



Immunoproteomic characterization of a *Dermatophagoides farinae* extract used in the treatment of canine atopic dermatitis



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ABSTRACT

Background: Canine atopic dermatitis is a pruritic allergic skin disease. House dust mites have been identified as the main non-seasonal responsible agent. Unlike in human allergic patients, groups 1 and 2 antigens have been described as minor allergens in dogs, while groups 15 and 18 are considered the major allergens. Despite these differences, allergic dogs have traditionally been treated using extracts intended for human immunotherapy.

Objectives: To investigate the immunological characteristics and the allergen reactivity of dogs with atopic dermatitis using a *Dermatophagoides farinae* commercial extract.

Methods: Eighteen dogs diagnosed with atopic dermatitis and 3 healthy control dogs from the Iberian Peninsula were included in the study. All the animals were older than 12 months, from both sexes and different breeds and showed positive specific IgE against *D. farinae* (>2500 ELISA Absorbance Units). The *D. farinae* allergenic extract used in this study was manufactured and characterized. The allergenic profile of the dogs was investigated by immunoblot and specific IgE, IgG, IgG1 and IgG2 measured by direct ELISA. Allergen identity was confirmed by immunoblot inhibition and mass spectrometry analyses.

Results: The results confirmed the relevance of groups 15 and 18 antigens, but also groups 1, 2 and other medium molecular weight allergens in the sensitization of dogs with atopic dermatitis. Immunoblot inhibition and mass spectrometry assays confirmed these results. Relevant allergens were quantified by scanning densitometry (Der f 1: 17 µg/mg, Der f 2: 20.3 µg/mg, Der f 15: 18.1 µg/mg and Der f 18: 9.4 µg/mg). Concerning immunoglobulins profile, differences in IgE and IgG1 levels were observed between non-atopic and atopic dogs.

Conclusions: The commercial *D. farinae* extract characterized in this study contains the major allergens involved in the sensitization of dogs with atopic dermatitis, representing a suitable candidate for its use in the diagnosis and immunotherapy of mite allergic dogs.

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1. Introduction

Canine atopic dermatitis (CAD) is a genetically predisposed inflammatory and pruritic allergic skin disease, associated to the production of IgE antibodies against environmental allergens,

mainly mites and pollens (Lian and Halliwell, 1998; DeBoer and Hillier, 2001; Hill and DeBoer, 2001; Jackson et al., 2002; Farmaki et al., 2010; Mueller et al., 2016).

House dust mites (HDM) have been identified as the main cause of non seasonal canine and human allergies (Nuttall et al., 2006). In Western countries, *Dermatophagoides farinae* is accountable for more than 70% of CAD cases identified (Codner and Tinker, 1995; Weber et al., 2003). Contrary to what happens in humans, dogs show lower positive responses against *Dermatophagoides pteronyssinus*, even in locations where this mite is more prevalent than *D. farinae* (Jackson et al., 2005; Nuttall et al., 2006; Farmaki et al., 2012; Fernández-Caldas, 2013).

Significant differences between the human and canine HDM allergens profile have also been reported. Whereas the major allergens for humans are proteins of relatively low molecular weight

Abbreviations: CAD, canine atopic dermatitis; HDM, house dust mites; LMW, low molecular weight; HMW, high molecular weight; AD, atopic dermatitis; EAU, ELISA Absorbance Units; MW, molecular weight; OD, optical density; ASIT, allergen specific immunotherapy.

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(LMW) (Thomas and Smith, 1999), this is not the case in dogs. The most important allergens of *D. farinae* in atopic dogs are included in groups 15 and 18, which belong to the high molecular weight (HMW) fractions of HDM extracts (Nuttall et al., 2001). Preliminary immunological studies have shown that Der f 15 presents two IgE-binding capacity bands at 98 kDa and 109 kDa, whereas Der f 18 presents as a single IgE-binding band of about 60 kDa (McCall et al., 2001; Weber et al., 2003; Hou et al., 2008). Based on the published studies, the major allergens described in allergic human patients, Der f 1 and Der f 2 with IgE binding to 25 kD and 14 kD bands respectively, are recognized by less than 50% of atopic dogs, and therefore should be considered as minor allergens for this species (Bard and Esch, 1992; Noli et al., 1996; Masuda et al., 1999; Thomas et al., 2002; Goicoa et al., 2008).

Several hypotheses have been postulated to explain the cause of the discrepancies observed between dogs and humans, including genetic, immunologic and metabolic theories (Fernández-Caldas, 2013). One of these hypotheses is based on the differences in T-cell epitopes and subsequent T-cell responses (Nuttall et al., 2006). Other authors blame this discrepancy on the route of sensitization to mite allergens (Marsella et al., 2006), that, in dogs (unlike in humans) could be partially the ingestion route due to the contamination of dog food with mites (Brazis et al., 2008). In spite of these important differences in allergen recognition between species, *D. farinae* allergens included in allergen specific immunotherapy (ASIT) used to desensitize both human and canine patients, are usually the same. Normally, *D. farinae* immunotherapy is standardized regarding Der f 1, to determine activity and potency.

Major mite allergens for humans have been intensively studied, however, the information of major mite allergens for dogs available is more limited (Noli et al., 1996; Masuda et al., 1999; McCall et al., 2001; Hou et al., 2008). Further research of mite allergic dogs is required to clarify remaining questions such as the role of mite allergens in the development of CAD or the quality of mite extracts used for the diagnosis and treatment of these animals.

