

# Comparison of Allergenic Properties of Salmon (*Oncorhynchus nerka*) between Landlocked and Anadromous Species

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## ABSTRACT

**Background:** Salmon is one of the most widely consumed seafoods in Japan and many other countries around the world. Due to the confirmed cases of salmon-induced allergy, the food sanitation law in Japan stipulates salmon as one of the specific food items for which labeling is recommended when used as an ingredient of processed foods. However, trout, the landlocked form of anadromous salmon, is not subject to the allergen-labeling requirements, even though both populations belong to a single species. Since no supporting data have been demonstrated to make a clear distinction between these two populations in terms of allergenicity, we comparatively examined their allergenic properties using sera from patients allergic to fish.

**Methods:** Extracts of *Oncorhynchus nerka* from different habitats were obtained: kokanee (landlocked) and red salmon (anadromous). Control extracts were derived from four other species. This study focused on the (1) IgE-binding capacity of the fish extracts in patients' sera ( $n = 50$ ), (2) ELISA inhibition test ( $n = 6$ ), and (3) inhibition immunoblot test ( $n = 8$ ) between the kokanee and red salmon.

**Results:** The extracts from kokanee and red salmon showed the highest correlation with each other in terms of the IgE-binding capacity, and showed complete (100%) reciprocal cross-inhibition in the ELISA inhibition test. On immunoblotting, there was no marked difference in the staining pattern between the two extracts, and each IgE-binding band gradually disappeared when the patients' sera were preincubated with the counterpart antigen in a dose-dependent manner.

**Conclusions:** These results suggest that kokanee has similar allergenic properties to red salmon.

## KEY WORDS

allergenicity, ELISA, fish allergy, food allergy, IgE

## INTRODUCTION

Most salmon are born in rivers, migrate to the sea, and return to the same rivers for spawning after spending several years at sea. However, some salmon do not migrate to the sea, but remain in the rivers. The former are called anadromous and the latter landlocked. As well-known examples, kokanee and red salmon are anadromous in *Oncorhynchus nerka*, and rainbow trout are landlocked and steelhead are anadromous in *Oncorhynchus myk-*

*iss*. Thus, salmon and trout belong to a single species.

Salmon and trout are consumed worldwide, and are reportedly a cause of allergy.<sup>1-5</sup> In Japan, 24 and 8 cases of salmon-induced immediate food allergy were reported in 2001-2002 and 2005, respectively.<sup>6</sup> Currently, the Japanese Allergic Food Sanitation Law includes salmon in specified food ingredients, and the labeling of foods containing salmon is recommended. In contrast, labeling is not obligatory for trout, despite salmon and trout belonging to the same species living in different habitats.

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Received 11 November 2008. Accepted for publication 5 January 2009.

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**Table 1** Profile of 6 patients with allergic reactions to salmon and 2 patients with a high salmon-specific IgE level

Patient No.	Age (year)	Sex	Salmon-induced symptoms	Specific IgE (UA/ml, score)
1	1	Male	Urticaria	2.25 2
2	1	Female	Urticaria	2.20 2
3	2	Male	Exanthema, itching	5.55 3
4	4	Female	Abdominal pain, vomit	75.5 5
5	5	Male	Abdominal pain, diarrhea	> 100 6
6	9	Female	Urticaria	6.17 3
7	1	Male	Avoidance	37.7 4
8	6	Male	Avoidance	24.4 4

There has been no report on the allergenicity of the two habitat types, and so there is no evidence to distinguish their allergenicity. We investigated the difference in allergenicity between the two habitat types based on binding with patients' IgE.

