

ORIGINAL ARTICLE

Experimental Allergy and Immunology

Allergens in dog extracts: Implication for diagnosis and treatment

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Funding information

The study was funded by the Swedish Asthma and Allergy Association's Research Foundation, the Cancer and Allergy Foundation and Karolinska Institutet.

Abstract

Background: Five to ten percent of the population in affluent countries are allergic to dog. Diagnosis and treatment is based on allergen extracts from natural sources where composition and concentration are poorly defined.

Objective: We aimed to quantify six dog allergens (Can f 1-6) in commercial skin prick test (SPT) solutions and to determine individual allergen profiles in dogs.

Method: The allergen content of SPT solutions from five vendors and allergen source material from three anatomical sites were analyzed. Fur and saliva samples were collected from a mixed population of 120 dogs. Can f 1-6 were quantified by inhibition ELISA using purified recombinant or natural allergens and polyclonal or monoclonal antibodies. Allergenicity was analyzed by basophil activation test.

Results: Extensive variation in allergen composition was observed in commercial SPT vials resulting in a patient-dependent ability to activate basophils. Extract heterogeneity depended on collection site and allergen composition in individual dogs and source materials. Can f 2 and Can f 6 exhibited low levels in fur and SPT solutions, whereas Can f 4, which was the dominating allergen in fur samples, did not display similar high proportions in SPT solutions. Can f 3 varied most among SPT solutions.

Conclusion: There is a great variation of dog allergens in natural extracts raising questions of source, sampling, processing and ultimately of standardization and minimum allergen levels for accurate diagnosis and treatment.

KEYWORDS

allergy, dog allergen extract, dog allergens, skin prick test

1 | INTRODUCTION

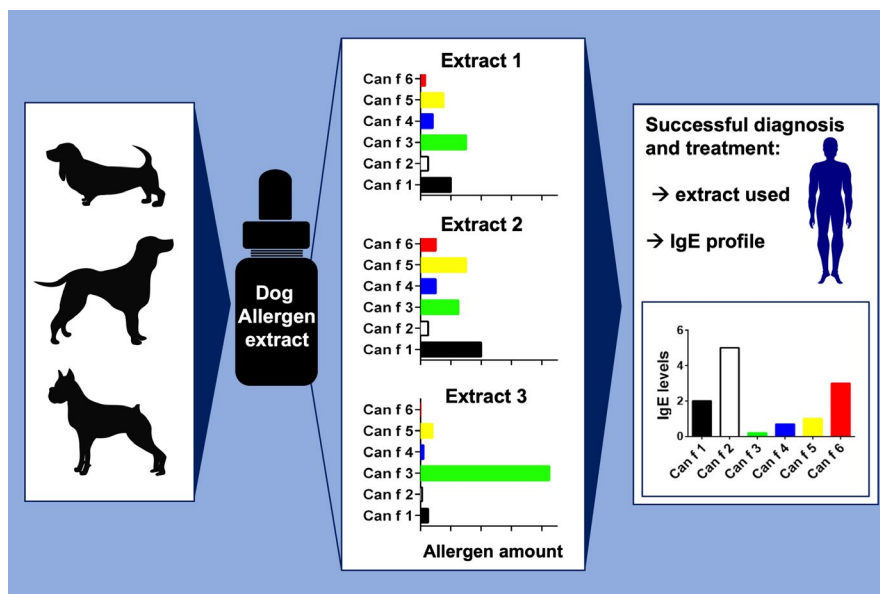
Dogs are common in society. Only in the UK, the dog population is estimated to 11.6 million¹ and in Australia the number equals 4.2 million.² Dogs are often kept as pets, but may also serve in assistance by, for example, the police, health care, and customs, effectively

placing them in many public places. Dogs produce allergens which easily become airborne and can cause sensitization and subsequently allergy.³ This is a steadily increasing affliction worldwide, being responsible for respiratory symptoms such as rhinitis and asthma.⁴ This in turn leads to reduced professional performance and quality of life for affected individuals.⁵

Dog allergen source material is excreted in individually unique compositions and concentrations at distinctly different anatomical sites, the skin (dander/hair), mouth cavity (saliva), and

Abbreviations: AIT, allergen-specific immunotherapy; HSA, human serum albumin; SPT, skin prick test.

Gafvelin and Grönlund shared last authorship.



GRAPHICAL ABSTRACT

Raw material for dog allergen extracts comes from dogs with various allergen profiles. Commercial dog allergen extracts vary in allergen content. Successful diagnosis and treatment of a dog allergic patient depends on the extract used for diagnosis/treatment and on the IgE profile of the patient.

prostate/bladder (urine).⁶ The composition complexity increases by cross-contamination of these sources within the dog. To date, seven dog allergens have been identified. Can f 1, Can f 2, Can f 4, and Can f 6 are members of the lipocalin protein family. Can f 1 is considered a major dog allergen to which 50%-70% are sensitized.^{7,8} High IgE levels to Can f 1 early in life have been proposed as the most important predictor for developing dog allergy later in childhood.⁹ Can f 2 causes sensitization in around 25%.¹⁰ Both these proteins are abundant in saliva,⁶ but Can f 1 is also found in fur.¹¹ Can f 3, dog serum albumin, displays a high homology to other mammalian albumins. It is present in dog dander, saliva, and urine in addition to serum.¹² Can f 4 appears in at least two isoforms.¹³ The prevalence of sensitization varies between geographic regions from 46% to 81%, indicating that there might be a difference in reactions to the isoforms.^{13,14} Can f 5 is a prostatic kallikrein homologous to human prostate-specific antigen (PSA). It is found in the urine of intact male dogs causing IgE responses in up to 70% of dog allergic patients, thus classified as a major allergen.¹⁵ Can f 6 cross-reacts with the cat allergen Fel d 4 and horse allergen Equ c 1, indicating symptomatic reactions in additional species. The prevalence of sensitization is around 38% in patients with allergy to dog.¹⁶ The latest discovered allergen, Can f 7, or NPC2 protein, belongs to the MD-2-related lipid recognition (ML) family. The prevalence of sensitization is 10%-20%.¹⁷ The amount and composition of allergens has been shown to differ between dogs, and the difference is greater between individuals than breeds.¹¹ Likewise, each patient displays a unique IgE profile and will thus respond to different combinations of dog allergens. This is a likely reason to why some individuals state that they react to certain dogs but not to others.

Skin prick test¹⁸ and allergen-specific IgE (sIgE) serologic measurements¹⁹ are the most common methods to aid diagnosis of allergy.²⁰⁻²² Diagnosis, as well as treatment by allergen-specific immunotherapy (AIT), is almost exclusively performed with extracts from natural allergen sources such as dander or epithelia.²³ This poses an uncertainty since not all of the allergens are present in skin. Moreover, those that are present may vary in quantity and quality depending on the starting material and extraction procedure. Of note, manufacturers use in-house methods for quality assessment and do not provide information on individual allergen content or composition in their products.²⁴ This obstructs evaluation and comparison of extracts.²⁵ A previous study has indeed shown qualitative differences between Can f 1 and Can f 3 in extracts intended for SPT from different manufacturers,²⁶ and others have pointed out inconsistency in reproducibility of extracts.²⁷⁻²⁹ Here, we have systematically characterized and compared the allergen content in dogs and different commercial dog allergen extracts in order to evaluate the implication of extract quality on diagnosis and treatment of dog allergic patients.

2 | MATERIAL AND METHODS

2.1 | Dog fur and saliva samples

Randomly included dog owners ($n = 120$) sampled their dogs. The sampling method is detailed in the supplement. In short, fur samples were collected from a defined area (35×30 mm) on the dog's neck and groin using a sampling kit according to instructions (Medi-Tec Research & Development AB, Stockholm, Sweden). A saliva swab (Oracol, Malvern

Medical Developments, Worcester, UK) was applied on the inside of the dog's cheeks until saturated (30–60 seconds). Fur and saliva samples were collected in parallel from each individual dog. Fur samples were prepared by extraction in 1 mL PBS for 1 hour. The saliva samples were centrifuged at 1000 g for 10 minutes.

2.2 | Dog allergen extracts

Diagnostic dog allergen extracts were obtained from ALK (ALK-Abelló Nordic, Hørsholm, Denmark), Bencard (München, Germany), Greer (Cambridge, MA, USA), Immunotek (Madrid, Spain), and Stallergenes (Antony, France), randomly labeled no 1–5 (a and b for different batches from same manufacturer), Table S2. Evaluation was made on ready-to-use solutions for SPT, except for one that was for skin scratch testing. In this report, they are all categorized as “SPT solutions.” From two manufacturers, Allergon (Ängelholm, Sweden) and Greer, lyophilized extracts not intended for clinical use were purchased. These were denoted as dog source material, that is of different anatomical origin, hair, epithelia, and dander, and were randomly labeled extract no 6–9. The source materials were reconstituted in phosphate buffered saline, pH 7.4 (PBS). Protein concentration was determined using Pierce™ BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA).