The objectives of this study were to characterise a commercial *D. farinae* extract, identifying key allergens involved in the sensitization of dogs suffering from CAD, in order to evaluate whether *D. farinae* extracts normally used in canine immunotherapy contain these allergens, and to investigate the allergenic profile of sensitization and the immunological characteristics of dogs suffering from atopic dermatitis (AD). For this objective, different immunological assays were performed with sera from both healthy and atopic dogs using a commercially available *D. farinae* extract.

2. Materials and methods

2.1. Canine serum samples

Serum samples were collected following standard veterinary procedures from 18 dogs clinically diagnosed with AD by private veterinarians. Selected animals fulfilled the following inclusion criteria: (1) Dogs older than 12 months of age, of both sexes and any breed, with a clinical diagnosis of non-seasonal AD according to the criteria of Favrot et al. (2010); (2) Dogs with a positive serological test (ELISA test from Greer Laboratories [Lenoir, NC; USA]) for detection of allergen specific IgE against *D. farinae*, evidencing ELISA Absorbance Units (EAU) higher than 2500.

None of the following exclusion criteria were fulfilled by any of the animals included in the study: pregnancy or lactation, seasonal signs of AD involving flea infestation or food allergens (based on response to treatment for flea infestation and to an elimination diet [at least 6 weeks]), presence of bacterial or *Malassezia* sp. skin infections or other infestations causing pruritus (e.g. mites), and having been treated with glucocorticoids or oral corticosteroids within

4 weeks of enrolment and/or injectable glucocorticoids within 8 weeks of enrolment.

Negative control samples were collected from 3 healthy dogs with no dermatological lesions and showing less than 50 EAU after assessing *D. farinae* specific IgE determination by serological ELISA test.

The characteristics of the patient population are described in detail in Table 1.

2.2. Characterization of *D. farinae* extract

D. farinae extract was prepared according to internal manufacturing procedures (Laboratorios LETI, Madrid, Spain). Briefly, the extract was prepared using semipurified mite bodies ($\geq 80\%$ mite bodies) in 0.01 mol/L of phosphate buffered saline (1:20 wt/volume) and extracted for 24 h. Afterwards the solution was centrifuged, and the supernatant collected, dialyzed, filtered, and freeze-dried. The extract was characterized as follows.

2.2.1. Protein content

The protein content was determined using the Bradford method (Thermo Scientific, Waltham, MA, USA) following the manufacturer's instructions.

2.2.2. Protein profile

Protein profile of the extract was investigated by SDS-PAGE under reducing conditions following two different strategies: (1) 15%T acrylamide:bis-acrylamide gels, with LMW range standard for the identification of proteins in a range between 10 and 70 kDa; (2) Any kD™ Mini-PROTEAN® TGX™ Gels (BioRad, Hercules, CA, USA) with a HMW range for the characterization of proteins between 45 and 200 kDa. In both cases gels were Coomassie stained. Gel images were analysed using the ImageQuant TL 2005 software (GE Healthcare, Uppsala, Sweden).

Concentration of each individual band compared to the whole protein composition was calculated by scanning densitometry, to quantify the content of individual allergens compared to the whole protein concentration.

2.3. Allergenic profile

Allergenic profile of each serum sample was determined by immunoblot using *D. farinae* extract in solid phase. Briefly, 50 µg of allergen extract/serum sample were run in a gel. After electrophoresis, gels were electrotransferred onto a Trans-Blot® Turbo™ Transfer Pack (Bio-Rad). Membranes were blocked with 5% skimmed milk in PBS 0.01 mol/L; Tween 0.1% for 1 h and incubated overnight with individual sera (diluted 1/10 in PBS 0.01 mol/L). After washing, the membranes were incubated with goat anti dog IgE:HRP diluted 1:10000 (AbD Serotec, Bio-Rad). Finally, membranes were developed with luminol solution (Clarity™ Western ECL Substrate, Bio-Rad) and detected by chemiluminescence (ChemiDoc XRS, Bio-Rad). The molecular weight (MW) of the detected bands was determined using Image Quant TL 2005 (GE Healthcare).

2.4. ELISA assays

Specific IgE, IgG, IgG1 and IgG2 to *D. farinae* were measured in the individual serum samples by direct ELISA. Briefly, microplates (Immulon IV; Thermo Scientific) were coated with *D. farinae* extract (20 µg/ml) and incubated overnight at room temperature. Plates were blocked for 1 h with 1% BSA in PBS 0.01 mol/L; Tween 0.1%. Serum samples were serially diluted and incubated for 2 h. After washing, the secondary antibody (diluted 1:10000) consisting on goat anti dog IgE:HRP, sheep anti dog IgG:HRP, goat anti dog

Table 1

Characteristics of the dog population included in the study.

