Original paper

Allergy to grass pollen: mapping of *Dactylis glomerata* and *Phleum pratense* allergens for dogs by two-dimensional immunoblotting

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Abstract

Introduction: Much less is known about grass-pollen allergens to dogs, when compared with humans. Genetic-based patterns might play an important role in sensitization profiles, conditioning the success of allergen-specific immunotherapy.

Aim: Mapping of *Dactylis glomerata* (*D. glomerata*) and *Phleum pratense* (*P. pratense*) allergens for grass pollen-sensitized atopic dogs, for better understanding how individual allergograms may influence the response to grass-pollen immunotherapy.

Material and methods: To identify *D. glomerata* and *P. pratense* allergoms for dogs, 15 individuals allergic to grass pollen and sensitized to *D. glomerata and P. pratense* were selected. *D. glomerata* and *P. pratense* proteomes were separated by isoelectric focusing (IEF), one-dimensional (1-D) and two-dimensional (2-D) sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were blotted onto *Polyvinylidene difluoride* (PVDF) membranes and allergens were identified by patient sera IgE in Western Blotting (WB).

Results: In *D. glomerata*, 17 allergens were identified from IEF and 11 from 1-D SDS-PAGE, while from *P. pratense*, 18 and 6 allergens were identified, respectively. From 2-D SDS-PAGE 13 spots were identified from *D. glomerata* and 27 from *P. pratense*.

Conclusions: Several similarities were found between dog and human *D. glomerata* and *P. pratense* sensitization profiles but no relationship between clinical signs and a specific pattern of allergen recognition was observed. Similarities were found in each patient pattern of sensitization between *D. glomerata* and *P. pratense*, also suggesting cross-reactive phenomena. Further molecular epidemiology approach is needed to understand the role of the sensitization pattern in allergen-specific immunotherapy effectiveness in grass-pollen allergic dogs.

Key words: allergens, atopy, dog, grass pollen allergens, immunoblotting.

Introduction

After a clinical diagnosis of atopy, which criteria have been successively improved [1], extensive laboratory data are very useful to understand sensitization patterns and for a better clinical follow-up [2]. Intradermal skin testing (IDT) and specific IgE determinations are also useful to establish eviction measures and consider allergen-specific immunotherapy (ASIT), in order to obtain clinical improvement [3]. In fact, allergen-specific immunotherapy, especially for aeroallergens, has proven to be useful for allergy control, by promoting substantial clinical improvement in 65–75% of the cases [4] and should be prescribed as directed as possible [3, 5]. Nevertheless, those figures may be safely improved by using more specific allergen pools for specific immunotherapy, which is already being studied for human allergy management [6–10].

Studies performed by Kubota *et al.* [11] with *Cryptomeria japonica* pollen allergens, using sera from sen-

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sitized dogs with atopic dermatitis, showed important differences in terms of individual allergen recognition between humans and dogs. In fact, a molecule that has shown to be a major allergen for humans may be a minor one for dogs and vice versa. Sometimes, an allergen for a given species may even not be so for another. More recently, Ognjenovic *et al.* [12] reported several similarities, as well as differences, between dog and human sensitization to the *Ambrosia artemisiifolia* pollen, with 81% recognition of the 38 kDa band, belonging to the Amb a 1 group, between sensitized dogs.

For a precise allergy diagnosis, or indication of allergen-directed eviction measures [2], as well as to improve the efficacy of allergen-specific immunotherapy [13], further fine tune laboratory information on the molecular diagnosis seems to be truly helpful.

Following very useful concepts, such as component-resolved diagnosis and immunotherapy (CRD and CRIT) [14–16], characterization of patient serum IgE spectrotypes (individual allergograms) by WB techniques, with electrophoretic-separated molecular allergens, may provide relevant information, especially for an immunotherapy decision [13].

For the determination of dog-specific serum IgE, ELISA has been the routine laboratory method, but when comparing with the latest-generation methods, sensitivity and specificity has shown to be lower [17]. However, the lack of standardization of those new methods for veterinary proposal is still an obstacle. An extraordinary advance for allergy complementary diagnosis is based on the protein microarray systems [15]. The Immune Solid-phase Allergen Chip method (ISAC – Thermo Fisher Scientific, USA), despite its solely qualitative and semi-quantitative ability, allows the detection of very small amounts of specific IgE, which may nevertheless be of clinical significance [8]. While protein microarray systems are still far from veterinary routine, the characterization of patient IgE sensitization profile by WB over electrophoretic-separated allergens may provide relevant information, especially to understand particular conditions related to cross-reaction phenomena and immunotherapy effectiveness [2, 8, 13].

In this work the authors intended to further map *Dactylis glomerata* (*D. glomerata*) and *Phleum pratense* (*P. pratense*), two well-known allergen sources for humans [18, 19], allergens for grass pollen-sensitized atopic dogs, in order to settle the necessary conditions to start understanding how individual allergograms may influence the response to ASIT.

