

Identification of major and minor chicken allergens in dogs

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Abstract

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Background

Allergens targeted by serum-specific immunoglobulin E (sIgE) in dogs clinically allergic to chicken have not been reported.

Objectives

To characterise the allergens targeted by sIgE in dogs sensitised and allergic to chicken.

Animals

Sera from three dogs not sensitised to chicken, from 10 chicken sensitised dogs and from 12 chicken allergic dogs.

Methods and materials

Enzyme-linked immunosorbent assay (ELISA) and immunoblotting with a commercial chicken extract were utilized. The bands identified on immunoblotting were sequenced by mass spectrometry for allergen characterization.

Results

Using ELISA, we detected chicken-sIgE above the positive threshold in zero of three (0%) nonsensitised dogs, five of five (100%) chicken-sensitised dogs (a selection criterion), and in seven of 12 (58%) chicken-allergic dogs. Immunoblotting performed with the same extract revealed IgE-bound protein bands in 100% of all chicken-sensitised and -allergic dogs, respectively. To identify the allergens, we excised the corresponding bands on the electrophoretic gel, and submitted them for sequencing by mass spectrometry. We conclusively identified seven major allergens (serum albumin, pyruvate kinase M, enolase

3, creatine kinase M, lactate dehydrogenase A, glyceraldehyde-3-phosphate dehydrogenase and triose-phosphate isomerase) and one minor allergen (troponin C), which are relevant to dogs.

Conclusions and clinical relevance

We identified herein seven major chicken allergens for dogs, several of which are known to be cross-reactive allergens for humans. Based on their degree of sequence identity, these allergens exhibit the theoretical potential to be cross-reactive between poultry and mammalian meats; six of these allergens already are known to be cross-reactive between chicken and fish species. Future studies should address the clinical relevance and cross-reactivity potential of these chicken allergens in dogs.

Introduction

Food allergy is the diagnosis given to between 15% and 30% of dogs and cats with pruritus, allergic skin diseases, or atopic dermatitis (AD).¹ At this time, the diagnosis of food allergies in small animals relies on often-lengthy restriction dietary trials,² followed by one or more oral food challenge(s) with the previously fed diet or its component(s).³

Despite more than 20 years of development, serological tests for food allergen-specific immunoglobulin E (sIgE) still have insufficient sensitivity and specificity to permit their routine use in the diagnosis of food allergy in daily veterinary practice.⁴ This lack of diagnostic accuracy could be a consequence of a combination of various factors. These factors include, at least: (a) the possibility that some canine and feline food allergies might not be IgE-mediated; (b) the presence of IgE against cross-reactive carbohydrate determinants in serum samples; and (c) the standard extracts used for enzyme-linked immunosorbent assays (ELISAs) lack sufficient concentrations of the offending allergen(s) or their pathogenic transcription infidelity variants.⁵

With the increasing recognition of clinically relevant allergenic proteins in human food sources (see Matricardi *et al.* for an extensive review),⁶ the era of molecular allergology has now arrived. First called “component-resolved diagnosis (CRD)”⁷ and now renamed “precision allergy molecular diagnosis applications (PAMD@)”,⁸ this concept involves the use of purified or recombinant allergens, alone or in combination, as substrates for sIgE serological testing. Molecular allergology thus permits a more detailed profiling of the individual hypersensitivities of each allergic patient. At this time, single-allergen assays seem to have a lower or equal sensitivity – but a higher specificity – than first-line serological tests that use standard (whole) extracts for the diagnosis of food allergy.⁹ Nevertheless, molecular approaches seem particularly useful to help predict the severity of food allergic reactions in some human patients.⁹ With an expansion of the number of tested allergens – for example, in multiplex molecular arrays – both the sensitivity and accuracy of sIgE serological testing for the diagnosis of human allergies are expected to increase substantially.

The food sources that historically induced most cases of cutaneous reactions in dogs are beef, dairy products, chicken, wheat and lamb.¹⁰ Among these, only rare allergens have been characterised at the molecular level in dogs with detectable sIgE against the parent extract (i.e. "sensitised dogs").¹¹⁻¹⁶ Of all the allergens identified in the references above, only bovine serum albumin (ALB, Bos d 6) and three egg white proteins [ovomucoid (Gal d 1), ovalbumin (Gal d 2) and ovotransferrin (Gal d 3)] were identified in the serum isolated from a small number of dogs with food allergies proven by a positive oral challenge. Thus, there is a need to expand both this list of molecularly characterised food allergens and the number of sera collected from dogs with provocation-proven food allergies.

In this report, we identify seven major and one minor chicken allergens using the sera of dogs that were either IgE-sensitised or proven allergic to chicken; seven other proteins are considered as additional allergen candidates.

Methods and materials

Ethics

Sera from the chicken-sensitised (CS) and nonsensitised (NS) dogs were retrieved from an archive, while those from the chicken-allergic (CA) group were obtained with their owner's consent. Where necessary, the approval of serum collection also had been secured beforehand from the local institutional animal use committee (Louisiana State University). Otherwise, all methods were carried out in accordance with relevant local guidelines and regulations.

Canine sera

We assembled three groups of canine sera:

Nonsensitised (NS) group

For ELISA testing, we used individual sera from three laboratory dogs previously shown to have a negative sIgE serology to chicken with Avacta's SENSITEST assay (Avacta Animal Health Ltd; Wetherby, Yorkshire, UK). Of importance is that the serum supplier had reported all dogs having eaten a chicken-containing pet food. For immunoblotting, these three sera were pooled together owing to volume limitation.

Chicken-sensitised (CS) group

In this group, sera had been collected from dogs with cutaneous signs compatible with an allergy – including AD – and in which a previously performed SENSITEST ELISA had revealed medium-to-high serum levels of chicken-specific IgE. Whether or not these dogs truly had a food – or chicken – allergy was not known. For ELISA, we used three individual sera and two serum pools grouped because of the low amount of residual individual sera. For immunoblotting, we expanded the number of tested CS individual sera to 10. This group was

composed of five males and five females from various breeds, aged 1.1 to 8.0 years (median 2.3 years); these sera were different from those tested by ELISA as a result of the low amounts of serum that remained after testing (see Supporting Information Table [S1](#) for further details).

Chicken-allergic (CA) group

Finally, we collected sera from 12 dogs with a proven clinical allergy to chicken (Table [S1](#)). There were six dogs from the USA, which had been included in a study reported previously,¹⁵ five dogs were from Germany and one from Sweden; all had been diagnosed as chicken-allergic by a board-certified dermatologist. Ten of 12 dogs (83%) were pure-bred, including four Labrador retrievers. There were seven females and five males, aged between 1.0 and 11.0 years (median 4.5 years). Ten of 12 dogs (83%) had cutaneous signs, and the remaining two had cutaneous and digestive manifestations. All dogs were pruritic, five (42%) exhibited an erythematous eruption, four (33%) had AD, two (17%) a superficial pyoderma and two (17%) otitis externa associated with other skin diseases.

