IgE reactivity to fish allergens in Pacific cod (*Gadus macrocephalus*) in atopic dogs

Ichiro Imanishi
  Tokyo University of Agriculture and Technology

Jumpei Uchiyama
  Azabu University

Keijiro Mizukami
  Azabu University

Junichi Kamiie
  Azabu University

Keigo Kurata
  ITEA Inc

Masato Fujimura
  Fujimura Animal Hospital

Kuniyoshi Shimakura
  Tokyo University of Marine Science and Technology

Koji Nishifuji
  Tokyo University and Agriculture and Technology

Masahiro Sakaguchi
  Azabu University

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Abstract

Background: IgE reactivity to fish allergens in atopic dogs, which are used as models for food allergy, has not been elucidated to date. We investigated IgE reactivity to crude extracts and purified Pacific cod (*Gadus macrocephalus*) allergens in atopic dogs to identify the allergenic proteins of cod.

Methods: Specific IgE to crude cod extracts in the sera of 179 atopic dogs, including 27 dogs with cod allergy, were measured using enzyme-linked immunosorbent assay (ELISA). The allergens of crude cod extracts were analyzed by ELISA, immunoblotting, and liquid chromatography-tandem mass spectrometry (LC-MS/MS). IgE reactivity to parvalbumin, collagen, and tropomyosin was evaluated using the sera of atopic dogs that were positive for specific IgE to crude cod extracts.

Results: Specific IgE to crude cod extracts were present in 36 (20%) of the 179 atopic dogs and 12 (44%) of the 27 dogs with cod allergy. In allergen component analysis, IgE reactivity to tropomyosin and enolase was observed in the sera of dogs with cod allergy. Among the 36 dogs with IgE reactivity to crude cod extracts, 9 (25%), 14 (39%), and 18 (50%) dogs had specific IgE to parvalbumin, collagen, and tropomyosin, respectively.

Conclusions: The dogs exhibited IgE reactivity to the cod allergens which observed in humans, providing support for the use of atopic dogs with fish allergy as a model for fish allergy in humans.

Background

The prevalence of fish allergy, which affects approximately 0.2% of the human world population [1], is over ten times higher in geographic regions where fish is an essential dietary component such as Japan [2,3]. Fish allergy is typically known to be a life-long condition in contrast to other food allergies [3]. Since clinical cross-reactivity to different fish species is a widely accepted feature of fish allergy, affected individuals have to avoid all fish species for long periods, and inadvertent exposure to fish, and the consequent severe or fatal reactions remain a grave risk for fish-sensitive individuals [3]. The components inducing allergic reactions are mostly immunoglobulin (Ig) E-binding proteins, which are called allergens. Among the fish allergens, parvalbumin is the best characterized major allergen that is found in many species [4-6]. In a previous study, we identified fish gelatin (type I collagen) as an allergen [7]. More recently, other proteins such as tropomyosin, enolases, and aldolases were also considered as relevant allergens in fish [8]. To resolve the issue of quality of life in fish-allergic patients, it is useful to experiment on animals which focus on the management of fish allergenicity.

Animal models are beneficial to test food allergies as they provide a more rapid and extensive evaluation of allergenicity of certain foods [9]. Dogs are under intense investigation as a useful model to study IgE-mediated hypersensitivity by demonstrating the production of specific IgE to crude extracts and positive oral challenges similar to those observed in human subjects [10-12], as they are one of the species other than humans in which allergies develop naturally following environmental exposure to a broad spectrum of allergens, including foods [13,14]. Disease states in dogs include atopic dermatitis, gastroenteric

inflammation, and anaphylaxis [15,16]. Based on the many similarities between canine atopic dermatitis and humans [17], atopic dogs have been utilized as animal models for food allergies to cow's milk [11], corn [18], and nuts [12].

The allergens present in atopic dogs' food are assumed to correspond with those in humans [19]. However, Kubota *et al.* reported that IgE reactivity to allergens for dogs were different from those for humans [20]. To develop atopic dogs into suitable models of food allergy in humans, it is important to analyze the homologies of IgE reactivity in food allergy between humans and dogs. Cod is not only one of the most commonly consumed fish species in Europe and Japan [21], but also one of the most characterized fish species with allergen components [8]. Nevertheless, little is known about IgE reactivity to cod allergy in dogs. Therefore, in the present study, we investigated IgE reactivity for atopic dogs to crude extracts and purified allergens present in Pacific cod (*Gadus macrocephalus*).

Methods

Sera of atopic dogs

To examine IgE reactivity in atopic dogs, we obtained surplus sera from 179 dogs that were clinically diagnosed with atopic dermatitis based on the criteria by Willemse [22] and Prelaud *et al* [23] among dogs visiting Fujimura Animal Hospital (Osaka, Japan), which is clinically compatible with atopic dermatitis in humans [17]. Twenty samples from laboratory dogs were used as negative controls. The dogs were housed indoors as experimental laboratory animals and had never been exposed to fish antigens. None of the laboratory dogs exhibited signs of atopic dermatitis. Enzyme-linked immunosorbent assay (ELISA) for parvalbumin or collagen from salmon (Atlantic salmon; *Salmo salar*), sardine (Japanese pilchard; *Sardinops melanostictus*), and mackerel (Chub mackerel; *Scomber japonicus*) were performed using individual sera of nine negative control dogs that provided a large amount of serum. All sera were stored at −80°C before use. Oral informed consent was obtained from the dog owners. All experimental procedures were carried out in accordance with Japanese law and approved by the animal care and user committee of Azabu University.