Aim

By mapping *D. glomerata* and *P. pratense* allergens for grass pollen-sensitized atopic dogs, the authors intended to disclose the allergens from those two relevant sources for dogs, for further understanding how individual allergograms may influence the response to grass-pollen immunotherapy.

Material and methods

Patient selection and characterization

At the dermatology and allergy outpatient consultation from the University of Évora Veterinary Hospital (Évora, Portugal), all the patients were submitted to an extensive query for anamnestic and clinical parameters and selected by filling the clinical criteria defined by the International Task Force on Canine Atopic Dermatitis for atopy diagnosis [1]. For further selection, patients were submitted to IDT with different allergen sources, such as grass, herbs and tree pollens, mites and fungi, and positive (histamine solution) and negative (solvent extract) controls from Bial Aristegui (Bilbao, Spain). Specific IgE for a panel of the most common mites and pollens was also determined according to Lee *et al.* [20] (Univet, Barcelona, Spain). Fourteen of those, mostly indoor patients and presenting atopic dermatitis, which tested positive in the IDT and presented with specific IgE for *D. glomerata* and *P. pratense*, were selected. Only one patient (no. 6) was already undergoing specific immunotherapy.

Allergen extract preparation

Lyophilized pollens from *D. glomerata* and *P. pratense* (Allergon, Angelholm, Sweden) were submitted to extraction by 2 h orbital stirring at 1 : 10 in 4°C double-distilled water, for IEF, and in phosphate buffered saline (PBS) for SDS-PAGE. The extract was centrifuged at 3500 g for 10 min at 4°C and supernatant protein concentration measured according to the Warburg Christian Method [21] in a spectrophotometer (Beckman DU530 UV-VIS, Beckman Coulter, Brea, USA).

Protein separation

Isoelectric focusing (IEF)

Dactylis glomerata and *P. pratense* extracts (protein concentration of 4.6 mg/ml) were subjected to pH 3–10 IEF (Ampholytes from GE Healthcare, USA) in 5% polyacrylamide gel (Acrylamide/Bisacrylamide from Bio-Rad, Hercules, USA). Separated proteins were then blotted onto methanol-activated 0.2-µm-pore PVDF membranes (Bio-Rad) according to the technique described by Martins *et al.* [22] for isoelectric-focused protein transference. Both extracts were also subjected to IEF, to produce 1-D gel strips for the 2-D gel electrophoresis.

SDS-PAGE

Dactylis glomerata and *P. pratense* extracts were treated with SDS, under non-reducing conditions, and submitted to 12% PAGE. Separated proteins were blotted under electrotransference [23] onto 0.2 µm-pore PVDF membranes in a Trans-Blot Semi-Dry Transfer Cell

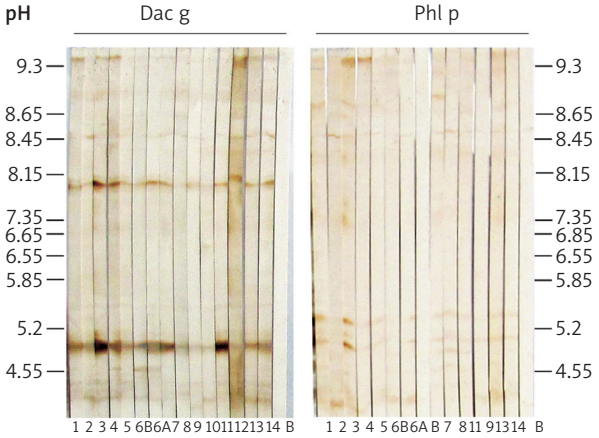

Figure 1. IgE immunoblots from *D. glomerata* and *P. pratense* IEF, revealed with horseradish peroxidase-conjugated goat anti-dog-IgE. Patients/lanes no. 1–14. Patient no. 6 before (6B) and after (6A) specific immunotherapy B – Blank controls without serum

(Bio-Rad). Two-dimensional separations were performed by 12% SDS-PAGE of the 1-D IEF strips and separated proteins were also blotted onto methanol-activated 0.2 µm-pore PVDF membranes.

Antigen detection – immunoblotting

One-dimensional blotted membranes were cut in 3-mm-wide strips and immersed for 2 × 0.5 h in blocking buffer (1% non-fat dry milk – 0.1% Tween-20 in Tris-buffered saline, at pH 7.4) at room temperature and each strip was incubated overnight with individual patient sera, diluted at 1 : 10 in 1 ml blocking buffer. Fourteen patients' (no. 1 to 14) sera were tested with *D. glomerata* and *P. pratense* strips, from IEF and from SDS-PAGE. Two samples were used from patient no. 6: one before immunotherapy (6B) and another after a year of immunotherapy (6A). For specific IgE detection, strips were incubated for 2 h with horseradish peroxidase-conjugated goat anti-dog-IgE antibody (Bethyl Laboratories, Inc., Montgomery, USA) at 1 : 1000 in blocking buffer, followed by 4 × 5 min washes in 0.9% NaCl – 0.1% Tween-20. Another 4 × 5 min washing cycle was carried out and specific IgE visualization performed by chromogenic reaction with 3.3'-diaminobenzidine/$NiCl_2$ (Sigma-Aldrich, St. Louis, USA) in Tris-buffered saline at pH 7.4.

