

Specific *Dermatophagoides farinae* extract for canine immunotherapy

Raquel Moya* , Laura Ramió-Lluch† , Nuria Parody*, Pilar Brazis† , Anna Puigdemont‡ and Jerónimo Carnés* 

*R&D Allergy & Immunology Unit, Laboratorios LETI, S.L, Calle del Sol no. 5, Tres Cantos, Madrid 28760, Spain

†Animal Health B.U, Laboratorios LETI, S.L, Gran Via de les Corts Catalanes 184, Barcelona 08038, Spain

‡Department of Therapeutic Pharmacology and Toxicology, Universitat Autònoma de Barcelona, Edifici M, Bellaterra, Barcelona 08193, Spain

Correspondence: Jerónimo Carnés, Laboratorios LETI, S.L., Calle del Sol no. 5, 28760 Tres Cantos, Madrid, Spain. E-mail: jcarnes@leti.com

Background – Canine atopic dermatitis (cAD) is a pruritic allergic skin disease most often caused by *Dermatophagoides farinae*. Differences in the sensitization profile to *D. farinae* have been reported between people and dogs. However, allergic dogs traditionally have been treated with extracts intended for human immunotherapy.

Hypothesis/Objectives – To develop a specific allergen immunotherapy for veterinary practice enriched in canine major allergens and to demonstrate its *in vitro* efficacy.

Animals – Twenty privately owned dogs, clinically diagnosed with cAD, and three healthy dogs.

Methods and materials – A veterinary *D. farinae* allergen extract was manufactured and characterized compared to *D. farinae* extract used for human immunotherapy. The protein profile was analysed by SDS-PAGE and size exclusion chromatography and Der f 15 and Der f 18 allergens quantified by mass spectrometry. The allergenic profile was studied by immunoblot and the biological potency by enzyme-linked immunosorbent assay-inhibition assays. The extract's capacity to induce cytokine production [interleukin (IL)-10, interferon (IFN)- γ] by peripheral blood mononuclear cells also was evaluated.

Results – The veterinary extract showed a higher content of high molecular weight proteins, preferentially recognized by atopic dog sera. The fold-increases in Der f 15 and Der f 18 with respect to the human extract were 2.07 ± 0.32 and 1.63 ± 0.15 , respectively. The veterinary extract showed higher biological potency (0.062 versus 0.132 μg required for 50% inhibition of dogs sera) compared to the human extract and induced significantly higher levels of IL-10 (1,780 pg/mL) and IFN- γ (50.4 pg/mL) with respect to the negative control.

Conclusions and clinical importance – A veterinary *D. farinae* extract with a higher content of dog major allergens was developed and *in vitro* efficacy demonstrated by immunological parameters.

Introduction

Canine atopic dermatitis (cAD), an inflammatory and pruritic allergic skin disease, is highly prevalent in the field of veterinary medicine.¹ Atopic dogs often present high allergen-specific immunoglobulin (Ig)E levels against environmental allergens such as house dust mites (HDM), which are one of the most important causes of allergic reactions, not only in dogs but also in other species such as people and cats. However, although allergic sensitization of dogs resembles that of people, there are some differences. *Dermatophagoides pteronyssinus* is the main

species involved in human sensitization, whereas *D. farinae* is the most prevalent in dogs.^{2–4} In fact, based on our experience, high levels of *D. farinae*-specific IgE are present in approx. 60% of atopic dogs, and other authors have reported an even higher prevalence (70–90%).^{5,6}

Other differences between human and canine HDM sensitization have been reported regarding specific allergen sensitization. Although low molecular weight (LMW) proteins (Der f 1 and Der f 2) are considered to be major allergens in people,⁷ in dogs there is controversy about the determination of these proteins as major allergens. Historically, these proteins were considered to be minor allergens,^{8,9} although recent studies performed in Japan and European countries suggest greater importance for Der f 1 and Der f 2,^{10–12} and that they are major allergens for dogs.

Apart from this, allergic dogs predominantly react to the high molecular weight (HMW) allergens Der f 15 and Der f 18.^{8,11,13,14} More recently, another HMW protein (Zen-1) also was reported as a dog major allergen.^{12,15}

Despite the differences between people and dogs in HDM sensitization, extracts used for allergen-specific immunotherapy (ASIT) in veterinary medicine usually are

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the same as those used for people. In fact, allergen preparations standardized and manufactured for their specific use in veterinary allergy have been requested for years.^{1,16} Although numerous studies^{8,12–15} have demonstrated the relevance of HMW proteins from mites in dog sensitization, veterinary allergenic extracts have not been characterized by either their canine major allergen content or their biological potency in sensitized dogs. Therefore, the efficacy of those extracts for treatment of cAD may be compromised.¹⁷ In this sense, it seems reasonable to treat veterinary cases with the appropriate allergenic extracts according to their immunological profile. Recently, the European Medicines Agency (EMA) has released draft guidelines to provide and update guidance for allergenic veterinary products that addresses all these questions.¹⁸

The aim of this study was to develop a specific *D. farinae* extract for canine immunotherapy, based on the concept of precision medicine, and characterize it according to its content of major allergens for dogs. The immunological efficacy of this veterinary extract was compared side-by-side with a *D. farinae* extract intended for human immunotherapy, using *in vitro* biological potency assays, mass spectrometry for allergen quantification and measurement of cellular immune responses in order to demonstrate that a veterinary-specific extract is more appropriate for treating cAD in dogs.

Methods and materials

Allergen extract manufacture

Two different *D. farinae* extracts were manufactured in compliance with Good Manufacturing Practices (GMP) according to internal procedures (Laboratorios LETI S.L.U., Madrid, Spain): (i) a conventional allergenic extract for the treatment of human allergic patients (human mite extract; HME), used as control, and (ii) an allergenic extract enriched in mite-specific dog allergens (veterinary mite extract; VME). Briefly, the manufacturing process was as follows. For each extract, 30 g of semipurified *D. farinae* mite bodies ($\geq 80\%$ mite bodies) were processed to extract proteins for 24 h at 4°C in 0.01 M phosphate buffered saline (PBS) (1:20 w/v) with continuous magnetic stirring. Solutions were centrifuged at 4°C for 30 min at 7,000g. Supernatants were collected and dialyzed against double-distilled water using a Pellicon system and ultrafiltration membrane cassettes (Millipore; Bedford, MA, USA) with cut-offs of 3 kDa for HME or 50 kDa for VME. The purpose of using membranes with different pore sizes for the HME and VME manufacturing process was to adapt the allergen composition of the extract to humans or dogs, respectively. Finally, both extract solutions were sterile-filtered, frozen and freeze-dried separately (Figure S1).

Canine serum samples and peripheral blood mononuclear cells (PBMCs)

This study involved client-owned dogs presented to the Universidad Autónoma de Barcelona, Spain. All owners gave informed consent before participation. The study was conducted in accordance with Universidad Autónoma de Barcelona Institutional Animal Care and Use Committee requirements, and safe blood sample volumes were obtained following their recommendations.¹⁹ The study also involved healthy dogs from Isoquimen S.L. (Barcelona, Spain) and a protocol was approved by its Ethics Committee.

Serum samples were collected from 16 animals selected for the study based on the following inclusion criteria: (i) dogs of any sex and breed older than 12 months of age, with a clinical diagnosis of non-seasonal cAD using published criteria;²⁰ and (ii) a positive ELISA test from Greer Laboratories (Lenoir, NC, USA) for detection of specific

IgE (sIgE) against *D. farinae*, with ELISA absorbance units (EAU) $> 1,000$.

Animals were excluded from the study based on: (i) pregnancy or lactation; (ii) flea infestation or known food allergy; (iii) presence of bacterial or *Malassezia* spp. skin infections or other infestations causing pruritus (e.g. fleas or scabies); and (iv) having been treated with glucocorticoids or oral corticosteroids within four weeks of enrolment and/or injectable glucocorticoids within eight weeks of enrolment.

PBMCs were obtained from four atopic animals fulfilling the above inclusion and exclusion criteria. In addition, PBMCs were obtained from three healthy controls with no dermatological lesions and showing < 50 EAU against *D. farinae* (Isoquimen S.L.). For this purpose, 20 mL of blood were obtained by venepuncture from the animals and kept in lithium heparin tubes at 4–25°C until PBMC isolation.

Characterization of extracts

Protein profile. Gel electrophoresis and size exclusion chromatography (SEC) analyses were performed to investigate the protein profile and molecular weight (MW) of the individual proteins present in the extracts. Samples of HME and VME containing 10 µg protein were separated by SDS-PAGE with reducing conditions in 2.67% C-15% T acrylamide:bis-acrylamide gels according to standard procedures and stained with Coomassie Brilliant Blue R-250.

