

Skin Prick Test Extracts for Dog Allergy Diagnosis Show Considerable Variations Regarding the Content of Major and Minor Dog Allergens

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Key Words

Dog allergens · Dog allergy diagnosis · Skin prick test extracts

Abstract

Background: Commercial skin prick test (SPT) extracts used for the diagnosis of dog allergy are prepared by extracting allergens from natural sources, e.g. dog hair and dander. Due to different starting material and extraction methods used, it is likely that extracts differ regarding their allergen contents. **Methods:** The total protein content and composition of dog SPT extracts from 5 European manufacturers were compared by silver-stained SDS-PAGE. Specific antibody probes were generated to detect major and minor allergens in each extract by immunoblotting. Additionally, sera of patients suffering from dog allergy were used to detect dog allergens in SPT extracts. **Results:** SPT extracts showed a 20-fold variation regarding the total protein content. The contents of the major dog allergen Can f 1 and of Can f 2 varied considerably between the extracts. In one of the extracts, neither Can f 1 nor Can f 2 could be detected by immunoblotting. The contents of the minor dog allergen Can f 3, albumin, also showed great variability. In one of the dog SPT

extracts, the presence of human serum albumin (HSA) was detected with HSA-specific antibodies. **Conclusion:** The observed variability of commercial dog SPT extracts regarding their allergen contents likely has a negative influence on the accuracy of diagnosis of dog allergy.

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Introduction

Together with house dust mites and cats, dogs are important sources of indoor allergens [1]. Up to 40% of asthmatic individuals are sensitized to dogs. Dog hair, dander, serum, saliva and urine were shown to contain several allergenic proteins [reviewed in 2, 3]. A lipocalin protein family member, Can f 1, has been identified as a major dog allergen. Between 52 and 75% of dog-allergic patients showed IgE reactivity against Can f 1 depending on the studied population [4–7]. Another member of the lipocalin protein family, Can f 2, represents a minor dog allergen with 20–30% of dog-allergic patients showing IgE reactivity to it [4, 6, 7]. Can f 3, i.e. dog serum albumin, is an important cross-reactive allergen, and depending on the tested population up to 35% of dog-allergic

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patients reacted against Can f 3 [8, 9]. Other dog allergens have been identified only recently, and they include a Fel d-1-like allergen [10] and prostatic kallikrein [11].

Diagnosis of dog allergy in daily practice relies on the clinical history of the patient together with skin prick tests (SPT) with commercial dog SPT extracts. SPT extracts are mostly prepared by extracting allergens from natural sources and contain a variety of allergenic and non-allergenic proteins [12]. Different manufacturers use different starting materials like dog hair, dander and/or epithelium [13] and different extraction methods [14]. Several studies with natural extracts from other allergen sources reported heterogeneity between the extracts of different manufacturers regarding their allergen contents and as a consequence their allergenic potency [15–17]. It was also shown that natural extract preparations might contain mite allergens potentially causing false-positive SPT results [18].

The importance of standardization of allergen extracts has been recognized early, and the international standard for dog hair dander extract was established in 1986 on the basis of the study conducted by 15 laboratories worldwide [19]. The international standard was assigned a unitage of 100,000 IU per ampule and refers to both the total allergenic activity of the ampule and the allergenic activity of the individual allergens.

However, allergen manufacturers still use different in-house standardization methods and display their results using a variety of company-specific units.

In this study, we raised highly specific antibodies against Can f 1, Can f 2 and Can f 3 and compared commercial allergen SPT extracts currently used for the diagnosis of dog allergy regarding the presence and relative amounts of major and minor dog allergens. In addition, we compared the IgE reactivity of dog-allergic individuals with the commercial SPT extracts.

Materials and Methods

Allergen Extracts

Commercial SPT solutions for diagnosis of dog allergy were purchased from 5 European manufacturers (1: Alyostal 0509, dog hair, 100 RI/ml, Ch.B. 74561; 2: Allergopharma 306, dog epithelium, 10,000 BE/ml, Ch.B. U7001689-AT; 3: ALK Abello B402-L553, dog hair, 10 HEP, Ch.B. C0161; 4: Bencard 3205-030-043, dog hair, 10,000 DU/ml, Ch.B. A11002LP; 5: Hal 15-01, 1% dog epithelium, Ch.B. B1734016). Extracts were stored at 4°C as recommended by the manufacturer.

