine/SDS) at 200 V for approximately 45 min in a Mini-Protean II system (Bio-Rad). The gels were stained with 0.1% Coomassie brilliant blue to visualize the protein bands of the allergenic extracts and standards (Bollag, Rozycki & Edelstein 1996).

Immunoblotting

The DEF and BLO extracts were separated by 12% SDS-PAGE under reducing and denaturing conditions and then transferred to a 0.45-µm pore size nitrocellulose membrane (Millipore). The gel in contact with the nitrocellulose membrane was mounted in a transfer cassette according to manufacturer instructions (Mini Trans-Blot, Bio-Rad). The blot was placed in a chamber containing transfer buffer (15.6mM Tris base, 120 mM glycine, and 20% methanol) and proteins were transferred at 100 V for 1 h. The quality of transfer was evaluated by staining the gel with 0.1% Coomassie blue to demonstrate the absence of proteins in the gel and the presence of the stained molecular weight marker (Full Range Rainbow™ Recombinant Protein Molecular Weight Marker, Amersham) in the membrane.

The membrane was blocked with 3% bovine serum albumin in phosphate-buffered saline (PBS) for 1 h under shaking. Next, the membrane was incubated with the sera (1:5) for 2 h under shaking at room temperature. After washing with PBS/Tween 20 (0.1%), the membrane was incubated with peroxidase-labeled canine anti-IgE conjugate (ICL GE-40P) (1:1000) for 1 h at room temperature. The membrane was again washed with PBS/Tween 20 (0.1%) and exposed to the luminol substrate (ECL Plus Western Blotting Detection System, Amersham), and the bands was detected by fluorescent scanning (Storm, Molecular Dynamics, Amersham).

RESULTS

SDS-PAGE of the DEF and BLO extracts

The results of SDS-PAGE of the DEF and BLO extracts are shown in Figure 1. Electrophoresis of the DEF extract under reducing conditions revealed proteins bands of approximately 15 kDa, 15 to 20 kDa, 25 kDa, 37 kDa, 50 kDa, 100 kDa, and > 100 kDa.

No significant differences in the protein band pattern were observed between BLO extracts run under reducing and non-reducing conditions (Fig.1). Protein bands of the following molecular weights were detected in this extract: < 15 kDa, approximately 15 kDa, 15 to 20 kDa, 25 kDa, 25 to 37 kDa, 50 kDa, 100 kDa, and 100 to 150 kDa.

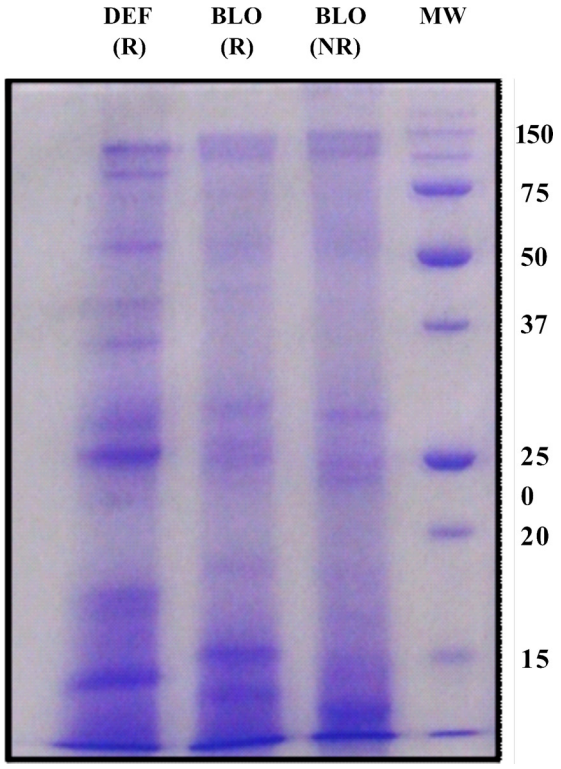

Fig.1. SDS-PAGE of the Dermatophagoides farinae (DEF) and Blomia tropicalis (BLO) extracts under reducing (R) and non-reducing (NR) conditions. MW: molecular weight standard (kDa).

Immunoblotting

Eight of the 10 sera from dogs allergic to DEF tested with canine anti-IgE conjugate recognized two protein bands with molecular weights of about 102 kDa, eight recognized two bands with molecular weights of 52 to 76 kDa, five recognized one band of approximately 76 kDa, five recognized one band of approximately 225 kDa, four identified one band of 31 to 38 kDa, and two identified one band of 12 to 17 kDa (Fig.2). Only one of the four sera from healthy dogs recognized bands with molecular weights of 52 to 102 kDa.