2.3 | Allergen production

Recombinant (r)Can f 1, Can f 2, Can f 4, and Can f 6 were produced with and without biotinylation tag and purified as previously described.^{10,13,16} rCan f 5¹⁵ was produced in HEK293 cells using FreeStyle™ 293 expression system (Thermo Fisher Scientific). Natural Can f 3 was purified from dog serum (a kind gift, Djurakuten, Stockholm, Sweden).³⁰ Can f 3 and Can f 5 were *in vitro* biotinylated (Sigma-Aldrich, Darmstadt, Germany).

2.4 | Antibody production

Can f 1, Can f 2, and Can f 3 monoclonal antibodies were kind gifts from Medi-Tec Research and Development Stockholm AB, Sweden. Polyclonal affinity purified mouse antibodies were obtained from mice immunized with rCan f 4 or rCan f 6, respectively (ethical permission from Stockholm Norra Animal Ethics Board). The antisera were passed over 1 mL HiTrap® NHS-activated columns (GE Healthcare, Uppsala, Sweden) with covalently linked rCan f 4 and 6, respectively, and washed with 20 column volumes of PBS, followed by elution with 0.1 M glycine-HCl, pH 2.5. The pooled antibodies were neutralized using 1 M carbonate buffer pH 9.6. Can f 5 monoclonal antibodies were a kind gift from Phadia, Uppsala, Sweden.

2.5 | ELISA

Allergen concentrations in extracts were measured by competitive inhibition ELISA, further described in the Data S1. In short, mouse antibodies directed to each dog allergen, Can f 1–6, were added

to ninety-six-well MaxiSorp plates (Thermo Fisher Scientific) pre-coated with goat-anti mouse IgG (Jackson ImmunoResearch, Ely, UK). The plates with allergen-specific antibodies were incubated with equal amounts of sample and competitor, corresponding to *in vivo* biotinylated recombinant Can f 1–2–4–6³¹ for Can f 1, Can f 2, Can f 4, and Can f 6 measurements. *In vitro* biotinylated native Can f 3 and *in vitro* biotinylated recombinant Can f 5 was used for the Can f 3 and Can f 5 measurement, respectively. To generate sensitive assays, the biotinylated antigens were titrated to an optical density (OD) value around 1.0. For detection of bound biotin-labeled antigen, the plates were incubated with streptavidin-horseradish peroxidase (Jackson ImmunoResearch) followed by incubation with substrate 3,3',5,5'-Tetramethylbenzidine super slow (Sigma-Aldrich) and finally absorbance was measured at 450 nm. Concentration was determined against a reference curve obtained with eight concentrations of recombinant or purified allergens, typically in the range of 2.5–320 ng/mL.

Human serum albumin (HSA) was assessed using a HSA-specific ELISA kit (Abcam, Cambridge, UK) according to manufacturer's instructions. The specificity of the assay was confirmed by using our internal Can f 3 standard curve as control for cross-reactivity.

2.6 | IgE assay

Patients' allergen-specific IgE antibodies were analyzed using ELISA, described in detail in the Data S1. In short, 96-well microtiter plates (Thermo Fisher Scientific) were coated with respective allergen and for the reference curve with polyclonal rabbit anti-human IgE (kind gift from MIAB AB, Uppsala, Sweden). Dilutions of chimeric anti-Bet v 1 human IgE (kind gift from Professor Rudolf Valenta, University of Vienna, Austria) were used to form the standard curve.

2.7 | CD-sens

Basophil activation was measured using the CD-sens method.³² Three dog allergic patients gave their informed written consent to donate blood (ethical permission from the Regional Ethical Review Board, Stockholm, Sweden). Whole blood was stimulated with 15 dilutions of the extracts (5000–0.0005 ng/mL), red blood cells lysed using ACK lysing buffer (Gibco, NY, USA), and the white blood cells stained using PE-conjugated anti-CD63 and APC-conjugated anti-CD203c antibodies (BD Biosciences). Activated degranulated basophils were analyzed by flow cytometry (FACS-Verse, BD Biosciences, Franklin Lakes, NJ, USA) and the percent of CD203c-positive basophils co-expressing CD63 out of the total number of CD203c-positive cells recorded.³³ Rabbit anti-human IgE (1000ng/ml, kind gift from MIAB, Uppsala, Sweden) was used as positive control and unstimulated cells as negative control. The CD-sens value was calculated as the allergen concentration giving 50% of maximum up-regulation of CD63 inverted and multiplied with 100.³⁴ A result was considered positive (“+,” without stating a value) when only the highest concentration of extract triggered a positive response, and thus, a value could not be calculated. The cutoff value for determining a

positive value was set to minimum 5% upregulated CD63 of total number of basophils.

2.8 | Statistical analysis

Statistical analyses were made with GraphPad Prism 6 (GraphPad Software, Inc); Mann-Whitney *U* test was used to compare gender differences, Sidak's multiple comparisons test to compare breeds, and Dunnett's multiple comparisons test to compare Can f 4 levels to other allergens. *P*-values <0.05 was considered significant.

3 | RESULTS

3.1 | Allergen content and composition of extracts

The total protein content in SPT solutions from five manufacturers ranged between 1.53 and 3.07 mg/mL (Table 1a). The six dog allergens, Can f 1 to 6, constituted between 0.26% and 10% of the total protein content. Can f 1 to 6 were present in all extracts, although the concentrations of each allergen varied greatly between SPT extracts from different manufacturers (Table 1 and Figure 1). The composition of extract number 3 singled out, having the highest total allergen content. Generally, Can f 2 and Can f 6 were detected at comparably low amounts, corresponding to minor proportions of the total allergen content (Table 1a, Figure 1A). Can f 3 showed the largest variation between manufacturers, ranging from 19% in extract 2 to 98% in extract 3. The detected Can f 3 did not correspond to HSA supplemented to the extracts. An assay specific for HSA confirmed that the extracts contained HSA, equivalent to 0%–0.6% of the total Can f 3 concentrations. Except for extract 3, the proportions of Can f 1, Can f 4, and Can f 5 each comprised at least 8% of the total allergen content (Figure 1A). Both the proportion and the amount of these three allergens still varied considerably between the extracts (Table 1a and Figure 1A).

Allergen composition in two different batches of SPT solutions from two of the manufacturers was investigated (Figure 1B). Comparison of the composition of two batches from each company showed more consistency than the composition of extracts from different manufacturers. Still, we observed some batch variation, especially for extracts from company 4, which could mainly be attributed to variation in Can f 3 content (Figure 1B).

In the source material obtained from epithelia, hair, and dander, that is extracts 6, 7, and 8, respectively, the total protein content varied between 0.8 and 2.7 mg/mL and the allergen content between 1.3% and 3.2% (Table 1b). The highest total proportion of allergens (28%) was found in extract no 9 (origin—dander). Comparison of the allergen composition of extracts from different origins exhibited distinct distributions of the six dog allergens (Figure 1C). The epithelial extract 6, similarly as SPT solution 3, was dominated by Can f 3 (99% of total allergen content), which also constituted the largest part of the dander extracts no 8 and 9 (60 and 54%, respectively). In contrast, the hair extract 7 mainly contained Can f 1 (67%), which was also a dominating allergen in extract 9 (43%).

3.2 | Basophil reactivity to dog allergen extracts

The allergenic activity of the different extracts was determined by CD-sens in a basophil activation assay. Three dog allergic subjects, displaying different profiles of IgE to Can f 1–6, were selected (Table 2b). The basophil activation capacity varied between extracts and between subjects (Table 2a). Patient 1, sensitized exclusively to Can f 6, displayed CD-sens values to extracts 7 and 8 and reacted to the highest allergen concentration of extracts 1, 2, and 5a. Patient 2, with low serum IgE levels to all of the tested allergens, reacted only to the highest concentration of two extracts. Finally, patient 3, highly sensitized to all the allergens, exhibited positive CD-sens values to all but one extract to which the subject responded to the highest concentration.

