Differential patterns of fish sensitization in Asian populations: implication for precision diagnosis

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Abstract

Background The current diagnostics of fish allergy lack sufficient accuracy such that more reliable tests such as component-resolved diagnosis (CRD) are urgently needed. This study aimed at identifying fish allergens of salmon and grass carp and evaluating the sensitization pattern towards the identified allergens in fish allergic subjects from two distinct populations in Asia. **Methods** One hundred and three fish allergic subjects were recruited from Hong Kong (67 subjects) and Japan (46 subjects). Western blot and mass spectrometry were used to identify allergens from salmon and grass carp. Fish allergens were purified and tested against 96 sera on ELISA to analyze patients' sensitization pattern. The protein profiles of salmon meat prepared under different cooking methods until core temperature reached 80°C were evaluated by SDS-PAGE and mass spectrometry. **Results** Three common allergens between salmon and grass carp, namely enolase, glycerldehyde-3-phosphate dehydrogenase (GAPDH) and parvalbumin, and two salmon-specific allergens collagen and aldolase were identified. Parvalbumin was the major allergen for both fishes showing an overall sensitization rate of 74.7%, followed by collagen (38.9%), aldolase (38.5%) and enolase (17.8%). Japanese subjects showed more diverse allergen sensitization pattern and more frequent IgE-binding to heat-labile salmon allergens. Compared with steaming and boiling, cooking by baking and frying retained more fish proteins inclusive of heat-labile allergens. **Conclusions** Fish allergic patients from different Asian populations show varying fish allergen sensitization profiles. The relevant extracts and components for diagnosis are population-dependent but parvalbumin and collagen are important biomarkers. Cooking methods modify allergen composition of salmon and appear to influence patients' allergic manifestations.

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Key words

Fish allergy; component-resolved diagnosis, parvalbumin; enolase; aldolase; collagen

Highlights

Parvalbumin and collagen are major fish allergens important for diagnosis. Grass carp parvalbumin presents higher IgE binding affinity than salmon parvalbumin but the relevant extract and component for diagnosis differ among populations that is partially dependent on dietary habits.

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Abstract

Background

The current diagnostics of fish allergy lack sufficient accuracy such that more reliable tests such as component-resolved diagnosis (CRD) are urgently needed. This study aimed at identifying fish allergens of salmon and grass carp and evaluating the sensitization pattern towards the identified allergens in fish allergic subjects from two distinct populations in Asia.

Methods

One hundred and three fish allergic subjects were recruited from Hong Kong (67 subjects) and Japan (46 subjects). Western blot and mass spectrometry were used to identify allergens from salmon and grass carp. Fish allergens were purified and tested against 96 sera on ELISA to analyze patients' sensitization pattern. The protein profiles of salmon meat prepared under different cooking methods until core temperature reached 80°C were evaluated by SDS-PAGE and mass spectrometry.

Results

Three common allergens between salmon and grass carp, namely enolase, glycerldehyde-3-phosphate dehydrogenase (GAPDH) and parvalbumin, and two salmon-specific allergens collagen and aldolase were identified. Parvalbumin was the major allergen for both fishes showing an overall sensitization rate of 74.7%, followed by collagen (38.9%), aldolase (38.5%) and enolase (17.8%). Japanese subjects showed more diverse allergen sensitization pattern and more frequent IgE-binding to heat-labile salmon allergens. Compared with steaming and boiling, cooking by baking and frying retained more fish proteins inclusive of heat-labile allergens.

Conclusions

Fish allergic patients from different Asian populations show varying fish allergen sensitization profiles. The relevant extracts and components for diagnosis are population-dependent but parvalbumin and collagen are important biomarkers. Cooking methods modify allergen composition of salmon and appear to influence patients' allergic manifestations.