As a complementary method, HME and VME protein profile and MW distribution also were determined by SEC using a Bio SEC-3 Column (Agilent Technologies; Santa Clara, CA, USA) on a 1260 series HPLC system (Agilent Technologies). Twenty microlitres of each sample were loaded onto the column and run at 1 mL/min in 150 mM phosphate buffer (pH 7.0). Detection was performed at UV–215 nm and Gel Filtration Standard (BioRad Laboratories; Hercules, CA, USA) was used as MW marker.

Allergen identification. Relevant allergens were detected and identified in VME by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). Briefly, bands corresponding to Der f 15 (~119/96 kDa) and Der f 18 (~57 kDa) in VME were cut from a SDS-PAGE gel (Coomassie stained), digested with trypsin, and the resultant peptide mixture was analysed in a nanoAcquity LC system (Waters Corporation; Milford, MA, USA) coupled to an LTQ-Orbitrap Velos (Thermo Scientific; Waltham, MA, USA) mass spectrometer. Raw data were collected with THERMOXALIBUR software (Thermo Scientific), and a database search was performed through the Mascot search engine using Thermo Proteome Discover against the Uniprot database.

Allergen relative quantification. Der f 15 and Der f 18 were quantified in three VME batches with respect to HME by MS. In short, VME and HME samples were digested individually with the endoproteinase Lys-C followed by trypsin. The resulting peptide mixtures were analysed by a nanoAcquity LC system (Waters Corporation) coupled to a LTQ Orbitrap Velos (Thermo Scientific) mass spectrometer. Raw data were collected with THERMOXALIBUR software (Thermo Scientific). A database search was performed by Sequest HT engine using Thermo Proteome Discover against the SwissProt Arthropoda database containing the two allergens of interest (Q9U6R7 = Der f 15; Q86R84 = Der f 18).

A targeted MS/MS method containing the m/z and retention times for the peptides selected for both allergens (Table 1) was performed. Three peptides from Der f 15 and one from Der f 18 were selected and synthesized as heavy labelled peptides to be used as internal standards in the quantification assay. Quantitative analysis of the targeted MS/MS data was performed using SKYLINE, open source software from MacCoss Lab (Department of Genome Science, University of Washington, Seattle, WA, USA). Peak areas of each peptide were normalized relative to the heavy labelled peptides. Query samples (three different batches) from VME were compared against the control sample (HME), and statistically significant differences were assayed using a Student's *t*-test with 95% confidence at the protein level. Each sample was analysed in three technical replicates.

Allergenic profile. Western blot was used to identify the IgE reactivity of the proteins, an indirect measure of the presence of allergens in the extracts. Briefly, 10 µg protein/extract sample were run in an SDS-PAGE under reducing conditions. Separated bands were

Table 1. List of the peptide m/z acquired for relative quantification of Der f 15 (Uniprot Q9U6R7) and Der f 18 (Uniprot Q86R84)

Protein	m/z	Peptide m/z	Sequence
Der f 15	539.277	1076.539	TIEDIDPFK
Der f 15	543.284	1084.553	TIEDIDPFK*
Der f 15	776.383	1550.750	VDPYTIEDIDPFK
Der f 15	780.390	1558.765	VDPYTIEDIDPFK*
Der f 15	813.895	1625.776	IWVGYYDLASISCK
Der f 15	817.902	1633.790	IWVGYYDLASISCK*
Der f 18	562.589	1684.746	TVCYYESVWHWR
Der f 18	565.925	1694.754	TVCYYESVWHWR*
Der f 18	554.226	1106.438	TVCYYESW
Der f 18	679.773	1357.532	TYCATYYECK
Der f 18	683.780	1365.546	TYCATYYECK*
Der f 18	954.921	1907.828	MDPEDIDTSLCTHIVY
Der f 18	962.919	1923.823	MDPEDIDTSLCTHIVY [†]
Der f 18	401.210	1200.630	FGIDAATHEIK
Der f 18	527.780	1053.560	GIDAATHEIK
Der f 18	682.840	1363.680	YFGIDAATHEIK
Der f 18	726.362	1450.709	SYFGIDAATHEIK
Der f 18	510.900	1529.678	TLSNEDVHVGCGDK
Der f 18	765.846	1529.678	TLSNEDVHVGCGDK
Der f 18	815.384	1628.754	AMIAVGGSTMSDQFSK
Der f 18	823.382	1644.749	AMIAVGGSTMSDQFSK [†]

*Heavy label peptide.

[†]Oxidized methionine peptide.

electrotransferred onto PVDF membranes by a Trans-Blot Turbo Transfer Pack (Bio-Rad). Membranes were blocked with 5% skimmed milk in 0.01 M PBS with 0.1% Tween (PBS-T) for 1 h, and incubated overnight with a pool of sera from atopic dogs (diluted 1:4 in 0.01 M PBS). After washing, membranes were incubated with HRP-conjugated goat anti-dog IgE (1:10,000 dilution; AbD Serotec, Bio-Rad). Finally, membranes were developed with luminol solution (Clarity Western ECL Substrate, Bio-Rad) and detected by chemiluminescence (Amersham Imager 600 RGB system, GE Healthcare; Uppsala, Sweden). Der f 15 and Der f 18 band intensities were determined using IMAGE QUANT TL 2005 software (GE Healthcare).

IgE reactivity of serum samples. Serum reactivities of atopic dogs ($n = 16$) against VME and HME were studied by analysing their IgE binding capacity by direct enzyme-linked immunosorbent assay (ELISA) assays. Briefly, microplates (Immulon IV; Thermo Scientific) were coated with VME or HME (8 µg protein/mL) and incubated overnight at room temperature. Plates were blocked for 1 h with 5% skimmed milk in PBS-T. Serum samples were diluted 1:4 in 5% skimmed milk in PBS-T, incubated for 2 h and washed with PBS-T, and then secondary antibody (HRP-conjugated goat anti-dog IgE; 1:10,000 dilution) was added. Finally, the microplates were washed, the reaction developed with chromogen, stopped with sulfuric acid and optical density (OD) at 450 nm was measured on an automated ELISA plate reader. OD values obtained for HME or VME were analysed statistically by PRISM 7 software (GraphPad Software; La Jolla, CA, USA).

Biological potency. The biological potencies of the VME and HME were compared by ELISA inhibition assay, using a pool of sera (containing equal volumes of each individual serum sample from above). Briefly, microplates (Immulon IV; Thermo Scientific) were coated with VME (2 µg/well) and incubated overnight at room temperature, then blocked for 1 h with 5% skimmed milk in PBS-T. The pool of sera was preincubated with serial dilutions of inhibitory extract (VME or HME) in a Nunc plate (Thermo Scientific) for 2 h. Then, the “inhibited” pool of sera was added to the Immulon IV plate and incubated for 2 h, washed with PBS-T, and secondary antibody (HRP-conjugated goat anti-dog IgE; 1:10,000 dilution) was added and incubated for 2 h. Finally, microplates were developed as described above. The percentage of sera inhibition was calculated relative to the log µg of extract to determine the 50% inhibitory dose; lower values of 50% inhibitory dose corresponded to a higher biological potency of the extract.

PBMC cytokine production. The PBMCs were isolated from the blood of atopic and healthy dogs using a Ficoll gradient. Briefly, whole blood was poured onto Ficoll solution and then centrifuged for 35 min at 1,800g. The white PBMC layer was collected and washed with PBS containing 5% fetal bovine serum (FBS). After erythrocyte lysis, PBMCs were washed and resuspended in RPMI containing 10% FBS. Finally, PBMCs were plated at a final concentration of 2.5×10^6 cells/mL and incubated in triplicate with VME or HME (40 µg protein/mL), Concanavalin A (Con A, 3 µg/mL) or lipopolysaccharide (LPS, 3 µg/mL) at 37°C in a 5% CO₂ atmosphere. Culture supernatants were recovered after 24 h and 48 h of incubation for cytokine determination. Supernatants from nonstimulated cells were used as negative control, and supernatants from cells stimulated with Con A or LPS were used as positive controls.

The cytokines interferon (IFN)-γ and interleukin (IL)-10 were measured in supernatants using a ELISA-based Milliplex Mag Dog kit (Merck Millipore; Darmstadt, Germany), performed in accordance with the manufacturer's instructions. Results were analysed statistically by PRISM 7 software (GraphPad).

Consistency between batches

Three VME batches manufactured from different raw materials were characterized and compared using SDS-PAGE, SEC, Der f 15 and Der f 18 MS identification and biological potency assays as above.