All dogs had had a positive oral challenge to chicken, which induced the recurrence of pruritus (10 of 12 dogs, 83%), erythema and oedema (six of 12, 50%), papules (one of 12, 8%) and gastrointestinal signs (two of 12, 17%; Table [S1](#)). The time-to-flare of clinical signs after the challenge had been recorded for nine dogs: it was 6–12 h in two (22%), 13–24 h in two (22%), 25–48 h in three (33%) and >48 h in the last two dogs (22%). The serum had been collected within the following durations after the last ingestion of chicken: zero to seven days in four of 11 dogs (36%), eight to 14 days in two dogs (18%), 15–21 days in three dogs (27%), 29–35 days in one dog and 36–42 days in one dog. Eleven of 12 dogs (92%) were not receiving any anti-allergic medication at the time of sampling; the last one was reported having taken an unspecified H1R antihistamine.

ELISA

All CS, CA and NS sera were tested for the presence of chicken-sIgE using a standard ELISA. Microtitre plates (4550 Kremsmünster, Greiner AG; Kremsmünster, Austria) were coated overnight at 2–8°C with 100 µL per well of a commercial chicken meat extract (F265, Stallergenes-Greer; Lenoir, NC, USA) at 10 µg/mL (saturating coat level) in a 0.05 M carbonate/bicarbonate coating buffer at pH 9.6. The remainder of the methodology was similar to that in our previous paper.¹⁴ In this chicken-specific ELISA, we determined the positive threshold to be 2.3 arbitrary units (AU), which was twice the mean optical density (405 nm) of the (no-analyte) negative control.

SDS-PAGE and immunoblotting

The same chicken meat extract used for ELISA was heated at 70°C for 20 min in the presence of lithium dodecyl sulfate (LDS) sample buffer (Invitrogen; Paisley, UK) and reducing agent (Invitrogen) before sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

The extract then was separated (5 µg per lane) on Bolt 4–12% Bis-Tris Plus precast gels (Invitrogen) at 200 V using MES (4-morpholinethane-sulfonic acid) running buffer in an Invitrogen Bolt Mini Gel tank. Molecular weight (MW) markers (3–198 kDa; SeeBlue Plus2 markers, Invitrogen) were run in parallel to the extract. Following electrophoresis, the gels were either stained using the SimplyBlue Coomassie stain (Invitrogen) to verify protein loading or the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Invitrogen). The membrane was cut into multiple strips, each containing one lane of markers and one lane of separated chicken extract. Strips then were blocked with tris-buffered saline (TBS) containing 0.5% polyvinylpyrrolidone 10 (PVP10) at room temperature (RT) for 2 h. Blocked membrane strips were incubated with serum diluted 1/10 in TBS with Tween (TBST) at room temperature (RT) overnight with continuous mixing. After washing three times with TBST, membrane strips were incubated with an alkaline phosphatase-conjugated anti-dog IgE antibody [clone 5.91 (same as ELISA and in a previous study)¹⁴ at 0.5 µg/mL in TBST] at RT for 2 h with shaking. Membrane strips were finally washed three times with TBST and rinsed twice with TBS before the addition of the NBT (nitro-blue tetrazolium)/BCIP (5-bromo-4-chloro-3-indolyl-phosphate) substrate (Thermo Fisher Scientific; Loughborough, UK). The reaction was stopped with deionised water, and blots dried at RT. Gel and blots were analysed using the Core Laboratory Image Quantification System (CLIQS, TotalLabs; Newcastle, UK).

Allergen identification by mass spectrometry

We used SDS-PAGE to separate the chicken meat extract at both 5 and 10 µg per lane along with the MW marker, as described above.

The 16 bands (C1–C16) recognised by at least one CS or CA serum [range 157 (C1) to 13 kDa (C16)], which were visible on one of the Coomassie blue-stained gels, were excised and sent to the Bioscience Technology Facility's Metabolomics and Proteomics Laboratory, Department of Biology, University of York, for mass spectrometry (MS; matrix-assisted laser desorption ionization time-of-flight tandem mass spectrometry, MALDI-MS/MS) sequencing, as we had done previously.¹⁴

The resulting MS data (i.e. linear amino-acid sequences) were searched against the *Gallus gallus* (domestic chicken) protein sequences in the Swissprot protein database (www.uniprot.org) using the Mascot programme (Matrix Science; London, UK). As reported previously,¹⁴ we only considered proteins whose threshold scores were above those needed for a conclusive identification.

Whenever the MS results only returned a single protein in a band, we deemed it a "confirmed" allergen. By contrast, when multiple protein sequences were found in an excised band, the proteins were labelled as "tentative" allergens, unless they had been identified already in other bands, as higher MW multimers or digested fragments.

Statistics

In dogs from the CA group, we calculated the Spearman's rank-order correlation between the ELISA AU values and the sum of the volumes of all immunoblotting-generated bands, as calculated by the CLIQS. For this calculation, we assigned an AU of 0.1 and 100.0 whenever the ODs were too low or too high, respectively, to enable an interpolation from the standard curve. We used a one-tailed calculation assuming the hypothesis that AU values and band volumes could only be positively correlated (PRISM 7, GraphPad Software; La Jolla, CA, USA).

Results

ELISA

None of the three NS laboratory dogs had detectable chicken-specific IgE (Figure 1, right).