Background

Fish is one of the most frequent causes of food-induced allergy alongside cow's milk, hen's egg, peanut, tree nut and shellfish, and has been included in the European mandatory labeling legislation together with 13 other allergens[1]. Fish is a valuable source of healthy nutrients such as omega-3 fatty acids with increasing global demand. Fish allergy affects 0.1-0.4% of the world's population[2,3], has an early onset and tends to persist throughout life[4]. Allergic reactions are mainly provoked through ingestion but also by skin contact or by inhalation of fish steam during fish processing. The allergic reactions triggered by fish can be life-threatening and even fatal resulting in a heavy socioeconomic burden and negative impact on quality of life[5,6]

β-parvalbumin represents the major fish allergen accounting for up to 90% of sensitization and has been described and characterized in a wide range of fish species[7-10]. Parvalbumins are 8-12kDa calcium-binding muscle proteins with extreme thermal stability and shared 55-95% amino acid sequence homology among various fish species. This might account for the cross-reactivity among fish species but case reports have shown mono-sensitization and clinical allergy to single fish species[11,12]. Alongside parvalbumin, collagen, aldolase, enolase, pyruvate kinase, glucose-6-phosphate isomerase (G-6-PI), creatine kinase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), triosephosphate isomerase (TPI) and tropomyosin are other registered fish allergens in the World Health Organization and International Union of Immunological Societies (WHO/IUIS) database[13]. Among these allergens, collagen, aldolase and enolase are more well-characterized. Collagen is another heat stable protein, with reports showing anaphylaxis provoked by collagen and illustrating IgE binding to collagen only but not parvalbumin[14,15]. Enolase and aldolase, on the other hand, are identified as major heat-labile fish allergens in cod, salmon and tuna[16].

The current diagnostic workup of fish allergy relies on skin prick test (SPT), measurement of specific IgE and when indicated, an oral food challenge[17]. Although a number of fish extracts are available on the ImmunoCAP platform, only parvalbumins from cod and common carp are available for IgE testing and other identified fish allergens have not been included for diagnosis due to the insufficient understanding of their allergenic properties and clinical relevance especially in the Asian populations with high fish consumption. Yet a considerable number of studies have illustrated the low specificity of SPT and specific IgE measurement while safety has always been a concern in oral food challenges[18]. Together with the reports of monosensitization to some fish species and parvalbumin-independent IgE sensitization[19], a testing panel inclusive of the relevant fish allergens for precision component-resolve diagnosis (CRD) will be of great value.

This study identified fish allergens of salmon (*Salmo salar*) and grass carp (*Ctenopharyngodon idella*), which are among the most commonly farmed and consumed seawater and freshwater fishes in Asia. We compared the sensitization pattern towards the four major allergens, β-parvalbumin, enolase, aldolase and collagen, from both salmon and grass carp in fish allergic subjects from Hong Kong and Japan. Based on our results, we further characterized the allergen profiles in salmon meat cooked at the same core temperature

by different methods to dissect the link between dietary preferences and allergen sensitization profiles.

Methods

Recruitment of fish allergic subjects

This study recruited subjects aged 1-18 years with reported history of IgE-mediated fish allergy from six regional hospitals in Hong Kong (HK; Prince of Wales Hospital, Queen Mary Hospital, Queen Elizabeth Hospital, Yan Chai Hospital, Princess Margaret Hospital and United Christian Hospital) and Sagamihara National Hospital in Kanagawa, Japan. The inclusion criteria include (1) subjects with convincing history of immediate fish allergy as defined by immediate allergic symptoms within 2 hours following fish ingestion within two years before recruitment and clinically diagnosed by an allergy specialist; and (2) subjects with evidence of IgE sensitization to fish by blood sIgE measurement against cod (f3), salmon (f41), tuna (f40) and/or grass carp (experimental ImmunoCAP developed in collaboration with Thermo Fisher) and/or SPT with commercial fish mix extract (ALK-Abelló). Exclusion criteria are subjects receiving maintenance immunosuppressive treatments that cannot be stopped for four weeks prior to study and who receive intravenous immunoglobulin treatment within three months. Severity of allergic symptoms to fish was calculated based on the Ordinal Food Allergy Severity Score (oFASS)[20]. Briefly, Grade 1 includes reactions restricted to oral cavity. Grade 2 to 5 may include oral symptoms but with other target organs affected, by which grades 2 and 3 reactions involve skin, eye/nose and/or digestive system. Involvement of larynx and/or bronchi reactions are graded 4 while grade 5 would involve cardiovascular and/or nervous systems. Participants and/or their legal guardians gave written informed consent. Ethical approvals were obtained from Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee (2019.034 & 2019.612) and the Sagamihara National Hospital Ethics Committee (2019-003).

