



Research paper

Immunoproteomic characterization of *Ambrosia artemisiifolia* pollen allergens in canine atopic dermatitis

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ABSTRACT

Canine atopic dermatitis (CAD) is an immune system disorder that affects 10–15% of the canine population. Short ragweed (*Ambrosia artemisiifolia*) pollen represents one of the major seasonal sources of allergenic pollen proteins in Europe, particularly in the Pannonian valley of the Balkan region. In Serbia, about 66% of atopic dogs showed a positive intradermal skin test with its pollen extract, which is second to house dust mites. Therefore, characterization of *Ambrosia artemisiifolia* pollen components, in terms of defining major and minor allergens that induce clinically manifested allergic reaction in dogs, is important for valid diagnosis and efficient therapy.

This study has, for the first time, characterized and identified major *Ambrosia artemisiifolia* allergens in CAD, using an immunoproteomic approach. To assess the prevalence of specific IgE in electrophoretically separated ragweed pollen proteins, individual reactivity of sera from dogs with CAD was analyzed and compared to the reactivity of sera from healthy dogs in the non-reducing conditions, which were found optimal for specific canine IgE detection. A specific IgE band (38 kDa) was recognized as the most dominant allergen in CAD, occurring in 81% of positive dog's sera. 2-D immunoblotting followed by a mass spectrometry peptide fingerprint analyses with pooled canine and human atopic sera, revealed that 38 kDa major *Ambrosia artemisiifolia* allergens in CAD were all five isoallergens of the Amb a 1 group (antigen E), including the previously named Amb a 2 (antigen K). In contrast to canine sera, human atopic sera also recognized lower mass allergens such as the β fragment of Amb a 1 and profilins (Amb a 8 variants).

The most prominent ragweed proteins in CAD, represent, as in humans, variants of all five isoallergens of the Amb a 1 group (pectate lyase): Amb a 1.0101 and its natural variant E1XUL2, Amb a 1.0202, 1.0304, 1.0402 and the natural variant of Amb a 1.0501, E1XUM0, as well as the α fragment of pollen allergen Amb a 1.0201.

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Abbreviations: AD, atopic dermatitis; CAD, canine atopic dermatitis; CBB, Coomassie brilliant blue; ID, internal diameter; IDST, intradermal skin test; LTQ, linear ion trap with quadrupole mass filter; MS, mass spectroscopy; nLC–MS/MS, nano-liquid chromatography coupled to tandem mass spectroscopy; PAA, polyacrylamide; PNU, protein nitrogen unit; TTBS, Tris buffered saline with 0.1% Tween 20 (pH 7.4).

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1. Introduction

Canine atopic dermatitis (CAD) is one of the most common skin diseases in dogs (Willemse, 1986; Scott et al., 2001). It is defined as a genetically predisposed inflammatory and pruritic allergic skin disease, with characteristic clinical features associated with IgE antibodies most commonly directed against environmental allergens (Halliwell, 2006). Numerous environmental allergens have been incriminated in the pathogenesis of CAD with house dust mites and pollens being on the top of the list (Hill and DeBoer, 2001). The diagnosis of atopic dermatitis (AD) in dogs relies upon a careful evaluation of history provided by the owner, the presence of appropriate clinical signs, and exclusion of other pruritic skin diseases (Willemse, 1986; Prelaud, 1998; Favrot et al., 2010) and may be confirmed with *in vivo* or *in vitro* tests to demonstrate the presence of allergen-specific IgE.

Some of the clinically important environmental allergens, such as pollens (e.g. trees, grass, weeds) vary with the season, the climate and the geographic region (Sture et al., 1995; Hill and DeBoer, 2001). Pollen of short ragweed (*Ambrosia artemisiifolia*) is the single most seasonal allergen in North America and parts of Europe, affecting up to 36 million human individuals (Wopfner et al., 2009), causing more allergic reactions and related diseases in humans, than all other plants together (Bagarozzi et al., 1998). Short ragweed is becoming a major source of weed pollen allergen in central and southern Europe (Weber, 2005; D'Amato et al., 2007). Recent phyto-geographical and aerobiological studies of the distribution and abundance of short ragweed populations have shown that Serbia is severely affected by this plant and threatened by its airborne pollen (Janjic and Vrbnicanin, 2007; Sikoparija et al., 2009), with pollination periods extending to more than 100 days (Janjic and Vrbnicanin, 2007). The sensitization rate of human subjects allergic to ragweed pollen in the European population is quite high and still increasing; being above 2.5% in all European countries, highest in Hungary (50%), the Netherlands (15.2%) and Germany (14.2%) as shown in a pan-European GALEN study (Jager, 2000; Asero et al., 2006; Burbach et al., 2009).