TABLE 1 Concentration (ng/mL) of six dog allergens and total protein concentration (mg/mL) in (a) ready-to-use skin prick test (SPT) solutions from five suppliers (1–5) and (b) source material extracts from dog, epithelia (6), hair (7), and dander (8 and 9)

	Can f 1	Can f 2	Can f 3	Can f 4	Can f 5	Can f 6	Total protein concentration	Allergens of total protein (%)
(a) SPT solution								
1	9050	597	10 000	2530	3720	874	1.55	1.7
2	1210	58.7	1530	1920	3190	157	3.07	0.26
3	14.6	14.4	177 000	2560	786	10.7	1.72	10
4a	6950	52.1	5280	6540	3290	191	1.53	1.5
5a	8980	167	38 500	12 900	5300	284	2.28	2.9
(b) Source material								
6	86.8	7.12	40 600	450	29.1	36.9	2.41	1.7
7	17 100	230	6660	461	151	895	0.80	3.2
8	2180	767	20 900	206	7640	3390	2.70	1.3
9	95 000	911	119 000	20.6	6020	49.7	0.80	28

The allergen content in extracts is calculated as percentage (%) of aggregated concentration of the six allergens out of total protein content

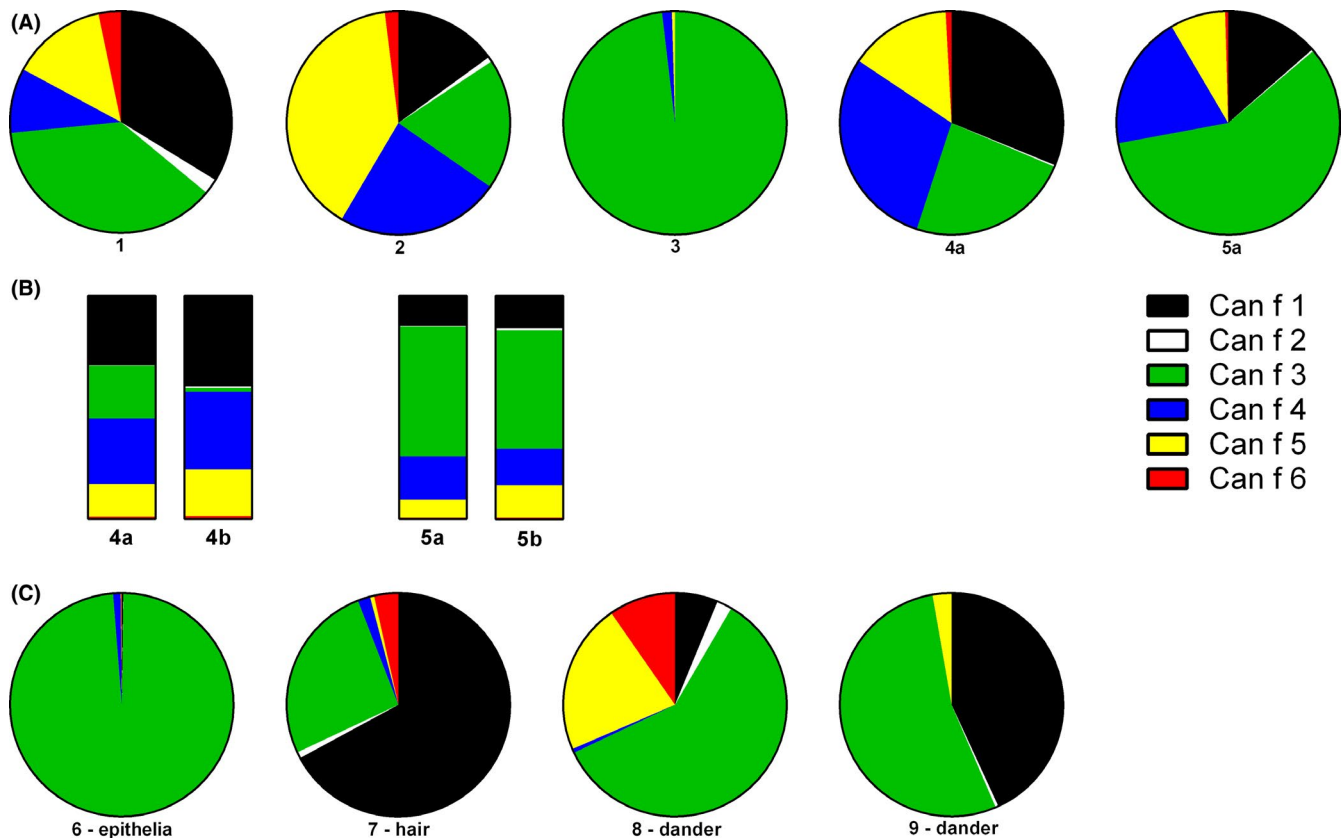


FIGURE 1 Distribution of six dog allergens, Can f 1-6, in ready-to-use SPT solutions (1-5) from five suppliers (A), two batches (a and b) of skin prick test (SPT) solutions from two manufacturers (B) and in allergen source material extracts (6-8) from one vendor and (9) from another supplier (C)

TABLE 2 (a) Basophil reactivity (CD-sens) to skin prick test (SPT) solutions (no. 1-5) and source material extracts (no. 6-8) of three dog allergic patients (Patients 1-3). Plus (+) indicates a positive result below the CD-sens limit criterion. (b) Serum IgE reactivity (kU/L) to six dog allergen allergens for Patients 1-3

	Patient 1	Patient 2	Patient 3
(a) Extract			
1	+	–	0.1
2	+	–	+
3	–	–	1.0
4a	–	–	0.1
5a	+	–	0.1
6	–	–	1.8
7	1.0	+	0.8
8	0.2	+	1.0
(b) Allergen			
Can f 1	0.0	0.6	21
Can f 2	0.0	0.3	8.8
Can f 3	0.0	0.0	75
Can f 4	0.0	0.1	19
Can f 5	0.0	0.0	1.8
Can f 6	7.4	0.2	8.1

3.3 | Allergen profiles of dogs

Can f 1 to 6 were quantitatively analyzed in fur and saliva samples from 120 dogs (Table S1). Higher allergen levels were detected in saliva than in fur, and the significantly highest concentrations were recorded for Can f 4 (Figure 2).

All allergens exhibited great individual variation. Can f 2 was exclusively found in saliva and Can f 5 in fur (Figure 2). Can f 5 was only present in trace amounts in samples of female dogs, while significantly higher Can f 5 levels ($P < 0.0005$) were found in fur samples from the groin of male dogs (Figure 3A). Such gender difference could not be detected for the other allergens (shown for Can f 1, Figure 3B). Allergen profiles of individual dogs varied both within and between breeds, exemplified by a short haired (Labrador retriever) and a long haired (Border collie) breed (Figure 4 and Figure S1).

4 | DISCUSSION

Exposure to dog allergens is a common worldwide cause of allergy. Allergen extracts are routinely used for diagnosing and treating allergy. However, the content of the extracts remains poorly defined, which may lead to a fragmentary diagnosis and insufficient treatment. In this study, we evaluated ready-to-use SPT ampules from five vendors and four nonclinical grade allergen source materials for

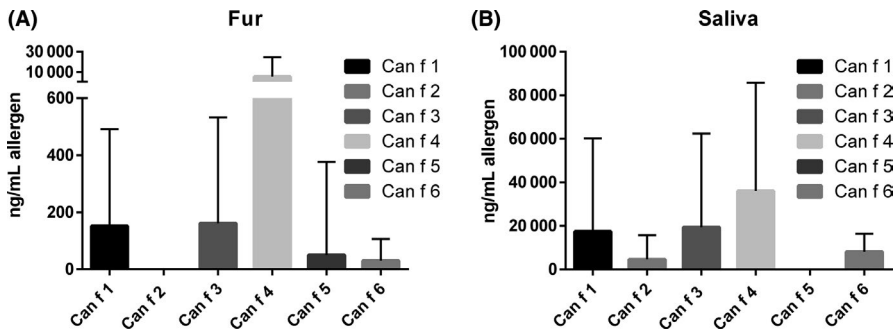


FIGURE 2 Concentration (ng/mL, y-axis) of six allergens (x-axis) analyzed in extracts of fur sampled from the neck (A) and of saliva (B) collected from 120 dogs. Mean with standard deviation shown. Can f 2 is not detectable in fur. Saliva concentration is approximately 100-fold higher compared to fur

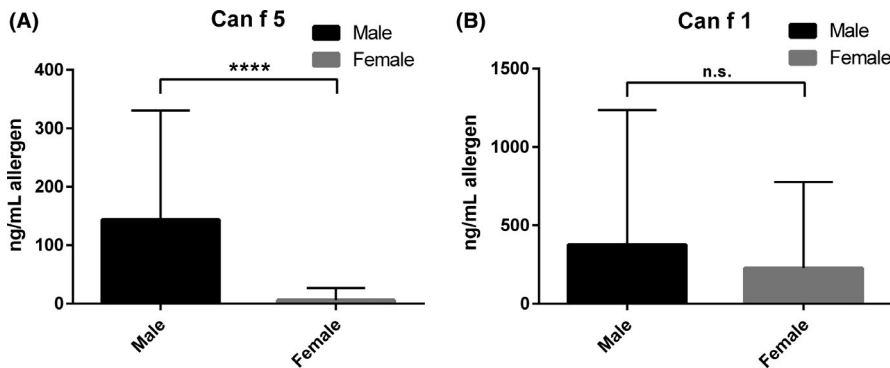


FIGURE 3 Concentration (ng/mL) of Can f 5 (A) and Can f 1 (B) in extract of fur sampled from the groin of male ($n = 44$) and female dogs ($n = 76$). Mean with standard deviation, **** $P < 0.0005$; ns, no significant difference

their levels of the six dog allergens Can f 1-6. The processed commercial dog allergen extracts were compared with allergens extracts of fur samples from a representative number of dogs ($n = 120$).

Here, we show a remarkable range of allergen component concentrations in the different SPT and allergen source solutions. In most cases, the allergen proportion out of the total protein content did not constitute more than a few percent.