## METHODS

**Antigen extractions:** Fish antigens were extracted from raw fish meat using 1 M KCl buffer, as previously reported.<sup>7</sup> Antigens were extracted from landlocked *Oncorhynchus nerka*, kokanee, and its anadromous type, red salmon, belonging to *Salmoniformes*, *Salmonidae*, *Oncorhynchus*. For controls, different species of the same genus, rainbow trout (*Oncorhynchus mykiss*) and silver salmon (*Oncorhynchus kisutch*), and species of a different order (*Perciformes*), Japanese jack mackerel (*Trachurus japonicus*) and bluefin tuna (*Thunnus thynnus*), popular foods in Japan, were selected.

## ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Each fish antigen-specific IgE was measured in sera of patients allergic to fish ( $n = 50$ ), as follows:

The freeze-dried samples were dissolved (0.1 mg/ml) with PBS buffer and placed (0.1 ml/well) in each well of Nunc-Immuno Plate I (Nunc A/S, Roskilde, Denmark) for 1.5 hours at room temperature. Samples were discarded and SuperBlock Blocking Buffer in PBS (0.15 ml/well, Pierce, Rockford, IL, USA) was added and stored overnight at 4°C. Each well was washed with 0.2 ml/well of PBS-Tween and 0.1 ml/well of the serum diluted by SuperBlock Blocking Buffer (1 : 5) was added and stored overnight at room temperature. After being washed with PBS-Tween, Goat Anti-Human IgE BIOT (1 : 1,000, 0.1 ml/well, Vector Laboratories, Inc., Burlingame, CA, USA) was added for 1 hour at room temperature. This was washed well, and then streptavidin-HRP (1 : 5,000, 0.1 ml/well, Southern Biotechnology Associates, Bir-

**Table 2** Correlation coefficient of IgE-binding rate ( $n = 50$ )

	Red salmon	Kokanee	Silver salmon	Rainbow trout	Jack mackerel
Kokanee	0.885				
Silver salmon	0.851	0.882			
Rainbow trout	0.706	0.746	0.849		
Jack mackerel	0.723	0.824	0.700	0.685	
Bluefin tuna	0.182	0.281	0.198	0.101	0.332

mingham, AL, USA) was added for 1 hour at room temperature. This was washed well, followed by incubation with 0.1 ml/well of TMB (ICN Biomedicals, Aurora, OH, USA) for 30 minutes under a light shield. The reaction was stopped by adding 0.1 ml/well of 1 N HCl, and measured with LSPLATE manager 2001 (Wako, Osaka, Japan).

Using patients allergic to non-fish substances as controls ( $n = 30$ ), the measured ELISA values of IgE antibodies against the fish antigens were compared with the mean control ELISA values. The values were divided by the SD of the control, and presented as Z scores. The IgE Z score against each fish meat was calculated using the equation below:

$$Z \text{ score against fish meat} = (\text{measured value of patients allergic to fish} - \text{mean measured value for control}) / \text{SD of the measured values of the control}$$

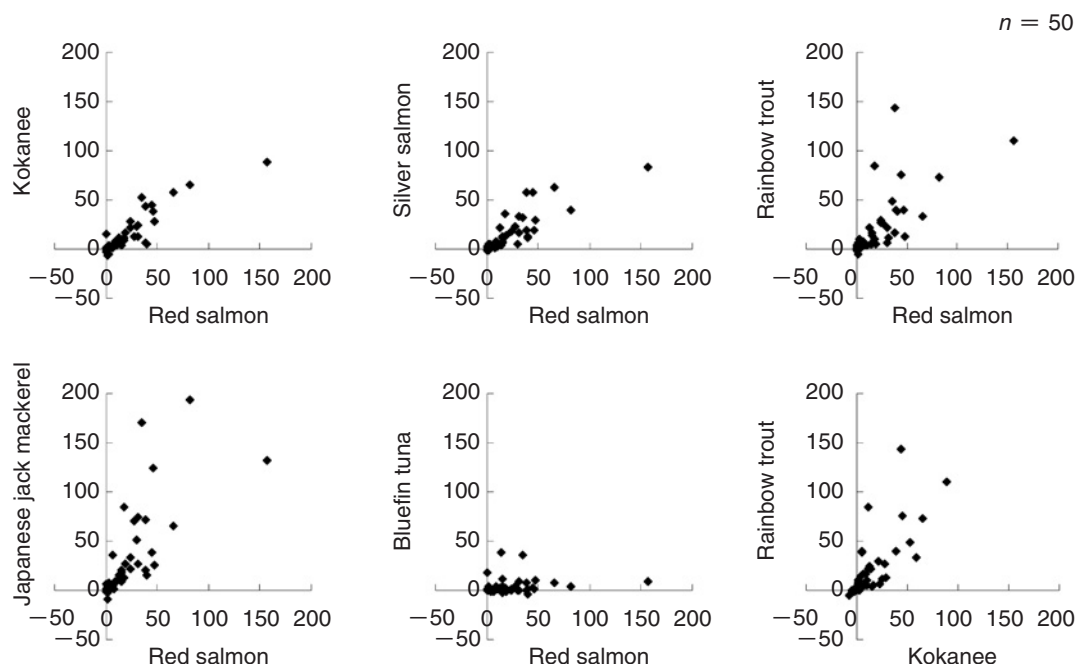
The IgE antibody titers against the fish species were compared with regard to the Z score.