There is no similar, multiregional study regarding the prevalence of dog's sensitization to ragweed pollen, and this could be due to its wide geographical variation. While some American authors agree that short ragweed is the most significant weed pollen allergen in CAD (Reedy et al., 1997; Scott et al., 2001; Zur et al., 2002), in the Scandinavian countries and Poland, such reports are still absent due to the climate restricted spread of this plant (Taszkun, 2011; Cunze et al., 2013). According to the intradermal skin test (IDST) results from the USA (Scott, 1981; Nesbitt et al., 1984), France (Beco and Fontaine, 1995) and Greece (Saridomichelakis et al., 1999), ragweed pollen causes a positive reaction in 7.7–59% of dogs with AD (Hill and DeBoer, 2001) and 77% of atopic dogs have elevated IgE levels against this pollen, as revealed by ELISA (Kleinbeck, 1994; Hill and DeBoer, 2001). Results from Serbia indicate that ragweed allergens are important in CAD development, since 66% of atopic dogs showed a positive IDST with its

pollen extract, right after house dust mites (Milcic-Matic et al., 2010).

Up to date, seven groups of ragweed allergens have been identified in human allergies: the Amb a 1 group or antigen E (pectate lyase) with 5 isoallergen subgroups (<http://www.allergen.org/viewallergen.php?aid=32>), Amb a 3/Amb a 7 group (plastocyanine), Amb a 4 (defensin-like protein) (Leonard et al., 2010), Amb a 5 homologues, Amb a 6 (lipid transfer protein), Amb a 8 (profilin) and Amb a 9/Amb a 10 (polcalcin/polcalcin-like protein). About 95% of ragweed-sensitized human patients display IgE antibodies to the Amb a 1 major allergen (Adolphson et al., 1978; Løwenstein and Marsh, 1983; Asero et al., 2006; Tamarcaz, 2006). Included in this group is the previously named Amb a 2 allergen group (antigen K), being now assigned as isoallergen Amb a 1.05, which is recognized in about 70% of human patients (Griffith et al., 1991; Rogers et al., 1991). The remaining groups represent minor human allergens.

Progress in immunological characterization of major allergens in human allergies has enabled the development of advanced diagnostic methods and new approaches toward specific immunotherapy of allergies. As with the human atopic population, component-resolved diagnosis and therapy could also result in an increased quality of life in the canine atopic population, by improving the pace and level of recovery. Besides, such an investigation could contribute to a better understanding of the mechanisms responsible for CAD development.

Identification and characterization of ragweed allergens in CAD have not been investigated so far and this is the first study to address this subject. Unlike humans, dogs are very susceptible to parasitic infections, either inherent or acquired, and the defense against numerous parasites accounts for the presence of high serum concentrations of IgE antibodies in healthy animals (Meeusen, 1999; Ledin et al., 2006). As a result, detection of very low concentrations of specific IgE antibodies to allergens is very complex and difficult in terms of methodology. With the new methodological approach using immunoproteomic research with a crude ragweed pollen extract, the chapter of *A. artemisiifolia* allergen characterization in CAD has been opened. We present the major ragweed allergens against which specific-IgE antibodies were detected in the sera of dogs with AD by the means of 2-D PAGE electrophoresis, immunoblotting and nano-liquid chromatography coupled with tandem mass spectroscopy (nLC-MS/MS).

2. Materials and methods

2.1. Study population

Dogs with AD were selected from the Dermatology clinic at the Faculty of Veterinary Medicine, Belgrade University, Serbia, during the period of the short ragweed pollination season. Diagnosis was based on a combination of a compatible history and clinical signs, and exclusion of other pruritic dermatoses (Willemse, 1986; Prelaud, 1998; Favrot et al., 2010). Coat brushings, skin scrapings and trial therapy were used to eliminate ectoparasites. All dogs underwent a 6-week, commercial hypoallergenic diet to eliminate

possible adverse food reactions. Coincidental seborrhea, staphylococcal pyoderma, otitis externa and *Malassezia* infections were managed appropriately.

As part of the clinical evaluation, these animals were intradermally tested, one to three months after completion of the ragweed pollination season with its pollen aqueous extract (1000 PNU/ml, Institute of Virology, Vaccines and Sera, “Torlak”, Belgrade, Serbia) as previously described (Milcic-Matic et al., 2010, 2013). No anti-inflammatory medication was given for at least 3 weeks prior to the intradermal skin test. A score of 2 or greater was regarded as positive. Finally, 16 dogs with AD and positive IDST values of mixed breeds and sexes with a mean age value of 3.53 years were recruited for serum collection.

Control samples were taken correspondingly, from 8 healthy dogs (mixed breeds with an average age of 9.5 years) set for euthanasia at the Faculty of Veterinary Medicine, Belgrade University, with no previous history and clinical signs of pruritus or conditions likely to alter the immune function.

2.2. Canine and human serum sample collection

Blood samples from dogs with AD ($n=16$) were collected by jugular venopuncture and from healthy dogs ($n=8$) by cardiac puncture immediately after euthanasia. Sera were separated by centrifugation and stored at -80°C .

Human serum samples from 6 individuals allergic to *A. artemisiifolia* pollen (positive skin prick test and documented clinical history of allergic reactions), were obtained from the Institute of Virology, Vaccines and Sera, “Torlak”, Belgrade, Serbia. Sera of these 6 patients, as assessed to ragweed proteins with ImmunoCAP® 100 system code w1 (Pharmacia Diagnostics, Uppsala, Sweden), were grade 4 (3 samples) and grade 6 (3 samples). These samples were pooled for 2-D PAGE and subsequent immunoblot experiments.