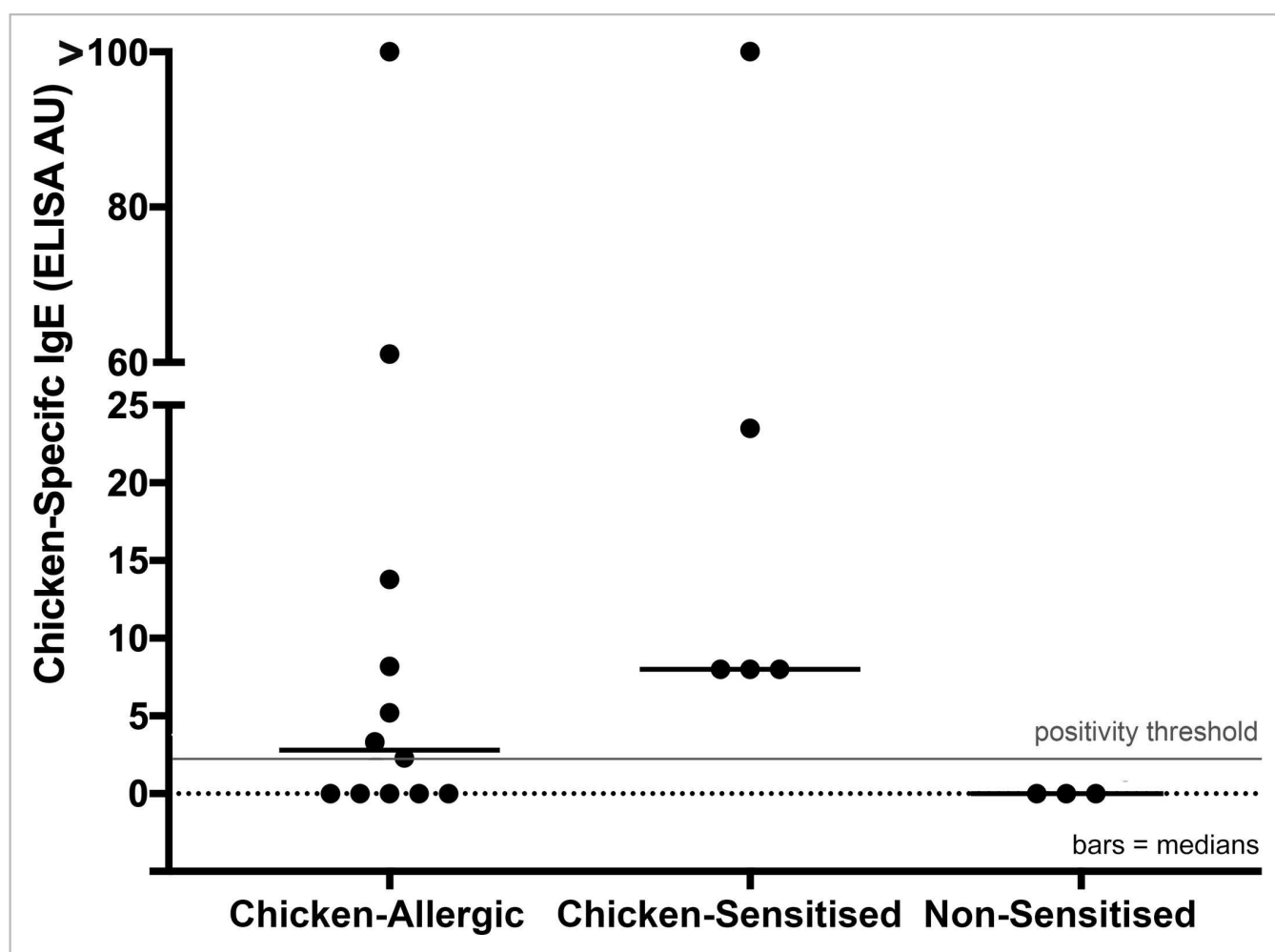


Figure 1

Scatter plot of chicken-specific immunoglobulin (Ig)E values in enzyme-linked immunosorbent assay (ELISA).

All chicken-sensitised (100%), seven of 12 (58%) chicken-allergic and none of three nonsensitised canine sera had detectable chicken-specific IgE.

Following our selection criteria, which required a previously positive SENSITEST assay to chicken, we confirmed that the three individual and the two pooled sera from CS dogs had chicken-specific IgE above the chosen positive threshold of 2.3 AU. The ELISA values were 8.0, 8.0 and 5.0 AU for the three individual sera, and 23.5 and >100 AU for the pooled sera (Figure [1](#), centre).

Seven of 12 CA dogs (58%) had serum chicken-specific IgE levels higher than our threshold for positivity (median 8.2 AU; average 27.8 AU; range 2.3 to >100.0 AU; Figure [1](#), left; Table [S1](#)).

Because of the low number of CA, CS and NS sera, AU values were not compared statistically between groups.

SDS-PAGE and immunoblotting

We separated the proteins from the chicken extract according to their MW using PAGE under reduced and denatured conditions. Multiple bands from approximately 13–220 kDa were visible, with distinct ones noticed at ~65, 63, 58, 48, 43 and 38 kDa (Figures [2](#) and [3](#), second lanes from the left).

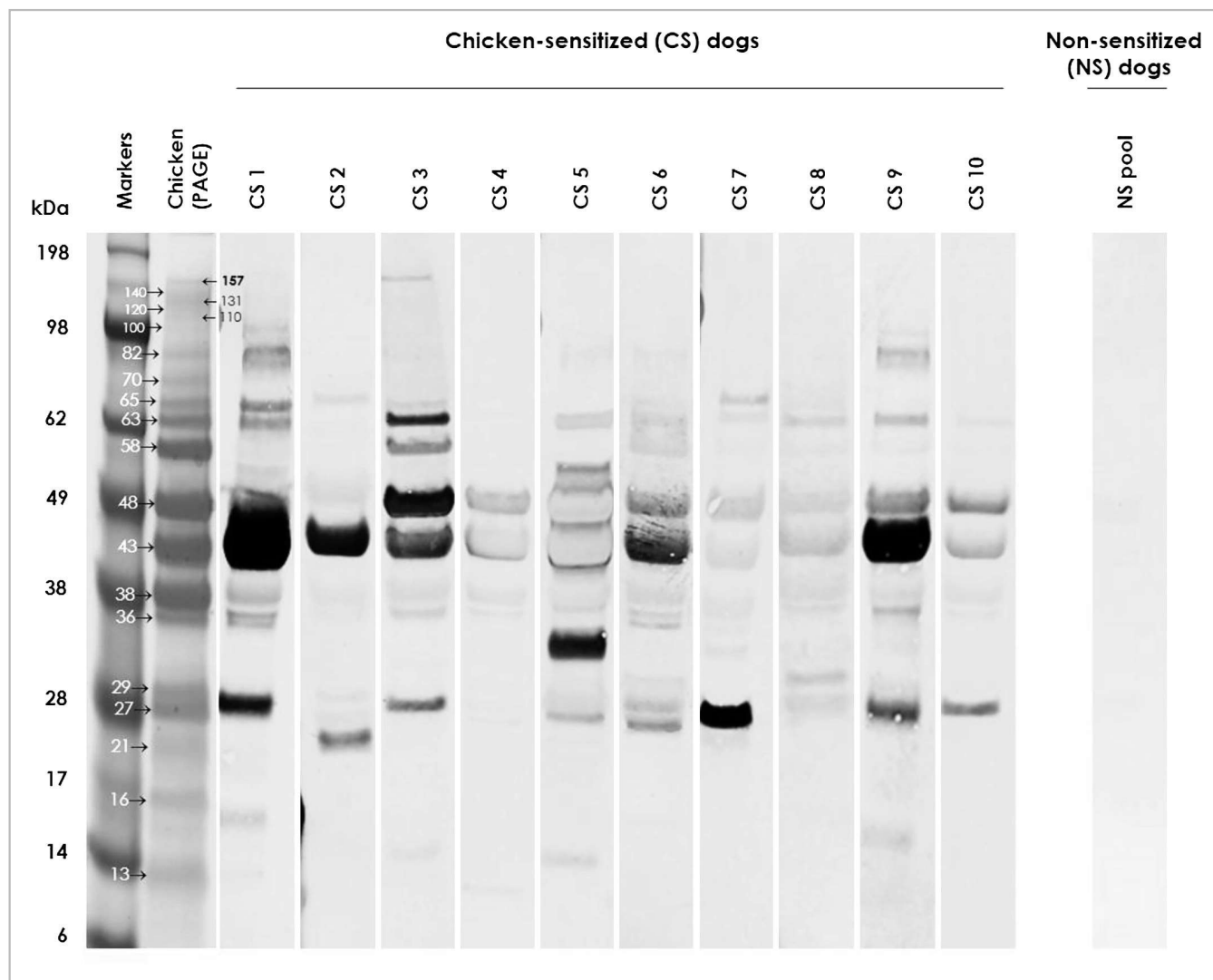


Figure 2

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Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with chicken nonsensitised and chicken-sensitised canine sera.