## ELISA INHIBITION

Before addition to an ELISA plate precoated with extracts of red salmon or kokanee, serum samples were pre-incubated with solutions containing extracts (red salmon, kokanee, silver salmon, rainbow trout, Japanese jack mackerel, and bluefin tuna) at 4 different concentrations (0, 0.001, 0.01, 0.1, and 1.0 mg/ml) as inhibitors at room temperature. The subsequent procedure was the same as that for ELISA described above. To compare the rates of inhibition of IgE binding to the red salmon and kokanee antigens on the addition of each fish antigen, sera of 6 patients with salmon allergy (patients 1–6 in Table 1) were pooled and used.

## TRANSFER AND IMMUNOBLOTTING

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 4–20% Trisglycine precast gel (Tefco Corporation, Machida, Japan) according to the Laemmli method under reducing conditions. Each sample was separated at 120 V for 2 hours. After electrophoresis, proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA), as previously reported.<sup>8</sup> For the detection of IgE bound to the protein bands, the blot was reacted with biotin-labeled anti-human IgE antibody used in ELISA (1 : 1,000) for 3 hours, washed,



**Fig. 1** Correlation of IgE binding to red salmon and kokanee antigens with that to other fish species antigens ( $n = 50$ ). The IgE binding capacity of sera of 50 patients allergic to fish with each fish antigen was presented as the Z score and compared.

and reacted with streptavidin-HRP (1 : 5,000) for 1 hour. After being washed, the blot was subjected to color development using the ECL™ Western Blotting Analysis System (GE Healthcare UK, Little Chalfont, UK).

Sera of the 6 patients allergic to salmon and 2 fish-allergic patients avoiding salmon ingestion because of a high salmon-specific IgE level, 8 sera in total (patients 1–8 in Table 1), were investigated. For the control, sera from patients allergic to non-fish substances were used.

### IMMUNOBLOT INHIBITION

After each protein was transferred to the Immobilon-P membrane, pooled sera showing a high IgE antibody titer to multiple fish antigens preincubated with each extracted solution (0, 1, and 100  $\mu$ g) as inhibitors were added. The detection of bound IgE was the same as described above.

The pooled serum of the 8 patients applied to immunoblotting was investigated.