2.3. Short ragweed pollen extract preparation

A. artemisiifolia pollen samples, obtained from the Institute of Virology, Vaccines and Sera, “Torlak”, Belgrade, Serbia, were collected in the surrounding areas of Belgrade. Pollen samples (5 g) were defatted by ethyl ether, suspended in 50 ml of distilled water with 2% polyvinylpyrrolidone and shaken at 4°C for 4 h. Each suspension was centrifuged at $10,000 \times g$ for 30 min. The protein content in the pollen extract was determined by the method of Bradford (Bradford, 1976).

2.4. 1-D SDS-PAGE and Western blot

SDS-PAGE was carried out according to Laemmli (1970), using a 14% Tris-glycine polyacrylamide (PAA) resolving gel. The short ragweed extract was diluted in either a reducing (60 mM Tris-HCl, 2% SDS, 25% glycerol, 0.1% bromophenol blue and 14.4 mM 2-mercaptoethanol) or non-reducing (without 2-mercaptoethanol) loading buffer and heated at 95°C for 5 min. The extract was then loaded into a single broad well with the molecular weight marker alongside. Separated proteins were transferred to

a nitrocellulose membrane (0.45 mm pore size; Millipore, Billerica, MA, USA) by a semi-dry electroblotter system (Serva, Germany), according to the manufacturer's instructions. Success of Western blot transfer was checked with Ponceau S staining.

2.5. Immunoblotting

The membrane was cut longitudinally into 5-mm wide strips that were blocked with 1% gelatin during the night at 4°C . Serum samples were diluted to 1:10 with 0.1% gelatin in Tris buffered saline (pH 7.4) with 0.1% Tween 20 (TTBS) and incubated for 3 h at room temperature. After washing with TTBS three times for 5 min, strips were incubated with a secondary antibody mouse anti-canine IgE coupled to biotin (Abd Serotec, UK) diluted 1:2000 in 0.1% gelatin/TTBS buffer for another 2 h at room temperature. Blanks and control strips with no serum or no anti-canine IgE were included in each assay. Strips were washed as described above and incubated with 1:50,000 Extravidin AP (Sigma-Aldrich, Germany) dilution in TTBS for 1 h. After extensive washing, the binding patterns were visualized with a substrate solution consisting of 1.5 mg BCIP (5-bromo-4-chloro-3-indolyl phosphate) and 3 mg NBT (nitro blue tetrazolium; Sigma-Aldrich) in 10 ml of 100 mM Tris buffer, containing 150 mM NaCl and 5 mM MgCl_2 (pH 9.6).

2.6. 2-D polyacrylamide gel electrophoresis

Isoelectrofocusing was performed in 5% PAA slab gel in the presence of 2% Ampholytes (Pharmacia), pH 3–10, in a Multiphor electrophoretic system (Pharmacia) according to the manufacturer's instructions. The proteins (35 μg per lane) were applied to an isoelectric focusing gel and further separated by SDS-PAGE under the previously described conditions. Pooled human and canine sera were diluted 1:10 and 1:2, respectively, in 0.1% gelatin/TTBS, before the experiment. As a secondary antibody, an alkaline phosphatase labeled monoclonal anti-human IgE (Sigma-Aldrich, Germany) with 1:1000 dilution was used, while for canine IgE detection the same antibody as in the 1-D immunoblot was applied.

2.7. Identification of proteins in the short ragweed extract by nano-liquid chromatography coupled to tandem mass spectrometry (nLC-MS/MS) analysis

After 2-D electrophoresis, gels were stained with Coomassie Brilliant Blue (CBB) R250 dye (Pharmacia Biotech, United Kingdom). Protein spots of interest were cut from the gels and in gel trypsin digested as previously described (Shevchenko et al., 2006). Finger print peptide analyses was done with an Easy nano-Liquid Chromatography II mass spectrometry system coupled with a linear ion trap and a quadrupole mass filter (LTQ) Orbitrap XL (Thermo Scientific Inc., Waltham, Massachusetts, USA).

Separation of peptides was achieved with an EASY-Precolumn (C18-A1), 2 cm, internal diameter (ID) 100 μm with 5 μm particle size and an EASY-Column, 10 cm, ID 75 μm with 3 μm particle size. Eluent A was a 0.1% aqueous solution of formic acid and eluent B was 0.1% of formic acid

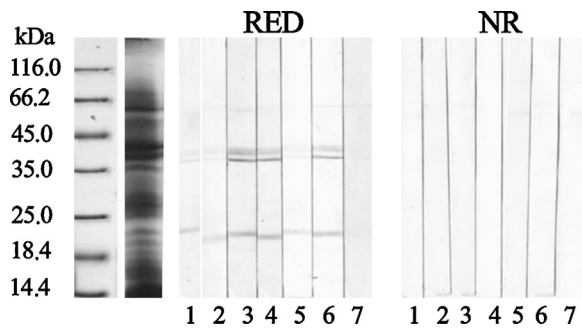


Fig. 1. Comparison of the western blots of *Ambrosia artemisiifolia* pollen extract under reducing (RED) and non-reducing (NR) conditions probed with 1:10 diluted individual sera of 5 healthy dogs (lanes 1–5). In the both lanes 6, serum is omitted and in the lanes 7, secondary antibody is omitted. The lane next to the molecular weight markers in reducing conditions represents 1-D SDS-PAGE separation of the crude ragweed pollen extract.