There were no bands clearly visible with the nonsensitised (NS) serum pool, as was expected from the ELISA results. By contrast, all chicken-sensitised sera (CS1–CS10) contained immunoglobulin (Ig)E that recognised numerous chicken protein bands. This figure contains lanes that were not adjacent in the original gels, and they are shown here as separated by a white space; however, the samples were tested on the same experiment and processed in parallel.

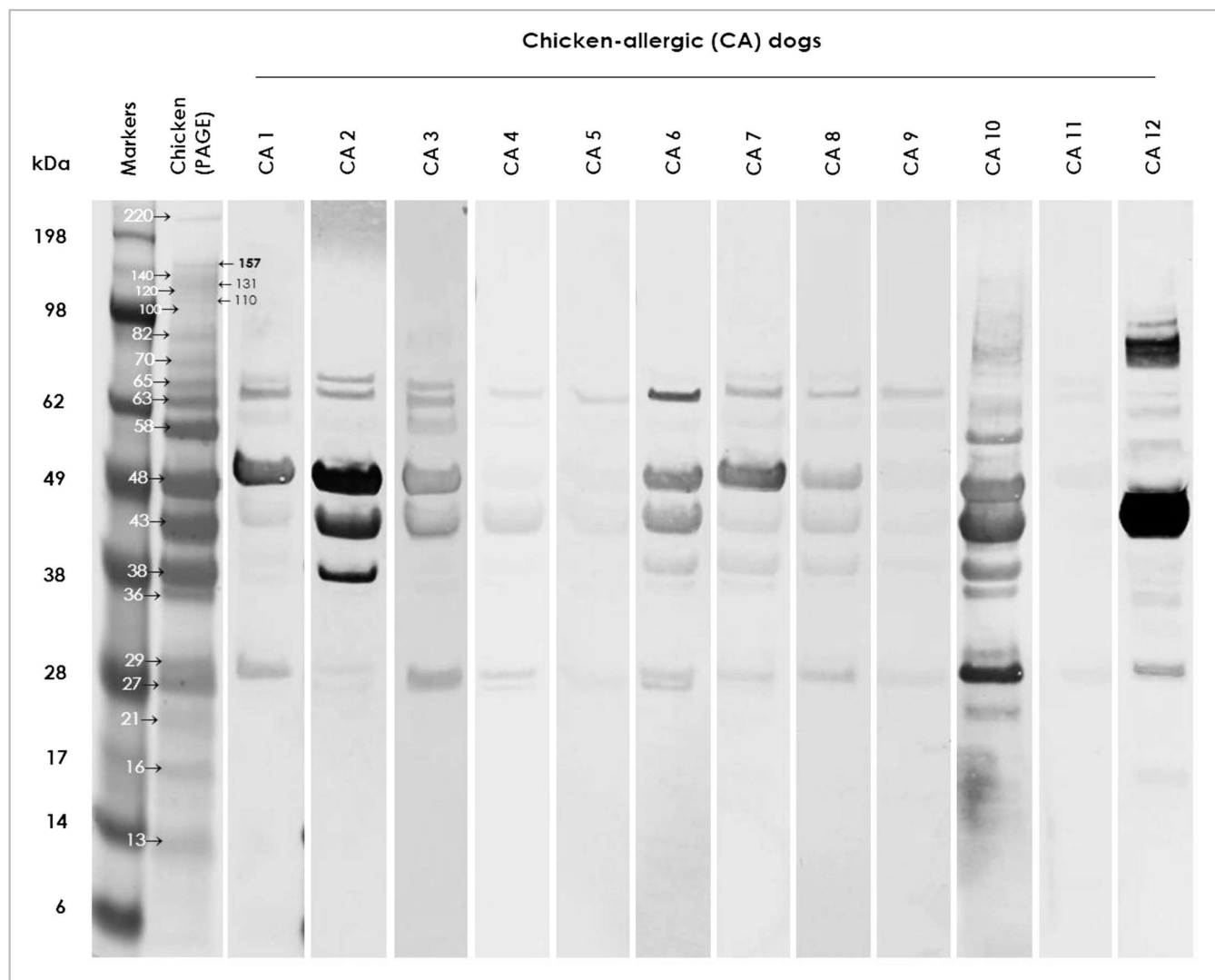


Figure 3

[Open in figure viewer](#) | [PowerPoint](#)

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with chicken-allergic canine sera.

All chicken-allergic sera (CA1–CA12) contained immunoglobulin (Ig)E that bound numerous chicken protein bands. The pattern of chicken allergen recognition mirrors that of the chicken-sensitised (CS) dogs (Figure 2). This figure contains lanes that were not adjacent in the original gels, and they are shown here as separated by a white space; however, the samples were tested on the same experiment and processed in parallel.

Immunoblotting performed with the NS canine serum pool did not reveal any detectable bands, as was expected from the undetectable chicken-specific IgE by ELISA using the same extract (Figure 1 and right-most lane of Figure 2).

Immunoblotting performed with the 10 CS and 12 CA sera confirmed that each contained IgE binding to multiple bands with weak-to-strong intensities (Figures 2 and 3; Table S2). Although the sIgE of all dogs recognised some bands (e.g. those of 43, 48 and 63 kDa), others were uniquely targeted in a single dog (e.g. 120 and 157 kDa bands).

Altogether, the protein recognition pattern was remarkably similar between chicken-sensitised dogs and those proven to be clinically allergic, as shown by the comparable percentage of IgE reactivity to the various bands (Figure 4). *In toto*, we identified nine major allergens of 27, 29, 36, 38, 43, 48, 58, 63 and 65 kDa MW (Figure 4), a major allergen being defined as one recognised by >50% of sera collected from dogs allergic to the parent source.¹⁷