Results

Characterization of *Dermatophagoides farinae* extracts

The protein profile of VME (Figure 1a) predominantly showed bands >40 kDa, different from HME which presented bands throughout the whole range analysed (10–100 kDa). Expected bands were found including Der f 15 and Der f 18, identified in a previous study¹¹ as bands of 119/96 and 57 kDa, respectively, as well as allergens Der f 1 and Der f 2. Figure 1b shows the allergenic profile obtained with the pool of sera from cAD dogs against the two extracts. The dog sera had a greater recognition of the HMW bands, and a higher intensity reaction with VME. The Der f 15 and Der f 18 band intensities were 2.3- and 2.5-fold greater in VME compared to HME, respectively (Figure 1c).

The difference in MW distribution of the protein content in the two extracts also was demonstrated by SEC (Figure 2). VME presented >50% of the chromatographic area before 10 min of elution, corresponding to proteins between 40 and 1,500 kDa, whereas HME had >50% of the chromatographic area after 10 min, which corresponds to proteins <40 kDa.

Mass spectrometry analyses

Mass spectrometry analysis of the bands corresponding to major allergens Der f 15 and Der f 18 in VME confirmed the presence of these allergens. Twenty different peptide sequences were identified for the 96 and 119 kDa bands (corresponding to Der f 15), covering 37% of the sequence, and 17 peptide sequences were obtained for the 57 kDa band (corresponding to Der f 18), covering 32% of the sequence.

Relative quantification analysis revealed a 2.07 ± 0.32 - and 1.63 ± 0.15 -fold increase of the HMW allergens Der f 15 and Der f 18, respectively, in VME batches relative to HME. Table 2 shows the fold-change at the protein level for both allergens when comparing query and control samples.

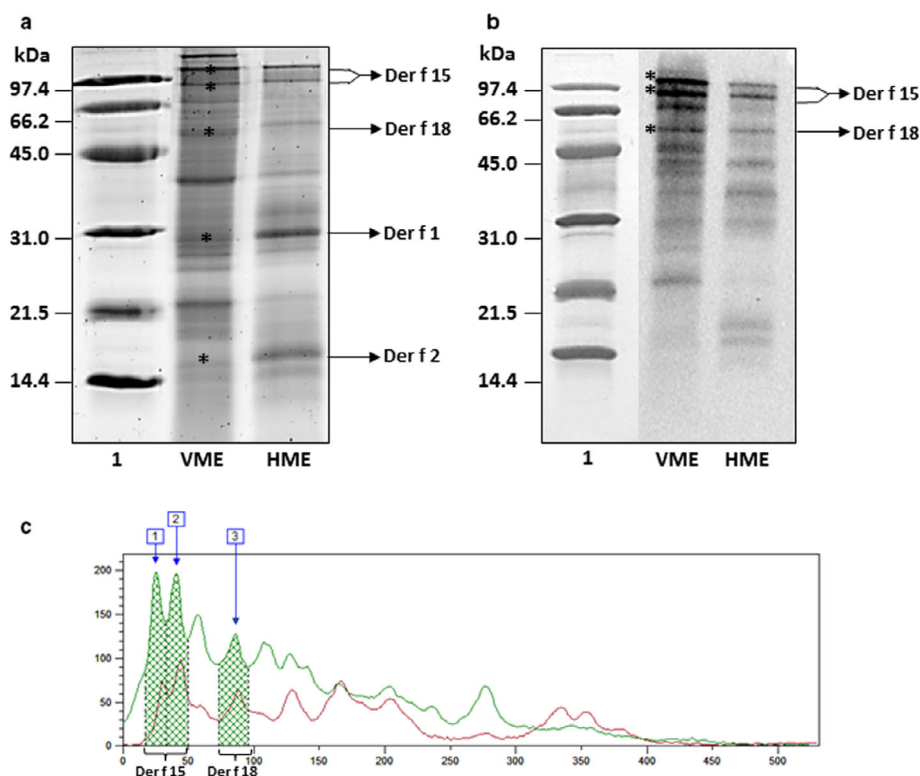


Figure 1. Protein profile (a) and immunoblot (b) of *Dermatophagoides farinae* extracts in reducing conditions.

Low Range Molecular Weight Standard (lane 1), veterinary mite extract (VME, 10 µg protein) and human mite extract (HME; 10 µg protein). Immunoblots were performed using a pool of sera from atopic dogs. (c) Protein profile determined by scanning densitometry comparing VME (green line) and HME (red line). Der f 15 (bands numbers 1 and 2), Der f 18 (band number 3), Der f 1 and Der f 2 are highlighted.

Biological potency

The individual levels of sIgE were significantly ($P < 0.05$) higher for VME than those observed for HME (Figure 3a), confirming that VME contained a higher concentration of the most relevant allergens to which allergic dogs are sensitized (Der f 15 and Der f 18).

This higher reactivity of atopic dog sera with VME was confirmed by a biological potency assay using pooled sera (Figure 3b). The amount of HME necessary to achieve 50% inhibition was 0.132 µg, 2.13-fold higher than that of VME (0.062 µg). These results confirmed that VME was approximately two times more potent than HME in terms of allergenic potency.

Cellular studies

When PBMCs were incubated with VME and HME, a similar induction of cytokines IFN-γ and IL-10 was observed in both atopic and control dogs (Figure 4). After 24 h, HME and VME induced significantly higher ($P < 0.01$) levels of IL-10 than the negative control [median (IQ) of 1,170 (559–1,502) pg/mL for HME and 1,780 (1,122–1,998) pg/mL for VME]. After 48 h of incubation, IFN-γ also was significantly higher ($P < 0.01$) than the negative control in cells from atopic dogs treated with HME [52.1 (15–113.2) pg/mL] and VME [50.4 (20–76.3) pg/mL].

Results from positive controls (ConA and LPS) were in all cases significantly higher ($P < 0.01$) than the negative control (data not shown). Atopic and nonatopic dogs also were compared. Higher levels of both cytokines were

observed in nonatopic dogs with HME and VME stimulation than in atopic dogs.

Consistency batches

There was a high consistency in the manufacturing process between the three different batches of VME. An identical protein profile was observed by SDS-PAGE (Figure S2). Der f 15 and Der f 18 were identified in all three batches by MS analysis, with sequence coverage percentages of 16–56% for Der f 15 and 18–28% for Der f 18 (Figure S3). Regarding biological potency, 50% inhibitory doses of 0.077, 0.063 and 0.056 µg were obtained for the three VME consistency batches, respectively (Figure S4).

Discussion

The efficacy of ASIT for treatment of some allergic diseases has been clearly documented in people,^{21,22} whereas in dogs cAD caused by allergic reactions usually is treated with alternative treatments such as glucocorticoids, cyclosporine, oclacitinib or biologicals. However, these treatments have shown several limitations in terms of adverse effects and cost, in only targeting disease clinical signs and in being unable to modify or reverse disease course. For these reasons, ASIT is a promising alternative for control of cAD. Different ASIT studies in dogs using conventional human allergenic extracts, most with an open and uncontrolled design, have demonstrated success rates of 50–80%.^{1,23} Nevertheless, many questions