Two-dimensional immunoblottings were performed by incubating blotted membranes with a pool of 7-patient sera (no. 1, 2, 3, 5, 6A, 13 and 14), selected for their representative IgE binding in IEF and 1-D SDS-PAGE blots, diluted to 1 : 10 in 18 ml blocking buffer. Specific IgE was detected by anti-dog-IgE biotin-labeled monoclonal antibody (AbD Serotec, Kidlington, UK) at 1 : 500 in blocking buffer, followed by 4 × 5 min washes in 0.9% NaCl – 0.1% Tween-20 and 1 h incubation with ExtrAvidin-alkaline phosphatase (Sigma-Aldrich) at 1 : 5000. Another 4 × 5 min washing cycle was done and specific IgE visualization was performed by chromogenic reaction with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich) in substrate buffer. Controls (B – blank) were performed with sera from 4 non-atopic dogs, negative for specific IgE to grass pollens.

Statistical analysis

Data distribution was verified with the Kolmogorov-Smirnov test and homogeneity of variance assessed by the Levene's test. As there was no normal distribution, the correlation between two variables was determined by the Spearman's rank correlation coefficient, considering $p < 0.05$ as significant.

Results

All patients presented with specific IgE and positive IDT for *D. glomerata* and/or *P. pratense*. Mean specific IgE was 753.0 Elisa absorbance units (EAU) to *D. glomerata* (min. of 92 and max. of 3204) with a standard deviation (SD) of 738.4, and 874.8 EAU to *P. pratense* (min. of 89 and max. of 3426; SD = 894.0).

From *D. glomerata* IEF separation, at least 17 allergens were identified from pI 3.7 to 9.1 (Figure 1 and Table 1). From these 17, 10 bands (pI 3.9, 4, 4.2, 4.3, 4.4, 4.8, 5.4, 7.6, 7.7 and 9.1) showed major recognition (Table 1 and Figure 1). The mean number of *D. glomerata* recognized IEF allergen bands/patient was 9.2 (min. of 3 and max. of 15; SD = 3.38). Two allergen bands (pI 5.4 and 7.6) were recognized by more than 85% of the patients and another one (pI 4.8) was recognized by all patients, while two others (pI 3.7 and 6.5) were only recognized by one patient, each (no. 9 and no. 8, respectively). From *P. pratense* IEF separation, at least 18 allergens were identified from pI 4 to 9.8. From these 18, seven bands showed major recognition (pI 4, 4.5, 4.8, 4.9, 5.4, 8.1 and 9.8), while three bands (pI 7.3, 7.6 and 9.4) were only recognized by one patient (no. 3) and patient no. 9 only recognized two allergens (pI 4 and 6.1) (Table 2 and Figure 1). The mean number of *P. pratense* recognized IEF allergen bands/patient was 6.9 (min. of 2 and max. of 17; SD = 4.15).

From *D. glomerata* 1-D SDS-PAGE, 12 allergens were identified by patient sera, with molecular weights (MW) from 11 to 110 kDa. Seven of these allergen bands (MW close to 16, 23, 27, 35, 42, 45 and 65 kDa) showed major recognition. The mean number of *D. glomerata* recognized PAGE allergen bands/patient was 6.9 (min. of 4 and max. of 8; SD = 1.09). Six allergen bands (16, 27, 35, 42, 45, 65 kDa) were recognized by more than 85% of the patients and two bands (MW close to 43.5 and 110 kDa) were only recognized by one patient (no. 12). From *P. pratense* 1-D SDS-PAGE, eight allergen bands were recognized from 11 to 136 kDa and the mean number of recognized PAGE allergen bands/patient was 4.4 (min. of

Table 1. Allergens recognized from *D. glomerata* IEF

Patients	*Dactylis glomerata* allergens (estimated pI*)																
	3.7	**3.9**	**4**	**4.2**	**4.3**	**4.4**	**4.8**	**5**	**5.4**	**5.7**	**6.5**	**6.8**	**7.6**	**7.7**	**8.3**	**8.7**	**9.1**
1		x	x	x	x	x	x	x	x				x	x			x
2		x	x	x			x		x			x	x				
3		x	x	x	x	x	x	x	x	x		x	x	x	x	x	x
4		x	x	x	x	x	x	x	x	x		x	x	x	x		x
5		x	x	x			x		x			x	x	x			
6B		x		x	x	x	x		x				x	x		x	x
6A		x		x	x	x	x	x	x				x	x			x
7			x				x	x	x			x	x			x	x
8			x				x		x		x					x	x
9	x			x			x						x				
10		x					x						x				
11			x	x	x	x	x	x	x				x			x	
12		x	x	x	x	x	x		x			x		x	x	x	x
13		x	x	x		x	x		x				x		x		x
14		x	x	x	x	x	x	x	x				x	x	x		x