Food elimination and oral provocation tests

About the 179 dogs with clinical signs of atopic dermatitis, the elimination diet trials were performed for 6−8 weeks with commercial hydrolyzed diets to which ingredients the dog has never been exposed before. Based on diet history, thirty-one dogs were analyzed to have cod reactivity by the oral provocation tests after the elimination diet trial with; consent for food provocation test was obtained from the dog owners as previously described [24]. When the veterinary physician recognized the complete resolution of the clinical signs during the food elimination test, the dog was admitted to the animal hospital and challenged with various cod ingredients, including grilled cod meat and cod-containing dog foods. The cod provocation test was discontinued immediately upon the relapse of the clinical signs including vomiting, diarrhea, erythema, pruritic, urticaria, and conjunctival hyperemia.

Preparation of crude cod extracts

Pacific cod was purchased from a fish market in Japan to be used in the study. The fresh, raw meat of four fishes (500 µg) was homogenized in 500 µl phosphate-buffered saline (PBS, 10 mM pH 7.2) and rotated overnight at 4°C. After centrifugation at 21500 g for 5 min at 4°C, the supernatant was collected, and the protein was quantified using the BCA protein assay (Bio-Rad, Hercules, CA, USA).

Purification of parvalbumin, collagen, and tropomyosin

Fish parvalbumin [4] and collagen [25] were purified as described previously. Tropomyosin was purified from the freeze-dried powder by Bailey's method with slight modification [26]. Briefly, freeze-dried fish powder was stirred in a beaker with 75 ml extraction buffer containing 15 mM Tris HCl pH 7.6 (Sigma Aldrich, St Louis, MO, USA), 1 M KCl (Kanto Kagaku, Tokyo, Japan), and 2 mM dithiothreitol (Sigma Aldrich) overnight at 4°C. The extract was collected by centrifugation at 5400 g for 10 min at 4°C. The supernatant pH was adjusted to 4.5 with 1 N HCl to precipitate tropomyosin, and the precipitation was collected by centrifugation at 5400 g for 10 min at 4°C. The isoelectric precipitation was repeated once, and the precipitated material was dissolved in the extraction buffer. The supernatant after the extraction was collected by centrifugation and fractionated by ammonium sulfate at a concentration of 50%. The sample precipitated by ammonium sulfate was dissolved and dialyzed against PBS. The obtained protein extracts were confirmed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

SDS-PAGE and immunoblotting

SDS-PAGE was performed according to the method of Laemmli [27]. Precision plus protein standards (Bio-Rad, Hercules, CA, USA) were used as molecular-mass markers. Crude cod extracts were electrophoretically separated using 5%–20% gradient polyacrylamide gels, and proteins were visualized either by Coomassie brilliant blue R250 (Bio-Rad) or transferring onto polyvinylidene difluoride membranes (GE Healthcare, Chicago, IL, USA). Immunoblotting was performed as described previously [7]. IgE in patient dog sera were used as primary antibodies, which were diluted 1:10 in tris buffered saline containing 0.1% Tween-20 and 5% nonfat dried milk. Mouse monoclonal anti-dog IgE antibodies (0.5 µg/ml) were used as secondary antibodies [28]. Detection was performed using an enhanced chemiluminescence immunoblotting detection reagent (GE Healthcare).

Liquid chromatography (LC)-tandem mass spectrometry (MS/MS)

For LC- MS/MS, the protein bands detected by immunoblotting were excised, and in-gel digestion was performed with 0.5 mg N-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (Promega, Madison, Wisconsin) at 37°C for 16 h. Tryptic digests were acidified using formic acid (pH < 2.0) and centrifuged at 21500 g for 15 min. The supernatants were analyzed using high-performance LC (Advance System; AMR, Tokyo, Japan) connected to an electrospray ionization triple quadrupole mass spectrometer (4000 QTRAP; AB Sciex, Framingham, MA, USA). Extracts were injected into a reversed-phase column (electrospray ionization column [octa decyl silyl]; particle inner diameter, 75 mm; length, 100 mm;

diameter 3 mm; LC Assist, Tokyo, Japan) that was eluted with a 5%–45% gradient of acetonitrile containing 0.1% formic acid for 60 min at 300 nl/min. Ionization was performed using an ion-spray voltage of 2000 V at a capillary temperature of 200°C. The mass spectrometry instrument was operated in the positive ion mode over the range of 450–1200 m/z. The MS/MS spectra were obtained in the enhanced production scan mode, and two higher-intensity peaks in each mass spectrometry scan were chosen for collision-induced dissociation.

The MS/MS data were used to search in entries under the *Liza aurata* category of the UniProt database using the Mascot peptide search engine. An MS tolerance of 1.0 Da for-precursor ion and an MS/MS tolerance of 0.8 Da were set as windows of processing parameters for matching peptide mass values.