Number	<i>D. farinae</i> IgE (EAU)	Breed	Age (years old)	Sex
1	2526	Shar Pei	4	Female
2	2531	Crossbred	5	Male
3	3347	Staffordshire Bull Terrier	5	Male
4	3157	Yorkshire Terrier	7	Female
5	3136	German Shepherd	3	Female
6	2551	Beagle	3	Female
7	3408	Shih-Tzu	6	Male
8	3857	Fox Terrier	5	Female
9	2627	Pitbull Terrier	6	Female
10	2946	West Highland White Terrier	10	Female
11	2807	American Pitbull	1	Male
12	3247	Spanish Waterdog	5	Female
13	3423	Labrador	6	Male
14	2564	Labrador	6	Male
15	3403	Labrador	7	Male
16	2959	Catalonian Sheepdog	4	Female
17	2520	Argentine Dogo	1	Male
18	2910	Crossbred	2	Female
C1	39	Chinese Crested	1	Male
C2	30	Shiba-Inu	2	Female
C3	0	Beagle	2	Female

IgG1:HRP or sheep anti dog IgG2:HRP (AbD Serotec, Bio-Rad) was added. Finally, microplates were washed, the reaction developed and optical density (OD) measured at 450 nm on an automated ELISA plate reader. A cut-off of 0.35 OD (mean plus 2 standard deviations of control dogs values) was established for specific IgE, while values ≥ 0.1 were considered positive for specific IgG, IgG1 and IgG2.

For the determination of specific IgE to the allergens Der f 1 and Der f 2 the microplates were coated (100 ng protein/well) with purified Der f 1 or Der f 2 (Indoor Biotechnologies, Charlottesville, VI, USA) and incubated overnight at room temperature. Plates were blocked for 1 h with BSA in PBS 0.01 mol/L; Tween 0.1%. Then, plates were washed and 100 μ l of serum samples diluted 1:10 vol/vol in PBS 0.01 mol/L added. After 2 h, plates were then washed and incubated for 2 h with goat anti dog IgE:HRP (AbD Serotec, Bio-Rad). Finally, microplates were developed as previously described. OD values ≥ 0.1 were considered positive.

2.5. Immunoblot assays

Immunoblot assays were carried out to confirm the recognition of allergens Der f 1 and Der f 2. For this purpose, the serum samples from atopic dogs (diluted 1/10 in PBS 0.01 mol/L) were individually incubated with the membrane containing 2 μ g of Der f 1 or Der f 2 (Indoor Biotechnologies) in solid phase.

Additionally, immunoblot inhibition assays were performed with two different pools of sera prepared from the serum samples with the highest OD in ELISA assays for Der f 1 and Der f 2. These pools (diluted 1/10 in PBS 0.01 mol/L) were individually incubated for 2 h with 40 μ g of Der f 1 or Der f 2 (Indoor Biotechnologies) before the incubation with the membrane containing 50 μ g of *D. farinae* extract in solid phase. Both pools of sera were also incubated directly with the purified allergens in the solid phase (5 μ g). All the immunoblots were developed as previously described.

2.6. Allergen identification

The most relevant bands identified by the immunoblot experiments were sequenced by mass spectrometry to confirm their identity. Bands were cut from an SDS-PAGE gel (Coomassie stained), digested with trypsin and individually identified by LC-MS/MS (Liquid Chromatography-Mass Spectrometry/Mass Spectrometry).

3. Results

3.1. *D. farinae* extract characterization

The *D. farinae* extract analysed showed a protein content of 219 μ g/mg. Protein profiles for the two SDS-PAGE conditions are showed in Fig. 1. When HMW gels were used (Fig. 1A), 8 protein bands in the MW range of 175–29 kDa were identified by ImageQuant software. On the other hand, the LMW gel (Fig. 1B) was more suitable for the identification of 21 proteins in the MW range of 89–11 kDa.

Canine major allergens, Der f 15 and Der f 18, as well as Der f 1 and Der f 2 were quantified by comparing protein profiles in both conditions and identifying them in the LMW condition profile (Fig. 1C). Der f 15 was identified as bands numbers 1 and 2 which constituted the 8.2% of the total of protein whereas Der f

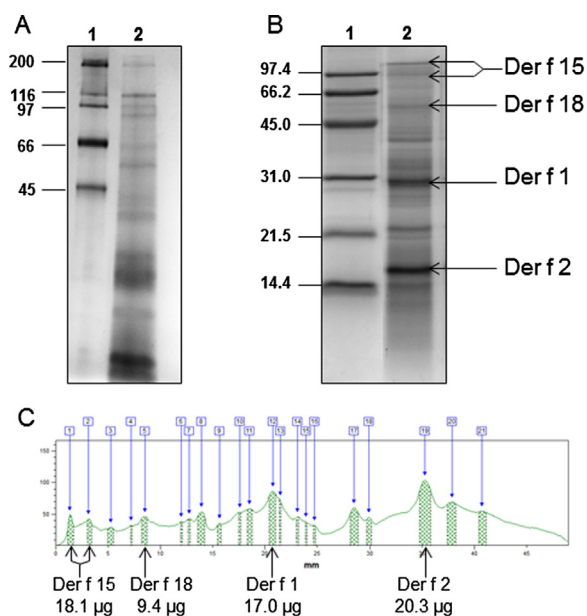


Fig. 1. SDS-PAGE stained with Coomassie of HMW (A) and LMW gels (B). 1: MW marker; 2: *D. farinae* extract (8 μ g of protein). (C) Band analysis of LMW gel with ImageQuant software; μ g of allergen/mg of lyophilized extract of Der f 15, Der f 18, Der f 1 and Der f 2 are indicated below their corresponding band.