**Isoelectric point.*

Table 2. Allergens recognized from *P. pratense* IEF

Patients	*Phleum pratense* allergens (estimated pI*)																	
	4	**4.5**	**4.6**	**4.8**	**4.9**	**5.1**	**5.4**	**5.6**	**6.1**	**7.1**	**7.3**	**7.6**	**8.1**	**8.8**	**9**	**9.4**	**9.5**	**9.8**
1		x	x		x	x	x	x	x	x			x	x	x			x
2	x	x												x	x			
3	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
4	x	x			x		x											x
5					x	x	x								x			
6B	x			x													x	x
6A	x			x	x		x											
7		x		x	x		x						x	x	x		x	x
8		x		x	x		x						x					
9	x								x									
11				x	x		x						x	x			x	x
13	x	x	x		x		x			x			x	x			x	x
14	x	x		x	x		x						x					x

**Isoelectric point.*

1 and max. of 6; SD = 1.28). Three allergen bands (MW close to 27, 35 and 42 kDa) showed major recognition, with the 27 kDa band being recognized by all the patients.

Patient no. 6, who had undergone 1-year ASIT, with a significant clinical improvement, changed his pattern of recognition towards the *D. glomerata* allergom (Tables 1 and 3), by failing to recognize the pI 8.7 (Figure 1) and the 47 kDa (Figure 2) bands and beginning to recognize the pI 5 band (Figure 1). Patient no. 6 also changed his pattern of recognition regarding *P. pratense* (Tables 2 and 4), by failing to recognize the pI 9.5 and 9.8 (Figure 1) and the 11 kDa (Figure 2) bands, and beginning to recognize the pI 4.9 and 5.4 bands (Figure 1).

Table 3. Allergens recognized from *D. glomerata* SDS-PAGE 1-D

Patients	*Dactylis glomerata* allergens (estimated MW* [kDa])											
	11	16	23	27	32	35	42	43,5	45	47	65	110
1			x		x	x					x	
2		x	x	x		x	x		x		x	
3		x	x	x		x	x		x	x	x	
4		x		x		x	x		x		x	
5		x		x		x	x		x		x	
6B		x	x	x		x	x		x	x	x	
6A		x	x	x		x	x		x		x	
7		x		x		x	x		x	x	x	
8		x		x		x	x		x		x	
9	x	x		x		x	x		x		x	
10	x	x		x		x	x		x		x	
11	x	x	x	x		x	x		x		x	
12		x	x		x			x	x	x	x	x
13	x	x	x	x		x	x		x		x	
14			x	x		x	x		x		x	

**Molecular weight.*

Two dimensional separation from *D. glomerata* disclosed 13 allergenic spots, from pI < 3.85 to > 7.9 and from 7 to 112 kDa (Figure 3), while to *P. pratense*, 27 allergenic spots were disclosed from pI < 3.85 to > 9.7 and from 9 to 85 kDa (Figure 4).

Table 4. Allergens recognized from *P. pratense* SDS-PAGE 1-D

Patients	*Phleum pratense* allergens (estimated MW* [kDa])							
	11	16	27	35	42	47	55	136
1			x	x	x		x	
2	x		x	x				x
3			x	x	x		x	x
4			x	x	x		x	x
5			x	x	x			
6B	x	x	x	x	x			x
6A		x	x	x	x			x
7	x		x	x	x		x	
8	x	x	x			x		x
9			x					
11			x	x	x	x	x	
13			x	x	x		x	
14			x	x	x		x	
15	x		x	x	x	x	x	

**Molecular weight.*

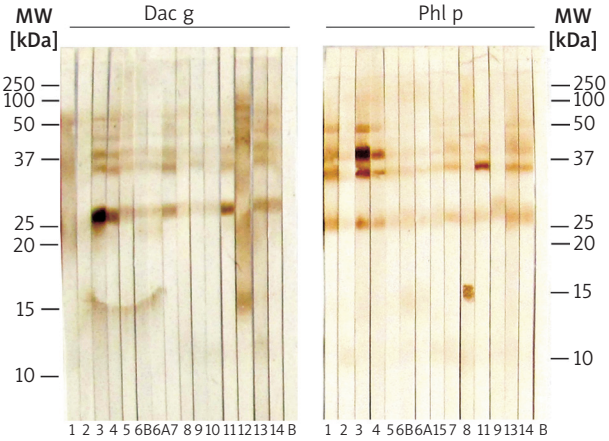

Figure 2. IgE immunoblots from *D. glomerata* and *P. pratense* SDS-PAGE, revealed with horseradish peroxidase-conjugated goat anti-dog-IgE. Patients/lanes no. 1–15. Patient no. 6 before (6B) and after (6A) specific immunotherapy. B – Blank controls without serum. Note: no lane from patient no. 15 in the *D. glomerata* set and no lanes from patients no. 10 and 12 in the *P. pratense* set

In a range from 1 to 4, the mean IDT score to *D. glomerata* was 2.3 (min. of 2 and max. of 4; SD = 0.602). To *P. pratense*, the mean IDT score was 2.0 (min. of 1 and max. of 3; SD = 0.516).