An in-house dog allergen extract was prepared by incubating dog hair in water for 90 min in the presence of protease inhibitors (Sigma, Vienna, Austria) with occasional vortexing. The total

protein concentration of commercial and in-house made dog extracts was determined using the Bradford assay with bovine serum albumin as a standard [20]. For this purpose, commercial SPT extracts were dialysed against distilled water using Slide-A-Lyzer Dialysis Cassettes with a cut-off at 3.5 kDa (Pierce, Rockford, Ill., USA). Since the volumes changed during dialysis, these changes were recorded exactly and used to calculate the final protein concentration. SDS-PAGE/immunoblotting experiments were performed with the original extract solutions.

Antibodies and Human Sera

The rabbit anti-Can-f-1 and anti-Can-f-2 antisera were raised against recombinant Can f 1 and recombinant Can f 2 which were produced in *Escherichia coli* as described [21] but without the GST portion (Charles River Breeding Laboratories, Kisslegg, Germany).

A rabbit anti-albumin antiserum cross-reacting with dog albumin was raised against a synthetic peptide, LAKEYEATLEKC-CATDDPPACYAHVFDEFK, from cat albumin. The peptide was synthesized using Applied Biosystems peptide synthesizer Model 433A (Foster City, Calif., USA), coupled to keyhole limpet haemocyanin and used for immunization of a rabbit (Charles River Breeding Laboratories). The rabbit antiserum reacted with dog albumin but not with human serum albumin (HSA).

A monoclonal anti-HSA antibody (clone HSA-11, A6684) was purchased from Sigma.

Human sera were obtained from patients with a positive case history of type I allergy to dogs and IgE antibodies to dog dander extract as determined by ImmunoCAP (Phadia, Uppsala, Sweden).

SDS-PAGE and Immunoblotting

The total protein compositions of dog SPT extracts were compared analysing equal volumes (15 µl/lane) of each extract by SDS-PAGE (15% separating gel) [22] and subsequent silver staining. For immunoblotting, equal volumes (7 µl/lane) of dog SPT extracts were separated by SDS-PAGE and transferred to nitrocellulose membrane (Schleicher & Schüll, Dassel, Germany) [23].

For specific detection of the allergens Can f 1 and Can f 2 in the extracts, blots were probed with rabbit anti-Can-f-1 or anti-Can-f-2 serum in a dilution of 1:2,000. For the detection of dog albumin, the rabbit anti-albumin antiserum reacting with dog albumin was used in a dilution of 1:1,000. The corresponding pre-immune sera were used as negative controls. Bound rabbit IgG antibodies were detected with a ¹²⁵I-labelled donkey anti-rabbit antiserum (GE Healthcare, Chalfont, St. Giles, UK) in a dilution of 1:4,000 and visualized by autoradiography, which was performed at –80°C for 24 h using Kodak X-Omat™ Blue (XB-1) films (Kodak, Heidelberg, Germany) and an intensifying screen.

HSA was detected using the monoclonal mouse anti-HSA antibody in a dilution of 1:2,500. Bound mouse IgG was detected with an alkaline-phosphatase-coupled goat anti-mouse antiserum (Pierce) in a dilution of 1:1,000. The blot was then developed with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate.

Sera of 4 allergic patients with a history of dog allergy were used in this study. All patients experienced at least one of the typical clinical symptoms (rhinitis, conjunctivitis, atopic dermatitis and asthma) upon exposure to dog and also had IgE antibodies to e5 (dog dander) of at least class 4 as determined by ImmunoCAP. Sera were used to probe blotted extracts in a dilution of 1:10.

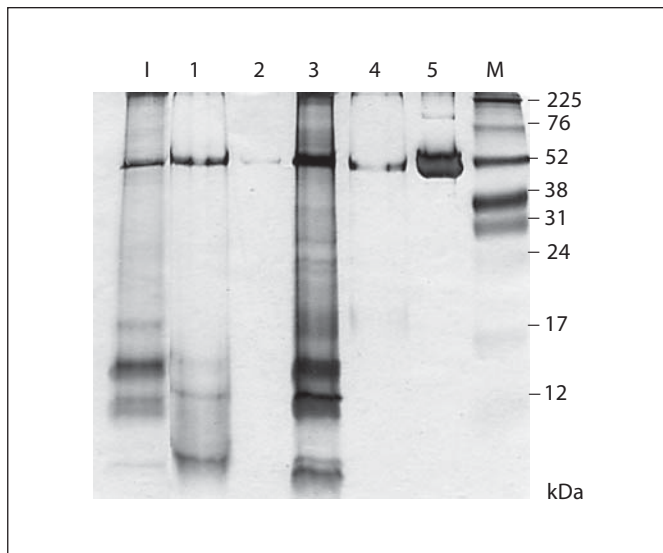


Fig. 1. Variability of total protein composition between commercial SPT extracts. Proteins of in-house (I) and commercial SPT extracts for dog allergy diagnosis from 5 European manufacturers (lanes 1–5) were separated by SDS-PAGE and visualized by silver staining. M = Molecular weight marker.