in 98% acetonitrile/H₂O. The linear gradient was from 5% to 95% of solvent B (70 min), with a flow rate of 300 nl/min, in 100 min of total run time. The injection volume was 2 µl. The spray voltage was set at 1.8 kV, the capillary voltage at 6 V and the capillary temperature to 250 °C. nLC-LTQ Orbitrap mass spectrometry was done according to VanDuijn et al. (2010). For fragmentation, a collision-induced dissociation was performed with collision gas normalized energy of 35% and duration of 30 ms. Data acquisition was performed using the Xcalibur (version 2.1) software. MS data analysis was performed in the Proteome Discoverer 1.3.0339. Search was performed in the UniProtKB/Swiss-Prot database with the SEQUEST algorithm. Two missed trypsin cleavages were allowed per peptide. The peptide tolerance and MS/MS tolerance were set to 10 ppm and 0.5 Da, respectively. Results were filtered to find minimum two peptides per protein with a high confidence.

3. Results

3.1. Non-reducing conditions of electrophoretic separation of ragweed pollen proteins result in less non-specific binding of the canine IgE

In our previous study related to CAD and ragweed allergy, several 1-D immunoblot parameters were optimized, such as the choice of membrane blocking agent, concentrations of secondary antibodies and streptavidin, since their non-specific binding for the proteins of ragweed extracts was noticed (Milcic-Matic et al., 2013). Certain non-specific binding was also observed in the most commonly used protein separation method (prior to immunoblotting), SDS-PAGE under reducing conditions (Milcic-Matic et al., 2013).

Thereby, in our initial experiments we have tested SDS-PAGE conditions that are more suitable for ragweed allergen characterization in the atopic dog population. In order to get the highest signal/noise ratio, we have opted for further 1-D immunoblot analyses under non-reducing conditions since it results in substantially less non-specific IgE binding compared to reducing SDS-PAGE conditions (Fig. 1). Comparison of immunoblots probed with

individual sera of healthy dogs under reducing and non-reducing conditions is shown in Fig. 1. It can be observed that under reducing conditions, nonspecific binding in the lane 6 originates from a secondary antibody (mouse anti-canine IgE), while in the lane 7 where it is omitted, non-specific binding is absent. Also, it can be noticed that bands around 22, 36 and 38 kDa present in reducing conditions as non-specific IgE bindings are almost absent in its corresponding non-reducing blot.

3.2. Identification of the most prevalent ragweed pollen allergens in canine AD by 1-D immunoblot analysis

Major allergens of an allergic source are defined as those recognized by more than 50% of allergic patients (Hauser et al., 2010). In order to assess the prevalence of specific IgE to electrophoretically separated ragweed pollen proteins, we analyzed 16 individual dog's atopic sera and compared reactivity to 8 healthy dog's sera in the non-reducing conditions that we found suitable for the detection of specific IgE antibodies (Fig. 2).

Individual sera showed a considerable variation in the reaction intensity, as well as in the different number of proteins it had reacted to. Several ragweed pollen proteins appeared reactive with canine specific IgE. Bands at the Mr of 100, 60, 45, 38, 36, 27, 17 and 15 kDa were recognized by several of the tested sera. The specific IgE band around 38 kDa was visible in 13 out of 16 positive sera (e.g. in 81% cases), thus being the most prominent ragweed protein in the CAD population tested in our study. In addition, the specific IgE reactivity present in 6 out of 16 sera from atopic dogs was seen with a protein band around 45 kDa. Other specific IgE binding related to a minor antigenic protein component of approximately 27 kDa (2/16), with a very strong and diffuse signal. Protein bands around 60 and 100 kDa, are presently considered as nonspecific binding due to their presence in the healthy population (although very faint, but visible), with 100 kDa being positive in conjugate control samples (Fig. 2). The nature of the 60 kDa protein band needs further investigation.

From 16 dogs with positive results on IDST, only sera 9 and 10 did not react positively with ragweed proteins analyzed in 1-D immunoblots and were excluded from further experiments with pooled samples.

3.3. Molecular characterization of the ragweed pollen allergens by 2-D SDS-PAGE and immunoblot analysis with pooled sera of dogs allergic to ragweed

Pollen extracts are complex mixtures of proteins, many of them having similar masses, but different pI values, such as Amb a 4 and the Amb a 1 α fragment (26 kDa), evolving as a proteolytic product during pollen extraction (King et al., 1974; Wopfner et al., 2009), that cannot be differentiated by 1-D electrophoresis. In order to resolve better and characterize allergen proteins and their isoforms in the ragweed pollen extract, we have employed 2-D gel electrophoresis with the first dimension of isoelectric focusing and then the second dimension with SDS-PAGE under non-reducing conditions. Respective Western blots were probed with sera of dogs allergic to ragweed, and the specific IgE