A significant positive correlation (r = 0.902) was verified between specific IgE to *D. glomerata* and the respective IDT scores, with a marked variability in individual specific IgE levels (SD = 738.4). A moderate positive correlation (r = 0.422) was found between specific IgE to *P. pratense* and the respective IDT scores, also with a marked variability in individual specific IgE levels (SD = 894.0). A significant positive correlation was verified between specific IgE, either to *D. glomerata* or to *P. pratense*, and their respective number of recognized IEF allergens (r = 0.570 and r = 0.710). A significant positive correlation was also found between IDT scores to *D. glomerata* and to *P. pratense*, and to their respective number of recognized IEF allergens (r = 0.649 and r = 0.660). No significant correlation was verified between IDT scores to *D. glomerata* and to *P. pratense*, and their respective number of recognized PAGE allergens (r = –0.070 and r = –0.216). No significant correlation was also verified between specific IgE to *D. glomerata* or to *P. pratense* and the number of their recognized PAGE allergens (r = 0.009 and r = –0.036, respectively).

A significant positive correlation was found between specific IgE levels to *D. glomerata* and *P. pratense* (r = 0.706), and between the number of *D. glomerata* and *P. pratense* recognized IEF allergens (r = 0.604). Between the number of *D. glomerata* and *P. pratense* recognized PAGE allergens, no significant correlation was observed (r = 0.152), whereas between IDT scores to both species, a moderate positive correlation was observed (r = 0.428). Besides, there was no significant correlation between the number

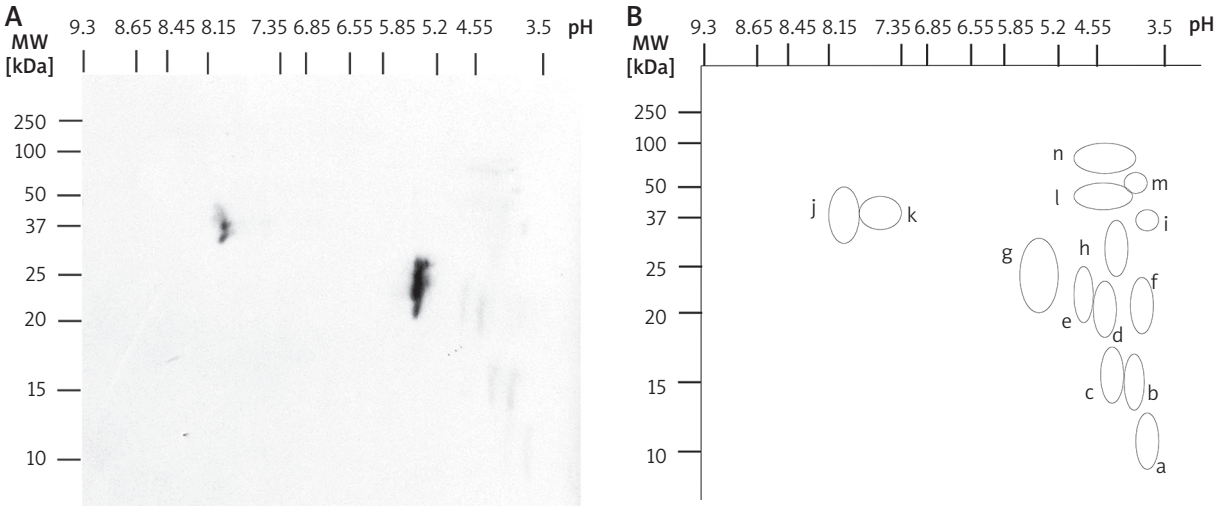

Figure 3. A – IgE immunoblot from *D. glomerata* 2-D separation, revealed with biotin-ExtrAvidin-alkaline phosphatase anti-dog-IgE. Dog *D. glomerata* allergogram with a pool of sera. **B** – Two-dimensional diagram from dog *D. glomerata* allergogram in Figure 3 A. Recognized allergen spots are identified from *a* to *n*