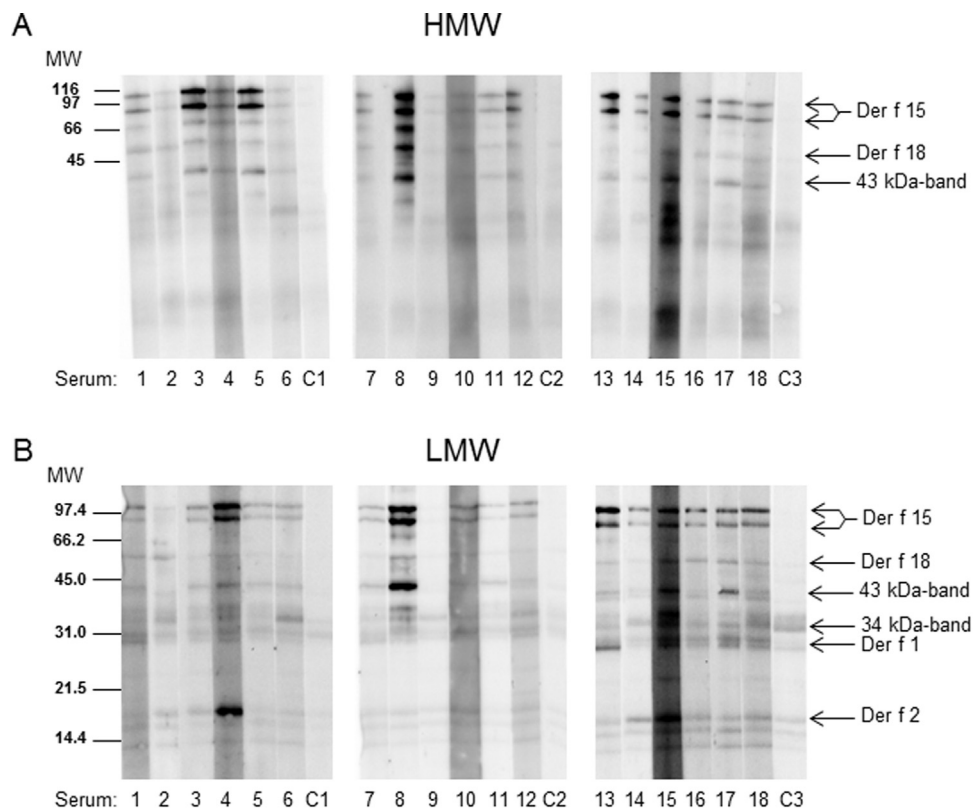


Fig. 2. Immunoblot of 50 µg of *D. farinae* extract/serum sample run in HMW (A) and LMW (B) gels and incubated with positive sera 1–18 and controls C1–C3. MW marker is indicated on the left. The most relevant allergens identified in both MW conditions, Der f 15, Der f 18, 43 and 34 kDa-bands, Der f 1 and Der f 2, are marked on the right.

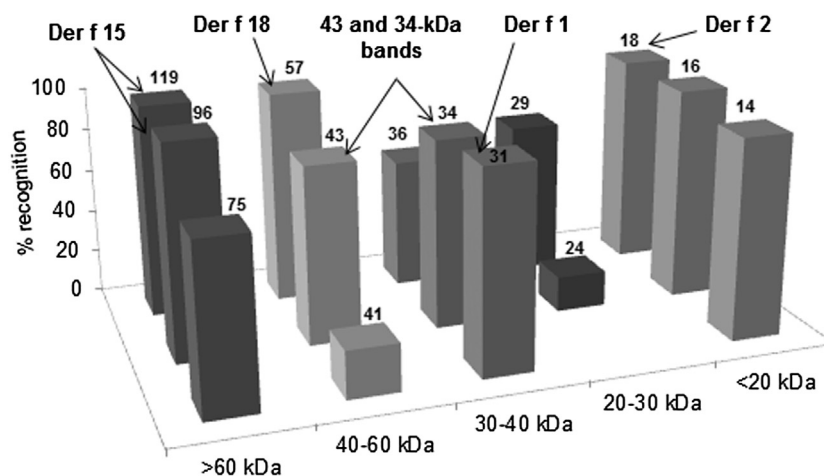


Fig. 3. Bands identified in immunoblot experiments under both SDS-PAGE conditions and percentage of atopic dogs that recognized them. MW is indicated on top of each bar. Most relevant allergens identified in both MW conditions, Der f 15, Der f 18, 43 and 34 kDa-bands, Der f 1 and Der f 2, are marked with an arrow.

18 (band 5), represented a 4.3% of the total. This means that the protein content of these allergens in the *D. farinae* extract analyzed was approximately 18.1 and 9.4 µg/mg of lyophilized extract, respectively. Quantification values obtained for Der f 1 and Der f 2 allergens were 17 and 20.3 µg/mg, respectively (which corresponds to band percentages of 7.8 and 9.4).

3.2. Allergenic profile

Allergenic profiles of the 18 atopic dogs sensitized to *D. farinae* and from the 3 negative controls (C1–3) were investigated. Fig. 2 shows the immunoblot results from LMW and HMW gels.