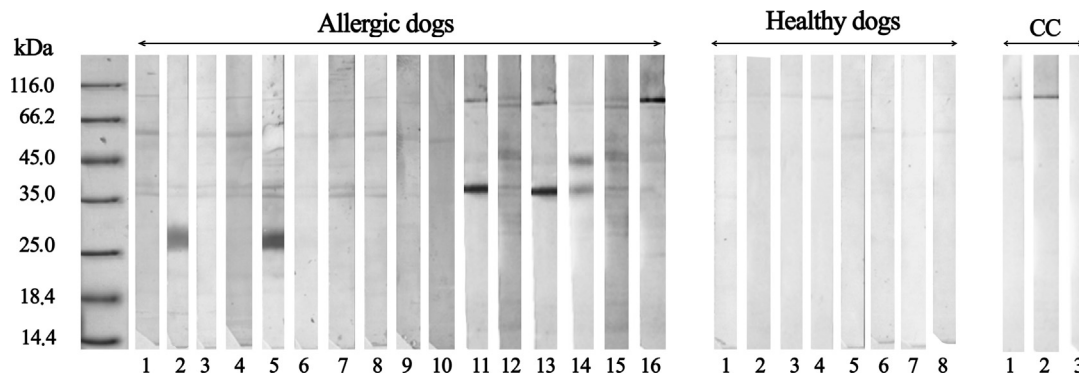


Fig. 2. The western blot of *Ambrosia artemisiifolia* pollen extract under non-reducing conditions probed with 1:10 diluted individual sera of atopic and healthy dogs. CC – conjugate control. In the lane CC 1, a serum is omitted; in the lane CC 2, a secondary antibody is omitted and in the lane CC 3 ExtraAvidin is omitted.

reactivity is presented in Fig. 3. In parallel, ragweed pollen proteins were probed with pooled sera of humans allergic to ragweed and probing was done with anti-human IgE. Contrary to ragweed allergens in canine allergies, the pattern of immune-reactivity of human sera with ragweed pollen proteins is well known and it was thoroughly studied. It is known that the most prevalent ragweed allergen in humans is the Amb a 1, present in five isoallergen forms, all of them being immunoreactive in the human ragweed allergy (Wopfner et al., 2005). In Fig. 3, the 2-D immunoblot analysis of pooled human sera shows strong recognition of several spots of Mr 38 kDa and pI (from 4.5 to 6) thus pointing to the Amb a 1 isoallergens. A very similar pattern was observed in the 2-D blot probed with canine sera,

suggesting similar immunoreactivity. Yet, canine sera do not recognize antigens in the lower Mr range (below 30 kDa), whereas humans do. It is also evident that both pooled sera do not bind to the basic and neutral pH range of allergens. These allergens in the human population appear to be of minor significance and less frequently recognized by a patient's IgE. In pooled sera, reactivity is usually seen for the most prominent allergens, i.e., those to which specific IgE is present in most of the individual sera. Thereby these allergens could be masked by acidic ones which appear to be more prevalent in both tested populations.

Comparison of the results from individual allergic canine sera with the pooled ones, showed that all specific bindings detected in 1-D SDS-PAGE around 27, 38 and 45 kDa were present in 2-D immunoblots as several dots; in some instances appearing like “tails”, probably representing numerous variants of the Amb a 1 isoallergens, known to have similar and/or overlapping pI values. The identity confirmation of the most prominent IgE binding proteins at 38 kDa was expected, by comparing data obtained with in gel digestion peptide fingerprint analyses generated by nLC-MS/MS.

3.4. Identification of ragweed protein spots from 2-D gel by nLC-MS/MS that showed immunoreactivity in 2-D immunoblots

We have analyzed and identified 8 spots marked in Fig. 3 on the non-reducing gel that were recognized by canine and human atopic sera. Similar to humans, major ragweed allergens that atopic dogs reacted to, were all 5 isoallergens with certain variants of the Amb a 1 group, as revealed by identities of the corresponding protein spots (Fig. 3, Tables 1 and 2). Thus, the specific IgE reactivity seen on 38 kDa in 1-D immunoblots, corresponded to intact Amb a 1 isoforms: Amb a 1.0101, 1.0202, 1.0304, 1.0402 and the natural variant of Amb a 1.0501, E1XUM0 (Table 1, spots: 1–3). We were able to distinguish between two pairs of Amb a 1 variants (amino acid sequence lengths of 396 and 398), that differed in just one amino acid residue (Fig. 4).

Based on the sequence coverage distribution (Fig. 4) and the molecular mass position of protein spots in the gel (Fig. 3), α and β fragments of the Amb a 1 allergen group

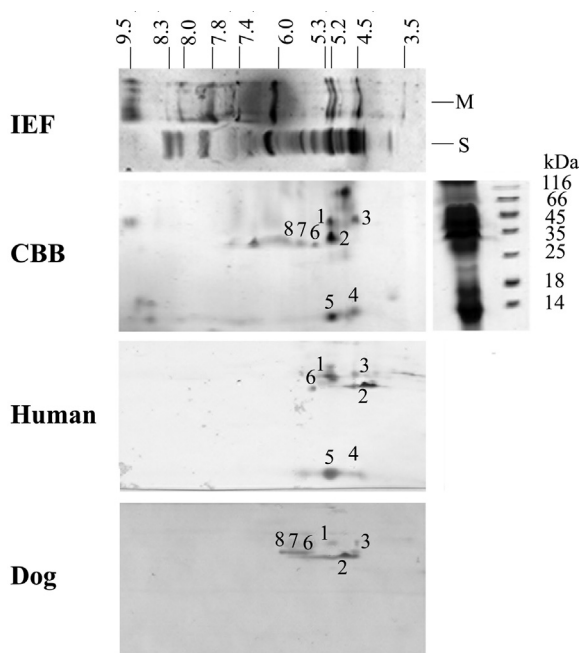


Fig. 3. 2-D Western blots of the short ragweed pollen extract probed with the atopic pooled sera of dogs and humans. IEF – isoelectric focusing; CBB – Coomassie brilliant blue; M – markers of isoelectric focusing dimension; S – isoelectrofocussed sample of the short ragweed crude extract; pH scale corresponds to pI values of IEF markers.