Preparation of raw and cooked fish extracts

Frozen salmon (*Salmo salar*) and fresh grass carp (*Ctenopharyngodon idella*) were purchased from local supermarket. Two milligrams of raw fish meat were manually homogenized in 8mL ice-cold phosphate-buffered saline (PBS) until a smooth paste was achieved. Protein was then extracted overnight at 4°C with constant stirring. The protein extract was centrifuged and supernatant was filter-sterilized through a 0.22 μm polyethersulfone membrane. The fish protein extracts were stored at -20°C until use.

Several cooking methods including steaming (at 100°C), boiling (at 100°C), baking (at 150°C), lightwave cooking (at 180°C), frying (at 180°C) and air-frying (at 200°C) were used to prepare cooked salmon and grass carp for comparison of its protein profile. Raw salmon meat, weighed 20 g per pack, was cooked until the core temperature of salmon meat reached 80°C as measured using digital thermometer. After cooking, all samples were weighed and 1/10 of the raw and cooked fish samples were extracted in 8mL PBS as described above. All protein extracts were stored at -20°C until use.

SDS-PAGE and western blotting

Four microliters of raw or cooked fish extracts were separated on a 13.5% SDS-polyacrylamide gel according to their molecular weights using a Mini PROTEAN SDS-PAGE system (Bio-Rad). Protein bands were stained with SimplyBlue SafeStain (Thermo Fisher). Western blotting was performed using the surf-blot antibody system (Idea Scientific). One hundred fifty microliters of raw fish extracts were resolved in 13.5% SDS-PAGE and transferred to PVDF membranes (Bio-Rad) with the Trans-Blot Turbo system (Bio-Rad). Non-specific binding of the membranes was blocked with 5% non-fat dry milk in 0.05% Tween-20 in Tris-buffered saline (TBS-T, blocking solution) for 1 hour at room temperature. Patient sera diluted at 1:10, 1:20 or 1:30 (adjusted for sIgE levels) and HRP-conjugated anti-human IgE (Southern Biotech) diluted at 1:2000 in blocking solution were used for probing IgE-binding proteins. IgE-binding fish proteins were visualized by incubating the membranes with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher), and the signal was acquired with the ChemiDoc MP Imaging System (Bio-Rad).

Mass spectrometry

The IgE-reactive bands of raw fish extracts were excised from the SimplyBlue SafeStain-stained SDS-PAGE gel, destained and digested with trypsin following our laboratory's established protocol[21]. The tryptic digests were analyzed on the UltrafleXtreme MALDI-ToF/ToF System (Bruker) for obtaining the peptide mass fingerprints. Searches against the entire protein sequence database (NCBI and UniProt) using the MASCOT search engine were then performed for protein identification.

Preparation of recombinant fish allergens

The protein sequences of enolase (Sal s 2; Uniprot ID: B5DGQ7) and aldolase (Sal s 3; Uniprot ID: B5DGM7) of *Salmo salar were*downloaded from the Uniprot database and reverse translated by MEGA 11.0. Enolase (Accession no: GBKA01005072.1) and aldolase (Accession no: GEUQ01036650.1)