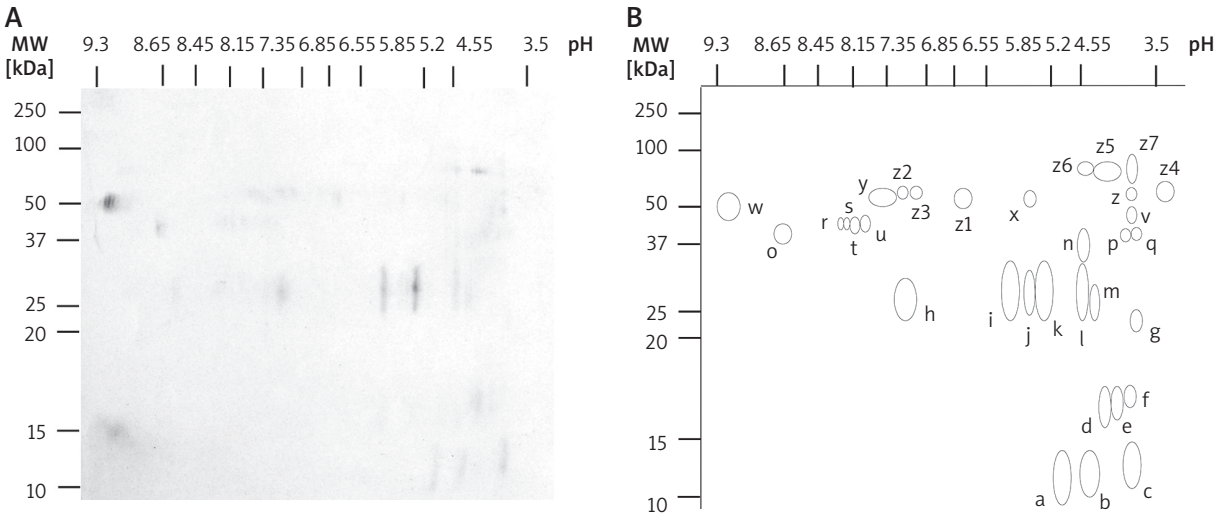

Figure 4. A – IgE immunoblot from *P. pratense* 2-D separation, revealed with biotin-ExtrAvidin-alkaline phosphatase anti-dog-IgE. Dog *P. pratense* allergogram with a pool of sera. **B** – Two-dimensional diagram from dog *P. pratense* allergogram in Figure 4 A. Recognized allergen spots are identified from *a* to *z7*

of recognized allergens from IEF and from PAGE, by each patient, in either *D. glomerata* or *P. pratense*. Although, a tendency to recognize a higher number of IEF allergens, when compared to PAGE allergens, was individually seen.

Two-dimensional separation disclosed multiple allergen spots from *D. glomerata* and *P. pratense*. Fourteen spots (*a* to *n*) were identified from *D. glomerata* (pI 3.8 to 7.9 and MW 7 to 75 kDa) (Figures 3 A and B) and 33 (*a* to *z7*) from *P. pratense* (pI < 3.5 to 9.1 and MW 8 to 83 kDa) (Figures 4 A and B). Diagrams from Figures 3 B and 4 B show clearer representations of *D. glomerata* and *P. pratense* 2-D allergoms. Controls with the non-atopic dogs, negative for specific IgE to grass pollens, showed no recognition in WB.

Discussion

No clear clinical differences were possible to identify among patients. Hence, no specific IgE level, IDT score or pattern of spectrotype was possible to relate to a specific clinical pattern. Similarly to house-dust mite allergy [24], IDT scoring seemed to be a reliable parameter in terms of correspondence to the level of specific IgE to *D. glomerata* and *P. pratense*. However, such high correlation may

not occur between specific IgE levels or IDT scores and the number of recognized allergens from PAGE.

From the individual allergograms observed among this clinically homogeneous group of patients, sensitization profiles were found to be rather diverse. In fact, a great diversity of individual spectrotype patterns was revealed – patient no. 9 recognized only 1 allergen band from *P. pratense* PAGE, while others, such as patients no. 6 and 14, recognized up to 6 bands. From *P. pratense* IEF, patient no. 9 recognized only 2 bands, while patient no. 3 recognized 17. The same trend was registered with regard to *D. glomerata* PAGE, where patient no. 1 recognized just 4 allergen bands, while patients no. 3, 6, 11, 12 and 13 recognized up to 8 bands. From *D. glomerata* IEF, the number of bands recognized ranged from 3 (patient no. 10) to 15 (patient no. 3). The number of recognized IEF-separated allergens was definitely lower in individual dogs than in humans, where more than 30 bands were individually recognized [25].

Dog patients with higher specific IgE levels also showed a higher allergen recognition signal in WB, as described for humans by Peltre *et al*. [18, 25], using the radioactive protocol for IgE detection.