Sequence difference	Identity (%)
P27759 > 1AA (1%)	100
E1XUL2 > 19%	99
E1XUL9 > 28%	81
P27760 > 1AA (1%)	76
E1XUL3 > 14%	76
E1XUL4 > 29%	76
E1XUM0 > 29%	76

Fig. 4. Amb a 1 allergen isoforms sequence alignment and sequence coverage by high confidence peptides generated by tryptic digestion, followed by nLC-MS/MS. Among many high confidence peptides (labeled light gray) are the unique peptides that enabled unambiguous delineation between extremely similar isoform sequences, such as those framed by black rectangles. The difference in the sequence of 1 amino acid between 2 isoform pairs is labeled with dark gray color. The sequence alignment and homology (pBLAST) was done against UniProt database release 2013.03 updated on 6th March 2013. AA – amino acid; Accession – an unique protein accession number from database. *** all amino acid residues in column are identical; * – a conserved amino acid residue; “-” – a semi-conserved amino acid residue.

Table 1Intact Amb a isoforms identified by nLC–MS/MS in spots excised from non-reducing 2-D gel of *Ambrosia artemisiifolia* pollen extract.

Spot no	Accession no (UniProtKB)	Protein name/allergome	Score/coverage (%)	No of peptides	No of unique peptides	Mr – gel (kDa)
1	P27759	Putative pectate lyase/Amb a 1.0101	708/48	14	3	40
	E1XUL4	Putative pectate lyase/Amb a 1.0304	197/52	13	9	
	E1XUL2	Putative pectate lyase/Amb a 1	169/37	11	1	
	E1XUL3	Putative pectate lyase/Amb a 1.0202	66/30	8	4	
	E1XUL9	Putative pectate lyase/Amb a 1.0402	37/19	5	4	
	E1XUM0	Putative pectate lyase/Amb a 1	25/11	3	3	
2	P27759	Putative Pectate lyase/Amb a 1.0101	450/58	18	17	34
	E1XUL9	Putative pectate lyase/Amb a 1.0402	449/63	17	16	
	E1XUL3	Putative pectate lyase/Amb a 1.0202	50/27	7	7	
	E1XUM0	Putative pectate lyase/Amb a 1	52/11	3	3	
	P27759	Putative Pectate lyase/Amb a 1.0101	934/51	15	14	
3	E1XUL9	Putative pectate lyase/Amb a 1.0402	88/34	9	8	38
	E1XUM0	Putative pectate lyase/Amb a 1	44/15	4	4	
	E1XUL4	Putative pectate lyase/Amb a 1.0304	31/16	5	2	
	E1XUL3	Putative pectate lyase/Amb a 1.0202	26/21	6	3	
	Q2KN23	Profilin/Amb a 8.0102	35/37	3	2	
4	Q2KN24	Profilin/Amb a 8.0101	28/37	3	2	12
	Q64LH0	Profilin-3/Amb a 8	24/27	3	2	

were proposed (King et al., 1974; Wopfner et al., 2009). The specific binding appearing on approximately 27–30 kDa in the one dimension immunoblot corresponds to the α fragment of pollen allergens Amb a 1.0201 (spots: 6–8), rather than to Amb a 4 (Table 2). Human pooled sera also reacted with the β fragment of Amb a 1 and Amb a 8.0101, 8.0102 and profilin-3 (Table 2, spot 5 and Table 1, spot 4).

All isoallergens of the Amb a 1 group were identified with high scores, which is an excellent result, bearing in mind that all Amb a 1 variants are highly homologous in sequence, thus having extremely similar masses and isoelectric points. As shown in Fig. 4, their sequence difference ranges from 1% to 29%. Due to plenty of unique peptides that captured differences in the isoform sequence, we were the first to demonstrate the existence of several Amb a 1 variants on the protein level, which were identified on the transcript level within the UniProt database: E1XUL2–E1XUL4, E1XUL9 and E1XUM0 (Fig. 4).

These results suggest that nLC–MS/MS is a powerful, reliable and fast method to identify proteins from crude pollen extract, including the one from *A. artemisiifolia*. They point to the superiority of MS immunoproteomic antigen characterization over 1-D or even 2-D immunoblotting methods.