sequences of *Ctenopharyngodon idella* were retrieved from the transcriptome shotgun assembly deposited at GenBank (Accession no: PRJNA325430) with tBLASTn search using Sal s 2 and Sal s 3 as the search templates. The nucleotide sequences encoding the full-length allergens were commercially synthesized and cloned into the His-tag expression vector pET30(a)+. His-tagged recombinant allergens were then expressed in *Escherichia coli* [BL21 (DE3)] by culturing in MagicMedia (Invitrogen). Allergens were then purified using the HisPur cobalt spin columns (Thermo Scientific) as per the manufacturer's instructions. For parvalbumin purification, raw extracts extracted in Tris-HCl (pH 8.0) were heated at 95°C – 100°C for 15 mins, followed by centrifugation to remove precipitated heat labile proteins. Supernatant containing soluble proteins were loaded into a MonoQ anion exchange column (Cytiva) connected to a NGC 10 FPLC system (Bio-Rad). Fractions containing pure parvalbumin were separated by a gradient elution of 0 – 1M NaCl in Tris-HCl (pH 8.0) and buffer exchanged into PBS for downstream applications with an Amicon centrifugal filter unit. Acid-soluble collagen was extracted from the muscle of salmon and grass carp as described previously[22]. Briefly, diced fish meat was resuspended in 10 volumes of 0.1mol NaOH overnight at 4°C with gentle agitation to remove non-collagenous proteins. Upon centrifugation at 3000 rpm for 10 mins and pH neutralization with ultrapure water, fish meat was soaked in 10% butanol and incubated for 24 h at 4°C with gentle agitation. After centrifugation and washing in ultrapure water, samples were resuspended in 10 volumes of 0.5M acetic acid for 4 days at 4°C with gentle agitation. The supernatant were then salted-out by adding NaCl to a final concentration of 2M. The resultant precipitate was dissolved in 0.5M acetic acid and dialyzed against distilled water.

The concentration and purity of purified fish allergens were determined on the NanoDrop OneC spectrophotometer (Thermo Scientific) and SDS-PAGE, respectively. Protein identity of the allergens was also confirmed by mass spectrometry. All purified allergens were stored at -20°C until use.

Enzyme-linked immunosorbent assay (ELISA)

Purified fish allergens diluted in coating buffer (100 mM Na_2CO_3. 100 mM $NaHCO_3$, pH 9.6) were coated onto MaxiSorp microtiter plates (Nunc) and incubated overnight at 4°C. After washing the plates with 0.05% Tween-20/PBS (PBS-T) and blocking the plates with 5% fetal bovine serum (Gibco) diluted in PBS (blocking buffer) at room temperature for 2 hours, serum samples diluted at 1:10 in blocking buffer were added for overnight incubation at 4°C. Intensity of IgE binding was detected by incubating the plates with biotinylated anti-human IgE antibodies (1:1000 dilution, Vector Labs), HRP avidin D (1:1000 dilution, Vector Labs) and TMB substrate (BD Biosciences). Upon terminating the reaction with 0.1M sulfuric acid, the optical density (OD) at 450 nm was measured using a microplate reader (BioTek). Results were considered positive only if the OD is 3-fold higher than the mean of non-atopic negative controls.

Statistical analysis

GraphPad Prism (version 8.0, GraphPad Software) was used for graphical presentation and statistical analyses. Quantitative variables were described by median, range and interquartile range whereas qualitative variables were described by absolute frequencies and percentages. $P < .05$ was considered as statistical significance.