As observed with humans [26], a wide range of identified *D. glomerata* and *P. pratense* allergens was disclosed for dogs and several similarities were also found between dog and human *D. glomerata* and *P. pratense* sensitization profiles. The pI range of the identified *D. glomerata* allergens for dogs (pI 3.7–9.1) was similar to the humans' (pI 4.1–10.5) and the *P. pratense* pI range of identified allergens for dogs (pI 4–9.8) was even closer to the human's range (pI 4.2–9.5). Towards *D. glomerata* an allergen band with pI 5.9, probably Dac g 1, was the most recognized by humans [19], whereas in dogs the closest pI of a recognized allergen band was 5.7 and it was only recognized by 2 patients (no. 3 and 4). Although regarding *P. pratense*, 2 allergen bands (pI 4.9 and 5.4), which may correspond to Phl p 5 (pI 4.2–7.5), a highly recognized allergen by humans [19], also showed highly recognition by individual dogs (10 out of 12 dogs).

Some similarities were also found between dog and human sensitization profiles, regarding PAGE-separated *D. glomerata* and *P. pratense* allergens. The 27 kDa and the 11 kDa *D. glomerata* allergens, probably corresponding, to Dac g 5 and Dac g 2, respectively, two major allergens for humans, were also identified by dogs, but only the 27 kDa band presented major recognition by dogs (12 out of 14 dogs). The 27 kDa and the 11 kDa *P. pratense* allergen bands, probably corresponding to Phl p 5 and Phl p 2, respectively, were also recognized by dogs and the 27 kDa band was even recognized by all of the sensitized dogs. Yet, regardless of the highly cross-reactive condition observed between different grass pollen species in humans that was not quite demonstrated among dogs. In fact, considerable differences were found between each patient *Dactylis glomerata* and *Phleum pratense* allergograms in the study population, as it was also reported with regard to *Cryptomeria japonica* [11] and *Ambrosia artemisiifolia* [12].

As in humans [26], mean specific IgE, either to *D. glomerata* or *P. pratense*, also presented a very high variability, with no correspondence to the severity of clinical signs. Nevertheless, a significant positive correlation was found between specific IgE and IDT scores, especially regarding *D. glomerata*, which may be understandable since the cutaneous reaction upon intradermal inoculation of the allergen extract seems to depend on the level of existent specific IgE. However, no correspondence was verified between IDT scores and the severity of the clinical signs. The relevant positive correlation verified between the level of specific IgE to *D. glomerata* and *P. pratense*, and the respective number of recognized IEF allergens, but not with regard to PAGE allergens, could be associated with a more conformational condition of humoral epitopes, which may be further preserved by the IEF, as a native method. Observing the correlations, early verified between IDT and specific IgE, and between specific IgE and the number of IEF-separated allergens, a significant positive correlation between the IDT scores to *D. glomerata* and the number of *D. glomerata* IEF-separated allergens was expected, by the same reasoning. Although, such conditions were not verified with respect to the number of PAGE allergens, either from *D. glomerata* or *P. pratense*, where no significant correlation was found with the respective levels of specific IgE and IDT scores. No relevant correlation was also found between the number of *D. glomerata* or *P. pratense* IEF and PAGE-separated allergens.

Individual spectrotypes, mainly 2-D allergograms, has already shown great utility for the identification of the implicated allergen molecules in human latex allergy, as demonstrated by Chardin and Peltre [2]. The interpretation of molecular allergen recognition may also be of great importance for an efficient immunotherapy approach, since the use of rather specific molecular allergens, instead of traditional commercial extracts, resulted in an additional 60% improvement following immunotherapy in humans [13], and it is honest to predict the same trend in dog. Therefore, previous knowledge of the individual sensitization allergograms should play a key role for more efficient allergen-directed immunotherapy in dog. Improvement in immunotherapy effectiveness may also be expected in dogs by the use of individual-specific vaccines with molecular allergens.

The usefulness of molecular epidemiology had also been defined by Bessot and Pauli [6], concerning mite allergy in humans, because it would lead to a better understanding of the cross-reaction phenomena between different mite species, either from the same family or not. Further knowledge on this subject will probably open new prospects for a more specific immunotherapy, based on genetically hypoallergenic variants of major allergens [6].

In our study, after a 1-year course of specific immunotherapy, patient no. 6 showed a remarkable clinical improvement, with a change in *D. glomerata* and *P. pratense* spectrotype complexity, which could be associated with several Th2-like immunological mechanisms, occurred in the initial phase of immunotherapy [27–29], and suggesting a lack of absolute correlation between sensitization and allergy. Such observations were also made by Martins *et al.* [24], with an allergic patient, regarding *Dermatophagoides pteronyssinus* mite spectrotypes, but so far it has not been possible to detect any correlation between that and the efficacy of immunotherapy. More patients should be studied to confirm any possible early/late evolution in sensitization during the course of specific immunotherapy. However, until a more "tailor-made" immunotherapy becomes available, based on a true component-resolved molecular diagnosis [15, 16], extract mixes for immunotherapy are certainly useful, because species such as grass pollens are widely spread throughout the world, and thus, those mixtures undoubtedly tend to reproduce the natural exposure environment, frequently with a beneficial clinical effect [9]. Nevertheless, immunotherapy may be safely improved by using more accurate allergen pools for specific immunotherapy, which is already being studied for human allergy management [6–10].