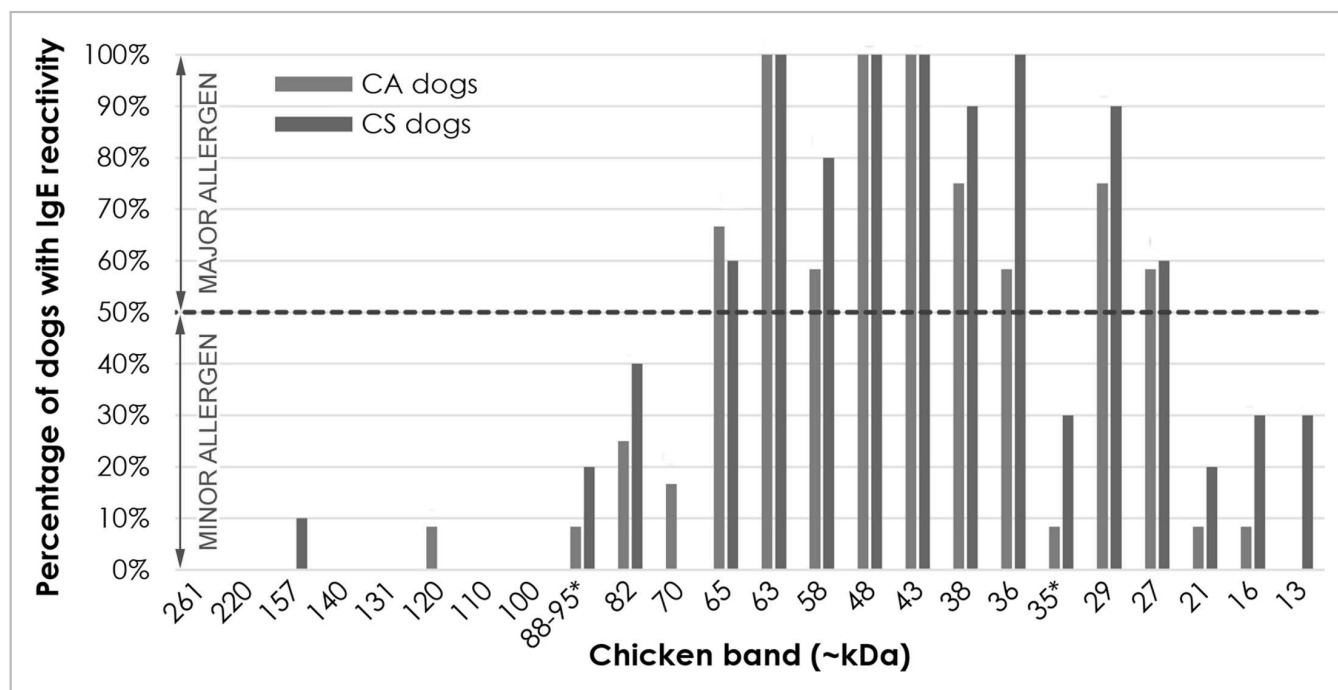


Figure 4

[Open in figure viewer](#) | [PowerPoint](#)

Frequency of band recognition by immunoglobulin (Ig)E from chicken-sensitized and -allergic sera.

Most chicken-sensitized (CS, red) and chicken-allergic (CA, blue) sera contained IgE that recognised a similar pattern of allergens (e.g. 100% recognition of the 43, 48 and 63 kDa bands). Every band recognised by at least one serum was sent out for sequencing by mass spectrometry, except those of 88–95 and 35 kDa (asterisks), which were not visible on the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels (i.e. the amount of protein in that band was lower than the visibility limit of the stain).

Even though only seven of 12 CA dogs (58%) had detectable chicken-specific IgE by ELISA, all were found to have sIgE that recognised chicken proteins by immunoblotting (Figure 3). This observation confirms the latter method as being more sensitive for the detection of sIgE than the former technique. Nevertheless, the ELISA sIgE AU values of CA dogs were strongly correlated with the sum of the volumes of all bands recognised by sIgE on immunoblotting (Spearman's correlation coefficient $\rho = 0.83$; $P = 0.009$; Figure 5).

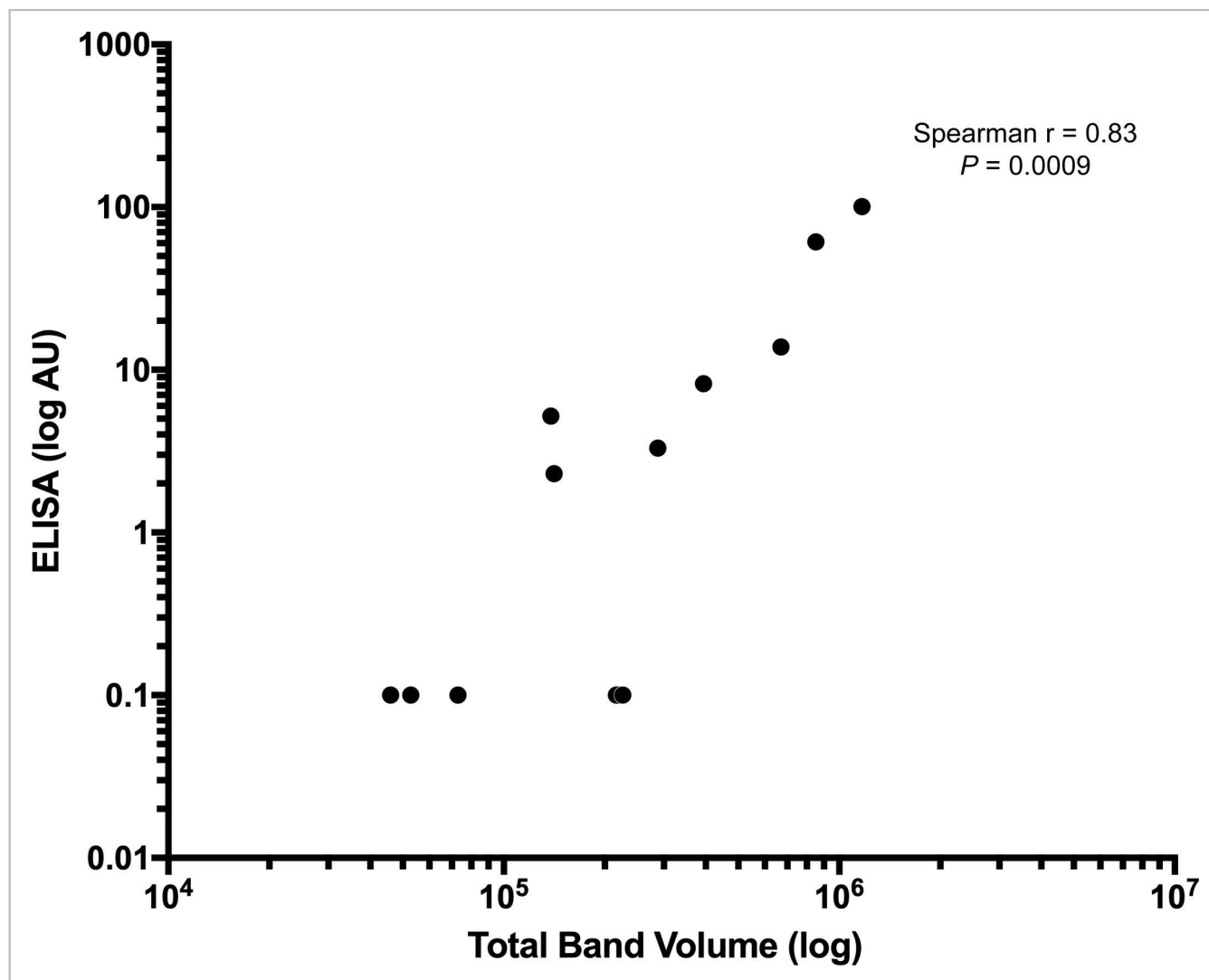


Figure 5

[Open in figure viewer](#) | [PowerPoint](#)

Correlation between enzyme-linked immunosorbent assay (ELISA) and immunoblotting results.