One of the SPT solutions, no 3, contained a considerably higher allergen content than the other extracts. Notably, 98% corresponded to the minor allergen albumin, Can f 3, suggesting the use of an alternative allergen source material or supplementation of serum albumin for stabilization purpose. However, no HSA could be detected in this extract. Moreover, solution no. 3 contained a particularly low concentration, $0.01 \mu\text{g/mL}$, of the major allergen Can f 1. Such low level may result in reduced diagnostic sensitivity. It should however be noted that solution no 3 is labeled for skin scratch test, a slightly more invasive test than SPT. The SPT solutions from three

of the five manufacturers contained Can f 1 concentrations in the range $7\text{--}9 \mu\text{g/mL}$, extracts that also showed consistency in the major dog allergen Can f 5 levels (range $3\text{--}5 \mu\text{g/mL}$). Yet, the other four allergens varied greatly between these extracts.

In contrast to other allergens, Can f 2 and Can f 6 were found at low amounts in SPT solutions. Can f 2 is primarily present in dog saliva.⁶ The low Can f 2 levels can thus be expected since the extracts derive primarily from hair and dander. Still, one fourth of the dog allergic population reacts to Can f 2.¹⁰ These patients may not be reliably diagnosed with currently available SPT extracts. The concentrations of Can f 6 were detected at 20 to 100 times lower concentrations than Can f 1 and even lower in extract 3. Can f 6 is an important minor allergen due to its cross-reactivity to other furred animals.¹⁶ Low amounts of Can f 6 in the extracts are therefore troublesome. The widely varying allergen composition in SPT solutions prompts the question; which allergen level should be present in an extract to be diagnostically reliable?

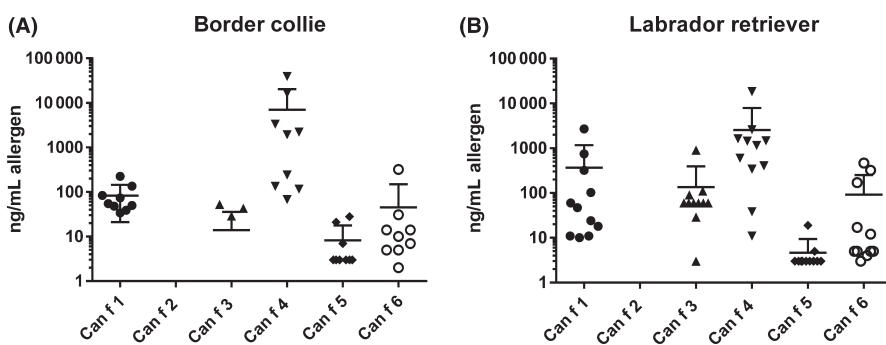


FIGURE 4 Concentration of allergens (ng/mL) in fur samples from different breeds, Border collie ($n = 9$) (A) and Labrador retriever ($n = 11$) (B). Mean values with standard deviation shown, y-axis is logarithmic

Inter batch variation was investigated for two manufacturers. Even batches from the same manufacturer varied in allergen content, especially for Can f 3, although to less extent than variation between manufacturers. This indicates that differences in source material and extraction methods impact the final allergen composition.

One reason for the allergen variation might be choice of extraction method. Different precipitation and extraction protocols may lead to varying yield and integrity, affecting each allergen independently. One convenient method is protein concentration by acetone precipitation (21), which might lead to partial denaturation and selective loss of antibody binding. Our results support the assumption that the extraction process may lead to partial loss of activity. Can f 4 was found to be the most abundantly detected allergen in fur of dogs. This observation is remarkable since Can f 4 did not exhibit a similar dominant feature in any of the SPT solutions or source materials. Thus, the activity of important allergens may be lost during manufacturing. An indirect proof of the importance of nCan f 4 is the reported prevalence of sensitization of more than 80%,¹⁴ suggesting that sensitization is underestimated when diagnosed with commercially available SPT extracts.

Allergen source material from epithelia, hair, and dander was examined. The analysis revealed that anatomical origin has a major impact on allergen composition. For example, epithelia were mainly composed of Can f 3 while hair contained a large proportion of Can f 1.

Moreover, the heterogeneity in the source materials may also reflect differences in allergen profiles between dogs. To investigate this, we analyzed samples collected from fur and saliva of 120 randomly selected dogs. The sampling was performed according to a well-specified procedure. A weakness of the method is that allergen levels are not related to sampling recovery. As the allergens were collected from a defined area on the neck, no statement can be made about the total allergenicity of particular dogs. Nevertheless, the relative allergen levels in samples from each dog provide individual allergen profiles irrespective of protein recovery. Moreover, although data on absolute allergen levels should be interpreted with caution, the large individual heterogeneity in allergen levels observed in our dog cohort is in line with previously published data on Can f 1 in dog hair and coat samples.¹¹ Quantification of six dog allergens in fur samples from the mixed dog population revealed individual variations in the distribution of allergens regardless of breed. Our data support that there are no hypoallergenic breeds thus extending previous data for Can f 1.¹¹ Furthermore, we show that the length of the coat does not influence on allergenicity. Gender mattered only for Can f 5, which was expected, since Can f 5 is a prostatic protein. Can f 5 was also detected in low amounts in a few female dogs, speculatively due to male dog kallikrein cross-contamination. Hence, not only does the individual dogs used for allergen collection matter, but also the origin of material, anatomical site, and method of extraction affects the final allergen composition of the SPT solution.

Basophil activation analyses were performed to determine the allergenic potential of extracts in relation to patients' sensitization profiles. For this purpose, three patients with widely different sensitization profiles were chosen and basophil degranulation measured. The allergenic activity to the extracts varied greatly for each patient,

essentially mirroring the content of allergens in the extract. For instance, the patient monosensitized to Can f 6 reacted to the extracts with highest content of this allergen, while the polysensitized patient, highly sensitized to Can f 3, also was particularly responsive to extracts with high content of this allergen. Basophil reactivity demonstrated that the content of single allergens may be crucial for extract allergenicity. Accordingly, patient 2, with low IgE levels to four of the six allergens, did not respond to any of the SPT solutions.

The same type of naturally derived extracts as used for SPT also forms the basis for vaccine formulation. Reported varying success to treat allergy to dog by AIT may speculatively be ascribed to poor or uneven quality of the vaccines.^{35,36} Successful treatment is ultimately dependent on how the amount and composition of allergen components in the extracts matches treated patients' sensitization profile.^{37,38}

In conclusion, here we show that natural dog allergen extracts intended for clinical use are highly unreliable. Factors such as individual dog allergen production within and at different anatomical sites and producer unique manufacturing all contribute to the demonstrated enormous variability and complexity in the mixture of allergens. Our data imply an urgent need for content declaration of dog allergen extracts, which would form the basis in a harmonized standardization and determination of minimum allergen levels for accurate diagnosis and effective treatment.

CONFLICT OF INTEREST

Hans Grönlund is co-founder of Medi-Tec Research & Development Stockholm AB; further, AW, KA, EH, and GG have acted as scientific advisors. The other authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

HG conceived the study and designed the experiments together with AW and GG. OBN and EH produced recombinant allergens. AW, KA, and JB performed immune analyses. AW, GG, and HG were the main responsible for data analysis and interpretation, with input from all co-authors. AW wrote the manuscript under supervision of HG and GG, and all co-authors reviewed and approved the final version.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Wintersand A, Asplund K, Binnmyr J, et al. Allergens in dog extracts: Implication for diagnosis and treatment. *Allergy*. 2019;74:1472-1479. <https://doi.org/10.1111/all.13785>

Supplementary Information

Allergens in dog extracts: implication for diagnosis and treatment

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Material & methods

Dog fur and saliva sampling

Allergen sampling kits (Medi-Tec Research & Development AB, Stockholm, Sweden) were sent out to participating dog owners. The owners sampled their dogs (n=120, supplementary table SI) according to the instructions given in the test kit. The kit contained two brushes for fur sampling, one saliva collector (Oracol, Malvern Medical Developments, Worcester, UK) and a template for defining the fur sampling area (35x30mm). The template was placed on the dog's neck and groin and a sample was collected from each site by brushing the defined area for 30 seconds at the pace of 1 second per stroke. The saliva collector was rotated under the lip and in the back jaw of the dog thoroughly until the swab became completely saturated (30-60 seconds). The samples were mailed to the lab where they were stored frozen at -20°C until extraction and analysis.

Dog fur and saliva extraction

Extraction was done by adding 1ml 1x PBS to the fur sample tubes, which were subsequently placed on a roller mixer (SRT9D, Stuart, Stone, UK) and rotated at 30 rpm for 1 hour. The tubes were then centrifuged at 840g for 2 minutes and the brushes discarded. The saliva tubes were centrifuged at 1000g for 10 minutes and the collection vials removed. All samples were stored at -80 degrees until analysis.