Results

Demographics of fish allergic participants

Sixty-seven and forty-six subjects fulfilling the inclusion criteria were recruited from the pediatric allergy clinics in HK and the Sagamihara National Hospital in Japan respectively (Table 1, Supplementary Tables E1, E2). The median (range) age of fish allergic subjects at evaluation was 6 (1-16) years and 10 (5-18) years for these two populations, respectively. The most common clinical features shared among the two populations were itchy mouth/throat (61.1%, 69/113) and urticaria (51.3%, 58/113) while subjects from HK also commonly displayed angioedema (59.7%, 40/67) upon fish ingestion (Supplementary Tables E1, E2). The two populations did not differ in terms of the symptom severity as reflected from oFASS ($p = 0.5251$). SPT to fish mix extract was only available for subjects recruited in HK with median (range) SPT wheal size of 5 (0-11) mm, and 48 subjects (84.2%) had positive SPT (cutoff at 3mm). The sIgE results for cod, tuna, salmon and grass carp are summarized in Table 1 and detailed in supplementary Tables E1 and E2. No difference was detected in sIgE levels to these fish species between the two populations, except for grass carp that HK subjects had significantly higher sIgE level comparing to Japanese subjects (Mann-Whitney test, $p = 0.0020$). Of note, 65/67 (97.0%) and 34/37 (91.8%) subjects from HK and Japan had positive sIgE to salmon (cutoff at 0.35kUA/L), of whom 72.3% and 70.6% had high sIgE level and above class III-VI, respectively. As for grass carp, 63/67 (95.5%) and 25/30 (83.3%) subjects from HK and Japan had positive sIgE, of whom 87.3% and 56.0% had high sIgE level and above class III-VI, respectively. An overall positive correlation between sIgE levels to salmon and grass carp was observed for both populations, by which a stronger correlation was found among the Japanese subjects ($r = 0.69$, $p = 0.0002$) than HK subjects ($r = 0.33$, $p = 0.0061$) based on Fisher r-to-z transformation ($z = 2.01$, $p = 0.04$). Sera of Japanese subjects yielded high sIgE response to salmon with an average grass carp/salmon sIgE ratio of 0.70 (0.02-1.93) while sera of HK subjects yielded higher sIgE response to grass carp with average ratio of 3.39 (0.02-20.8) (Supplementary Figure 1). Pairwise comparison between grass carp- and salmon-specific IgE levels also showed statistically higher grass carp-specific IgE level among HK subjects but statistically higher salmon-specific IgE level among Japanese subjects (Wilcoxon matched-pairs signed rank test, $p = 0.0008$ and 0.0315 respectively).

Identification of IgE-binding proteins in salmon and grass carp

The extracted salmon and grass carp proteins were analyzed on SDS-PAGE (Figure 1A). Sera from 45 subjects (30 samples from HK and 15 samples from Japan; marked with asterisks* in Supplementary Tables E1 & E2) and non-atopic controls ($n = 2$) were used to investigate IgE-binding to salmon and grass carp proteins by western blotting (Figure 1B & C). No IgE binding was observed for control sera. Serum samples from Japanese fish allergic subjects showed more diverse IgE binding compared to those from HK, including to grass carp extract despite of the lower IgE level measured on ImmunoCAP. For both populations, the 11kDa band in salmon and 10kDa band in grass carp represented the major IgE-binding protein. In HK fish allergic subjects, 18/30 (60.0%) and 16/30 (53.3%) showed IgE binding to this 10kDa protein in grass carp and salmon respectively, while 10/15 (66.7%) subjects in Japan had a positive reaction to the 10kDa proteins in salmon and grass carp extracts. Six (40.0%) Japanese subjects reacted to proteins with molecular sizes 130 kDa and 58 kDa.

Protein bands that showed IgE reactivity to at least three serum samples were then excised from SDS-PAGE for allergen identification on mass spectrometry. These included the 130kDa, 43kDa, 34kDa, 25kDa and 11kDa bands from salmon extract, as well as the 43kDa, 34kDa and 10kDa bands from grass carp extract. For salmon extract, the 130kDa protein was identified as α-collagen, 58kDa band as β-enolase, 32kDa band as aldolase A, 25kDa band as GAPDH and the 11kDa band as β-parvalbumin (Supplementary Table E3). Similarly, the 58kDa, 36kDa and 10kDa bands from grass carp extract corresponded to β-enolase, GAPDH and β-parvalbumin, respectively.

Differential sensitization patterns to fish allergens between populations

To understand the IgE sensitization pattern, we purified the well-characterized fish allergens including collagen, β-enolase, aldolase and β-parvalbumin from both salmon and grass carp. All allergens had >90% purity as validated by SDS-PAGE (Figures 2A & B). IgE binding to these allergens coated onto ELISA plates was

evaluated using 46 serum samples from each of HK and Japan subjects. The respective sensitization rates are summarized in Table 2.