There was a strong correlation (83%) between the ELISA arbitrary units (AUs) and the sum of the band volumes estimated by the CLIQs method.

Protein identification by MS

The proteins identified by MS are included in Table 1. Most proteins were extracted from a single band, for example, the 48 kDa ENO3 (enolase 3). Others were found in multiple ones because the reduced, denatured conditions of the SDS-PAGE had separated protein dimers [e.g. CKM (creatine kinase M) and PKM (pyruvate kinase M)] or multimers [GAPDH (glyceraldehyde-3-phosphate dehydrogenase), a tetramer]; the latter also was found in bands lower than its 37 kDa monomer, suggesting that these represented digested GAPDH fragments.

Table 1. Protein identification by mass spectrometry

Immunoblotting	Chicken-Allergic		Chicken-Sensitized		Altogether		Protein Name	Gene Name	Comment
MW	#	%	#	%	#	%			
157	0	0	1	10	1	5	Docking protein 3	DOK3	
							Fatty acid synthase	FAS	
120	1	8	0	0	1	5	Pyruvate kinase M	PKM	
							Myomesin-2	MYOM2	
82	3	25	4	40	7	32	Creatine kinase M	CKM	Protein dimer
							Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Protein multimer
70	2	17	0	0	2	9	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Protein multimer
65	8	67	6	60	14	64	Heat shock protein 70 kDa	HSPA2	
							Heat shock cognate 71 kDa protein /	HSPA8	

The molecular weight (MW) in the first column represents that calculated on the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel; it may differ from the theoretical MW that does not include the proteins' glycosylation. In the second and third columns are the number and percentage of chicken-allergic (CA) and chicken-sensitized (CS) sera. The protein and gene names provided are those from the Uniprot database.

Altogether, we discovered eight allergens, the sequences of which were determined indisputably (Table 2). Of these, seven proteins were major allergens (Table 1).¹⁷ In descending MW order, these were ALB (serum albumin), PKM, ENO3, CKM, LDHA (lactate dehydrogenase A), GAPDH and TPI1 (triosephosphate isomerase 1). Three major allergens were targeted by sIgE from all tested sera (ALB, ENO3 and CKM). We also found one minor allergen, troponin C (TNNC2). Among these, all except the ALB and TNNC2 already had been identified as fish–chicken cross-reactive allergens in our previous study.¹⁴ Importantly, all eight proteins are known to be allergenic to people, and they are found in multiple phylogenetically different food sources (Table 2).

Table 2. Chicken allergen identification

	Gene Name	Allergen Name	Theoretical MW (kDa) of protein monomer	Allergen in Bexley <i>et al.</i> 2019?	Major or minor allergen?	Chicken allergen in humans?	Allergen source
Confirmed allergens							
Serum albumin	ALB	Gal d 5	70	No	Major	Yes	Man mea
Pyruvate kinase M	PKM	-	58	Yes	Major	No	Whit shrir tiger prav Thai catfi salm
Enolase 3 (beta-enolase)	ENO3	Gal d 9	47	Yes	Major	Yes	Man plan mok
Creatine kinase M	CKM	-	43	Yes	Major	No	Man spec

The molecular weight (MW) in the third column is the theoretical one of the protein. A major allergen is a protein recognised by immunoglobulin (Ig)E in $\geq 50\%$ of the sera from patients sensitised to the parent (whole) allergen source; it is a minor otherwise. The "confirmed" and "tentative" denominations are explained in the mass spectrometry section in the methods.

Also included in Table 2 are seven "tentative" allergens for which sequences had been found among two to three others after trypsin digestion of a single band. We are unsure if these are "true" chicken allergens for dogs, as other "confirmed" allergens also were detected in the same band. For example, the 120 kDa band yielded both myomesin-2 and the PKM dimer, whose monomer was the sole protein sequenced in the 58 kDa band (Table 1). An important observation is that only two of these seven "tentative" allergens (the 70 kDa heat shock protein HSPA2 and the troponin TNNT3) are recognised allergens for humans (Table

2); this is a stark contrast with the panallergen status of all the “confirmed” major allergens above.

Discussion

In this study of sera collected from dogs with known IgE sensitization to chicken, or in which clinical allergy to chicken had been proven, ELISA and immunoblotting followed by MS were used to identify several allergenic proteins from chicken meat.

In spite of the relatively low number of tested sera, using the criteria used for decades to characterise the relative importance of allergens,¹⁷ we propose seven chicken proteins to be major allergens for dogs. These are, in alphabetical order of their gene name: ALB, CKM, ENO3, GAPDH, LDHA, PKM and TPI1. Another protein, TNNC2, is a minor allergen (Table 1). Remarkably, three of these major allergens (ALB, CKM and ENO3) were targeted by sIgE in all 22 sera from CS and CA dogs. Whether or not each allergen, in purified or recombinant form, would induce a clinical reaction in the dogs with elevated sIgE was not determined, however.

The major allergens reported herein are neither surprising nor novel, as we had recently reported six of them (CKM, ENO3, GAPDH, LDHA, PKM and TPI1) as cross-reactive allergens between chicken, white fish (cod/haddock) and salmon.¹⁴ That these proteins are now found to be recognised by sIgE in a different set of 10 CS and 12 CA canine sera further support their relevance as major allergens for dogs. Two of these proteins (ENO3 and GAPDH) already are known chicken allergens for humans (Gal d 9 and Gal d GAPDH, respectively).^{18, 19}

In contrast to the results discussed above, we did not find aldolase A (ALDOA), one of the known fish–chicken cross-reactive allergens in people and dogs^{14, 19} to be IgE-targeted in any of our tested sera. This observation suggests that fish might be the primary sensitiser for this protein, which, when recognised by sIgE, could then induce a secondary sensitisation against – and possibly an allergy to – chicken.²⁰

A notable addition to the list of food allergens for dogs is the chicken serum albumin, a protein already known as an allergen (Gal d 5) in humans reacting to chicken meat and egg yolks.^{21–23} To the best of our knowledge, Gal d 5 has not yet been reported to be targeted by sIgE in CS dogs. It was, however, one of the suspected proteins recognised by serum IgG in six dogs experimentally sensitised to chicken.²⁴ The addition of Gal d 5 as a chicken allergen for dogs is noteworthy, as serum albumins are known panallergens, evolutionarily conserved and widely distributed proteins involved in important life processes of plants and animals.²⁵ Panallergens share high rates of sequence homology and 3D structures between species, which allow them to be recognised by cross-reactive IgE.²⁵ A more extensive discussion on serum albumin allergens and the potential risk of poultry-mammalian meat cross-reactivity is provided in Appendix S1.

Nevertheless, the question of putative cross-reactivity between chicken and other mammalian meats is not restricted to albumins, as the sequence similarity of the seven other allergens identified herein is always >85% between chicken and beef, which is extensive. Such a substantial sequence homology implies a very high theoretical risk of allergenic cross-reactivity between chicken and mammal meat allergens, which is deserving of further studies.