4. Discussion and conclusions

Canine atopic dermatitis is estimated to affect 10–15% of the canine population and its prevalence is increasing (Hillier and Griffin, 2001; Scott et al., 2001). The results of

a study from Serbia are in accordance with these findings (Milcic-Matic et al., 2010). In the United States, ragweed pollen represents the major seasonal source of allergenic protein compared to other pollen sources, with a prevalence of about 50% in atopic human individuals (Boulet et al., 1997; Wopfner et al., 2005). In Europe, ragweed allergy is rapidly increasing, particularly in the Pannonian valley of the Balkan region (Janjic and Vrbicanin, 2007). In Serbia, two-thirds of atopic dogs showed positive results on IDST against the ragweed pollen extract (Milcic-Matic et al., 2010). Therefore, characterization of *A. artemisiifolia* pollen components, in terms of defining major and minor allergens inducing a clinically manifested allergic reaction in dogs, is, among other reasons, important for valid diagnosis and efficient therapy.

There are 52 antigens identified in aqueous extracts of ragweed pollen, at least 22 of which are allergens as defined by reactivity with human IgE and induction of a type I hypersensitivity response (Löwenstein and Marsh, 1983). Among several allergens described in ragweed pollen, Amb a 1 has been identified as its major allergen (Wopfner et al., 2005). This group consists of several isoforms that are acidic, single-chain proteins with a molecular weight of approximately 38 kDa determined by SDS-PAGE (King et al., 1981). The natural protein undergoes proteolysis during purification, resulting in two chains, designated as α - and β -fragments (King et al., 1974). The 26 kDa α -chain has been reported to associate non-covalently with the shorter 12 kDa β chain (King et al., 1974, 1981; Wopfner et al., 2009).

Table 2Fragments of Amb a isoforms identified by nLC–MS/MS, in spots excised from non-reducing 2-D gel of *Ambrosia artemisiifolia* pollen extract.

Spot no	Accession No (UniProtKB)	Protein name/allergome	Score/coverage (%)	No of peptides	No of unique peptides	Mr – gel (kDa)
5	E1XUM0	Putative pectate lyase (β fragment)/Amb a 1	154/39	9	9	10
	E1XUL2	Putative pectate lyase (β fragment)/Amb a 1	75/10	2	2	
6	P27760	Pollen allergen Amb a 1.2 (α fragment)/Amb a 1.0201	438/68	18	18	28
7	P27760	Pollen allergen Amb a 1.2 (α fragment)/Amb a 1.0201	654/74	19	19	28
8	P27760	Pollen allergen Amb a 1.2 (α fragment)/Amb a 1.0201	818/74	21	21	30

The central question of this study was: what are the identities of the major allergens of ragweed pollen that dogs with AD are reacting to? In this report we have addressed this question by examining an individual dog's sera with 1-D immunoblot techniques, followed with 2-D immunoblots of the allergic dog's pooled sera as well as that of allergic humans. Since ragweed allergens were thoroughly studied in the human population, the results from human sera served as a reference point. The distinctiveness of this study population included recruitment of dogs with AD during the pollination season of *A. artemisiifolia* that showed a positive result to the intradermal skin test. In our investigation, 14 out of 16 (88%) dogs with a positive IDST result displayed the specific serum IgE *in vivo*. Yet, it is well known that even healthy dogs can display specific IgE against environmental allergens, such as house dust mites (Nuttall et al., 2006). Therefore, the next studied population should include more subjects and test all clinically manifested CAD cases within the time frame of short ragweed pollination, regardless of the result on IDST. The results of such a study would certainly contribute to a better understanding of the pathogenesis of AD.

Regarding the difficulties in the detection of allergen specific IgE antibodies, our previous study optimized the conditions for their detection in immunoblots by using a chromogenic substrate, streptavidin and gelatin as blocking/stabilizing agents instead of milk proteins (Milcic-Matic et al., 2013). In the present study, non-specific binding of IgE was also minimized by a Western blot of proteins separated under non-reducing conditions, and by high dilution of anti-canine IgE and streptavidin-AP. The choice to rely on data obtained from non-reducing gels stems from the observation that under reducing conditions there is an increase in non-specific IgE binding (Fig. 1). After SDS-PAGE in reducing conditions, fully unfolded proteins are transferred to a membrane where they can partially refold. However they do not refold to their native conformation due to the cleavage of disulfide bridges, by which most proteins are directed to refold in their native conformation. Thus, SDS-PAGE in reducing conditions very likely abolishes conformational epitopes, leaving mostly the sequential epitopes that IgE antibodies can bind to (Lombardero et al., 1990; Nuttall et al., 2001). If specific canine IgE antibodies prefer conformational epitopes, this leaves a space for non-specific IgE to bind. The second fact that contributes to non-specific IgE binding when assessing canine sera is that healthy dogs have a very high level of total IgE (1–41 µg/ml of serum), being a hundred times higher than in humans (Ledín et al., 2006). This high IgE level is a consequence of the presence of intestinal parasites (Meeusen, 1999). It should be noted however, that a high IgE level is retained even in serum of dogs regularly treated with anti-parasite drugs (Pullola et al., 2006). There is no significant difference in the level of total IgE between atopic and non-allergic dogs (Halliwell and Gorman, 1989; Halliwell, 1990; Hill et al., 1995; Reedy et al., 1997; Scott et al., 2001). The reason for this is that out of the total amount of IgE in atopic dogs a very low proportion of allergen-specific antibodies is present, which is nevertheless sufficient to induce an allergic reaction, but does not significantly increase the serum IgE level (DeBoer, 2004).