Bound human IgE was detected with ^{125}I -labelled anti-human IgE (IBL, Hamburg, Germany) in a dilution of 1:20 and visualized by autoradiography [24].

For IgE immunoblot inhibition experiments, patients' sera were pre-incubated with dog dander extract (70 $\mu\text{g}/\text{ml}$), rCan f 1 (10 $\mu\text{g}/\text{ml}$), rCan f 2 (10 $\mu\text{g}/\text{ml}$) and, for control purposes, with PBS for 12 h at 4°C. Thereafter, IgE immunoblotting was performed as described [24].

Results

Quantitative and Qualitative Heterogeneity of Proteins in Commercial SPT Extracts

The total protein contents in the 5 commercial extracts showed an approximately 20-fold variation. The protein content was the highest in the extract from manufacturer 3 (278.63 $\mu\text{g}/\text{ml}$) followed by manufacturer 5 (185.35 $\mu\text{g}/\text{ml}$), manufacturer 1 (145.42 $\mu\text{g}/\text{ml}$), manufacturer 4 (81.07 $\mu\text{g}/\text{ml}$) and manufacturer 2 (13.77 $\mu\text{g}/\text{ml}$). The qualitative protein composition of the extracts was compared by SDS-PAGE and subsequent silver staining (fig. 1). The extracts showed great heterogeneity regarding their composition. Only extract 3 contained densely packed bands between 3.5 and 225 kDa. The pattern of protein bands between 17 and 24 kDa corresponding to the size of the important dog allergens Can f 1 and

Can f 2 was highly heterogeneous, with extracts 2, 4 and 5 having no visible bands at all in this area (fig. 1). Each of the extracts contained 1 or more bands between 52 and 76 kDa where the minor dog allergen Can f 3 can be expected.

Commercial SPT Extracts Contain Varying Amounts of Major and Minor Dog Allergens

Specific antibodies against Can f 1 (fig. 2a) and Can f 2 (fig. 2b) were used to detect these allergens in the 5 nitrocellulose-blotted commercial SPT extracts (lanes 1–5) and in 1 in-house made extract (fig. 2, I). The intensities of Can f 1 and Can f 2 signals showed great variability between the commercial extracts. The SPT extract from manufacturer 3 showed the signals of highest intensities for both allergens followed by extract number 4. Can f 1 and Can f 2 signals in SPT from manufacturers 2 and 5 were weaker, but still detectable. The fact that the allergens could not be observed in SDS-PAGE in extracts 2, 4 and 5 but were detected by immunoblotting may be explained by the higher sensitivity of the immunoblot method. Surprisingly, in extract from manufacturer 1, the important allergens could not be detected at all by immunoblotting.