As a future trend we think that the concept of CRD [15] and consequent CRIT [16] may also be applied to veterinary allergology. A possible example of those concepts could be applied towards the rather dog-recognized band of 27 kDa, either from *D. glomerata* or *P. pratense*, which may correspond to group 5 of grass pollen allergens. Hence, future experiments should investigate the precise molecular weight mass and amino-acid sequence of those molecules, by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) Mass spectrometry (MS), as demonstrated by Leung and Pitts [30]. Additionally, the use of rather specific identifying antibodies in WB or WB inhibition, by previous incubation of patient sera with selected natural or recombinant allergen molecules, may also allow direct or indirect 2D molecular spot identification. In fact, CRD is already being applied to human allergy management, through the Immune Solid-phase Allergen Chip (ISAC – Thermo Fisher Scientific, USA) diagnostic resource. However, while this trademark method is still not available for veterinary purposes, we consider that the identification and characterization of the most relevant allergens, especially for dogs, should continue through other available methods. For a better characterization of these identified allergens, further studies using several allergen-specific antibodies, recombinant allergens and mass spectrometry techniques are presently in progress. Additionally, as proposed by Canonica *et al.* [31] in a consensus document for the World Allergy Organization, the microchip-based molecular epidemiology concept diagnosis will facilitate identifying (i) genuine versus cross-reactive sensitization in polysensitized patients, thereby improving the understanding of triggering allergens; (ii) the risk of severe systemic versus mild local reactions in food allergy, without the need for challenge testing with unnecessary consequences and (iii) patients and triggering allergens for immunotherapy.

In fact, etiological diagnosis of pollen sensitization is usually difficult, because of a wide variety of sensitizing pollens, which is also associated with food allergy, frequently configuring a poly-sensitization status, due to several panallergens [32]. Associated with this polysensitization status in humans, a low concordance between skin-prick-tests (SPT) was observed, especially with regard to patients sensitized to panallergens, such as profilin and polcalcin. In these cases, patients presented many more positive results in SPT. Additionally, patients sensitized to profilin or to lipid-transfer proteins have been clearly associated with food allergy [32]. This etiopathogenic relation between food allergy and atopy also needs clarification for dogs and other animals.

Evidence of an allergen epitope community within the *Pooideae* subfamily of grass pollens was also found for dogs, since *P. pratense* itself showed a large variety of allergens in the 2-D allergom spectrum, standing in agreement with Martínez-Cócera *et al.* [10] and Hejl *et al.* [9] observations.

Dog, as well as horse and cat allergy framework, harbor similar important parameters, such as IgE, IgE receptor repertoire and expression pattern, and the same involved cell types (mast cells, eosinophils and T-regulatory cells) for sensitization and for allergy diagnosis, suggesting to be possible models for translational studies [33].

As for humans, studies of molecular epidemiology in dog allergy will allow clarifying the evolution of allergic response during immunotherapy. Further characterization of *D. glomerata* and *P. pratense* identified allergens, using allergen-specific antibodies, as well as recombinant allergens and mass spectrometry techniques is in preparation. Hence, besides the very close clinical patterns between different patients, the individual pattern of sensitization for each allergenic source may also become of an outstanding relevance to the success of a real allergen-specific immunotherapy.

In conclusion, no clear clinical differences were possible to identify from among patients. Hence, no specific IgE level, IDT score or pattern of sensitization was possible to relate to a specific clinical pattern. Specific IgE to *D. glomerata* and to *P. pratense* presented high variability, with no correspondence to the severity of clinical signs. Sensitization profiles were found to be rather diverse from patient to patient, with a lack of absolute correlation between sensitization and allergy. However, patients with higher specific IgE levels tended to show a higher allergen recognition signal in WB. Several similarities were found in the patient pattern of sensitization between

D. glomerata and *P. pratense*, suggesting cross-reactive phenomena. Similarities were found between dog and human *D. glomerata* and *P. pratense* sensitization profiles. No correlation was found between specific IgE levels or IDT scores and the number of recognized allergens from PAGE. The IDT scoring seemed to be a reliable parameter in terms of correspondence to the level of specific IgE to *D. glomerata* and *P. pratense*. Characterization of a patient IgE sensitization profile by WB over electrophoretic-separated allergens may provide relevant information, especially to understand particular conditions related to cross-reaction phenomena and immunotherapy effectiveness. Further molecular epidemiology approach is needed to understand the role of the sensitization pattern in allergen-specific immunotherapy effectiveness in grass-pollen allergic dogs.

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Conflict of interest

The authors declare no conflict of interest.

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