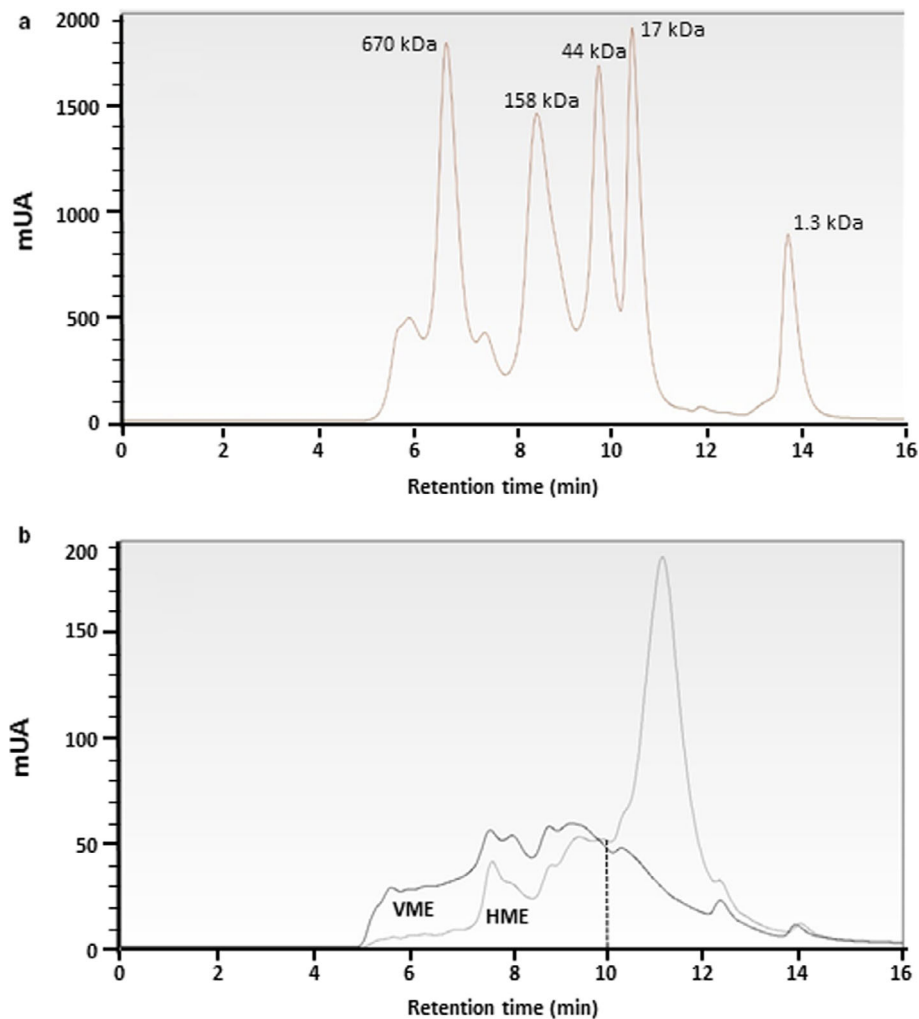


Figure 2. Size exclusion chromatograms of molecular weights of proteins in mite extracts. (a) Gel Filtration Standard. (b) Comparison of veterinary mite extract (VME; black line) and human mite extract (HME, grey line) chromatograms detected at 215 nm. Elution times given in minutes.

Table 2. Skyline output for differential abundance test at the protein level for Der f 15 and Der f 18 allergens when comparing veterinary mite extract (VME) samples against the control sample (human mite extract, HME)

Protein	Sample	Fold-change result	Adjusted <i>P</i> -value
Der f 15	VME 1	1.7 (95% CI: 1.57–1.85)	0.0001
	VME 2	2.3 (95% CI: 1.94–2.6)	0.0001
	VME 3	2.2 (95% CI: 1.9–2.6)	0.0001
Der f 18	VME 1	1.6 (95% CI: 1.37–2.28)	0.0203
	VME 2	1.8 (95% CI: 1.37–2.43)	0.0042
	VME 3	1.5 (95% CI: 1.05–2.10)	0.0335

remain about the efficacy of ASIT in dogs, and thus there is a need for specific, precise and standardized extracts for use in veterinary allergy and dose-optimization studies.

The efficacy of ASIT has been correlated with optimal amounts of the major allergens in immunotherapy extracts. However, traditionally dogs have been treated with human immunotherapy without taking into consideration compatibility between allergen composition and the species' sensitization profile. Regarding mites, the recognition profiles of humans and dogs have been reported to be different. Dogs mainly recognize allergens

corresponding to HMW proteins, such as Der f 15, Der f 18 and Zen-1,^{13–15} whereas major allergens for humans belong to LMW proteins, groups 1 and 2. In this sense, Der f 15 and Der f 18 may be poorly represented in human allergen extracts as their characterization is focused mainly on allergens from groups 1 and 2.

In a previous study, the presence of Der f 15 and Der f 18 allergens in a *D. farinae* extract used for human immunotherapy was demonstrated.¹¹ Based on this extract, we developed and patented²⁴ [WO/2019/025388] a new technology for producing a specific veterinary extract (VME) containing the appropriate composition of mite allergens for treating atopic dogs. To the best of our knowledge, this is the first time that an allergenic extract has been manufactured and characterized specifically for use in veterinary allergy treatment.

Design of the manufacturing process was based on reduction of LMW proteins while increasing those proteins to >40 kDa, thereby enriching it with allergens from groups 15 and 18. This relative modification in the MW distribution was confirmed by different *in vitro* assays. Additionally, based on MS tools, we developed a specific method for measuring Der f 15 and Der f 18 allergens, consisting of a relative estimation of the allergens in

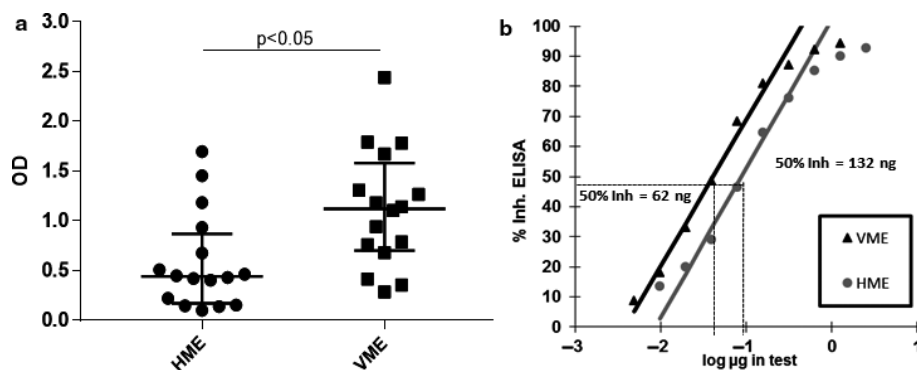


Figure 3. (a) Optical densities (OD) of specific immunoglobulin (IgE) levels in individual serum samples against human mite extract (HME) and veterinary mite extract (VME).

The graph shows median values with interquartile range. (b) Enzyme-linked immunosorbent assay (ELISA) for VME and HME biological potency determination; 50% Inh 50% inhibition.

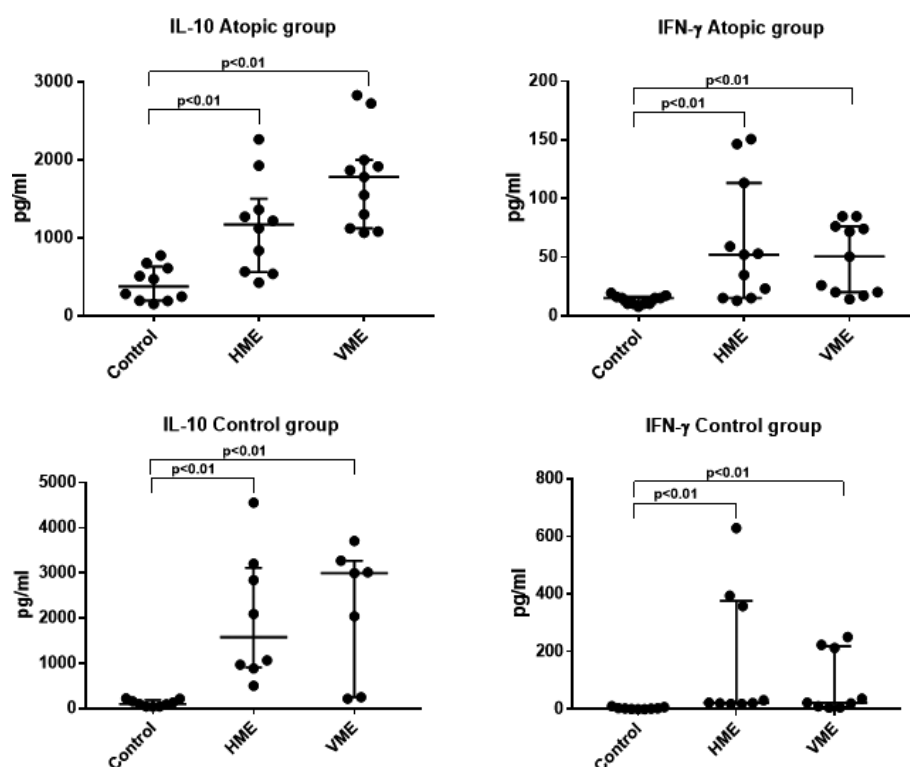


Figure 4. Interleukin (IL)-10 and interferon (IFN)- γ produced by peripheral blood mononuclear cells (PBMCs) from atopic and control groups after 24 h (IL-10) or 48 h (IFN- γ) of incubation with negative control, human mite extract (HME) or veterinary mite extract (VME).

Statistically significant differences (P -values) are indicated in each graph showing median value and interquartile range.

VME. The major human allergens (Der f 1 and Der f 2) usually are characterized by commercially available ELISA kits, yet such reagents unfortunately are unavailable for the major dog allergens. This is the first time that the content of *D. farinae* major allergens for dogs has been studied and quantified relative to HME.

Biological IgE potency is considered the main parameter for allergen extract characterization,²⁵ and thus a method for calculating the biological potency of VME using serum samples from allergic dogs was developed. All individual serum samples showed significantly higher sIgE against VME compared to HME, consistent with a higher content of allergens causing dog sensitization. This

was further demonstrated by biological potency determination using an ELISA inhibition assay.