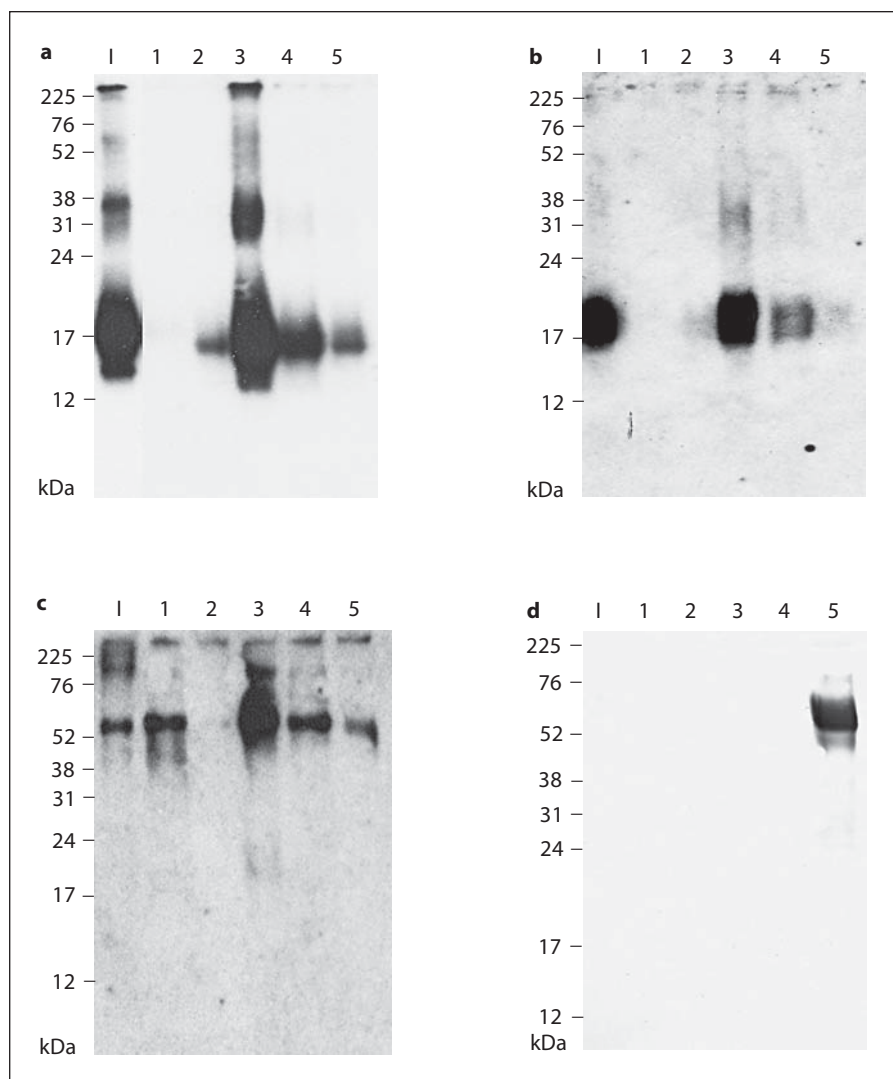
Additional bands between 31 and 38 kDa detected in some of the extracts may represent dimers of Can f 1 and Can f 2 [25].

The minor dog allergen Can f 3 was detected in all extracts but in variable amounts (fig. 2c). The signal was the strongest in extract 3 and almost not detectable in extract 2. In all immunoblotting experiments, probing with corresponding pre-immune rabbit sera gave no signal (data not shown). Since it has been suggested in a previous study [16] that SPT solutions of the manufacturer of extract 5 might contain HSA, we investigated whether the prominent band of approximately 69 kDa in extract 5 indeed represents HSA. By exposing immunoblots to an anti-HSA-specific antibody, HSA could be detected in extract 5 but not in the other commercial extracts (fig. 2d).

Commercial SPT Extracts Show Varying Reactivity with IgE Antibodies from Allergic Patients

To further compare the allergen content of different SPT extracts, sera of dog-allergic patients containing IgE antibodies against dog allergens were used to probe nitrocellulose-blotted commercial SPT extracts. Again, a considerable heterogeneity was detected between the extracts of different manufacturers. The IgE reactivity profiles of 4 representative patients are shown in figure 3a–d. The clear band between 52 and 76 kDa in figure 3a and c

Fig. 2. Allergen contents of commercial SPT extracts show considerable heterogeneity. Nitrocellulose-blotted in-house dog hair extract (I) and 5 commercial SPT extracts for dog allergy diagnosis (lanes 1–5) were probed with the following allergen-specific antibodies: anti-Can-f-1-specific rabbit serum (**a**); anti-Can-f-2-specific rabbit serum (**b**); anti-dog-serum-albumin-specific rabbit serum (**c**); anti-HSA monoclonal antibody (**d**). Molecular masses are indicated in the left margins.



represents dog albumin. The identification of bands between 12 and 24 kDa was more difficult and required inhibition experiments with rCan f 1 (data not shown). The band of 18 kDa was shown to be Can f 1 whereas bands of 12, 15 and 31 kDa represent dog allergens which are not known. The serum of a non-atopic individual was used as a negative control. It showed no signal upon testing with dog SPT extracts (data not shown).