All the positive sera showed similar binding patterns in HMW gels (Fig. 2A), recognizing bands of high MW. Four different bands (119–43 kDa) were identified when the image was analysed by ImageQuant and their MW calculated (Fig. 3). All sera recognized two bands at 119 and 96 kDa with high intensity. These bands represented the 50% of the total bands intensity in 9 of the 18 sera (data not shown). A band of 57 kDa was also recognized by all sera. No bands were detected in the 3 negative controls.

Concerning the LMW gels, numerous bands between 13 and 70 kDa, approximately, were identified in the 18 positive sera (Fig. 2B). A slight recognition of proteins at 30 and 14 kDa approximately was observed in negative controls, especially in C3. The

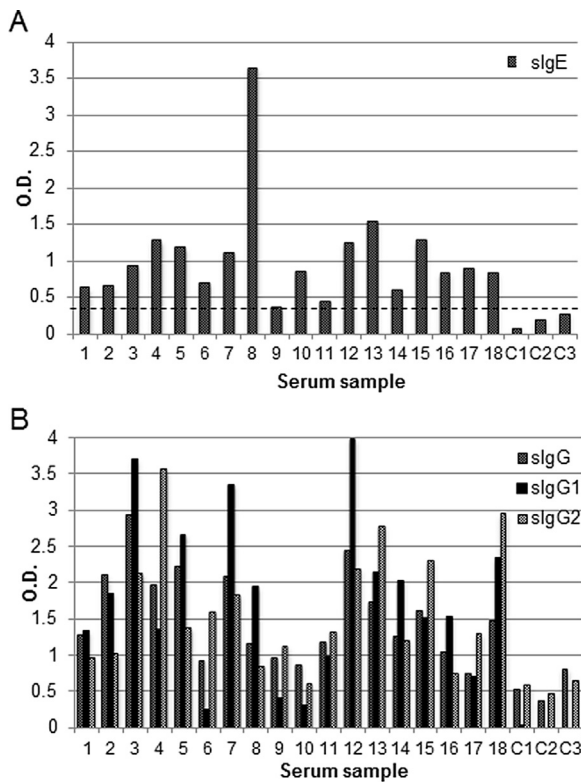


Fig. 4. Specific immunoglobulins levels measured by ELISA in serum samples of atopic (1–18) and control dogs (C1–3) using 2 μ g of *D. farinae* extract in the solid phase. (A) Specific IgE (serum samples diluted 1:8). Cut-off is marked with a dotted line. (B) Specific IgG, IgG1 and IgG2 (serum samples diluted 1:2000).

most recognized bands corresponded to proteins with a MW of about 16 kDa. Other bands of about 43 and 34 kDa were recognized by the 89% of sera. Comparing the allergenic profiles on both conditions, nine bands (43–14 kDa) different from the ones described for HMW gels were identified when the images were analysed by ImageQuant (Fig. 3). The 43 kDa-band was identified in both conditions.

3.3. Specific immunoglobulins determination

The 18 sensitized dogs showed a positive specific IgE against *D. farinae* extract, being the OD in the range of 0.4–3.6. The 3 negative controls showed negative absorbance values, below 0.25 (Fig. 4A).

Concerning specific IgG and IgG subclasses (Fig. 4B), the IgG1 was the only immunoglobulin where a difference was observed between the control and the sensitized group. Absorbance values obtained for specific IgG and IgG2 were in general higher in sera from sensitized dogs, although the negative controls sera were also found to be positive for these immunoglobulins.

When sensitization to the purified allergens Der f 1 and Der f 2 was analyzed by ELISA, positive low absorbance values (<0.6) were observed for all the canine sera in the case of Der f 1 allergen (Fig. 5A), except for the serum sample number 6 that showed a high binding to Der f 1 (OD of 3.9 ± 0.07). On the other hand, sensitization to Der f 2 (Fig. 5B) was in general higher than that observed for Der f 1, with OD ranging 0.4–3.2 in all the sera. No differences between the sensitized compared to the control group were observed in any of the two cases, being the values obtained for Der f 2 in C2 and C3 serum samples even higher than the values from some of the sensitized dogs.

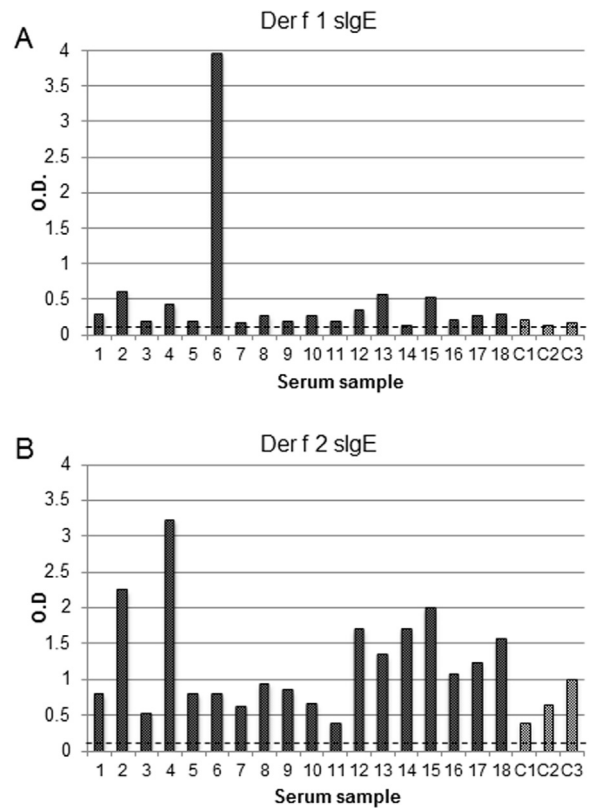


Fig. 5. Der f 1 (A) and Der f 2 (B) specific IgE levels measured by ELISA in serum samples (diluted 1:10) of atopic (1–18) and control dogs (C1–3) using 10 ng of purified Der f 1 or Der f 2 in the solid phase. Cut-off is marked with a dotted line.