Detection of dog allergens by competitive inhibition ELISA

Ninety-six well MaxiSorp plates (Thermo Fisher Scientific) were pre-coated with 5 µg/ml goat-anti mouse IgG (Jackson ImmunoResearch, Ely, UK) in PBS, pH 7.4 overnight at +4°C. Unbound antibodies were removed by rinsing five times with washing buffer (PBS and 0.05% Tween 20 (Sigma-Aldrich)). To reduce non-specific binding, the plates were incubated with 200 µl/well of blocking buffer (1% bovine serum albumin in PBS and 0.05% Tween 20) at room temperature for 1 hour. After wash with 5x300µl washing buffer, the plates were incubated 2 h at room temperature with 200µl per well of PBS pH 7.4 containing 1% lactose (Sigma Aldrich, Darmstadt, Germany), for stabilization of the pre-coated plates during storage. The buffer was aspirated and the plates left to dry overnight in room temperature covered by aluminum foil. The dried plates were stored in sealed foil bags containing Desiccant MiniPax (Sigma Aldrich, Darmstadt, Germany) at +4°C until use. Pre-coated plates that were stored this way performed equally well in ELISA analyses as freshly coated plates;

this was shown in separate validation experiments where one batch of plates were produced and tested in homogeneity tests using the same reagents and concentrations once per month over the course of one year.

At analysis the goat anti-mouse IgG pre-coated plates were washed 5x with 300 µl with washing buffer. For the Can f 1, Can f 2, Can f 3 and Can f 5 ELISA:s, each well was then incubated with 100µl of mouse monoclonal antibodies to Can f 1, Can f 2, Can f 3 and Can f 5, diluted 1:3000, 1:750, 1:50 and 1:2000 respectively in ELISA buffer containing PBS pH 7.4, 0.2% bovine serum albumin (Sigma Aldrich, Darmstadt, Germany) and 0.05% Tween-20 (Sigma Aldrich, Darmstadt, Germany), for 2 hours in room temperature. For detection of Can f 4 and Can f 6 the plates were incubated for 2 hours at room temperature with 100 µl/well of the polyclonal affinity purified antisera to Can f 4 and Can f 6 respectively diluted 1:50 and 1:400 in ELISA buffer.

To perform the competitive inhibition assay, samples and serial dilutions of standard allergens were premixed with an equal amount of competitor. The standard allergens rCan f 1, rCan f 2, nCan f 3, rCan f 4, rCan f 5 and rCan f 6 were diluted in ELISA buffer in 2-fold steps into 8 standard points. In house produced recombinant *in vivo* biotinylated Can f 1-2-4-6^{1,2} diluted in ELISA buffer to a working concentration of 100 ng/ml was used as competitor in the Can f 1, Can f 2, Can f 4 and Can f 6 assays. As competitor for the Can f 3-assay, *in vitro* biotinylated nCan f 3 was used at a working dilution of 100ng/ and for the Can f 5 assay *in vitro* biotinylated rCan f 5 at a working dilution of 1:500. Labeling of the *in vitro* biotinylated allergens was carried out using the EZ-Link™ Micro Sulfo-NHS-Biotinylation Kit (Thermo Fischer Scientific) according to the manufacturer's protocol. Negative control samples comprised of ELISA buffer only and positive control samples contained a known concentration of the respective allergen. To the ELISA plates, incubated with anti-allergen antibodies as described above and subsequently washed with 5x300µL washing buffer, 100 µl/well of the samples, standards and controls premixed with competitor (50 µl sample+50 µl competitor/well) was added. The plates were incubated for 2 hours at room temperature and washed 5x300 µl with washing buffer. Streptavidin-conjugated horseradish peroxidase (Jackson ImmunoResearch) diluted 1:4000 in stabilizing buffer (Thermo Fisher Guardian™ Peroxidase Conjugate Stabilizer/Diluent) was then added, 100 µl/well, followed by incubation for 1 hour at room temperature, wash with 5x300 µl washing buffer and addition of 100 µl/well of TMB substrate (Sigma Aldrich, Darmstadt, Germany). The plates were incubated with substrate for 10 minutes in dark at room temperature and the reaction was stopped by

100 µl/well of stop solution (0,5M H₂SO₄). Then the plates were read at 450 nm in an ELISA reader (SpectraMax PLUS (Molecular Devices, CA, USA)).

The detection range for the dog allergen competition ELISA:s were: 2.5-320 ng/ml for Can f 1, 2.5-320 ng/ml for Can f 2, 60-7680 ng/ml for Can f 3, 2.5-320 ng/ml for Can f 4, 2.5-320 ng/ml for Can f 5 and 2.5-320 ng/ml for Can f 6.

ELISA detection of antigen specific IgE

Rabbit anti-human IgE (kind gift from MIAB AB, Uppsala, Sweden) and the six canine allergens (Can f 1-6) were diluted to a final concentration of 5µg/ml in 1x PBS pH 7.4.

Ninety-six well ELISA plates (NUNC Thermo Scientific) were coated with 100µl per well of the anti-human IgE and allergen solutions. The plates were incubated overnight at +4°C.

A standard curve was created using chimeric anti-Bet v 1 human IgE (kind gift from Professor Rudolf Valenta, University of Vienna, Austria) diluted in ELISA buffer at seven dilutions ranging 38-0.08 kU/l with 0.08kU/l being the detection limit. The coated ELISA plates were washed with 5x300µl washing buffer and 200µl blocking buffer added to the wells. The plates were incubated at room temperature for 1 hour and washed with 5x300µl washing buffer.

Hundred µl per well of patients' serum samples diluted 1:2 in ELISA buffer and standard curve samples were then added and incubated for 2 hours in room temperature. After another wash with 5x300µl wash buffer, 100µl goat anti-human IgE (Novex by Life Technologies, CA, USA), diluted 1:2000 in ELISA buffer, was added and the plates were incubated for 1 hour at room temperature, followed by washing, 5x300 µl in washing buffer. Detection was performed by adding 100 µl TMB substrate (Sigma Aldrich, Darmstadt, Germany) per well and incubated 10 minutes in dark at room temperature. The reaction was stopped by distributing 100 µl 0.5M H₂SO₄ per well and the plate analyzed by reading at 450 nm.

Supplementary table SI

Characteristics of dogs sampled for allergen profiles in fur (neck and groin) and saliva (n=120) and individual results of dog allergen analyses. Allergen concentration in ng/ml. M=male, F=female. Age given in years (y). “High” indicating measurement over detection limit i.e. 320 ng/ml (Can f 1, 2, 4, 5, 6) or 7680 ng/ml (Can f 3). “Low” indicating measurement under detection limit e.g. 2.5 ng/ml (Can f 1, 2, 4, 5, 6) or 60 ng/ml (Can f 3). n/a refers to sample missing or insufficient amount for analysis.

Breed	Sex	Age (y)	Sample	Can f 1	Can f 2	Can f 3	Can f 4	Can f 5	Can f 6
Affenpinscher	M	1	Neck	175		72	834	3	14
Affenpinscher	M	1	Groin	116		60	20	5	18
Affenpinscher	M	1	Saliva	1882	682	9271	10495		2229
Airedale Terrier	F	9	Neck	194		127	52533	3	10
Airedale Terrier	F	9	Groin	64		60	611	3	5
Airedale Terrier	F	9	Saliva	4276	216	60	75482		2023
Airedale Terrier	F	5	Neck	17		374	20665	4	4
Airedale Terrier	F	5	Groin	197		939	2900	7	28
Airedale Terrier	F	5	Saliva	413	663	60	7569		944
American Staffordshire Terrier	F	6	Neck	9		60	229	3	3
American Staffordshire Terrier	F	6	Groin	54		83	3	3	3
American Staffordshire Terrier	F	6	Saliva	2824	1390	5549	755		2084
Australian Kelpie	M	4	Neck	14		60	104	3	3
Australian Kelpie	M	4	Groin	56		60	4	3	3
Australian Kelpie	M	4	Saliva	81677	9151	7431	90952		5816
Australian Shepherd	F	3	Neck	26		60	593	3	5
Australian Shepherd	F	3	Groin	50		60	237	3	10
Australian Shepherd	F	3	Saliva	18940	470	16260	653		5607
Australian Shepherd	F	2	Neck	223		60	1477	3	19
Australian Shepherd	F	2	Groin	135		68	1632	3	11
Australian Shepherd	F	2	Saliva	21390	369	3405	3904		2564
Australian Shepherd	F	4	Neck	63		80	389	3	6
Australian Shepherd	F	4	Groin	70		60	6	3	8
Australian Shepherd	F	4	Saliva	3437	3317	2213	2019		1183
Basset Fauve de Bretagne	F	10	Neck	140		175	2990	3	16
Basset Fauve de Bretagne	F	10	Groin	175		137	79	3	19
Basset Fauve de Bretagne	F	10	Saliva	16784	2467	12719	High		305
Beagle	M	1	Neck	20		60	2051	3	4
Beagle	M	1	Groin	11		60	10	22	3
Beagle	M	1	Saliva	2507	108	359359	2811		526
Bernese Mountain Dog	F	2	Neck	100		166	9	3	9
Bernese Mountain Dog	F	2	Groin	234		258	10	3	25
Bernese Mountain Dog	F	2	Saliva	11490	283	15135	256		4496
Bernese Mountain Dog	F	4	Neck	172		60	1910	3	24