Immunological efficacy of allergenic extracts and the mechanisms of action of immunotherapy in dogs remain poorly understood, although some results can be extrapolated from results reported for people. In both species, successful ASIT has been associated with an induction of regulatory T (Treg) cells and a shift from a Th2 to a Th1 response.^{23,26} Indirectly, the success of immunotherapy also has been related to the stimulation of certain cytokines such as IFN- γ and IL-10,²⁷ involved in Th1 and Treg responses, respectively, and a reduction of the Th2 response mediated by IL-4. In a study of 10 cAD cases

treated with HDM immunotherapy, the IFN- γ :IL-4 ratio increased significantly after ASIT.²⁸ In another study, atopic dogs receiving ASIT of different allergens presented higher Treg counts and serum IL-10 concentrations compared to the healthy control group.²⁹ In this study, we investigated the capacity of VME to induce IL-10 and IFN- γ in PBMCs from dogs sensitized to *D. farinae*, suggesting an induction of Th1 and Treg responses and, therefore, a beneficial immune response that could lead to tolerance in dogs treated with this extract. Similar results have been obtained with human PBMCs.^{30,31} Other studies in veterinary allergology have examined the effect of mite extracts and purified allergens on canine PBMC proliferation,³² or the effect of mite allergoids in canine dendritic cell uptake and cytokine production.³³ Although no statistically significant differences were found in the production of IL-10 between VME and HME, a tendency for higher production was observed with VME. The number of dogs included in this study was too low to confirm these results. For this reason, new cellular studies with increased numbers of animals are necessary in addition to *in vivo* clinical studies to demonstrate the improved efficacy of this veterinary extract.

A critically important issue when producing allergenic extracts for immunotherapy is consistency between batches, in order to guarantee the same allergen profile, allergen content and biological potency, as outlined in the recent draft guideline for "Use of veterinary extracts for ASIT".¹⁸ This guideline aims to provide principles and guidance for the manufacturing and quality control, safety and efficacy of allergen products for veterinary purpose. It is important to highlight that although this specific extract for cAD treatment was developed before the EMA document, the product is fully adapted to the EMA recommendations.

Additionally, in a recent study³⁴ evaluating allergen extracts used in veterinary settings, heterogeneity was demonstrated in the protein components of *D. farinae* extracts not only between manufacturers, but also within different batches from the same manufacturer. Our VME showed a high degree of consistency between different batches, and these studies have allowed us to select one as the in-house reference preparation (IHRP), to be used as a reference for control of active substances and intermediates in successive lots.

In summary, we developed a *D. farinae* extract enriched in HMW proteins according to the particular allergenic profile of dogs. All subsequent analysis performed in the veterinary extract, including major allergen (Der f 15 and Der f 18) composition and quantification, biological potency and induction of regulatory and Th1 cytokines, confirmed the suitability of the new VME for treating dogs with mite allergies and its improved efficacy with respect to the conventional HME. VME may be classified as a precision veterinary medicine because it contains the specific active principle related to allergic disease of dogs. This study opens up new possibilities for the identification of specific allergens for dogs and for the development of specific immunotherapy for other allergenic sources.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1. Scheme of the manufacturing process of VME and HME, methods used for extract characterization and main results obtained.

Figure S2. SDS-PAGE of VME showing batch consistency under reducing conditions.

Figure S3. MS results of major allergen identification in VME consistency batches.

Figure S4. ELISA inhibition assays comparing biological potency of VME (Standard) and VME consistency batches.

Résumé

Contexte – La dermatite atopique canine (CAD) est une maladie cutanée allergique prurigineuse souvent causée par *Dermatophagoides farinae*. Des différences dans le profil de sensibilisation à *D. farinae* ont été rapportées entre le chien et l'homme. Cependant, les chiens allergiques ont été traditionnellement traités avec des extraits conçus pour immunothérapie de humaine.

Hypothèses/Objectifs – Développer une immunothérapie spécifique d'allergènes vétérinaire enrichie en allergènes majeurs du chien et démontrer son efficacité in vitro.

Sujets – Vingt chiens de propriétaires diagnostiqués atopiques et trois chiens sains.

Matériels et méthodes – Un extrait allergénique vétérinaire de *D. farinae* a été conçu et caractérisé en comparaison avec l'extrait de *D. farinae* utilisé pour l'immunothérapie humaine. Le profil protéique a été analysé par SDS-PAGE et chromatographie d'exclusion de taille et les allergènes Der f 15 et Der f 18 quantifiés par spectrométrie de masse. Le profil allergénique a été étudié par immunoblot et le potentiel biologique par ELISA. La capacité des extraits à induire la production de cytokine [interleukine (IL)-10, interféron (IFN)- γ] par les cellules mononucléées sanguines périphériques a aussi été évaluée.

Résultats – L'extrait vétérinaire a montré un contenu de protéines à haut poids moléculaire plus élevé, préférentiellement reconnu par les séras de chiens atopiques. L'augmentation de Der f 15 et Der f 18 était respectivement multipliée par 2.07 ± 0.32 et 1.63 ± 0.15 comparé aux extraits de l'homme. L'extrait vétérinaire a montré un potentiel biologique plus élevé (0.062 versus 0.132 μ g nécessaire pour 50% d'inhibition des séras de chien) comparé à l'extrait humain et induit des taux d'IL-10 (1780 pg/mL) et IFN- γ (50.4 pg/mL) significativement plus élevés en comparaison avec les contrôles négatifs.

Conclusions et importance clinique – Un extrait vétérinaire de *D. farinae* avec un contenu plus élevé d'allergènes majeurs a été développé et une efficacité in vitro a été démontrée par paramètres immunologiques.

RESUMEN

Introducción – la dermatitis atópica canina (CAD) es una enfermedad cutánea alérgica pruriginosa causada con mayor frecuencia por *Dermatophagoides farinae*. Se han publicado diferencias en el perfil de sensibilización a *D. farinae* entre personas y perros. Sin embargo, los perros alérgicos tradicionalmente han sido tratados con extractos destinados a la inmunoterapia humana.

Hipótesis/Objetivos – Desarrollar una inmunoterapia con alérgenos específicos para la práctica veterinaria enriquecida en los principales alérgenos caninos y demostrar su eficacia *in vitro*.

Animales – veinte perros de propietarios privados, diagnosticados clínicamente con CAD y tres perros sanos.

Métodos y materiales – se fabricó y caracterizó un extracto de alérgeno veterinario de *D. farinae* en comparación con el extracto de *D. farinae* utilizado para inmunoterapia humana. El perfil de proteínas se analizó mediante SDS-PAGE y cromatografía de exclusión por tamaño y los alérgenos Der f 15 y Der f 18 se cuantificaron mediante espectrometría de masas. El perfil alergénico se estudió mediante inmunotransferencia y la potencia biológica mediante ensayos de inhibición por ensayo inmunoabsorbente ligado a enzimas. También se evaluó la capacidad del extracto para inducir la producción de citoquinas [interleuquina (IL)-10, interferón (IFN)- γ] por células mononucleares de sangre periférica.

Resultados – El extracto veterinario mostró un mayor contenido de proteínas de alto peso molecular, reconocidas preferentemente por sueros de perros atópicos. Los factores de aumento en Der f 15 y Der f 18 con respecto al extracto humano fueron $2,07 \pm 0,32$ y $1,63 \pm 0,15$, respectivamente. El extracto veterinario mostró una mayor potencia biológica (0,062 versus 0,132 μg requeridos para una inhibición del 50% de los sueros de perros) en comparación con el extracto humano e indujo niveles significativamente más altos de IL-10 (1780 pg/mL) e IFN- γ (50,4 pg/mL) con respecto al control negativo.

Conclusiones e importancia clínica – Se desarrolló un extracto veterinario de *D. farinae* con un mayor contenido de los principales alérgenos caninos y se demostró la eficacia *in vitro* mediante parámetros inmunológicos.

Zusammenfassung

Hintergrund – Die atopische Dermatitis des Hundes (CAD) ist eine juckende allergische Hauterkrankung, die am häufigsten durch *Dermatophagoides farinae* verursacht wird. Unterschiede zwischen Mensch und Hund im Sensibilisierungsprofil durch *D. farinae* wurden bereits beschrieben. Nichtsdestotrotz wurden Hunde traditionellerweise mit Extrakten behandelt, die für die Immuntherapie des Menschen gedacht waren.

Hypothese/Ziele – Die Entwicklung einer spezifischen Allergenimmuntherapie für die veterinärmedizinische Praxis, die durch wichtige Allergene bereichert wird und die Demonstration ihrer Wirksamkeit *in vitro*.