This work provided some methodological insights and limitations that could be valuable for future research on allergen characterisation in companion animals.

First, we were surprised to find that the pattern of allergens recognised on immunoblotting by sIgE in CS and CA dogs was nearly identical (Figure 4). This observation suggests that the search for allergens using archived sera from dogs with elevated food allergen-specific IgE, as we did for the determination of chicken-fish cross-reactive allergens,¹⁴ could be used, at first, as a proxy replacing the need for sera from dogs with a proven clinical food allergy.

Secondly, the ELISA performed with the commercial chicken meat allergen permitted the detection of sIgE in seven of 12 chicken-allergic dogs (58%); this apparently low sensitivity of the ELISA for the diagnosis of chicken allergy in dogs has been highlighted before.⁴ By contrast, immunoblotting revealed IgE-bound protein bands in all 12 CA sera, thus confirming the higher – and optimal – sensitivity of this method compared with ELISA. This observation is somewhat surprising, as allergens used in ELISA are in native form, while those used in immunoblotting are denatured and unfolded. In another study, however, immunoblotting to detect food allergen sIgE was found to have a very low specificity, as it could not help differentiate between dogs with or without food allergy.¹⁵ Nevertheless, in that study, the detection of positive bands in the absence of any canine serum suggests an insufficient blocking of the nonspecific binding of reagents to the membranes.

Finally, in the current study, the MS analysis occasionally returned two to three protein sequences digested out of a single gel band. This result was expected as proteins of different sequences can have the same MW and electrophoretic migration. As a result, we could not always determine if only one or all of the proteins sequenced in that band were targeted by sIgE. To address this issue, we recommend the preferential use of 2D electrophoresis, which separates the proteins vertically based on their MW and horizontally based on their charge (i.e. their isoelectric point). This method, which is more likely to return a single protein when sequencing one "dot," already has been employed to characterise *Dermatophagoides pteronyssinus* allergens for dogs.²⁶

Our research, even though it led to the identification of novel chicken allergens and the confirmation of previously reported ones,¹⁴ does raise some questions worthy of further investigations. First, one needs to better define which one(s) of the allergens identified herein are clinically relevant in dogs. Whether processing alters the allergens' immunogenicity could have implications for the generation of future, more "hypoallergenic"

pet foods. Second, studying whether the use of single allergens in ELISA (i.e. “singleplex assays”), or multi-allergen arrays (i.e. “multiplex assays”), improves the accuracy of current whole-extract serological tests for food-specific IgE is timely and technically feasible in veterinary medicine. Finally, there is a critical need to establish if chicken meat allergens are cross-reactive with homologous proteins in meats of other species (especially mammalian). Indeed, if this phenomenon were to be confirmed, it would raise serious concerns about the validity of our widely recommended practice of performing elimination (restrictive) diets with “novel” animal meats that could still be recognised by sIgE from dogs allergic to homologous proteins of other species.

In summary, we identified herein seven major and one minor chicken allergen for dogs, and several other proteins that also are possible allergen candidates. Additional studies are needed to confirm their relative importance, their usefulness for the serological diagnosis of chicken allergy, their pathogenicity, their potential for cross-reactivity between species, and whether food processing or cooking alters their immunogenicity.

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Author contribution

Thierry Olivry: Conceptualization; Data curation; Funding acquisition; Project administration; Supervision; Validation; Visualization; Writing-original draft; Writing-review & editing. **Cherie M Pucheu-Haston:** Resources; Writing-review & editing. **Ursula Mayer:** Resources; Writing-review & editing. **Kerstin Bergvall:** Resources; Writing-review & editing. **Jennifer Bexley:** Data curation; Formal analysis; Investigation; Methodology; Writing-original draft; Writing-review & editing.

Supporting Information

Filename	Description
vde13029-sup-0001-AppendixS1.docx Word document, 117.9 KB	Appendix S1. Serum albumin as cross-reactive panallergens.

Filename	Description
vde13029-sup-0002-TableS1.pdf PDF document, 61.3 KB	Table S1. Characteristics of the CS and CA canine sera.
vde13029-sup-0003-TableS2.docx Word document, 350.5 KB	Table S2. Serum IgE-binding chicken protein bands in CA and CS dogs.

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Appendix S1. Serum albumin as cross-reactive panallergens

At this time, seven serum albumins currently are officially included in the WHO/IUIS Allergen Nomenclature Sub-Committee database (www.allergen.org, page last accessed June 7, 2021), yet, in total, 24 animal serum albumins are known allergens (figure S3 in Chruszcz et al.)¹. The risk of allergic cross-reactivity among animal albumins is very high,¹ and the probability of this happening depends upon the degree of albumin sequence similarity between species (see Figure S1, below).¹⁻³

When species are phylogenetically close, the protein homology is extensive (Figure S1). For example, the bovine serum albumin (ALB, Bos d 6) is one of the top beef meat allergens responsible for the cross-reactivity regularly noted among mammalian meats;^{2,3} chicken ALB is responsible for the same phenomenon among poultry and game bird meats.⁴

For phylogenetically distant species, the risk of albumin cross-reactivity is low yet not negligible. Indeed, most bovine ALB/Bos d 6-sensitised human individuals allergic to beef meat and milk normally will not clinically react when eating chicken.² A similar situation exists for chicken-reactive patients who cannot eat turkey and usually tolerate mammal meats.⁵ Such a lower risk of cross-reactivity between poultry and mammals likely is due to the medium sequence homology that exists between bovine and chicken ALB (Figure S1). Still, rare patients suffering from cross-reactive clinical allergies between poultry, mammal and fish meats have detectable sIgE against several animal serum albumins.⁶ Finally, Gal d 5 (chicken ALB) is one of the two allergens involved in the “chicken-pork” allergy syndrome caused by its cross-reactivity with the porcine serum albumin (Sus s 1).^{7,8}

We would be remiss not to remind the reader that Bos d 6 has been reported as an allergen in a dog with beef allergy in Japan,⁹ and in some food-allergic dogs in the USA.¹⁰ This protein also is one of the known culprits responsible for immediate allergic reactions to some vaccines in dogs.^{9,11} Such sensitisation to bovine ALB after vaccination is not surprising due to its presence in the fetal calf serum used to grow the cells necessary for the culture of vaccinal virus strains.¹² Because of the known cross-reactivity between animal serum albumins in humans, the specter of vaccination sensitising some dogs to bovine ALB, then predisposing them to a clinical allergy to beef – and perhaps for some, even to chicken – does not seem such an unrealistic hypothesis.