For both fishes, parvalbumin was the major allergen that showed an overall sensitization rate of 74.7% (71/95) in fish allergic subjects (Figures 2C-F) while enolase is the least common allergen (17.8% sensitization). Interestingly, the sensitization rates to salmon enolase and aldolase, the heat-labile allergens, were higher among subjects from Japan (15.2% and 23.9% respectively) than HK (4.3% and 15.2% respectively). On the other hand, the sensitization rates to salmon and grass carp collagen were higher among subjects from HK (43.5% and 30.4% respectively) than Japan (30.4% and 17.8% respectively). No remarkable difference in parvalbumin sensitization rate could be found between the two study cohorts. Mono-sensitization to salmon or grass carp parvalbumin was the most common in both populations (28.3% to 37.0%). Only 7/96 subjects did not show IgE binding to parvalbumin but other fish allergen, and collagen represented the leading sensitizer in these patients (6/7 patients reacted to collagen). IgE-binding to both parvalbumin and collagen was the most common form of co-sensitization (13.0% to 23.9%).

For both populations, the sensitization rates to grass carp parvalbumin (68.9% for Japan and 78.3% for HK) were higher than that to salmon parvalbumin (63.0% and 56.4%) (Table 2). The IgE avidity (i.e. OD450nm) of grass carp parvalbumin was also higher than salmon parvalbumin as noted in the heatmap (Figure 2). Despite of the significantly higher salmon-sIgE level than grass carp-sIgE level, IgE avidity to grass carp parvalbumin was significantly higher than that to salmon parvalbumin among the Japanese fish allergic subjects (Wilcoxon matched-pairs signed rank test, $p < 0.0001$). However, no difference could be detected for grass carp parvalbumin binding intensity between the two populations regardless of the significantly higher grass carp-sIgE level for the HK subjects. In contrast, both the sensitization rates and IgE avidity of salmon collagen were higher than those of grass carp collagens.

Analyses of specific sensitization were summarized in Table 3. Out of the 36 HK subjects with positive grass carp parvalbumin-specific IgE, ten were sensitized to grass carp parvalbumin only giving a monosensitization rate to grass carp at 27.8%. On the contrary, none with positive salmon parvalbumin-specific IgE ($n = 26$) showed IgE binding to salmon parvalbumin only. When taking both populations into consideration, the overall rate of mono-sensitization to grass carp parvalbumin (20.9%) was higher than that to salmon parvalbumin (5.5%). On the contrary, mono-sensitization to salmon collagen (44.1%) was remarkably more common than to grass carp collagen (9.1%). The two fish species did not differ with respect to sensitization rate, IgE avidity and rate of mono-sensitization to enolase and aldolase.

Relationship between dietary habit and sensitization patterns

Noting that Japanese subjects had higher salmon-sIgE level and a more diverse sensitization profile to salmon fish allergens, we sought to investigate if it might be related to different fish cooking preferences. Salmon meat was cooked using different methods until its core temperature reached 80°C. Equal volumes of the extracted fish proteins were then resolved on SDS-PAGE. A significant portion of proteins were lost upon cooking (Figure 3). Interestingly, retention of salmon proteins was the highest after baking followed by frying and airfry and the lowest after steaming, boiling and lightwave cooking. When salmon extracts were analyzed by mass spectrometry, protein bands at 130kDa, 37kDa and 8kDa were retained following all cooking methods, and these bands were identified as α-collagen, GAPDH and parvalbumin, respectively (Supplementary Table E4). On the other hand, protein bands at 55kDa, 43kDa, 40kDa, 38kDa, and 34kDa were visualized most abundantly in baked salmon extract, less after fry/airfry and absent in steamed or boiled extracts. By mass spectrometry analysis, these proteins corresponded to serum albumin, β-enolase, actin, aldolase A and tropomyosin, respectively.