Fluorometric ELISA for allergen-specific serum IgE

Specific IgE levels to cod crude extracts were measured using a fluorometric ELISA as previously described [20]. A microplate (NUNC Immuno Plate Maxisorp F96; Nalge Nunc International, Roskilde, Denmark) was coated with crude cod extracts (10 µg/ml) or the purified allergens (parvalbumin, collagen, or tropomyosin; 1 µg/ml) at 4°C overnight. After washing, the plate was incubated with diluted sera (1:10) in PBS with 10% (v/v) fetal calf serum and 0.05% (v/v) Tween 20 at room temperature for 3 h. The plate was then washed and incubated at 4°C overnight with a mouse monoclonal anti-dog IgE antibody (0.5 µg/ml) [28]. After washing with PBS containing 0.1% (v/v) Tween 20 (PBS-T), the plate was incubated with a biotinylated rat monoclonal anti-mouse IgG1 (Zymed Laboratories, San Francisco, CA, USA) at room temperature for 1 h. After washing, the plate was incubated with b-D-galactosidase-conjugated streptavidin (Zymed Laboratories) at room temperature for 1 h. After the final washing, the plate was incubated with 0.1 mM 4-methylumbelliferyl- b-D-galactopyranoside (Sigma Aldrich) at 37°C for 2 h. The enzymatic reaction was stopped with the addition of 0.1 M glycine-NaOH (pH 10.2). The fluorescence intensity was measured as fluorescence units (FU) on a microplate fluorescence reader (Fluoroskan; Flow Laboratories, McLean, VA, USA). The absorbance was measured at 355 nm with a 460 nm reference filter. All the washing steps were performed three times for 5 min in PBS-T. The cutoff value was determined as the average + three standard deviations (SDs) of FU in serum samples of 20 dogs used as negative controls. All tests were performed in triplicate.

Results

Clinical characteristics of atopic dogs with cod allergy

The present study included 179 atopic dogs from 34 breeds, and there were 79 males and 100 females (age, 3.9± 3.2 years; range, 2 months–11 years). Elimination diet trials improved the atopic symptoms of 144 dogs (80%). Among 31 atopic dogs with cod oral provocation analysis, the clinical reactivity to cod meat was confirmed in 27 atopic dogs (i.e. dogs with cod allergy). The mean age of these 27 dogs with cod allergy was 3.4 ± 2.6 years (range, 2 months–8 years), which contained 12 males and 15 females. Among these 27 dogs with cod allergy, eight dogs were administered grilled cod meat and 19 dogs were administered cod-containing dog foods. The clinical signs responses to provocation test were observed

as skin symptoms in all dogs (pruritus 27 dogs, erythema 16 dogs, and urticaria 3 dogs), and concurrent gastrointestinal signs in 3 dogs (11.1%: vomiting 3 dogs and diarrhea 1 dog). Among these 27 dogs with cod allergy, eighteen dogs were immediately provided symptoms within 3 hours later, whereas 9 dogs were provided symptoms after a few days later. Among nine dogs with late-onset reaction, one dog showed vomiting and diarrhea.

IgE reactivity to crude cod extracts among atopic dogs

Specific IgE reactivity to crude cod extracts were examined in the atopic dogs using ELISA. Twenty percent (36/179) of the dogs increased levels of specific IgE to crude cod extract (Figure 1). Among the atopic dogs with positive reactions on food elimination trials, 25% (36/144) of the dogs also increased levels of specific IgE to crude cod extracts. Among the dogs with cod allergy, IgE reactivity to crude cod extract was positive in 44% (12/27) (Figure 1). All the dogs with late-onset reaction showed negative reactions about IgE reactivity to crude cod extracts (9/9). Among 4 dogs with non-cod allergic dogs, one dog showed a false-positive level of specific IgE reactivity to crude cod extracts.

IgE reactivity to cod parvalbumin and collagen among atopic dogs exhibiting IgE reactivity to crude cod extracts

IgE reactivity to the purified cod allergens parvalbumin and collagen were tested by ELISA using the sera of the 36 dogs with IgE reactivity to crude cod extracts. IgE reactivity to parvalbumin was observed in 25% (9/36) of the dogs, whereas IgE reactivity to collagen was present in 33% (12/36) of the dogs. IgE reactivity to both parvalbumin and collagen was present in 16% (6/36) (Figure 5). However, 58% (21/36) of the dogs showed no IgE reactivity to these purified cod allergens (Figure 2A), indicating that reaction occurred to other fish proteins. We also compared IgE reactivity to parvalbumin and collagen in four fish species (cod, salmon, mackerel, and sardine) in six dogs that were reactive to cod parvalbumin and eight dogs that were reactive to cod collagen. IgE reactivity to parvalbumin was present to cod, salmon and sardine, but not mackerel in these dogs (Figure 2B), whereas IgE reactivity to collagen from all fish species occurred in 100% (8/8) of the dogs (Figure 2C).

Identification of other cod allergen components in atopic dogs with cod allergy

Among the 20 atopic dogs with IgE sensitivity to crude cod extracts that did not react to parvalbumin or collagen, sera were used from two dogs (no. 34 and no. 128) that provided a large amount of sera and reacted to crude cod extract but not cod parvalbumin or collagen, as confirmed with the provocation test using cod-containing foods (Table S1). After the separation of crude cod extract proteins using SDS-PAGE, IgE immunoblotting experiments detected IgE-reactive protein bands at around 35 and 55 kDa (Figure 3). LC-MS/MS performed for identification of the corresponding proteins in SDS-PAGE showed that the protein band around 35 kDa in weight matched tropomyosin from golden gray mullet (*Liza aurata*), and the protein band around 55 kDa in weight matched a enolase of python (*Python regius*) (Table 1).

We next purified cod tropomyosin from crude cod extract and confirmed with SDS-PAGE (Figure 3). The IgE levels for fish tropomyosin in the serum of dog no. 34 was measured using ELISA (Figure S1), and resulted that the IgE level to cod tropomyosin in the serum of dog no. 34 was significantly higher than those in the sera of the 20 control dogs. The levels of specific IgE to other fish tropomyosins were also higher in dog no. 34 compared to the negative controls.