Tiere – Zwanzig Hunde in Privatbesitz, die klinisch mit einer CAD diagnostiziert worden waren und drei gesunde Hunde.

Methoden und Materialien – Es wurde ein veterinärmedizinischer *D. farinae* Allergenextrakt hergestellt und im Vergleich zu einem *D. farinae* Extrakt, der in der Humanmedizin zur Immuntherapie Verwendung findet, beschrieben. Das Proteinprofil wurde mittels SDS-PAGE und Größenausschlusschromatografie analysiert und Der f 15 und Der f 18 Allergene mittels Massenspektrometrie quantifiziert. Das Allergenprofil wurde mittels Immunoblot und die biologische Potenz mittels Enzym-linked Immunosorbent Assay-Inhibitionsassays untersucht. Die Fähigkeit des Extraktes eine Zytokinproduktion [Interleukin (IL)-10, Interferon (IFN)- γ] zu induzieren wurde mittels peripherer mononukleärer Zellen des Blutes ebenfalls evaluiert.

Ergebnisse – Der veterinärmedizinische Extrakt zeigte einen höheren Gehalt an hochmolekularen Proteinen, welche vorzugsweise durch atopische Sera von Hunden erkannt wurden. Das Ausmaß der Zunahme von Der f 15 und Der f 18 in Bezug auf den humanen Extrakt betrug das $2,07 \pm 0,32$ bzw. das $1,63 \pm 0,15$ fache. Der veterinärmedizinische Extrakt zeigte im Vergleich zum Humanextrakt eine höhere biologische Potenz (0,062 versus 0,132 μg waren nötig um eine 50%ige Inhibition der Hunde Sera zu erlangen) und induzierte signifikant höhere Werte an IL-10 (1789 pg/mL) und IFN- γ (50,4 pg/mL) im Vergleich zur Negativkontrolle.

Schlussfolgerungen und klinische Bedeutung – Es wurde ein veterinärmedizinischer *D. farinae* Extrakt mit einem höheren Gehalt an caninen hochmolekularen Allergenen und seine Wirksamkeit *in vitro* mittels immunologischer Parameter demonstriert.

要約

背景 – 犬アトピー性皮膚炎 (CAD) は、しばしば *Dermatophagoides farinae* が原因となる掻痒性アレルギー性皮膚疾患である。 *D. farinae* に対する感作プロファイルの違いは、人と犬の間で報告されている。しかしながら、アレルギー犬は伝統的にヒト免疫療法を目的とした抽出物で治療されてきた。

仮説/目的 – 本研究の目的は、犬の主要アレルゲンが豊富な獣医療向け特定アレルゲン免疫療法を開発し、*in vitro* 効果を実証することであった。

供試動物 – 臨床的にCADと診断された20頭の私有犬および3頭の健常犬。

材料と方法 – 獣医療向けD. farinaeアレルゲン抽出物を製造し、ヒト免疫療法に使用されるD. farinae抽出物と比較して特徴付けられた。タンパク質プロファイルはSDS-PAGEおよびサイズ排除クロマトグラフィー法で分析し、Der f 15およびDer f 18アレルゲンを質量分析法で定量した。アレルゲンプロファイルは、イムノブロット法および酵素結合免疫吸着アッセイ阻害アッセイ法による生物学的効力によって研究された。末梢血単核球によるサイトカイン産生[インターロイキン (IL) -10、インターフェロン (IFN) - γ]を誘導する抽出物の能力も評価された。

結果 – 獣医療向け抽出物は高分子量タンパク質の含有量が高く、アトピー犬の血清によって優先的に認識された。ヒト抽出物に対するDer f 15およびDer f 18の倍率増加は、それぞれ 2.07 ± 0.32 および 1.63 ± 0.15 であった。獣医診療向け抽出物は、ヒト抽出物と比較してより高い生物学的効力（犬血清の50%阻害に必要な $0.062 \mu\text{g}$ 対 $0.132 \mu\text{g}$ ）を示し、ネガティブコントロールと比較して有意に高いIL-10 (1780 pg / mL) およびIFN- γ (50.4 pg / mL) を誘導した。

結論と臨床的重要性 – 犬の主要アレルゲン含有量が高い獣医療向けD. farinae抽出物が開発され、in vitroでの有効性が免疫学的パラメーターによって実証された。

摘要

背景 – 犬特応性皮炎 (CAD) は一種の痒痒過敏性皮膚病、通常由粉塵蟎 (*Dermatophagoides farinae*) 引起。据报道, 人和犬对粉塵蟎的敏感特征存在差异。然而传统上, 过敏犬用人类免疫治疗的提取物进行治疗。

假设/目标 – 开发兽医临床中的特异性过敏原免疫疗法, 富集犬主要过敏原, 并证明其体外疗效。

动物 – 临床诊断为CAD的20只私家犬, 以及三只健康犬。

方法和材料 – 生产兽医用粉塵蟎过敏原提取物, 并与用于人免疫治疗的粉塵蟎提取物进行特征比较。通过SDS-PAGE和分子排阻色谱法分析蛋白图谱, 并通过质谱法定量测定Der f 15和Der f 18过敏原。通过免疫印迹研究过敏原特征, 通过酶联免疫吸附试验-抑制试验研究生物效价。还评价了提取物诱导外周血单核细胞产生细胞因子[白细胞介素(IL)-10、干扰素(IFN)- α]的能力。

结果 – 兽医用提取物显示高分子量蛋白质含量较高, 可优先得到特异性犬血清的识别。Der f 15 和 Der f 18 的折叠增加值与人用提取物的分别是 2.07 ± 0.32 和 1.63 ± 0.15 。与人用提取物相比, 兽医用提取物显示出较高的生物效力 (0.062 对 $0.132 \mu\text{g}$, 达到犬血清50%抑制), 并且, 与阴性控制相比, 诱导IL-10 (1780 pg/mL) 和 IFN- γ (50.4 pg/mL) 水平显著提高。

结论和临床重要性 – 开发兽医用粉塵蟎提取物, 其中犬主要过敏原含量较高, 并通过免疫学参数证明其体外功效。

Resumo

Contexto – A dermatite atópica canina (DAC) é uma doença cutânea alérgica pruriginosa, mais frequentemente causada por *Dermatophagoides farinae*. Diferenças no perfil de sensibilização a *D. farinae* foram relatadas entre pessoas e cães. No entanto, os cães alérgicos tradicionalmente têm sido tratados com extratos destinados à imunoterapia humana.

Hipótese/Objetivos – Desenvolver uma imunoterapia alérgeno-específica para a prática veterinária formulada com os principais alérgenos caninos e demonstrar sua eficácia *in vitro*.

Animais – Vinte cães de propriedade privada, com diagnóstico clínico de DAC, e três cães saudáveis.

Métodos e materiais – O extrato de alérgeno veterinário de *D. farinae* foi fabricado e caracterizado em comparação com o extrato de *D. farinae* usado para imunoterapia humana. O perfil proteico foi analisado por SDS-PAGE e cromatografia de exclusão por tamanho e os alérgenos Der f 15 e Der f 18 quantificados por espectrometria de massa. O perfil alérgico foi estudado por *immunoblot* e a potência biológica por ensaio de imunoabsorção enzimática. A capacidade do extrato de induzir a produção de citocinas [interleucina (IL) -10, interferon (IFN) - γ] por células mononucleares do sangue periférico também foi avaliada.

Resultados – O extrato veterinário apresentou maior teor de proteínas de alto peso molecular, preferencialmente reconhecidas por soros de cães atópicos. Os aumentos de Der f 15 e Der f 18 em relação ao extrato humano foram $2,07 \pm 0,32$ e $1,63 \pm 0,15$, respectivamente. O extrato veterinário mostrou maior potência biológica ($0,062$ versus $0,132 \mu\text{g}$ necessário para 50% de inibição de soros de cães) em comparação com o extrato humano e induziu níveis significativamente mais elevados de IL-10 (1780 pg/mL) e IFN- γ ($50,4 \text{ pg/mL}$) com relação ao controle negativo.

Conclusões e importância clínica – Um extrato alérgico veterinário de *D. farinae* com uma maior quantidade dos principais alérgenos para cães foi desenvolvido e a eficácia *in vitro* demonstrada por parâmetros imunológicos.