Figure S1. Allergen relative identity, similarity and cross-reactivity (A-RISC) indices for serum albumins



Herein, we extracted the relevant albumin A-RISC indices, the average (as a percentage) sequence identity and similarity for each pair of proteins, from figure 8 in Chruszcz et al.¹ We then calculated additional values for salmon, duck, turkey and alligator using the same methodology and the SIAS tool (<http://imed.med.ucm.es/Tools/sias.html>; page last accessed August 18, 2020) with its default parameters. An A-RISC >50% (0.50) increases the likelihood of cross-reactivity between allergens; it is highest if the index is $\geq 75\%$. To facilitate comprehension, we remind readers that albumins from chicken and beef are Gal d 5 and Bos d 6, respectively.

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Supporting Information 1: Characteristics of the chicken-sensitised and chicken-allergic canine sera

Chicken-Sensitized (CS) dogs

Dogs	Breed	Sex	Age (yrs)	Cutaneous or Digestive?	Cutaneous Signs
CS1	German shepherd	FS	4.3	cutaneous	AD, E
CS2	German shepherd	MI	1.6	cutaneous	OE, P
CS3	crossbred	FI	5.0	cutaneous	P
CS4	Labradoodle	FS	2.0	cutaneous	OE
CS5	Labrador retriever	MC	8.0	cutaneous	P
CS6	Dogue de Bordeaux	MI	1.3	cutaneous	OE, P
CS7	Boxer	FI	3.0	cutaneous	OE, P
CS8	Basset Hound	MC	1.1	cutaneous	OE
CS9	French Bulldog	MC	2.5	cutaneous	?
CS10	Labrador crossbred	FS	1.5	cutaneous	P
		median	2.3		
		mean	3.0		

Chicken-Allergic (CA) dogs

Chicken-Allergic (CA) dogs				BEFORE CHALLENGE		AFTER CHICKEN CHALLENGE					
Dogs	Breed	Sex	Age (yrs)	Cutaneous or Digestive?	Cutaneous Signs	Challenge Type	Cutaneous Signs	Time to Flare (hrs)?	Anti-Allergic Drugs During Challenge?	Time (days) Serum Collection After Challenge?	Chicken ELISA (AU)
CA 1	Labrador retriever	MC	1.0	cutaneous	Co, E, P, Py	Cd, Cm	Ee, P	13-24	none	not specified	-
CA 2	Labrador retriever	FS	2.0	cutaneous	E, P	Cd	P	15-21	none	15-21	13.8
CA 3	CKCS	MC	5.0	cutaneous	OE, P, Py	Cd	Pa, P	20-40	none	8-14	-
CA 4	crossbred	FS	1.0	cutaneous	E, P	Cd	Ee, P	-	none	0-7	5.2
CA 5	Labrador retriever	FS	6.0	cutaneous	OE, P	Cd	Ee	> 48	none	1	-
CA 6	Pit Bull	MC	1.0	cutaneous	E, P	Cd	P	-	none	1	8.2
CA 7	Yorkshire	FS	11.0	both	AD, P	Cd, Cm	GI, P	6-12	none	0-7	3.3
CA 8	Poodle	FS	6.0	cutaneous	AD, CJ, P	Cd, Cm	Ee, P	25-48	none	0-7	2.3
CA 9	crossbred	FS	3.0	cutaneous	AD	Cd, Cm	P	13-24	none	36-42	-
CA 10	Malinois	FI	6.0	cutaneous	AD	Cd, Cm	Ee, P	25-48	none	0-7	61.1
CA 11	Labrador retriever	MC	7.0	cutaneous	AP	Cd, Cm	P	> 48	none	29-35	-
CA 12	Rottweiler	MI	4.0	both	E, P	Cd, Cm, Rm	Ee, GI	6-12	H1R	8-14	> 100
		median	4.5								
		mean	4.4								

FI: female intact

FS: female spayed

MC: male castrated

MI: male intact

AD: atopic dermatitis

CJ: conjunctivitis

Co: coughing

E: erythema

OE: otitis externa

P: pruritus

Py: pyoderma

GI: gastrointestinal signs

Ee: erythema, edema

P: Pruritus

Pa: papules

Cm: cooked meat

Rm: raw meat

Cd: Chicken petfood

H1R:

H1 receptor antihistamine

"-": OD too low to interpolate from the standard curve

Table S2. Serum IgE-binding chicken protein bands in CA and CS dogs

MW (kDa)	Molecular weight (kDa), approximate																					
	Chicken-allergic (CA) study dog												Chicken-sensitized (CS) study dog									
	CA 1	CA 2	CA 3	CA 4	CA 5	CA 6	CA 7	CA 8	CA 9	CA 10	CA 11	CA 12	CS 1	CS 2	CS 3	CS 4	CS 5	CS 6	CS 7	CS 8	CS 9	CS 10
261	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
220	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
157	-	-	-	-	-	-	-	-	-	-	-	-	-	-	155	-	-	-	-	-	-	-
140	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
131	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
120	-	-	-	-	-	-	-	-	-	126	-	-	-	-	-	-	-	-	-	-	-	-
110	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
88-95*	-	-	-	-	-	-	-	-	-	-	-	88	95	-	-	-	-	-	-	-	95	-
82	-	-	81	-	-	-	-	-	-	76	-	80	81	-	-	-	82	81	-	-	81	-
70	-	-	-	-	-	-	-	-	-	70	-	73	-	-	-	-	-	-	-	-	-	-
65	67	66	66	-	-	67	68	68	-	-	67	64	67	66	67	-	-	67	65	-	-	66
63	63	64	64	63	63	63	64	64	64	63	63	62	63	64	64	63	64	63	63	63	63	63
58	59	-	58	-	-	57	58	59	-	58	-	57	56	60	59	-	57	60	60	60	60	-
48	51	50	49	51	50	50	51	51	51	49	50	51	50	50	51	51	51	52	52	52	52	51
43	45	44	44	44	44	43	44	44	44	43	43	43	46	44	45	44	44	45	45	45	46	44
38	39	37	38	38	-	38	38	38	-	38	-	39	39	38	39	38	-	39	38	39	38	38
36	37	-	36	37	-	36	36	-	-	36	-	36	37	36	37	36	37	37	36	37	36	36
35*	-	-	-	-	-	-	-	-	-	-	-	35	-	-	-	-	33	36	33	-	-	-
29	29	29	-	28	29	28	-	28	28	30	28	-	28	29	28	28	-	29	28	32	28	29
27	-	27	27	27	-	26	27	-	-	28	-	27	-	27	-	27	26	25	25	28	-	-
21	-	-	-	-	-	-	-	-	-	22	-	-	-	25	-	-	23	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-	-	-	15	15	-	15	-	-	-	-	-	16	-
13	-	-	-	-	-	-	-	-	-	-	-	-	13	-	-	13	14	-	-	-	-	-

*not visible/distinct on Coomassie-stained gels. Where no cell is checked (-), IgE-binding bands of this MW were not visible. N.B. given the manual assignment of MW to the markers, some degree of inaccuracy in the MW of the bands, as given here, should be expected. Boxed value indicates one large band spanning MW ~43–51 kDa

Commento [RS1]: WILEY: change to table S2:
Dark green row: change to 'Approximate MW (kDa)'
Top light green row, col heads: change to 'CA study dog' and 'CS study dog'