Dog no. 34 presented with specific IgE and positive intradermal test to crude mite extract (House dust mite; *D. farinae*) (data not shown). We evaluated with IgE level for recombinant mite tropomyosin (*Der f* 10) in the serum of dog no. 34 using ELISA (Figure S1) and resulted that the IgE level to mite tropomyosin in the serum of dog no. 34 was significantly higher than those in the sera of the 20 control dogs.

IgE reactivity to cod tropomyosin in atopic dogs

Using the sera of 36 atopic dogs with IgE reactivity to cod extract, we determined IgE reactivity to cod tropomyosin using ELISA (Figure 4), which resulted that the IgE reactivity to tropomyosin was present in 50% (18/36) of the dogs. As shown in Figure 5, 67% (12/18) of the dogs with atopic dermatitis that had high IgE levels to crude cod extract and tropomyosin did not have those to cod parvalbumin or collagen. However, 25% (9/36) of the dogs still remained no IgE reactivity for any of these allergens.

Discussion

Atopic dermatitis affects approximately 10%–20% of the canine population [29], hence it could be useful as a spontaneous animal model with easily acquired. To assess the potential of atopic dogs with fish allergy as animal models, it is useful to characterize IgE reactivity for dogs with cod allergy. We reported here on the prevalence rate, each allergenicity, and association with a symptom for dogs with cod allergy.

First, the prevalence of fish allergy might be higher than expected. The rate of food allergy or intolerance due to fish has previously reported as only 1.3% (4/297) in dogs with food allergy or intolerance, which is diagnosed by food trial and provocation tests [30]. However, the present study showed that 20% (36/179) of the dogs exhibited increased levels of specific IgE to crude cod extract and 25% (36/144) of dogs with food allergy or intolerance, which was diagnosed by food elimination trials (Figure 1). Moreover, the dogs in Japan might be exposed to fish more frequently compared with dogs in other countries such as USA. Our field survey of commercial canine dry food products to estimate the difference in fish allergen exposure within and outside Japan found that 75% (117/157) of the Japanese canine dry food products contained fish. In contrast, 9% (7/82) of the products produced in Australia and the USA included fish. These evidences suggest that atopic dogs in Japan might be a higher risk of developing fish allergy due to an increase in the frequency of daily exposure for fish. These characteristics of atopic dogs might be mimic those of humans.

To our knowledge, this is the first study to describe the allergenic potency of parvalbumin and collagen in dogs as the property of being able to induce subsequent production of allergen-specific IgE antibodies. Parvalbumin has a higher allergenic potency than collagen in humans with cod allergy [2]. The present

study revealed that the rate of collagen allergy was higher than that of parvalbumin in dogs (Figure 5) and collagen elicited a stronger reactivity than that based on specific IgE levels compared with parvalbumin in these animals (Figure 2A). One possible interpretation of this discrepancy might be due to a loss of parvalbumin from dog food via physical and chemical steps in food processing, because parvalbumin is a water-soluble protein, unlike collagen [4,25]. Additionally, humans exhibit broad cross-reactivity to parvalbumin and collagen from distinct fish species [21,31], might show occurring in dogs with fish allergy as well. The current study suggested that the IgE reactivity to collagen and parvalbumin from cod in dogs were comparable with reactivity to those from the other fish species such as salmon, mackerel, and sardine (Figure 2B and 2C).

Of note, the rate of tropomyosin allergy was higher than those of parvalbumin or collagen in dogs (Figure 5) and the level of specific IgE to tropomyosin was higher than that for other allergenic components of cod, i.e., parvalbumin and collagen, in the dogs with cod allergy (Figure 2A and 4). Tropomyosin was demonstrated to be a fish allergen in a study using the sera of human patients with tilapia allergy [32]. Additionally, our comparison of the protein sequence of cod tropomyosin with those of other fish species revealed that cod tropomyosin exhibited 94%–99% sequence similarity with tropomyosin of other fish species that are commercially available on the market (Table S2). Tropomyosin is a major allergen that is the cause of many forms of crustacean allergy [33] as well as mite allergy [34] in humans. Although comparison of the tropomyosin protein sequence revealed a low sequence similarity between cod and shrimp (Table S2), fish-shrimp cross-reactivity was previously reported in humans [35,36]. Additionally, mite-crustacean cross-reactivity was widely reported in humans, and the tropomyosin sequence similarity between the two species is over 90% [37]. In dogs, mite is one of the most frequent sensitizing allergens, and mite tropomyosin is the allergenic component of mite allergy in canine atopic dermatitis [38]. Additionally, the serum of dog no. 34 exhibited reactivity to mite tropomyosin as strongly as cod tropomyosin (Figure S1). Taken together, these findings raise the possibility that mite allergy in dogs with IgE reactivity to mite tropomyosin might be associated with increased IgE reactivity to cod tropomyosin.

The results of the current study also revealed enolase is a potential allergen associated with canine fish allergy (Figure 3 and Table 1). Enolase was recently defined as a fish allergen that exhibited cross-reactivity to chicken in humans and dogs [36,39]. Numerous fish proteins, other than those purified proteins that are recognized as critical allergenic components in humans, have been registered in the International Union of Immunological Societies allergen database [40]. In some cases, minor allergens in humans can be dominant allergens in dogs [20,41]. Moreover, in the present study, 25% (9/36) of the dogs with specific IgE to crude cod extracts did not show IgE reactivity to any of the three major purified allergens by ELISA, implying that other purified cod allergens might underlie fish allergy in dogs. Future studies should focus on the identification of other allergens using the sera with IgE reactivity to cod extract in atopic dogs.