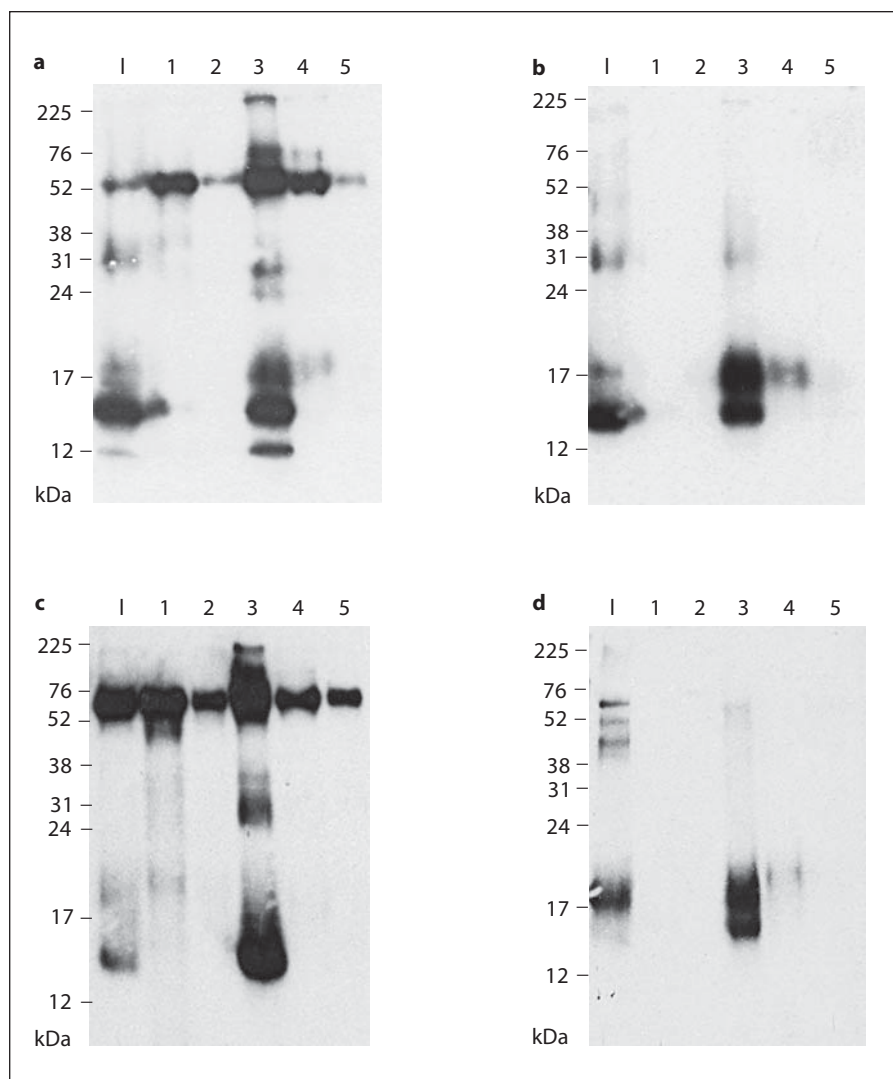
Discussion

Our analysis revealed that 5 commercial dog allergen SPT extracts differed 20-fold regarding the total protein contents. Furthermore, we could show by immunoblot-

ting that the extracts varied in the contents of major and minor dog allergens, and in one of the extracts, the allergens Can f 1 and Can f 2 were below the detection level. Although an immunoblot does not represent a quantitative method, the differences in the signal intensities for the tested allergens were obvious between the extracts of different manufacturers. Based on the different reactivity of the dog-allergic patients' sera to 5 commercial SPT extracts, it is very likely that the SPT extracts will give different results when applied for in vivo testing.

There may be several possible reasons for the observed heterogeneities of the extracts: (i) different starting material from which manufacturers extract allergens (hair, dander or/and epithelium) may contain different allergens [8, 26], (ii) the levels of the major dog allergen Can

Fig. 3. Commercial SPT extracts show differences in IgE reactivity when probed with the sera of allergic patients. Nitrocellulose-blotted in-house dog hair extract (I) and 5 commercial SPT extracts for dog allergy diagnosis (lanes 1–5) were probed with IgE antibodies from the sera of 4 dog-allergic patients (a–d). Molecular masses are indicated in the left margins.



f 1 may vary between dog breed and sex and between individual dogs of the same breed [27], (iii) different extraction techniques as well as the presence of stabilizers like glycerol and phenol might contribute to differences in extract composition [14, 28].

HSA has also been reported to influence the stability of natural allergen extracts [14, 28]. We detected HSA with highly specific antibody probes that can discriminate human from animal albumin in extract 5 which was derived from a company that adds HSA also to pollen extracts [16, 17]. Interestingly, this extract indeed contained all 3 tested allergens but in lower concentration than extracts 3 and 4.

In conclusion, natural dog allergen extracts show considerable heterogeneity in their allergen composition and

this will likely negatively affect the accuracy of SPT testing. These shortcomings of natural extracts can be overcome by replacing natural extracts with well-defined recombinant allergens.

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