## RESULTS

### COMPARISON OF RATES OF IgE BINDING TO THE FISH ANTIGENS (FIG. 1, TABLE 2)

On comparison of IgE binding shown in Table 2, the correlation between red salmon and kokanee, belonging to the same species, was the highest ( $r = 0.885$ ), and that between red salmon and bluefin tuna, belonging to different orders, was the lowest ( $r = 0.182$ ). As shown in Figure 1, the line representing the corre-

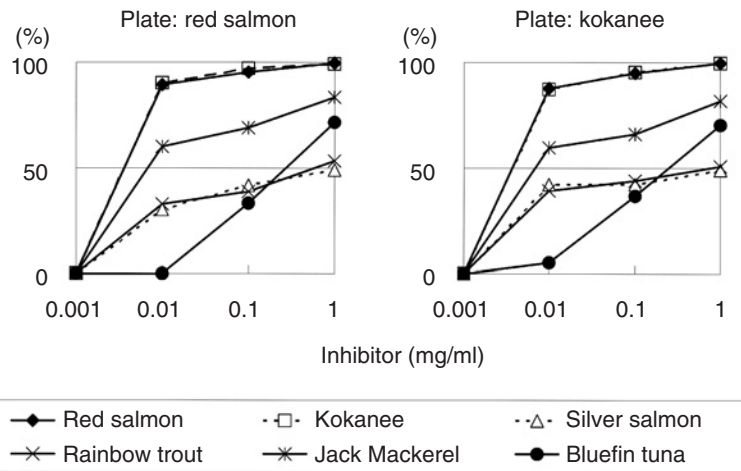
lation between red salmon and kokanee was slightly sloped toward the red salmon side.

### COMPETITION FOR IgE (FIG. 2)

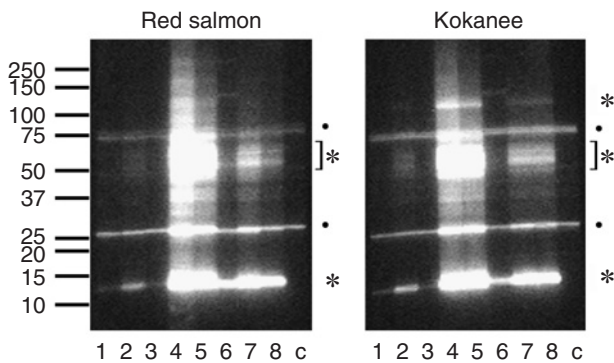
In the ELISA inhibition test, the rates of IgE-binding inhibition caused by different species in the same genus, silver salmon and rainbow trout, were about 50%, and those by species belonging to a different order, Japanese jack mackerel and bluefin tuna, were 83 and 70%, respectively. In contrast, between red salmon and kokanee, IgE binding was 100% inhibited by the counterpart antigen.

### IMMUNOBLOT AND IMMUNOBLOT INHIBITION (FIG. 3, 4)

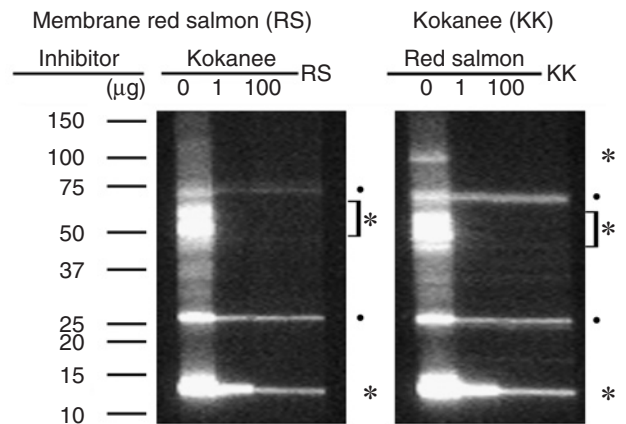
On immunoblotting, there was no marked difference in the staining pattern between the two antigens; however, a 94-kDa band was only stained in kokanee in 4 patients (patients 4, 5, 7, and 8 in Fig. 3). Staining of 13-kDa bands of red salmon and kokanee was positive in all sera excluding the control serum. This protein band was confirmed as parvalbumin using the mouse monoclonal antiparvalbumin antibody clone PARV-19 (1 : 3,000; Sigma-Aldrich, St Louis, MO, USA, data not shown). Staining of 44-54-kDa protein bands of the two habitat types was equivalent in 5 patients (patients 2, 4, 5, 7, and 8). All IgE binding to 13- and 44-54-kDa proteins of red salmon and kokanee and the 94-kDa protein of kokanee was inhibited by the addition of the antigen of the other species in a concentration-dependent manner (Fig. 4). Twenty-