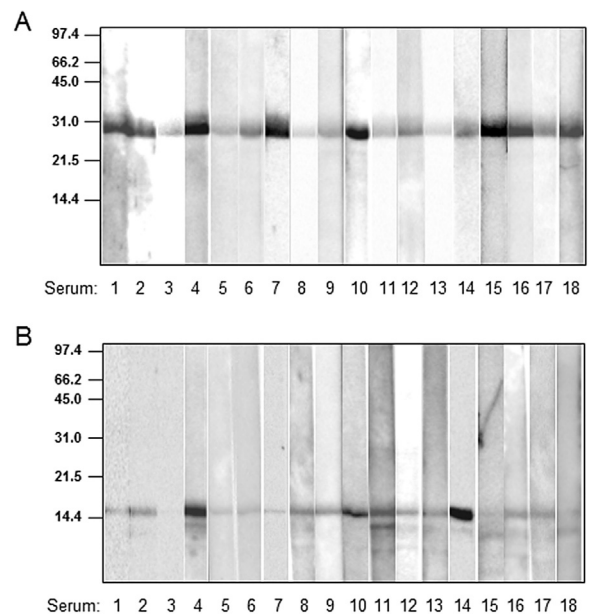


Fig. 6. Immunoblot experiment using 2 μ g of Der f 1 (A) or Der f 2 (B) in the solid phase and incubated with positive serum samples 1–18 (diluted 1:10). MW marker is indicated on the left.

3.4. Immunoblots

A hundred percent of the serum samples from atopic dogs recognized Der f 1 by immunoblot (Fig. 6A) whereas 17/18 recognized Der f 2 (Fig. 6B). Two different pools of sera with the highest OD in ELISA assays for Der f 1 and Der f 2 were prepared for the

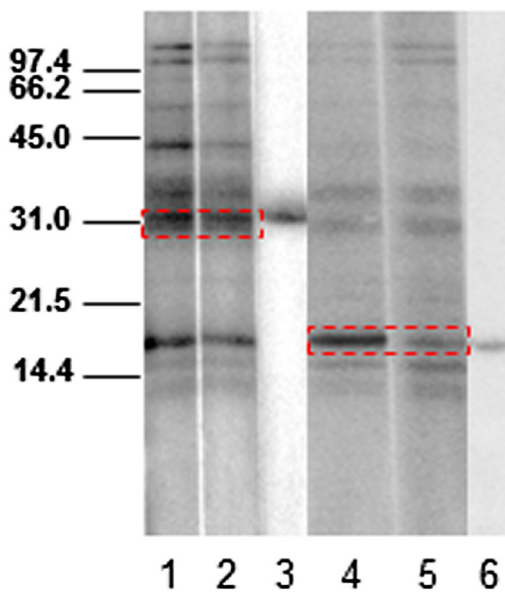


Fig. 7. Immunoblot inhibition experiment using 50 µg of *D. farinae* extract/serum sample in the solid phase and incubated with: 1–a pool of sera positive to Der f 1; 2–a pool of sera positive to Der f 1 previously incubated with 40 µg of Der f 1; 4–a pool of sera positive to Der f 2; 5–a pool of sera positive to Der f 2 previously incubated with 40 µg of Der f 2. Positive controls: 5 µg of Der f 1 (3) or Der f 2 (6) in the solid phase incubated with their corresponding pool of sera. MW marker is indicated on the left.

immunoblot inhibition assays. In the case of Der f 1, no inhibition of the band corresponding to this allergen (about 31 kDa) was observed. However, the pool recognized the purified allergen when it was used in the solid phase (Fig. 7, lanes 1–3). A strong inhibition in the intensity of a band of about 15 kDa was observed in the pool of sera sensitized to Der f 2, when it was previously incubated with this purified allergen. Moreover, this pool of sera also recognized the purified allergen (Fig. 7, lanes 4–6).

3.5. Allergen identification

Five protein bands of 119, 96, 57, 43, and 34 kDa approximately were sequenced by mass spectrometry to confirm their identity. The 96 kDa and 119 kDa bands (Table 2) were identified as Der f 15 according to the homology in the amino acid composition. Seven different peptide sequences were identified in the 96 kDa band, corresponding to a 98 kDa HDM allergen [*Dermatophagoides farinae*], GenBank AF178772, and identified as Der f 15. In the case of the 119 kDa band, also seven different peptide sequences were identified corresponding to Der f 15. Four peptide sequences were obtained for the 57 kDa band, corresponding to a 60 kDa allergen Der f 18p [*Dermatophagoides farinae*], GenBank AAM19082. In the case of the 43 and 34 kDa bands, peptides corresponding to more than one allergen were identified. All the information concerning allergens sequencing is summarized in Table 2. For the 43 and 34 kDa bands, identified allergens with the highest sequence coverage are shown.