Bernese Mountain Dog	F	4	Groin	151		60	344	3	15
Bernese Mountain Dog	F	4	Saliva	70695	1644	6663	12184		20068
Bichon Havanais	M	2	Groin	178		80	15	3	39
Bichon Havanais	M	2	Neck	17		60	1000	3	4
Bichon Havanais	M	2	Saliva	2330	1571	2110	6631		5154
Bichon Havanais	M	7	Neck	31		60	2730	73	3
Bichon Havanais	M	7	Groin	265		60	14	228	14
Bichon Havanais	M	7	Saliva	1787	77	4899	334		837
Mixed Breed	F	4	Neck	139		60	2480	3	41
Mixed Breed	F	4	Groin	53		116	68	3	35
Mixed Breed	F	4	Saliva	1940	1073	53010	1870		843
Mixed Breed	F	<1	Neck	82		60	304	3	13
Mixed Breed	F	<1	Groin	101		60	45	3	12
Mixed Breed	F	<1	Saliva	3245	43	6925	463		359
Border Collie	M	3	Groin	5424		527	63179	3	High
Border Collie	M	3	Neck	55		60	116	3	10
Border Collie	M	3	Saliva	3785	183	60	75919		7117
Border Collie	M	7	Neck	48		60	39155	28	14
Border Collie	M	7	Groin	102		158	55	216	26
Border Collie	M	7	Saliva	8420	247	21155	7633		3463
Border Collie	M	4	Neck	39		60	68	3	5
Border Collie	M	4	Groin	61		60	14	3	11
Border Collie	M	4	Saliva	4450	3152	4950	High		12424
Border Collie	M	6	Neck	50		60	240	3	7
Border Collie	M	6	Groin	1754		1156	68	3	16
Border Collie	M	6	Saliva	4406	478	5213	54570		4578
Border Collie	M	2	Neck	34		60	16391	7	3
Border Collie	M	2	Groin	155		60	213	419	19
Border Collie	M	2	Saliva	3235	220	1005	High		2453
Border Collie	F	3	Neck	84		60	134	3	5
Border Collie	F	3	Groin	12		60	15	3	3
Border Collie	F	3	Saliva	2328	497	4355	78296		17570
Border Collie	M	2	Neck	74		60	2198	21	14
Border Collie	M	2	Groin	561		903	174	551	39
Border Collie	M	2	Saliva	3569	3390	60	26845		4238
Border Collie	M	6	Neck	135		60	1922	3	High
Border Collie	M	6	Groin	167		104	66	565	17
Border Collie	M	6	Saliva	6097	3518	60	53282		7976
Border Collie	F	3	Neck	225		60	3322	3	31
Border Collie	F	3	Groin	440		185	1087	3	29
Border Collie	F	3	Saliva	41048	7675	3036	30907		20978
Border Terrier	M	9	Neck	58		173	16284	47	5
Border Terrier	M	9	Groin	50		60	57	112	5
Border Terrier	M	9	Saliva	13200	944	5700	n/a		971
Border Terrier	F	9	Neck	155		60	296	3	6
Border Terrier	F	9	Groin	119		271	16	3	8

Border Terrier	F	9	Saliva	16262	5321	843	65944	12697
Border Terrier	F	13	Neck	70		60	11	3 5
Border Terrier	F	13	Groin	135		60	10	3 12
Border Terrier	F	13	Saliva	5650	3271	10371	17738	7274
Border Terrier	F	2	Neck	12		60	2876	3 8
Border Terrier	F	2	Groin	7		60	317	3 3
Border Terrier	F	2	Saliva	4125	6832	1068	130867	8092
Boxer	F	10	Neck	167		60	687	3 7
Boxer	F	10	Groin	127		66	4	3 10
Boxer	F	10	Saliva	10665	448	13206	1768	1284
Bullmastiff	F	8	Neck	41		60	285	5 6
Bullmastiff	F	8	Groin	73		60	22	3 10
Bullmastiff	F	8	Saliva	11252	1820	60	80725	15408
Cavalier King Charles Spaniel	M	1	Neck	66		60	24	3 13
Cavalier King Charles Spaniel	M	1	Groin	145		60	107	100 20
Cavalier King Charles Spaniel	M	1	Saliva	3510	391	60	45583	2702
Cavalier King Charles Spaniel	M	8	Neck	99		82	20	123 59
Cavalier King Charles Spaniel	M	8	Groin	36		60	18	131 15
Cavalier King Charles Spaniel	M	8	Saliva	1796	1662	4240	20216	6282
Chihuahua	F	9	Neck	49		60	1318	3 5
Chihuahua	F	9	Groin	42		116	619	3 8
Chihuahua	F	9	Saliva	45010	39404	63421	n/a	30189
Cocker spaniel	F	6	Neck	13		60	13	3 3
Cocker spaniel	F	6	Groin	3		60	10	3 Low
Cocker spaniel	F	6	Saliva	1587	564	1820	3751	1729
Cocker Spaniel	M	12	Neck	22		60	633	3 3
Cocker Spaniel	M	12	Groin	260		60	6	3 45
Cocker Spaniel	M	12	Saliva	n/a	n/a	n/a	n/a	8374
Coton de Tuléar	M	2	Neck	165		192	6	36 14
Coton de Tuléar	M	2	Groin	177		143	14	502 24
Coton de Tuléar	M	2	Saliva	2192	44	12030	84	512
Coton de Tuléar	F	3	Neck	383		902	17	26 62
Coton de Tuléar	F	3	Groin	77		13909	5	3 18
Coton de Tuléar	F	3	Saliva	7120	1195	7864	22493	7134
Curly Coated Retriever	M	9	Neck	86		89	7	149 8
Curly Coated Retriever	M	9	Groin	91		81	3	239 18
Curly Coated Retriever	M	9	Saliva	3050	356	1208	228652	3109
Danish-Swedish Farmdog	M	2	Neck	7		60	229	142 3
Danish-Swedish Farmdog	M	2	Groin	11		60	4	44 5
Danish-Swedish Farmdog	M	2	Saliva	903	590	60	11171	2416
Dobermann	F	5	Neck	28		79	26	7 7
Dobermann	F	5	Groin	49		60	18	8 12
Dobermann	F	5	Saliva	727	1025	7981	7615	1238
Miniature Schnauzer	M	6	Neck	105		148	78	3 10
Miniature Schnauzer	M	6	Groin	53		60	19	5 7

Miniature Schnauzer	M	6	Saliva	4499	1702	28443	35348		11955
English Bulldog	M	3	Neck	54		60	11061	3	7
English Bulldog	M	3	Groin	129		190	141	3	8
English Bulldog	M	3	Saliva	2622	4023	3406	15047		5187
English Springer Spaniel	M	6	Neck	33		60	2276	3	8
English Springer Spaniel	M	6	Groin	60		60	21	3	12
English Springer Spaniel	M	6	Saliva	27908	91408	10123	High		19380
English Springer Spaniel	M	12	Neck	88		1922	1598	4	9
English Springer Spaniel	M	12	Groin	57		17214	241	44	127
English Springer Spaniel	M	12	Saliva	19409	3216	60	62649		3647
English Springer Spaniel	F	3	Neck	196		104	1388	6	17
English Springer Spaniel	F	3	Groin	35		60	8	3	5
English Springer Spaniel	F	3	Saliva	12140	7906	60	68490		11618
Finnish Lapphund	M	1	Neck	114		319	4856	High	19
Finnish Lapphund	M	1	Groin	3		60	6	3	3
Finnish Lapphund	M	1	Saliva	675	7936	1698	35686		2104
Flatcoated Retriever	F	8	Neck	21		60	27	3	7
Flatcoated Retriever	F	8	Groin	48		3709	18	3	11
Flatcoated Retriever	F	8	Saliva	11245	277	6940	365		1095
Flatcoated Retriever	F	1	Neck	73		60	1038	3	8
Flatcoated Retriever	F	1	Groin	66		60	51	3	9
Flatcoated Retriever	F	1	Saliva	3837	586	1999	High		7174
Flatcoated Retriever	F	10	Neck	190		1757	32859	8	11
Flatcoated Retriever	F	10	Groin	319		428	973	3	36
Flatcoated Retriever	F	10	Saliva	101	4167	2600	High		20969
Flatcoated Retriever	F	10	Neck	209		2984	16003	3	35
Flatcoated Retriever	F	10	Groin	1526		1891	232	3	64
Flatcoated Retriever	F	10	Saliva	6685	3509	38910	30910		3707
Flatcoated Retriever	F	2	Neck	165		198	2445	3	17
Flatcoated Retriever	F	2	Groin	504		12676	50	3	30
Flatcoated Retriever	F	2	Saliva	16861	16661	4935	High		12433
French Bulldog	F	2	Neck	14		60	10	10	3
French Bulldog	F	2	Groin	6		60	3	3	3
French Bulldog	F	2	Saliva	3527	8143	9753	22618		8174
Golden Retriever	M	<1	Neck	27		60	7	3	9
Golden Retriever	M	<1	Groin	67		60	3	3	3
Golden Retriever	M	<1	Saliva	4930	2056	53725	High		11113
Golden Retriever	M	5	Neck	43		60	1486	22	5
Golden Retriever	M	5	Groin	352		350	98	562	37
Golden Retriever	M	5	Saliva	11044	1127	15164	76874		14522
Golden Retriever	F	11	Neck	29		60	65	3	3
Golden Retriever	F	11	Groin	255		2593	35	3	17
Golden Retriever	F	11	Saliva	6096	315	60	68877		4557
Golden Retriever	F	5	Neck	71		60	1313	4	4
Golden Retriever	F	5	Groin	391		469	574	3	49