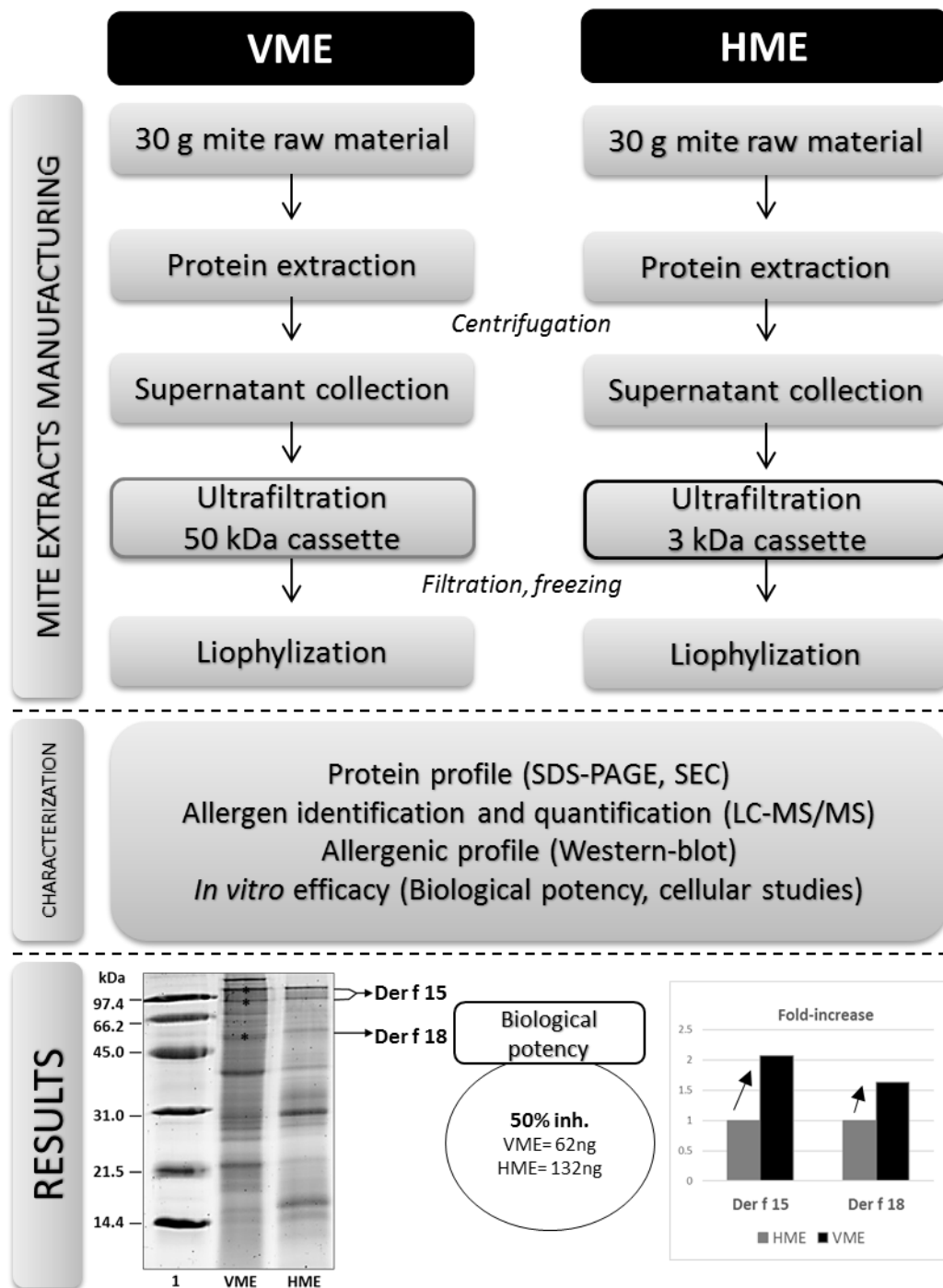


Figure S1. Scheme of the manufacturing process of VME and HME, methods used for extracts characterization and main results obtained.

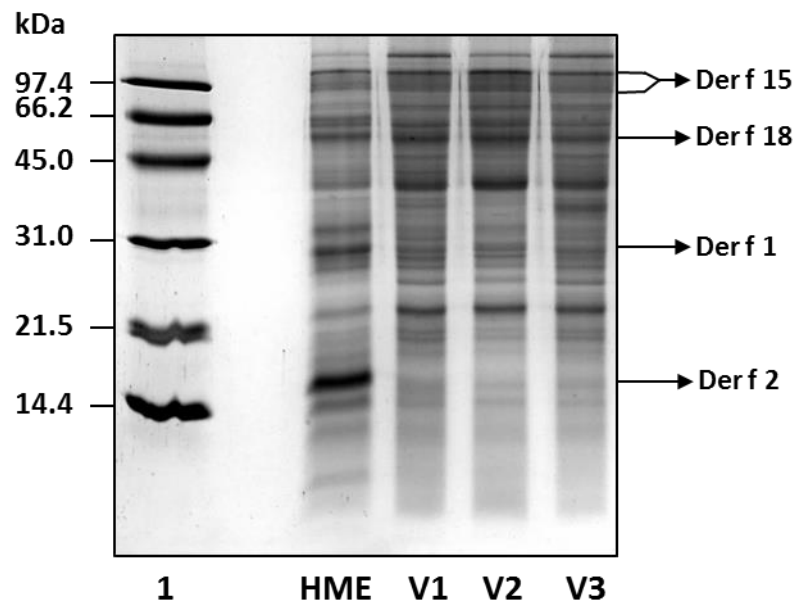


Figure S2. SDS-PAGE of VME consistency batches under reducing conditions. Low Range Molecular Weight Standard (lane 1), HME (10 μ g protein) and VME (10 μ g protein) batches 1 (V1), 2 (V2) and 3 (V3).

A

Protein sequence coverage: 29%
gi|5815436: Der f 15

1 MKTIYAILS MACIGLMNAS IKRDHNDYSK NPMRIVCYVG TWSVYHKVDP
51 YTIEDIDPFK CTHLMYGFAK IDEYKYTIQV FDPYQDDNHN SWEKRGYERF
101 NNLRLKNPEL TTMISLGGWY EGSEKYS DMA ANPTYRQQFI QSVLDFLQ EY
151 KFDGLDL DWE YPGSRLGNPK IDKQNYLALV RELKDAFEPH GYLLTA AVSP
201 GKDKIDRAYD IKELNKLFDW MNVMTYDYHG GWENFYGHNA PLYKRPDET D
251 ELHTYFNVNY TMHYLLNNGA TRDKLVMGVP FYGRAWSIED RSKLKLGDPA
301 KGMSPPGFIS GEEGVLSYIE LCQLFQKEEW HIQYDEYYNA PYGYNDKI WV
351 GYDDLASISC KLAFLKELGV SGVMVWSLEN DDFKGHC GPK NPLL NKVHNM
401 INGDEKNSFE CILGPSTTTP TPTTPTTPT TPTTPSPTT PTTTPSPTTP
451 TTTSPSTTPT TTPSPTTPT TPTTPAPTTS TSPSTTTEHT SETPKYTTYV
501 DGHLIKCYKE GDIPHPTNIH KYLVCEFVNG GWWVHIMPCP PGTIWCQEKL
551 TCIGE

B

Protein sequence coverage: 18%
gi|27550039: Der f 18

1 MTRFSLTVLA VLAACFGSNI RPNVATLEPK TVCYYESWVH WRQEGEKMDP
51 EDIDTSLCTH IVYSYFGIDA ATHEIKLLDE YLMKDLHDME HFTQHKGN AK
101 AMIAVGGSTM SDQFSKTA AV EHYRETFVVS TVDLMTRYGF DGVMIDWSGM
151 QAKDSDNFIK LLDKFDEKFA HTSFVMGVTL PATIASYDNY NIP AISNYVD
201 FMNVLSLDYT GSWAHTVGHA SPFPEQLKTL EAYHKRGAPR HKMVM AVPFY
251 ARTWILEKMN KQDIGDKASG PGPRGQFTQT DGFLSYNELC VQIQ AETNAF
301 TITRDHDNTA IYAVYVHSNH AEWISFEDRH TLGEKAKNIT QQGYAGMSVY
351 TLSNEDEVHGV CGDKNPLLHA IQSNYYHGVV TEPTVVTLP P VTHTEHVT D
401 IPGVFHCHEE GFFRDKTYCA TYECKKGDF GLEKTVHHCA NHLQAFDEVS
451 RTCIDHTKIP GC