Atopic dermatitis with food allergy can be a manifestation of an IgE- or a non-IgE-mediated reaction to food in humans [42,43]. Similarly, IgE- and non-IgE mediated reactions might also exist in atopic dogs. Approximately 10% of atopic dogs have shown positive results in the lymphocyte stimulation test to fish

[24,44]. Lymphocyte stimulation tests are used for the diagnosis of non-IgE-mediated food allergies in humans [45,46]. Moreover, non-IgE mediated cod allergy in dogs may be more common compared with those in humans. Among dogs with cod allergy, the rate of IgE reactivity to crude cod extracts (44%; Figure 1) was lower than that in humans (90–95%) [47]. Sixty percent of dogs with cod allergy, which did not show IgE reactivity to crude cod extracts, manifested clinical reactions after a few days in the oral provocation tests. Conversably, fish allergy in humans generally present with classical food-allergic symptoms short after intake of fish [21,47]. These characteristics of importance about non-IgE mediated reactions among atopic dogs might be independent from those of humans.

The present result suggested that IgE reactivity about cod allergy in dogs and humans are similar but different. Thus, we should use these dogs as animal models of cod allergy with adequate caution. Further analysis would be required to characterize more detail about cod allergy in dogs. As an illustration, the current study was not possible to examine the relationship between detailed clinical symptoms and responsiveness to each allergen using methods such as specific IgE component analysis. Our future research would focus on analyzing the aforementioned relationship.

List Of Abbreviations

AD Atopic dermatitis

ELISA Enzyme-linked immunosorbent assay

FU Fluorescence units

Ig Immunoglobulin

LC Liquid chromatography

MS/MS Tandem mass spectrometry

PBS Phosphate buffered saline

SD Standard deviation

SDS-PAGE Sodium dodecyl sulfate Poly-acrylamide gel electrophoresis

Declarations

Ethics approval and consent to participate

All experimental procedures were carried out in accordance with Japanese law and approved by the animal care and user committee of Azabu University.

Consent for publication

All authors read and approved the final manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

Masahiro Sakaguchi has received research funding from ITEA Inc., whereas other authors declare no conflicts of interest.

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Author's contributions

 I.Imanishi made substantial contributions to the study conception and design as well as data acquisition and analysis; he also drafted the article. K. Kurata, J. Kamiie, and M. Fujimura made substantial contributions to performance of the experiments. J. Uchiyama, K. Mizukami, K. Shimakura, and K. Nishifuji assisted in the study design and manuscript preparation. M. Sakaguchi organized all the experimental settings and manuscript editing. He also revised the article critically for important intellectual content.

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Tables

Table 1. List of suspected allergens detected in two dogs with IgE reactivity to crude cod extracts by LC-MS/MS

Band (kDa)	Accession number	Protein name/species	Theoretical		LC-MS/MS analysis		
			MW (kDa)	PI	Score	Peptide matches	% coverage
35	P84335	Tropomyosin/ *Liza aurata*	32.710	4.69	111	3	7
55	gi17367183	Alpha enolase/ *Python regius*	47.541	6.97	64	1	2

LC-MS/MS, liquid chromatography-tandem mass spectrometry; MW, molecular weight; PI, isoelectric point.

Additional File

Additional file 1 - Supporting Method and Supplementary Data

Supporting method about market research of canine dry food. Supplementary Table S1; Clinical characteristics of dogs with IgE reactivity to crude cod extracts. Table S2; Homology of the amino acid sequences of tropomyosin from different species to that of tropomyosin from *Liza aurata* using the protein basic local alignment search tool (BLASTP) data. Supplementary Figure S1; IgE reactivity to tropomyosin from four different fishes and house dust mite in dog no. 34.