Discussion

In this study, we delineated the allergen profiles of salmon and grass carp using sera from two Asian populations. With the purified allergens from both fish species, our study illustrates parvalbumin and collagen as important biomarkers for fish allergy diagnosis. Our results also indicate a wider allergen spectrum including sensitization to heat labile allergens, as well as stronger sensitization to salmon than grass carp among the

Japanese fish allergic subjects where grass carp is unavailable. Such differences highlights that the relevant fish species as diagnostic marker and allergen sensitization are distinct in different populations due to diverse dietary exposures and habits.

Ruethers et al. reported recently 10 allergens from catfish and 6 allergens from salmon[13]. Specifically, tropomyosin and TPI identified as fish allergens in their study are known shrimp allergens[23]. Despite positive shrimp-sIgE by ImmunoCAP in six HK subjects, none showed reaction to fish tropomyosin or TPI in our western blot. Our subjects also showed no detectable IgE binding to allergens including creatine kinase, pyruvate kinase, L-lactate DH and G-6-PI that were previously reported as catfish allergens. This might partly indicate that these are only catfish-specific allergens and/or the reactions are population-specific. Apart from the well-defined fish allergens (i.e. parvalbumin, enolase, aldolase and collagen), we consistently identified GAPDH as a putative fish allergen of both salmon and grass carp[12]. Such findings supported GAPDH to be an important fish allergen.

Our present report further focuses on studying the more well-defined fish allergens in terms of their sensitization rates. In agreement with most studies that parvalbumin accounted for 70-95% of allergic reactions[9,24], parvalbumin is the major allergen in grass carp and salmon with 74% sensitization rate determined by western blotting and ELISA. Collagen is the second commonest fish allergen with 37.5% of subjects being sensitized. The reported rates of IgE binding to collagen vary in different populations. For instance, Kalic et al. analyzed samples from 101 fish allergic patients in Australia and showed that 21% of subjects displayed IgE binding to collagen from tuna, barramundi and/or salmon[14]. Kuehn et al. reported 19.3% sensitization rate to gelatin of cod, salmon and/or tuna in 62 fish allergic patients[16]. With only 21 patients, Shimojo et al. reported a higher rate of 52.4% sensitization to collagen than to parvalbumin[25] while Kobayashi et al. reported also 50% of sensitization rate to mackerel collagen[26]. IgE binding to collagen was also more frequent in patients with more severe fish allergic symptoms than those with mild-moderate responses while independent reports have shown anaphylaxis provoked by gelatin/collagen[15,27]. On the other hand, Kalic et al. reported that 8/21 of collagen-IgE positive patients showed IgE binding to collagen only but not parvalbumin[14]. Comparatively, seven of our 37 collagen-IgE positive subjects demonstrated moderate IgE binding to collagen but absence of parvalbumin-specific IgE. Taken together, collagen is a prevalent allergen in Asian populations. Fish allergic patients can have IgE binding to collagen only, supporting that testing for collagen should be included in the diagnostic workup for fish allergy.

To our knowledge, only one study with 62 fish allergic subjects reported the sensitization rates of 62.9% and 50.0% to enolase and aldolase, respectively[16]. Another smaller study by Ruethers et al. showed that half of their 16 Australian patients showed weak to moderate IgE reactivity to enolase in commercial tuna and salmon SPT extracts[28]. In our cohort, IgE binding to aldolase was detected in 38.5% of subjects while that to enolase was present in 17.8% of subjects. We did not anticipate a major difference in the reactivity of natural and recombinant aldolase and enolase as the reason for lower sensitization as the recombinant enzymes were confirmed to be reactive consistently by ELISA (data not shown) and western blots. Such discrepancy might, on the other hand, be explained by differences in the ethnicity and age of the studied populations: this study only recruited children below 18 years old while many subjects studied by Kuehn et al. were young adults (mean age 28.7, range 13-62 years). Among seven subjects without parvalbumin-specific IgE in our cohort, only one Japanese subject showed IgE binding to salmon and grass carp aldolase. Testing of enolase and aldolase thus do not substantially improve the diagnostic yield for fish allergy in Asians while detecting IgE against parvalbumin and/or collagen might be sufficient to identify patients with fish allergy.