C

Protein sequence coverage: 16%
gi|5815436: Der f 15

1 MKTIYAILS MACIGLMNAS IKRDHNDYSK NPMRIVCYVG TWSVYHKVDP
51 YTIEDIDPFK CTHLMYGFAK IDEYKYTIQV FDPYQDDNHN SWEKRGYERF
101 NNLRLKNPEL TTMISLGGWY EGSEKYS DMA ANPTYRQQFI QSVLDFLQ EY
151 KFDGLDL DWE YPGSRLGNPK IDKQNYLALV RELKDAFEPH GYLLTA AVSP
201 GKDKIDRAYD IKELNKLFDW MNVMTYDYHG GWENFYGHNA PLYKRPDET D
251 ELHTYFNVNY TMHYLLNNGA TRDKLVMGVP FYGRAWSIED RSKLKLGDPA
301 KGMSPPGFIS GEEGVLSYIE LCQLFQKEEW HIQYDEYYNA PYGYNDKI WV
351 GYDDLASISC KLAFLKELGV SGVMVWSLEN DDFKGHC GPK NPLL NKVHNM
401 INGDEKNSFE CILGPSTTTP TPTTPTTPT TPTTPSPTT PTTTPSPTTP
451 TTTSPSTTPT TTPSPTTPT TPTTPAPTTS TSPSTTTEHT SETPKYTTYV
501 DGHLIKCYKE GDIPHPTNIH KYLVCEFVNG GWWVHIMPCP PGTIWCQEKL
551 TCIGE

D Protein sequence coverage: 18%
gi|27550039: Der f 18

1 MTRFSLTVLA VLAACFGSNI RPNVATLEPK TVCYYESWVH WRQEGGKMDP
51 EDIDTSLCTH IVYSYFGIDA ATHEIKLLDE **YLMKDLHDME HFTQHKGN**AK
101 AMIAVGSTMSDQFSKTA AV EHYRETFVVS TVDLMTRYGF DGVMIDWSGM
151 QAKDSDNFIK **LLDKFDEKFA** HTSFVMGVTL PATIASYDNY NIPAISNYVD
201 FMNVLSLDYT GSWAHTVGHA SPFPEQLKTL EAYHKRGAPR **HKMVMVAVPFY**
251 **ARTWILEKMN** KQDIGDKASG PGPRGQFTQT DGFLSYNELC VQIQAEATNAF
301 TITRDHDNTA IYAVYVHSNH AEWISFEDRH TLGEKAKNIT QQGYAGMSVY
351 TLSNEDVHGV CGDKNPLLHA IQSNYYHGVV TEPTVVTLPP VTHHTTEHVT
401 IPGVFHCHEE GFFRDKTYCA **TYECKKGDF GLEKTVHHCA NHLQAFDEVS**
451 RTCIDHTKIP GC

E Protein sequence coverage: 56%
gi|5815436: Der f 15

1 MKTIYAILSIMACIGLMNAS IKRDHNDYSK NPMRIVCYVG TWSVYHKVDP
51 **YTIEDIDPFK CTHLMYGFAK** IDEYKYTIQV FDPYQDDNHN SWEKRGYERF
101 NNLRLKNPEL **TTMISLGGWY EGSEKYS DMA ANPTYRQQFI QSVLDFLQ**EY
151 **KFDGLDLWE YPGSRLGNPK** IDKQNYLALV RELKDAFEPH GYLLTAAVSP
201 GKDKIDRAYD IKELNKLFDW MNVMTYDYHG GWENFYGHNA **PLYKR**PDET
251 ELHTYFNVNY TMHYLNGA TRDKLVMGVP **FYGRAWSIED RSKLKLGDPA**
301 **KGMSPPGFIS GEEGVLSYIE** LCQLFQKEW HIQYDEYYNA PYGYNDKIWV
351 **GYDDLASISC KLAFLKELGV SGVMVWSLEN** DDFKGHCGPK NPLLNKVHNM
401 **INGDEKNSFE CILGPSTTTP** TPTTPTPTPT TPTTPTPTT PTTTPTPTT
451 TPTTPTPTT TPTTPTPTT TPTTPTPTT TPTTPTPTT TPTTPTPTT
501 DGHLYKCYKE **GDIPHPTNIH** KYLVCEFVNG GWWVHIMPCP PGTIWCQEK
551 TCIGE

F Protein sequence coverage: 28%
gi|27550039: Der f 18

1 MTRFSLTVLA VLAACFGSNI RPNVATLEPK **TVCYYESWVH WRQEGGKMDP**
51 EDIDTSLCTH IVYSYFGIDA ATHEIKLLDE **YLMKDLHDME HFTQHKGN**AK
101 **AMIAVGSTMSDQFSKTA AV EHYRETFVVS TVDLMTRYGF DGVMIDWSGM**
151 **QAKDSDNFIK LLDKFDEKFA** HTSFVMGVTL PATIASYDNY NIPAISNYVD
201 FMNVLSLDYT GSWAHTVGHA SPFPEQLKTL EAYHKRGAPR **HKMVMVAVPFY**
251 **ARTWILEKMN** KQDIGDKASG PGPRGQFTQT DGFLSYNELC VQIQAEATNAF
301 TITRDHDNTA IYAVYVHSNH AEWISFEDRH TLGEKAKNIT QQGYAGMSVY
351 TLSNEDVHGV CGDKNPLLHA IQSNYYHGVV TEPTVVTLPP VTHHTTEHVT
401 IPGVFHCHEE GFFRDKTYCA **TYECKKGDF GLEKTVHHCA NHLQAFDEVS**
451 RTCIDHTKIP GC

Figure S3. MS results of major allergens identification in VME consistency batches. Identified peptides (in red) by MS analysis in allergen sequences of Der f 15 (A,C,E) and Der f 18 (B,D,F). VME consistency batches V1 (A,B), V2 (C,D) and V3 (E,F).

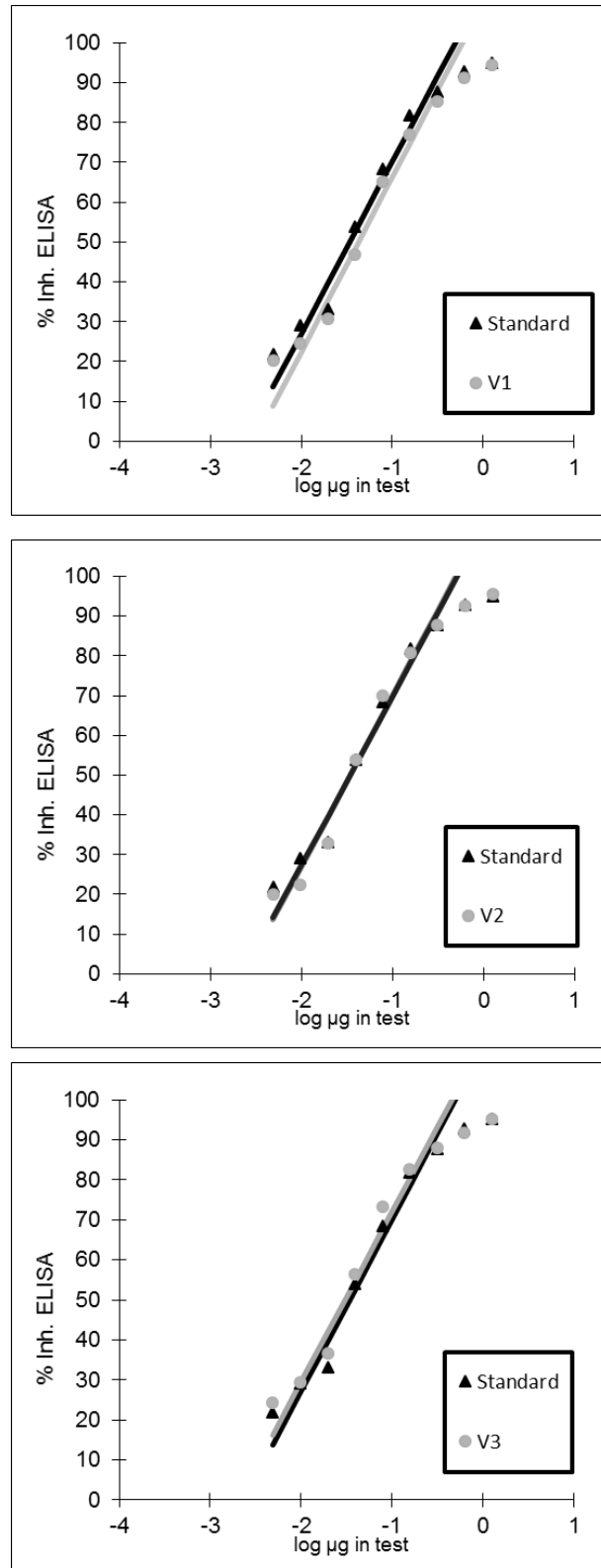


Figure S4. ELISA inhibition assays comparing biological potency of VME (Standard) and VME consistency batches (a) V1; (b) V2; (c) V3. 50% inhibition of the sera pool was obtained with 0.077 µg (V1), 0.063 µg (V2) and 0.056 µg (V3) compared to the VME batch used as standard (0.062 µg).