Golden Retriever	F	5	Saliva	18831	831	72059	5262		4444
Golden Retriever	F	1	Neck	201		315	881	29	41
Golden Retriever	F	1	Groin	111		60	37	3	10
Golden Retriever	F	1	Saliva	11958	3394	4118	70496		17166
Hamilton Hound	F	4	Neck	28		60	4	3	3
Hamilton Hound	F	4	Groin	102		60	6	3	19
Hamilton Hound	F	4	Saliva	9226	4253	2471	17353		14457
Jack Russel Terrier	M	11	Neck	30		60	18437	3	11
Jack Russel Terrier	M	11	Groin	25		60	74	3	5
Jack Russel Terrier	M	11	Saliva	4557	4041	60	High		7867
Labrador Retriever	F	<1	Groin	781		259	1922	3	352
Labrador Retriever	F	<1	Neck	322		60	1620	3	170
Labrador Retriever	F	<1	Saliva	11678	118	52364	37837		3540
Labrador Retriever	F	4	Neck	24		60	599	5	4
Labrador Retriever	F	4	Groin	13		60	7	4	3
Labrador Retriever	F	4	Saliva	6950	3288	10500	10359		9678
Labrador Retriever	M	<1	Neck	749		90	1467	3	468
Labrador Retriever	M	<1	Groin	1203		100	787	3	High
Labrador Retriever	M	<1	Saliva	16726	131	17162	40983		4472
Labrador Retriever	F	7	Neck	18		60	11	3	3
Labrador Retriever	F	7	Groin	13		599	4	3	4
Labrador Retriever	F	7	Saliva	26450	12169	20620	6470		13245
Labrador Retriever	F	4	Neck	10		60	38	3	5
Labrador Retriever	F	4	Groin	27		60	3	3	5
Labrador Retriever	F	4	Saliva	2836	439	5845	1594		2351
Labrador Retriever	M	<1	Neck	2693		900	405	3	High
Labrador Retriever	M	<1	Groin	900		60	603	3	High
Labrador Retriever	M	<1	Saliva	11442	54	180563	50636		3795
Labrador Retriever	F	9	Neck	47		60	339	3	5
Labrador Retriever	F	9	Groin	18		60	10	3	3
Labrador Retriever	F	9	Saliva	1912	5308	301	7215		3268
Labrador Retriever	F		Neck	60		60	1148	3	12
Labrador Retriever	F		Groin	87		60	90	3	14
Labrador Retriever	F	9	Saliva	5749	1944	85038	2319		3263
Labrador Retriever	F	12	Neck	102		111	18450	3	17
Labrador Retriever	F	12	Groin	37		60	49	3	6
Labrador Retriever	F	12	Saliva	1178	826	84534	3585		1440
Labrador Retriever	M	1	Neck	11		60	1429	3	5
Labrador Retriever	M	1	Groin	8		161	5	3	4
Labrador Retriever	M	1	Saliva	151114	13598	66856	101655		39245
Labrador Retriever	M	5	Neck	11		60	2590	19	5
Labrador Retriever	M	5	Groin	24		60	55	47	6
Labrador Retriever	M	5	Saliva	7694	438	1060	3044		4238
Mexican Hairless Dog	M	9	Neck	31		60	9	3	9
Mexican Hairless Dog	M	9	Groin	36		60	4	3	18
Mexican Hairless Dog	M	9	Saliva	14221	853	36751	94		3918

Pug	F	11	Neck	10		60	14	3	3
Pug	F	11	Groin	17		84	7	3	3
Pug	F	11	Saliva	25110	42224	173715	n/a		2897
Newfoundland Dog	M	3	Neck	49		75	1275	5	4
Newfoundland Dog	M	3	Groin	699		2771	543	301	66
Newfoundland Dog	M	3	Saliva	5275	2301	28885	24389		15702
Norwegian Buhund	F	6	Neck	22		60	3	3	3
Norwegian Buhund	F	6	Groin	19		60	4	3	3
Norwegian Buhund	F	6	Saliva	4553	2396	189	n/a		8716
Norwegian Buhund	F	2	Neck	53		60	839	3	9
Norwegian Buhund	F	2	Groin	43		60	13	3	13
Norwegian Buhund	F	2	Saliva	50797	5105	39478	106003		27215
Norwegian Elkhound	F	8	Neck	165		156	14378	91	12
Norwegian Elkhound	F	8	Groin	268		162	47	3	22
Norwegian Elkhound	F	8	Saliva	72104	27746	32672	101109		6706
Norwegian Elkhound	F	6	Neck	43		60	21497	3	6
Norwegian Elkhound	F	6	Groin	182		60	167	3	22
Norwegian Elkhound	F	6	Saliva	17702	3485	1216	70095		5903
Spanish Water Dog	F	1	Neck	10		60	15	3	3
Spanish Water Dog	F	1	Groin	3		60	9	3	3
Spanish Water Dog	F	1	Saliva	3176	71	10715	305		1261
Spanish Water Dog	F	3	Neck	51		60	3	3	7
Spanish Water Dog	F	3	Groin	147		60	5	3	25
Spanish Water Dog	F	3	Saliva	22744	3022	18247	n/a		22554
Spanish Water Dog	F	4	Neck	57		60	24	6	12
Spanish Water Dog	F	4	Groin	47		60	11	7	8
Spanish Water Dog	F	4	Saliva	532	71	15983	294		259
Spanish Water Dog	F	7	Neck	30		60	50	3	6
Spanish Water Dog	F	7	Groin	74		60	21	3	11
Spanish Water Dog	F	7	Saliva	4573	273	45523	2095		4855
Pomeranian	F	2	Neck	318		201	1422	20	18
Pomeranian	F	2	Groin	223		636	291	19	11
Pomeranian	F	2	Saliva	5751	1740	5108	5901		1783
Pomeranian	F	2	Neck	628		98	8542	4	59
Pomeranian	F	2	Groin	265		122	8	3	13
Pomeranian	F	2	Saliva	21015	1444	4374	101293		15904
Portuguese Water Dog	M	8	Neck	33		60	5	8	7
Portuguese Water Dog	M	8	Groin	167		222	5	3	18
Portuguese Water Dog	M	8	Saliva	5600	165	54150	295		3777
Portuguese Water Dog	F	6	Neck	50		60	20	12	15
Portuguese Water Dog	F	6	Groin	58		60	3	4	7
Portuguese Water Dog	F	6	Saliva	272722	657	1472	92554		29981
Miniature Poodle	F	3	Neck	54		60	480	3	8
Miniature Poodle	F	3	Groin	145		109	15	3	3
Miniature Poodle	F	3	Saliva	30028	2986	6176	95264		28970

Medium Poodle	F	3	Neck	78		60	2680	3	8
Medium Poodle	F	3	Groin	85		60	31	3	11
Medium Poodle	F	3	Saliva	42554	38609	1688	102075		11896
Medium Poodle	F	2	Neck	49		60	179	3	4
Medium Poodle	F	2	Groin	42		60	4	3	6
Medium Poodle	F	2	Saliva	2624	5334	3985	4098		4158
Standard Poodle	M	12	Neck	155		244	80787	905	17
Standard Poodle	M	12	Groin	864		514	930	522	45
Standard Poodle	M	12	Saliva	1900	595	1094	9475		1413
Standard Poodle	M	4	Neck	1175		547	High	3458	63
Standard Poodle	M	4	Groin	977		249	933	High	41
Standard Poodle	M	4	Saliva	4650	1093	2700	26202		6476
Standard Poodle	F	2	Neck	72		60	1463	3	13
Standard Poodle	F	2	Groin	22		60	14	3	3
Standard Poodle	F	2	Saliva	6730	1811	60	n/a		4246
Standard Poodle	F	5	Neck	661		703	7232	3	High
Standard Poodle	F	5	Groin	293		60	579	3	36
Standard Poodle	F	5	Saliva	213	119	60	16913		5264
Standard Poodle	M	13	Neck	283		70	High	60	22
Standard Poodle	M	13	Groin	177		60	1249	321	15
Standard Poodle	M	13	Saliva	1638	2141	1075	43311		1733
Standard Poodle	M	3	Neck	277		243	175432	94	24
Standard Poodle	M	3	Groin	255		286	2149	252	52
Standard Poodle	M	3	Saliva	3315	5063	679	303083		5830
Standard Poodle	M	3	Neck	38		60	25454	3	7
Standard Poodle	M	3	Groin	315		76	646	319	84
Standard Poodle	M	3	Saliva	3058	1523	1008	27388		4819
Standard Poodle	F	9	Neck	379		390	5890	7	83
Standard Poodle	F	9	Groin	40		60	51	3	9
Standard Poodle	F	9	Saliva	8284	3393	3854	10465		3617
Standard Poodle	M	2	Neck	19		60	559	3	3
Standard Poodle	M	2	Groin	15		60	25	62	3
Standard Poodle	M	2	Saliva	25573	3210	2820	80304		8240
Toy Poodle	F	<1	Neck	873		105	2333	4	High
Toy Poodle	F	<1	Groin	4424		87	554	4	High
Toy Poodle	F	<1	Saliva	27921	879	8926	7363		7704
Toy Poodle	F	<1	Neck	2021		95	370	17	High
Toy Poodle	F	<1	Groin	1220		280	931	4	High
Toy Poodle	F	<1	Saliva	57329	14634	75747	92083		38988
Toy Poodle	F	<1	Neck	279		60	44	3	77
Toy Poodle	F	<1	Groin	546		153	670	3	High
Toy Poodle	F	<1	Saliva	336531	38906	36719	106586		37013
Rhodesian Ridgeback	F	3	Neck	72		302	750	3	15
Rhodesian Ridgeback	F	3	Groin	108		181	57	3	11
Rhodesian Ridgeback	F	3	Saliva	4205	639	1615	1211		3782
Rhodesian Ridgeback	F	6	Neck	50		150	345	19	10