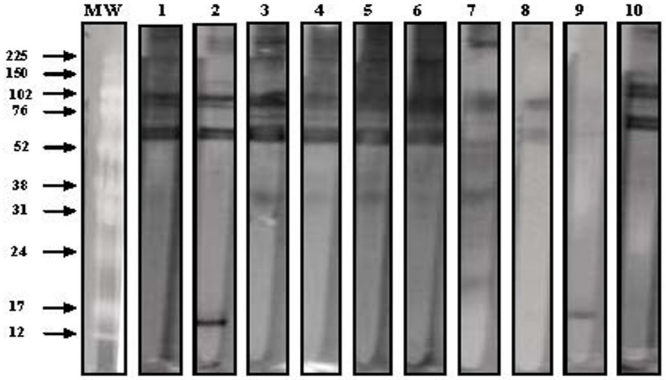

Fig.2. Immunoblot (anti-IgE) of the Dermatophagoides farinae (DEF) extract using sera from 10 dogs allergic to DEF. MW: molecular weight standard (kDa).

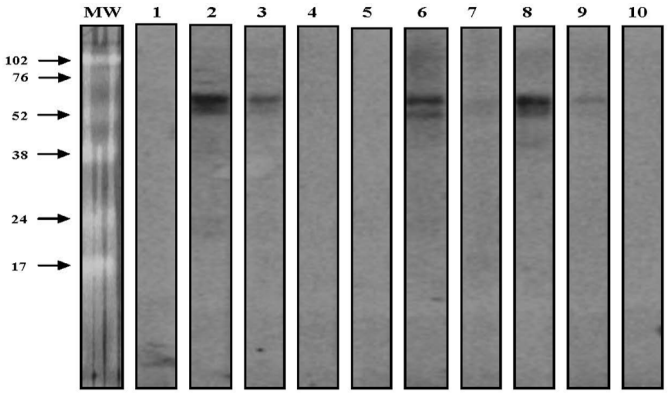

Fig.3. Immunoblot (anti-IgE) of the Blomia tropicalis (BLO) extract using sera from 10 dogs allergic to BLO. MW: molecular weight standard (kDa).

Five of the 10 sera from dogs allergic to BLO recognized two protein bands with molecular weights of 52 to 76 kDa (Fig.3). None of the four sera from healthy dogs recognized any protein band of this extract.

DISCUSSION

The identification of allergenic proteins in a particular species is an important step for the development of more accurate allergy tests and for the definition of more effective immunotherapy protocols. In this respect, more than 70 house dust mite allergens that are recognized by human sera have been described so far (Colloff 2009). However, only few allergens have been identified in dogs. The most important are: Der p 1 (25 kDa) and Der p 2 (14 kDa) from DEP, and Der f 1 (25 kDa), Der f 2 (14 kDa), Der f 10 (37 kDa), Der f 15 (98 kDa) and Der f 18 (60 kDa) from DEF (Noli et al. 1996, McCall et al. 2001, Weber et al. 2003).

In the present study, the results of the immunoblot assays using the DEF extract revealed the recognition of different bands with molecular weights ranging from 14 to >225 kDa. Sera from eight dogs with AD identified two bands with molecular weights of about 102 kDa. Two proteins, one of 98 kDa and one of 109 kDa, were recognized by most sera from dogs with AD tested in previous studies (Leung 2000, McCall et al. 2001). The N-terminal sequences of these two proteins were found to be identical and sequencing of internal peptides revealed homology to insect chitinases (McCall et al. 2001). These allergens were detected at the same frequency in immunoblots obtained under reducing and non-reducing conditions, suggesting that they are relatively resistant to reduction (Leung 2000). The 98-kDa protein was cloned and was called Der f 15. Sensitized dogs presented positive skin tests to the crude DEF extract and purified Der f 15 (McCall et al. 2001). Immunohistochemical staining detected Der f 15 in the mite intestine, but not in fecal pellets, suggesting a digestive enzyme (McCall et al. 2001).