It should also be noted that several reports deal with the immunological characterization of major allergens in canine atopic dermatitis. These are mostly dealing with Japanese cedar pollen and house dust mites (Yasueda et al., 1983; Sakaguchi et al., 1990; Masuda et al., 1999, 2000; Nuttall et al., 2001) and, to the best of our knowledge, none are on short ragweed allergen characterization.

Individual sera of atopic dogs, probed on a membrane from the non-reducing gel, differed in regard to the number and intensity of reactions to ragweed allergens (Fig. 2). The obtained results point to 3 different IgE binding proteins of approximately: 38 kDa (81% of sera), 45 kDa (38%) and 27–30 kDa (13%). The first SDS-PAGE molecular weight could correspond to the major human allergen in short ragweed pollen, Amb a 1, the most abundant allergen protein in its pollen, with up to 6% of total protein content (King et al., 1964; Rafnar et al., 1991). Other specific IgE bindings relate to minor antigenic protein components of approximately 45 kDa (7/16) and 27–30 kDa (2/16). SDS-PAGE molecular weight of the last one, showing a very strong and diffuse signal could correspond to the newly established Amb a 4 allergen (Leonard et al., 2010), or could be the result of specific IgE binding to the α fragment (26 kDa) of the Amb a 1 isoforms that are proteolytically generated during pollen extraction together with its shorter β fragment of approximately 12 kDa (King et al., 1974; Wopfner et al., 2009). Confirmation and determination of their identities was expected from further mass spectrometry peptide fingerprint analyses.

In the present study, we aimed at identification of the dominant, most abundant, protein spots and to focus on the major immunoreactive proteins with regard to canine IgE. With the immunoproteomic approach, we were able to identify Amb a 1 variants (AA length 396 and 398) that differed in just one amino acid (Fig. 4). Based on the sequence coverage distribution (Fig. 4) and the molecular weight position of protein spots in the gel (Fig. 3), α and β fragments of the Amb a 1 allergen group were identified, as proposed in King et al. (1974) and Wopfner et al. (2009). All isoallergens of the Amb a 1 group were identified with high scores, even though all Amb a 1 variants are highly homologous in sequence, and consequently, have extremely similar masses and pI values within 4.5–6. Their sequence differences range from 1% to 29% (Fig. 4). In addition to that, this is the first report to show via mass spectrometry analyses, the existence of certain Amb a 1 variants on a protein level, which were proposed and identified on the transcript level, due to high numbers of generated unique peptides (Fig. 4). Similar to humans, major ragweed allergens to which atopic dogs were reactive to, are all five isoallergens and their respective variant isoforms of the Amb a 1 group (alternative name: antigen E), including the Amb a 1.05 isoallergen (previously named Amb a 2 group or antigen K) (Tables 1 and 2). The specific binding appearing on approximately 27–30 kDa corresponds to the α fragment of the pollen allergen Amb a 1.0201 variant (Table 2, and Fig. 4). The recombinant Amb a 1 α fragment behaves as a hypoallergen with reduced IgE binding but preservation of the major T-cell reactivity in the human atopic population (Wopfner et al., 2009). Whether this could be a reason for a low recognition of this

fragment, by sera of atopic dogs (2/16), needs further investigation. Beside the Amb a 1 α fragment, pooled human sera also reacted with the β fragment of Amb a 1 (Table 2, spot 5) and Amb a 8 (Table 1, spot 4). The beta fragment bears major IgE binding epitopes of the natural, intact Amb a 1 allergen (Wopfner et al., 2009). Pooled canine sera did not recognize low mass proteins from the ragweed extract, including the aforementioned Amb a 1 β fragment. At first glance this fact raises caution, since it was reported that it has the same ability to bind human IgE compared to the natural counterparts. It seems that canine allergen-specific IgE recognize larger proteins and are more selective toward conformational epitopes. In support of this notion is the result of Nuttall et al. (2001) that, in atopic dogs, high molecular mass allergens are the most important *Dermatophagoides* allergens, rather than the low molecular mass group 1 and 2 proteins. The immunoreactive band on approximately 45 kDa, needs further investigation.

It is of great importance to define major and minor allergens in dogs, because intradermal skin testing could be performed only with the specific allergen (or group of allergens). This would increase the diagnostic value of intradermal tests in terms of increasing its specificity and sensitivity. In conclusion, this study has, for the first time, characterized and identified major *A. artemisiifolia* allergens in CAD, using the immunoproteomic approach. Thus, the specific IgE band of 38 kDa that is recognized by 13 out of 16 positive dog's sera is the most prominent protein group of short ragweed pollen allergens in CAD. It represents, as in humans, variants of all five isoallergens of Amb a 1 group (pectate lyase): Amb a 1.0101 and its natural variant E1XUL2, Amb a 1.0202, 1.0304, 1.0402 and the natural variant of Amb a 1.0501, E1XUM0, as well as the α fragment of the pollen allergen Amb a 1.0201. Human atopic sera also recognized lower mass allergens such as the β fragment of Amb a 1 and profilins (Amb a 8 variants), whereas pooled canine sera did not.

Conflict of interest statement

None of the authors has any financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the present paper, therefore the authors declare that they have no competing interests.

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