Rhodesian Ridgeback	F	6	Groin	311		639	50	5	32
Rhodesian Ridgeback	F	6	Saliva	1639	172	6628	355		2480
Rhodesian Ridgeback	M	<1	Neck	35		60	984	3	5
Rhodesian Ridgeback	M	<1	Groin	83		292	51	High	16
Rhodesian Ridgeback	M	<1	Saliva	2927	47	37056	371		594
Giant Schnauzer	F	3	Neck	58		60	45	3	10
Giant Schnauzer	F	3	Groin	56		60	10	3	8
Giant Schnauzer	F	3	Saliva	2064	3417	6103	42172		4929
Giant Schnauzer	F	8	Neck	64		60	1923	3	6
Giant Schnauzer	F	8	Groin	24		60	16	3	3
Giant Schnauzer	F	8	Saliva	6339	3920	34253	46078		11814
Shetland Sheepdog	F	2	Neck	22		60	1346	22	4
Shetland Sheepdog	F	2	Groin	134		60	68	186	15
Shetland Sheepdog	F	2	Saliva	4328	2177	4332	39995		5561
Shetland Sheepdog	F	8	Neck	14		60	446	3	3
Shetland Sheepdog	F	8	Groin	290		60	27	3	66
Shetland Sheepdog	F	8	Saliva	11248	1017	90366	21430		6372
Shiba	F	13	Neck	34		60	77	3	4
Shiba	F	13	Groin	49		60	14	3	7
Shiba	F	13	Saliva	5717	1375	2815	High		11399
Shiba	F	2	Neck	20		60	29	3	3
Shiba	F	2	Groin	46		60	5	5	5
Shiba	F	2	Saliva	482	2172	1716	27631		1780
Shiba	M	7	Neck	14		60	46	3	3
Shiba	M	7	Groin	156		60	17	17	19
Shiba	M	7	Saliva	5595	1835	1206	116827		11968
Shiba	F	2	Neck	24		60	293	3	3
Shiba	F	2	Groin	67		60	31	3	6
Shiba	F	2	Saliva	5568	1149	60	High		1776
Shiba	M	3	Neck	10		60	15	3	3
Shiba	M	3	Groin	158		60	19	29	12
Shiba	M	3	Saliva	2830	1312	60	High		6473
Shiba	F		Neck	13		60	199	3	3
Shiba	F		Groin	154		60	27	3	10
Shiba	F	11	Saliva	3277	890	1719	17039		4542
Shih Tzu	F	5	Neck	40		60	956	3	3
Shih Tzu	F	5	Groin	30		60	5	3	12
Shih Tzu	F	5	Saliva	13068	1749	3694	77028		19616
Hungarian Vizsla	F	13	Neck	27		60	667	3	3
Hungarian Vizsla	F	13	Groin	76		60	17	3	5
Hungarian Vizsla	F	13	Saliva	29594	1654	4659	88505		13693
Welsh Springer Spaniel	F	10	Neck	110		60	25	3	5
Welsh Springer Spaniel	F	10	Groin	220		133	16	3	10
Welsh Springer Spaniel	F	10	Saliva	1428	82	11414	232		1723
Welsh Springer Spaniel	F	4	Neck	63		60	18	3	6

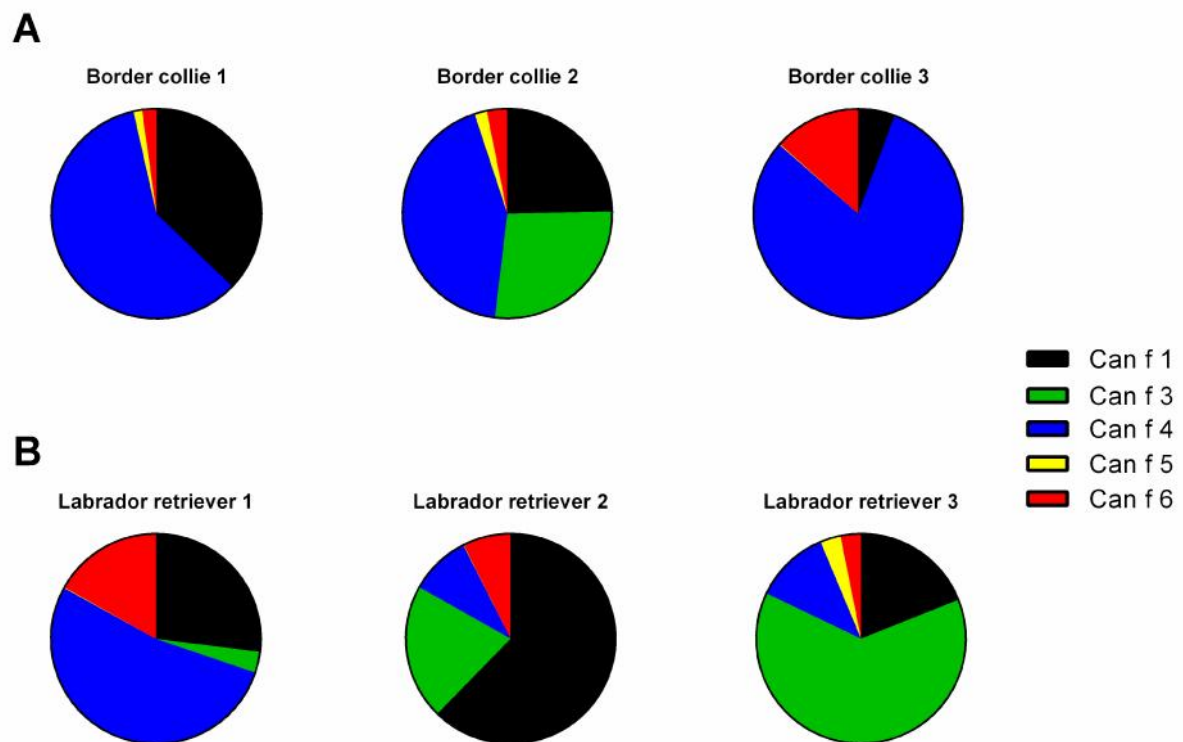
Welsh Springer Spaniel	F	4	Groin	740		250	91	3	63
Welsh Springer Spaniel	F	4	Saliva	14188	2562	10808	8277		12124
Whippet	F	8	Neck	238		60	2018	3	33
Whippet	F	8	Groin	47		60	28	3	7
Whippet	F	8	Saliva	7976	690	8001	426		3125
Swedish Vallhund	M	8	Neck	30		346	15876	3	4
Swedish Vallhund	M	8	Groin	103		217	19469	23	10
Swedish Vallhund	M	8	Saliva	11040	2244	9097	198658		15665

Supplementary table SII

SPT solutions and extract batches examined in the study, listed in randomized order.

Brand	Product	Batch
Greer	Dog epithelia	289477
Inmunotek	E802	14S02P
Bencard	Prick-Testlösung	A13852
ALK	Soluprick SQ 553	M2047_SE03
Stallergens	Alyostal 509	9715916
Source material	Product	Batch
Greer	Dog epithelia	210878
Greer	Dog hair	244666
Greer	Dog dander	209937
Allergon	Dog dander E5	228611901

Supplementary figure S1



Example of individual allergen profiles of three Border collies (A) and three Labrador retrievers (B).

References

1. Graslund S, Savitsky P, Muller-Knapp S. In Vivo Biotinylation of Antigens in E. coli. *Methods Mol Biol.* 2017;1586:337-344.
2. Nilsson OB, Neimert-Andersson T, Bronge M, et al. Designing a multimer allergen for diagnosis and immunotherapy of dog allergic patients. *PLoS One.* 2014;9(10):e111041.