4. Discussion

Mites are the main cause of allergy not only in humans, but also in dogs. However, the allergenic profile of reactivity presented by humans and dogs is different. Preliminary studies have evidenced that major allergens in dogs correspond to HMW bands, while in humans, major allergens correspond to LMW allergens. Additionally, clinical manifestations also vary between the two species. While in humans mite allergy is associated to asthma, rhinitis,

and/or AD, clinical manifestations in the dog are mainly displayed on the skin, by AD. Keeping this into consideration, the need to treat allergic dog patients with the appropriate ASIT seems clear. Dogs have been traditionally treated using human immunotherapy without taking into consideration factors such as allergen composition according to the sensitization profile; type of allergenic source, considering the species to be included; allergen concentration; biological standardization; and other important issues, clearly related with the efficacy of ASIT.

Regarding allergen composition, in the last years, the introduction of molecular diagnosis in the allergy field (Canonica et al., 2013) has demonstrated that the allergenic profile of sensitization varies amongst human individuals and is related to factors such as geographical distribution, genetic factors, allergen exposure, etc. This fact has not been clearly demonstrated in dogs yet. Taking into consideration the influence of these factors in the development of CAD, we selected a dog population in Spain composed by 18 animals with AD and 3 healthy dogs, with a similar pattern of exposure to allergens. In this particular geographic area, both HDM species (*D. farinae* and *D. pteronyssinus*) show a similar presence and abundance (Iraola and Fernández-Caldas, 2009) with a range between 100 and 500 mites/g of dust. Different breeds of dogs were included in the study, being 2 of the dogs among the atopic group cross-bred and the rest pure-breed. Our results agreed with those from previous studies which suggested that *Dermatophagoides* major allergens for dogs correspond to HMW proteins (Noli et al., 1996; Masuda et al., 1999), and specially to a chitinase (Der f 15) (McCall et al., 2001) and a chitin binding protein (Der f 18) (Weber et al., 2003). Der f 15 corresponds to two bands of about 98 and 109 kDa. Although the proteins identified in our study had a slightly different MW, we identified them as presumably Der f 15. This was confirmed by LC–MS/MS analyses. Der f 18 corresponds to a 60 kDa band, identified in our study as a 57 kDa-band. This protein is also a major allergen for both humans and dogs. All the sensitized animals included in the study recognized Der f 15 and these results are very similar to those obtained by other authors (Noli et al., 1996; Masuda et al., 1999). However, all the sensitized animals did also recognize Der f 18. These results differ slightly from those previously published by McCall et al. (2001) and Weber et al. (2003), who reported lower percentages of atopic dogs positive to this allergen. These differences could be attributable to the test (intradermal test and ELISA vs. immunoblot) performed, the concentration of this allergen in the extracts used or even to geographic factors as it has been clearly evidenced in humans. In relation to other allergens with high percentages of recognition, our atopic canine population recognized two bands of about 43 and 34 kDa. These bands were also sequenced and investigated, identifying more than one allergen in each case, indicating the presence of more than one protein in the selected bands. Many mite allergens have been described as glycosylated proteins that under reducing conditions can present very different MW (Le Mao et al., 1998), therefore, further studies would be necessary to correctly identify these proteins. Regarding LMW allergens, all the animals included in our study were also sensitized to Der f 1 and Der f 2, as demonstrated by ELISA and/or immunoblots results. In that case, these two allergens may also be considered major allergens in our population, although the intensity of recognition by ELISA was low and healthy dogs also recognized them. Until now, these two allergens had been described as minor allergens for dogs by different authors (Nuttall et al., 2006) although some studies have described that more than 50% of atopic dogs recognized group 1 and 2 allergens (Masuda et al., 1999; McCall et al., 2001). In our population (atopic and healthy dogs) these percentages were higher than 90% for immunoblot assay and 100% for ELISA. To determine if the recognition was specific, we investigated the reactivity by immunoblot experiments with purified Der f 1 and Der f 2. Even though it is true

Table 2*D. farinae* allergens identified by LC–MS/MS for each of the bands selected from the immunoblot experiments.

MW of sequenced band	Allergen identified	MW (kDa)	Biological function	GenBank	Uniprot	Number of peptides identified	Sequence coverage (%)	Score
119 kDa	Der f 15	98/109	Chitinase	AAD52672	Q9U6R7	7	26	225
96 kDa	Der f 15	98/109	Chitinase	AAD52672	Q9U6R7	7	25	354
57 kDa	Der f 18	60	Chitin-binding protein	AAM19082	Q86R84	4	6	152
43 kDa	Der f 14	177	Apolipoprotein	BAA04558	Q94507	6	20	427
	Der f 11	98	Paramyosin	AAK39511	Q96720	9	14	870
34 kDa	Der f 3	29	Trypsin	AAA99805	P49275	4	11	167
	Der f 14	177	Apolipoprotein	BAA04558	Q94507	7	6	83