This study found minimal binding of sera from HK subjects to allergens other than parvalbumin by western blotting. A possible explanation was the use of different serum samples for ELISA and western blot, different sensitivity of the assays, intrinsically low abundance of these allergens in fish extracts and the insoluble nature of collagen in aqueous solution[29]. Nevertheless, both ELISA and western blot assays robustly indicated a wider spectrum of allergen sensitization in Japanese than HK subjects. Japanese commonly consume raw fishes, but this is a less likely reason for this observation given the age-range of our recruited patients; the consumption of raw fish might not be a major attribute to the sensitization to a wider spectrum of allergen

including the heat labile allergens. Our present study showed, for the first time, differences in fish protein and allergen profiles in relation to cooking methods at the same core temperature. Despite higher cooking temperature with frying/baking compared with steaming/boiling, frying/baking retained more heat liable allergens in salmon meat. Steaming and boiling to make fish congee and soup are the preferred methods of fish cooking in Hong Kong while baking and frying of fish are more popular in Japan. For instance, 73.8% of the interviewed Japanese workers consumed fish by broiling (baking) comparing to only 14.1% for raw fish and 10% by stewing[30]. Although we are aware that our present study is limited by the lack of direct investigation into the dietary habits of our studied population, our results support the importance of dietary exposures, consumption and cooking preferences in determining fish allergen sensitization in particular when the two populations did not differ in terms of symptom severity. This factor should be considered during the selection of allergens for allergy testing and the interpretation of CRD results.

In the context of fish exposure, Japanese patients should not have eaten grass carp due to the unavailability of this freshwater fish. Thus, IgE binding to grass carp allergens in Japanese patients should be a result of cross-reactivity that can be partly reflected from the stronger positive correlation between grass carp and salmon sIgE levels than HK subjects whom could be independently sensitized to the two fish species. However, we observed that more Japanese patients reacted to grass carp parvalbumin with higher OD values in ELISA, although salmon-sIgE level was higher than grass carp-sIgE and dietary exposure to grass carp is lacking in this population. The higher salmon-sIgE level might thus be explained by IgE binding to other salmon allergens, particularly collagen, when considering the lower parvalbumin content in crude salmon extract, lower IgE affinity of salmon parvalbumin in ELISA, and the more diverse salmon allergen binding pattern in western blots. On the other hand, we also showed that one-fifth of subjects reacted only to grass carp parvalbumin, which indicated that grass carp parvalbumin has higher allergenicity than salmon parvalbumin. However, we are yet to know the clinical reactivity to grass carp among the Japanese subjects and so the question of improving assay sensitivity and specificity using fish allergen (parvalbumin) with higher allergenicity remains open. The varying allergenic potential of fish parvalbumins could be explained by the specific sensitization of patients from their clinical history[8]. With the lack of grass carp in the local Japanese diet, our results suggested the presence of higher diversity, distinct and/or species-specific IgE-binding epitopes for grass carp parvalbumin[31]. It is of note that IgE sensitization based on parvalbumin did not correlate well with patients' clinical allergy status[18], and we postulate that the identification of parvalbumin epitopes may help to resolve the inconsistency in cross-reactivity of different fish species.

In summary, this study extends our understanding on the allergen profile of two important fish species that parvalbumin and collagen are important components for fish allergy diagnosis. We also illustrated that the fish species extracts relevant for diagnosis and allergen sensitization pattern differ among populations. Overall, this study provides additional information for the implementation of relevant extracts and components for precise fish allergy diagnosis.

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Conflicts of Interest

All authors declared no competing interest exists.

Author Contributions

Performing laboratory assays and contributing to data analysis: CYYW, NYHL, KJYX; Subject recruitment and sample collection: ASYL, NF, SS, YSY, JSRD, MYWK, JWCHC, NF, WHC, GTC, QUL, DCKL, PKH, JSCW, ICSL; Writing – original draft: CYYW, NYHL; Funding acquisition and work supervision: GWKW, ME, TFL. All the authors have read and approved the manuscript.

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