Figures
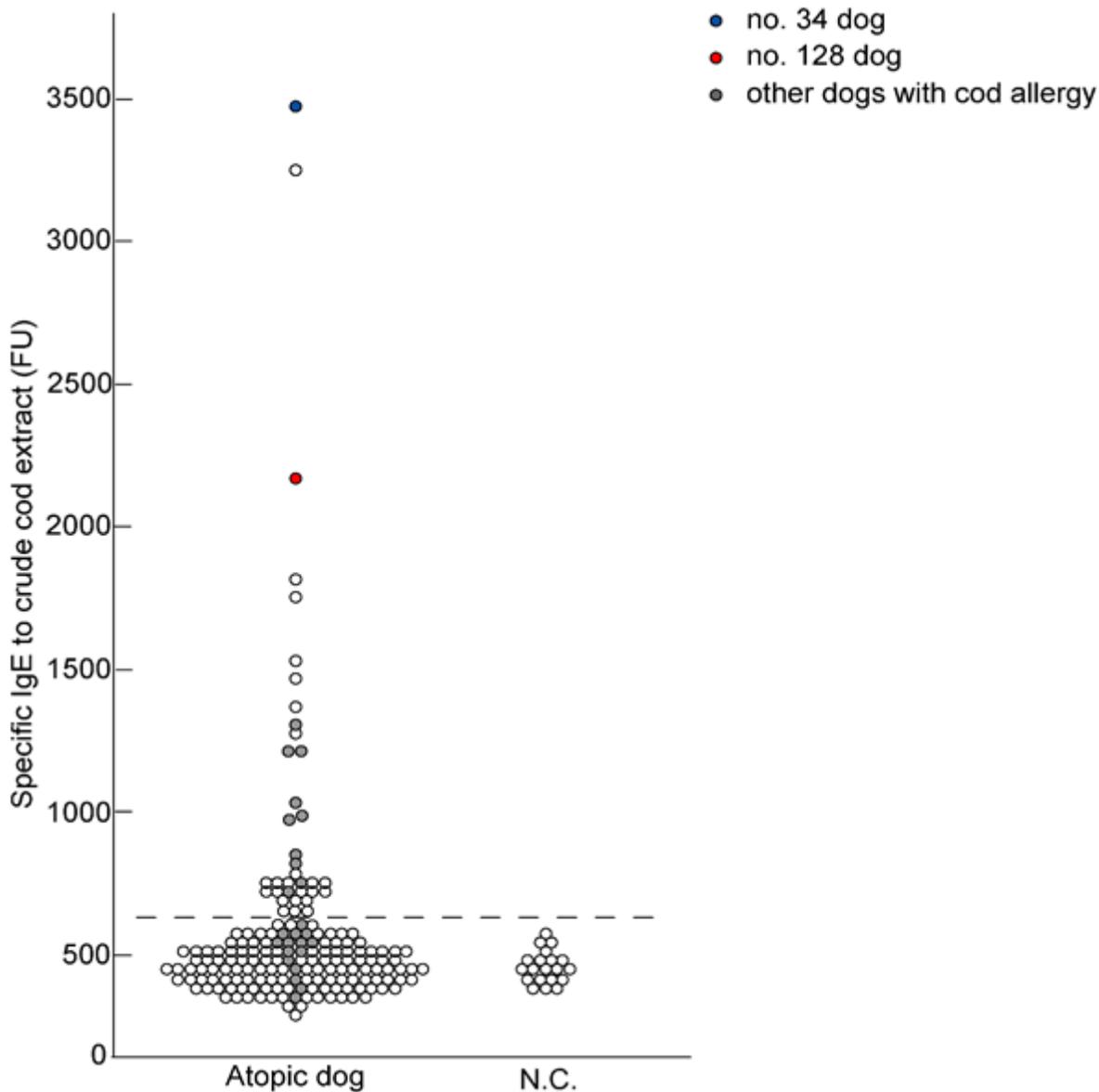
Figure 1

Immunoglobulin (Ig) E reactivity to cod crude extracts in atopic dogs based on the levels of specific IgE to crude cod extracts in 20 negative controls (N.C.). The mean fluorescence units (FU) ± standard deviation (SD) is 457 ± 61 FU, and the cutoff value (mean + 3SD) is 638 FU, shown as the dotted line. IgE reactivity to crude cod extracts among 179 atopic dogs. Thirty-six atopic dogs exhibited specific IgE reactivity to crude cod meat, with IgE levels ranging from 640 to 3483 FU. Blue circle indicates IgE reactivity in dog no. 34. Red circle indicates IgE reactivity in dog no. 128. Gray circles indicate IgE reactivity in other cod allergic dogs.
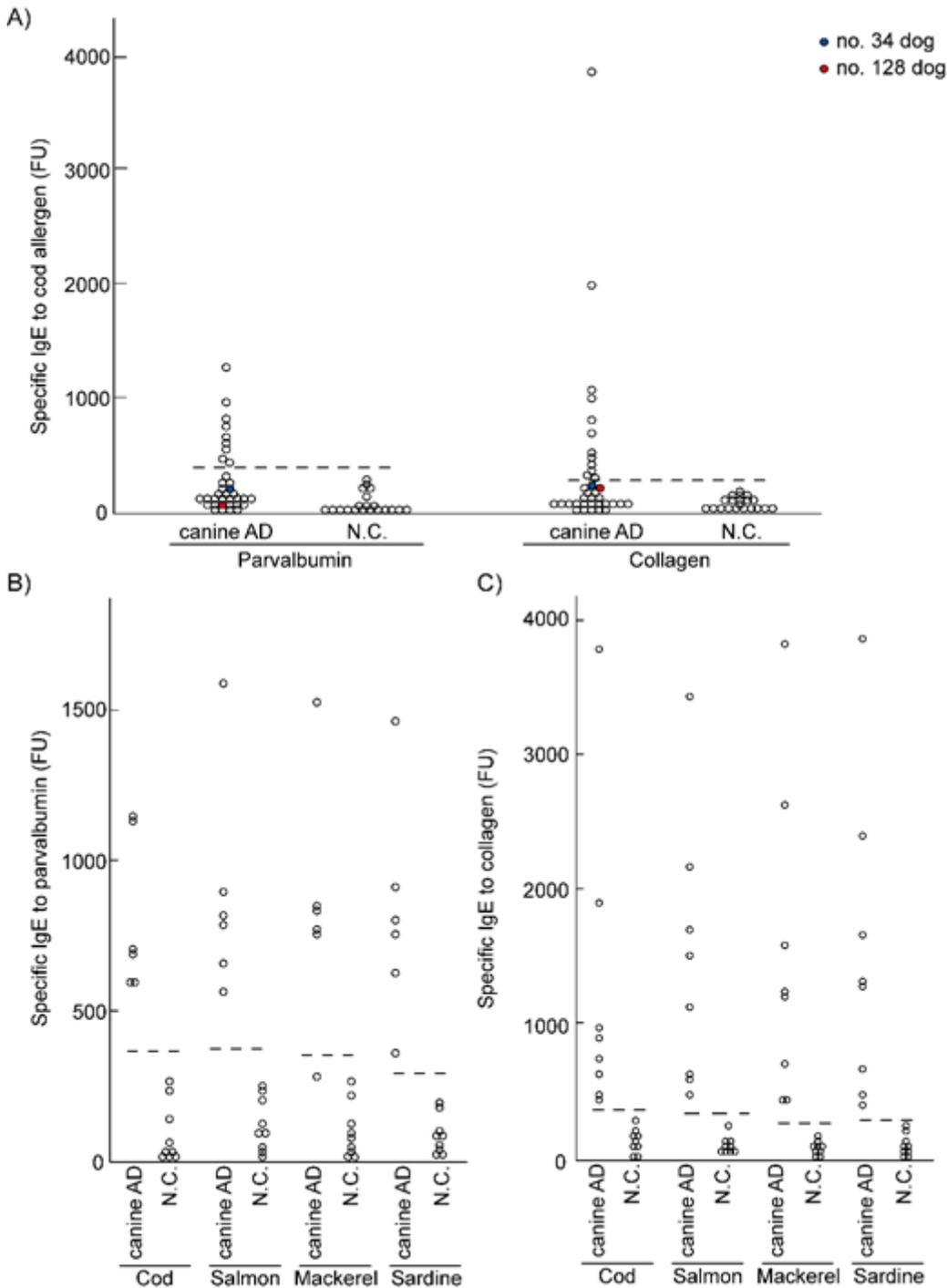
Figure 2