that inhibition of the band corresponding to Der f 1 was not clearly observed, it should be considered that in the *D. farinae* extract there are numerous proteins with a molecular weight around 30 kDa that could be recognized by the sera. This could explain why there is still recognition of the sera at this MW after the inhibition. However, both the pool and the individual serum samples specifically recognized the purified allergen Der f 1. Specific recognition was also observed for Der f 2, so we can conclude that our investigations evidenced that both Der f 1 and Der f 2 were specifically recognized by the serum samples selected for this assay. The fact that non-atopic dogs also showed recognition of these allergens by ELISA and immunoblot could suggest the involvement of these proteins in sensitization but not in the development of clinical symptoms. Additional studies would be necessary to elucidate these remaining questions. Based on these results, we can conclude that our canine population shows specific recognition to different *D. farinae* allergens, including Der f 15, Der f 18, other specific unidentified bands between 45 and 30 kDa, but also groups 1 and group 2. Although the intrinsic or extrinsic properties that make a protein an allergen remain still unknown, it seems clear that the immune system from dogs and humans are processing the proteins in a different manner and the interaction with the innate immune system in each species must be different. This suggests that the extracts used in immunotherapy for the treatment of mite allergy in dogs should contain all these allergens in the adequate proportion for an efficacious treatment. Traditionally, allergen composition for allergenic immunotherapies is based on the presence/existence of the offending allergens in the patient environment. In the case of mites, it is widely recognized that cross-reactivity between *D. pteronyssinus* and *D. farinae* is very high and some studies have suggested the non-necessity to use both of them or even their interchangeability in the case of human immunotherapy. However, both mite species show important differences concerning their ecology and life requirements, such as diet and endotoxins production (Valerio et al., 2005; Avula-Poola et al., 2012). It has been demonstrated that *D. farinae* consumes mainly bacteria and fungi while *D. pteronyssinus* ingests human dander and other death human tissues. This characteristic could be behind the fact that *D. farinae* is more allergenic for dogs. As a result, the selection of the species for the immunotherapy should be considered. In our geographical area of study both mite species are equally present as evidenced in the mite map of Spain (Iraola and Fernández-Caldas, 2009). However, previous studies have suggested that dogs with AD are more sensitized to *D. farinae* (Sture et al., 1995; Noli et al., 1996; Hillier et al., 2000; Nuttall et al., 2006). This represents a clear difference compared to humans where a high proportion of mite allergic patients are sensitized to both species of HDM.

As detailed above, the route of sensitization could also explain partially the different profile of allergen sensitization observed between humans and dogs or even the differences in clinical manifestations. Group 1 and group 2 allergens are present in faecal mite pellets and are potent inhalant allergens. On the contrary, immunohistochemistry studies (McCall et al., 2001; Weber et al., 2003) have

revealed that both Der f 15 and Der f 18, homologous to insect chitinases, are localized in the digestive system of mites, and they could sensitize the animals through the ingestion route. In addition, mites can also be found on dogs' hair (Jackson et al., 2005) which contributes to an epicutaneous route of sensitization and clinical exacerbation (McCall et al., 2001). Summarizing, there is no clear evidence about the route of sensitization and this is an additional factor to consider for the selection of the mites species to be included in the specific immunotherapy for dogs.

Finally, in this study we evaluated not only IgE but also specific IgG and IgG subclasses. Although there is little information about the role of these immunoglobulins in CAD development (Hites et al., 1989; Lian and Halliwell, 1998; Day and Corato, 1996; Hou et al., 2006) and defined IgG subclasses are not totally clear, we observed a clear difference in IgG1 levels in atopic dogs compared to healthy animals. Hou et al. (2006) evaluated IgG subclasses against *D. farinae* by immunoblot, finding that IgG1 levels were similar in healthy and atopic dogs, except for a 180 kDa protein that was significantly more recognized for atopic dogs. Further studies with a higher number of sera and with other allergens are necessary to confirm if IgG plays a major role in the pathogenesis of CAD and if any of the IgG subclasses could constitute a new and useful diagnostic tool for AD in dogs.

The differences described until now between human and canine mite allergy, have been addressed to answer the question of whether commercial mite extracts used for human diagnostics and immunotherapy are suitable for canine immunotherapy. In this study we calculated the proportion of major allergens for dogs (Der f 15 and Der f 18) with respect to the rest of *D. farinae* proteins, revealing that they were as much represented as other MW proteins. Taking into account our results, we consider that the mite extract characterized in the present study could be a suitable candidate for the diagnosis and immunotherapy of mite allergic dogs from the Mediterranean area although further studies with bigger populations, and different biologically standardized batch extracts would be desirable. Our study also concluded that the presence of allergens such as Der f 1, Der f 2 and other allergens with medium MW in the products for immunotherapy must not be overlooked. Currently, there is no clinical evidence regarding the µg of allergens required for the efficacy of the products. Even in human immunotherapy the researchers or clinicians do not agree on the quantity and concentration of major allergens needed. This issue remains controversial, and for sure these discrepancies will continue until, at least, the use of recombinant allergens.

To conclude, in this study we have characterized a commercial *D. farinae* extract confirming that it contains the major allergens described for dogs. We have also described the immunological characteristics of the canine population sera, finding differences in the levels of IgG1 between atopic and non-atopic dogs, which opens the door for future experiments of immunotherapy and diagnostic tests. In addition, we observed some discrepancies in minor allergens recognition compared to other studies that demonstrate the need to continue the investigation of the mechanisms

of AD development and the role of environmental allergens as important as mite allergens. New challenges for veterinary allergen immunotherapy are now under discussion and further studies with bigger populations and improved allergen extracts will establish the development of new veterinary allergen specific immunotherapy.

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