**Fig. 2** ELISA inhibition with red salmon and kokanee antigens. Sera of 6 patients allergic to salmon were pooled and used. The rate of inhibition by other fish species antigens were 48-83%, but red salmon and kokanee antigens inhibited IgE binding to the counterpart by 100%.



**Fig. 3** Immunoblotting of red salmon and kokanee antigens. IgE of most patients' sera bound to the 13-kDa band common in red salmon and kokanee. Lanes 1-6: sera of 6 patients allergic to salmon, lanes 7 and 8: sera of 2 patients with a high CAP level, c: serum of patients allergic to non-fish substances. \*: Specific binding, •: nonspecific binding.



**Fig. 4** Immunoblot inhibition between red salmon and kokanee. The pooled serum used in Fig.3 was subjected to the inhibition test between red salmon and kokanee. The specific IgE binding to 13- and 44-54-kDa proteins common in red salmon and kokanee and 94-kDa protein in kokanee was inhibited by the addition of the counterpart antigen at a low level. \*: Specific binding, •: nonspecific binding.

five- and 75-kDa protein bands were reacted with the control serum, and the binding was not inhibited by the addition of the identical antigen, suggesting that the binding was nonspecific.

### DISCUSSION

The Allergen Food Sanitation Law includes salmon in specified food ingredients, and the labeling of foods containing salmon is recommended. However, such labeling is not obligatory for trout, despite salmon and trout belonging to the same species living in different habitats, which may have been due to the numbers of reported cases, and not based on allergenicity. In our previous study on 38 patients with fish al-

lergy, 12 were allergic to salmon, but only one, a 4-year-old infant allergic to salmon, was allergic to trout.<sup>9</sup> We in Japan have very few occasions to eat trout compared to salmon, which may be a reason for the small number of reported cases of trout allergy. However, if their allergenicity is identical, those allergic to salmon may develop allergy when they eat non-labeled foods containing trout, which should be prevented. Thus, we investigated the difference in antigenicity between salmon and trout using sera of patients with salmon allergy.

Using sera of patients allergic to fish, the correla-

tion of IgE binding to red salmon and kokanee with those to other fish species was investigated. The correlation was highest between the same species, and 100% inhibition was achieved on the ELISA inhibition test, suggesting that the antigenicity of red salmon and kokanee was almost the same. A high-level correlation of IgE binding with a different species, Japanese jack mackerel, was also noted, but the ELISA inhibition rate did not reach 100% even at the highest salmon antigen concentration, suggesting the presence of a specific allergen shared by red salmon, kokanee, and Japanese jack mackerel, other than the major antigens. No correlation with bluefin tuna was noted in IgE binding, suggesting that tuna show little common antigenicity, but the inhibition reached nearly 70% with an increase in the concentration, indicating that protein with a common antigenicity is present in tuna, although the content is low.

On immunoblotting, a strongly stained 94-kDa protein was detected in kokanee, and this may have emerged due to differences in the habitat, but IgE binding to kokanee was inhibited by red salmon from a low concentration on the ELISA inhibition test, and, consistently, IgE binding to the 94-kDa protein was inhibited by red salmon at a low concentration on immunoblot inhibition. Based on these findings, it is unlikely that the variation in the expression level of this protein leads to a difference in allergenicity between the habitat types.

The allergenicity of red salmon and kokanee may be equivalent, and the labeling of foods containing trout is also recommended.

### ACKNOWLEDGEMENTS

This study was supported by Health and Labour Sciences Research Grants for Research on Food Safety

from the Ministry of Health, Labour, and Welfare of Japan.

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