IgE reactivity to parvalbumin and collagen among atopic dogs exhibiting significant elevations in cod-specific IgE levels. (A) IgE reactivity to cod parvalbumin and collagen in 36 dogs with IgE reactivity to crude cod extract was determined using diluted sera (1:10). The dotted line shows the cutoff value, which was calculated using sera from 20 negative controls (N.C.). Based on the levels of specific IgE to parvalbumin and collagen in the negative controls (mean ± SD, 84 ± 67 and 74 ± 25 FU respectively), the cutoff values (mean + 3SD) for specific IgE against parvalbumin and collagen were 286 and 149 FU, respectively. The specific parvalbumin and collagen IgE levels in atopic dogs were 299−1121 and 151−3774 FU, respectively. Blue circle indicates IgE reactivity in dog no. 34. Red circle indicates IgE reactivity in

dog no. 128. (B) Parvalbumin-specific IgE and (C) Collagen-specific IgE was measured in the sera of six dogs with specific IgE to parvalbumin and eight dogs with specific IgE to collagen and those of nine healthy dogs (N.C.). The cutoff values (mean + 3SD) of specific IgE were 379, 374, 351, and 372 FU for parvalbumin from cod, salmon, mackerel, and sardine, respectively, which are shown with the dotted lines. The cutoff values (mean + 3SD) of specific IgE were 393, 310, 231, and 372 for collagen from cod, salmon, mackerel, and sardine, respectively.
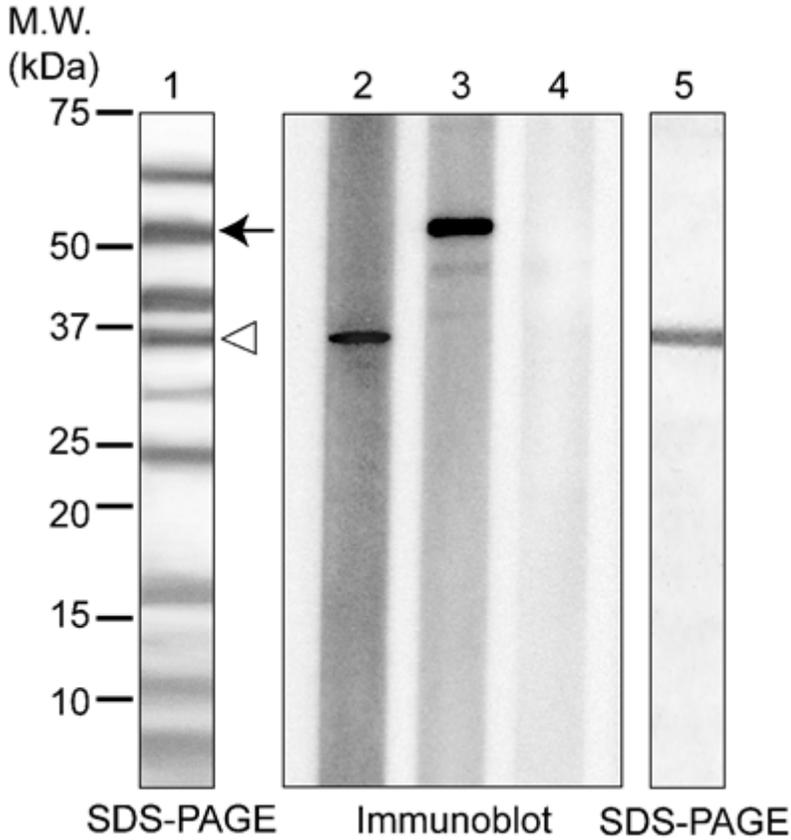
Figure 3