Eight other sera from atopic dogs recognized two bands of 52 to 76 kDa in the present study. One DEF protein of 60 kDa, called Der f 18, has been identified in a previous study as a major allergen for dogs (Weber et al. 2003). This allergen is a glycosylated protein comprising 437 amino acids, with a native molecular weight of 50 kDa. Purified Der f 18 identified 57 to 77% of sera from dogs sensitized to DEF (Weber et al. 2003). Like Der f 15, this protein showed homology to insect chitinases and was detected in the mite digestive system, but not in fecal pellets. This allergen was recognized by 85% of sera from atopic dogs in the study of McCall et al. (2001).

Sera from seven atopic dogs recognized two bands larger than 225 kDa. However, high molecular weight proteins are not adequately separated on 12% polyacrylamide gels and appropriate visualization of these bands was therefore not possible. At least five sera from dogs with CAD identified a band with a molecular weight of approximately 75 kDa and five other sera recognized a band of about 225 kDa. These bands were identified for the first time in the present study. Possible explanations for this finding are that the pattern of antigen recognition is characteristic of the dogs studied here or even that these antigens are absent in the allergenic extracts used in the previous studies.

Sera from four dogs with CAD recognized a band with a molecular weight of 31 to 38 kDa. In the study of McCall et al. (2001), a 37-kDa allergen was identified in the DEF extract and was called Der f 10. In that study, 29% of sera from dogs with CAD recognized this allergen, which was therefore classified as a minor allergen, as in the present study.

Two sera from dogs with CAD identified a low molecular weight allergen (a band of approximately 14 kDa). Hypothetically, this band corresponds to Der f 2, a 129-amino acid polypeptide with a molecular weight of 14 kDa (O'Hehir et al. 1993). None of the sera of the group of dogs with CAD recognized Der f 1, a major allergen for humans with a molecular weight of 24 kDa. In another study (Noli et al. 1996), 90% of dogs with CAD had positive skin tests. Specific IgGd antibodies against crude DEF extracts, but not against purified group 1 or 2 allergens, were detected in the sera of these animals. In the study of McCall et al. (2001), low molecular weight bands, such as those corresponding to group 1 and 2 allergens, presented a low frequency of recognition. Similarly, less than 10% of sera from dogs with AD identified group 1 and 2 allergens in other study (Nuttal et al. 2001). On the other hand, a Japanese study showed that four of six dogs presented positive skin reactions to purified group 1 and 2 allergens of *Dermatophagoides* (Masuda et al. 2000). These results suggest differences in the pattern of allergen recognition between Japanese and American or European dogs. In this respect, the present findings indicate that low molecular weight allergens such as Der f 2 are minor allergens for dogs with CAD in Brazil, in agreement with the results reported in European and American studies.

Only one control serum recognized protein bands in the immunoblot assay of the DEF extract. This serum recognized bands with the following molecular weights: two bands of 52 to 76 kDa, one band of approximately 76 kDa, two bands of 76 to 102 kDa, and at least one band > 225 kDa. One may speculate that this dog presented subclinical hypersensitivity at the time of the skin test and that the lack of a response in this test might have been due to the

exaggerated production of endogenous cortisol or to the recent unreported use of drugs that interfere with the skin response.

The clinical importance of BLO allergens for CAD is still unclear. BLO is a free-living mite found in tropical and subtropical climates, which is known to play a role in the pathogenesis of atopic diseases in humans (Thomas, Hales & Smith 2003; Zakzuk et al. 2009). In a study investigating the mite fauna of 72 homes in different regions of Brazil, BLO was detected in 100% of the samples and accounted for 79.5% of the total number of mites found (Rosa & Flechtmann 1979). The most important allergens for humans are Blo t 3 (Cheong et al. 2003), Blo t 5 (Arruda et al. 1997), Blo t 11(Ramos et al. 2001), and Blo t 12 (Puerta et al. 1996).

The results of the immunoblot assays using BLO extract showed that, as observed for humans, BLO allergens contribute to the pathogenesis of allergic diseases in dogs. Five of the 10 sera from dogs with AD allergic to BLO recognized two bands with molecular weights of 52 to 76 kDa. These protein bands were not identified by the four control sera. One allergen of 56 kDa, called Blo t 4, has been described as a minor allergen for humans and possesses α-amylase activity. α-Amylases are potent and highly conserved allergens found in mites, fungi, and cereals (Mills et al. 1999).

In conclusion, DEF and BLO, mite species commonly found in house dust from Brazilian cities, seem to contribute to the pathogenesis of CAD. Further biochemical and molecular studies are needed to confirm the true identity of the allergens identified in the present study.

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