Immunoblotting for crude cod extracts. In the left column, the molecular standard is shown. Lanes 1 and 5 stained by Coomassie brilliant blue R250. Lanes 2 and 3 show immunoblotting using the sera of two atopic dogs (lane 2, dog no. 34; lane 3, dog no. 128) and lane 4 show the serum of a healthy dog serum (negative control). The arrow and arrowhead next to lane 1 indicate the bands analyzed by liquid chromatography-tandem mass spectrometry, which corresponded to the band detected by immunoblotting (immunoblotting in lanes 2 and 3).
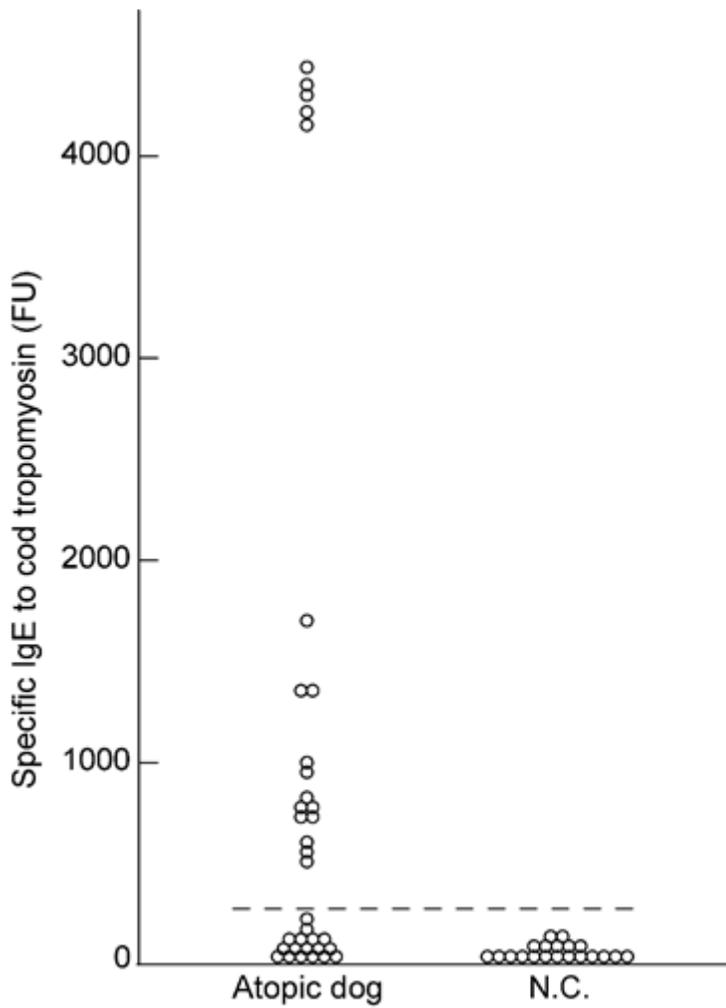
Figure 4

Determination of reactivity to cod tropomyosin among 36 dogs with specific IgE to crude cod extracts using enzyme-linked immunosorbent assay. The cutoff value (dotted line) calculated from 20 negative control samples. Based on the levels of specific IgE to tropomyosin in negative controls (mean ± SD, 33 ± 67 FU), the cutoff value (mean + 3SD) was determined as 234 FU.
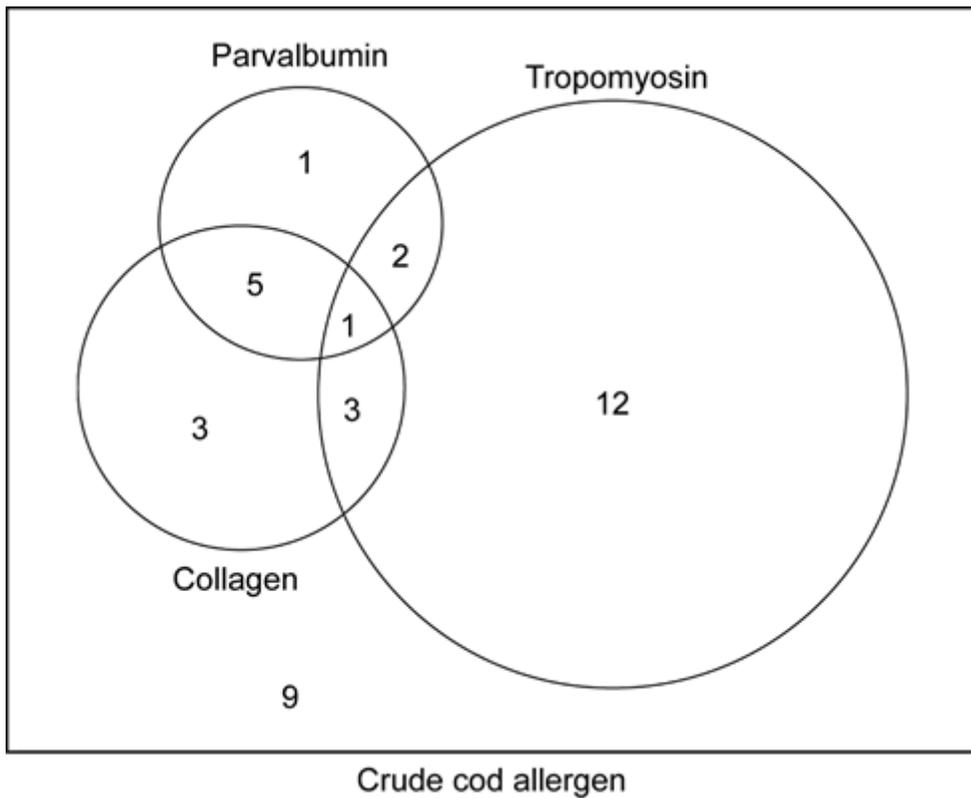
Figure 5

Venn diagram of the number of 36 dogs harboring fish allergens specific IgE. IeE reactivity to tropomyosin (12+2+1+3=18 dogs), parvalbumin (5+2+1+1=9 dogs), collagen (5+3+3+1=12 dogs), no allergen (9 dogs)