

Paper I

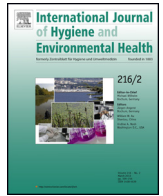
Molecular and immunological approaches in quantifying the air-borne
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Molecular and immunological approaches in quantifying the air-borne food allergen tropomyosin in crab processing facilities

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ABSTRACT

Tropomyosin is a cross-reactive allergenic protein present in ingested shellfish species. Exposure and sensitization to this protein via inhalation is particularly important in the crustacean processing industry where workers are continuously exposed to the aerosolized form of this allergen. The aim of this study was to develop an antibody-based immunoassay to enable the specific and sensitive quantification of aerosolized tropomyosin present in the environment of two crab processing facilities.

Anti-tropomyosin antibody was generated in rabbits against tropomyosins from four different crustacean species. These antibodies were purified using recombinant tropomyosin using an immuno-affinity column. The recombinant tropomyosin was also used as an allergen standard for the sandwich ELISA. In order to quantify aerosolized tropomyosin, air collection was performed in the personal breathing zone of 80 workers during two crab processing activities, edible crab (*Cancer pagurus*) and king crab (*Paralithodes camtschaticus*) using polytetrafluoroethylene filters. The purified antibody was able to detect tropomyosin selectively from different crustaceans but not from vertebrate sources. The limit of detection (LOD) for the developed sandwich ELISA was 60 picogram/m³ and limit of quantitation (LOQ) 100 picogram/m³. Immunoassay validation was based on linearity (R^2 0.999), matrix interference test ($78.8 \pm 6.5\%$), intra-assay CV (9.8%) and inter-assay CV (11%). The novel immunoassay was able to successfully identify working activities, which generated low, medium or high concentrations of the aerosolized food allergen.

We describe an IgG antibody-based immunoassay for quantification of the major food allergen tropomyosin, with high sensitivity and specificity. This modified immunological approach can be adapted for the detection of other aerosolized food allergens, assisting in the identification of high-risk allergen exposure areas in the food industry.

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Abbreviations: Tm, tropomyosin; rTm, recombinant tropomyosin; cAb- α TM, anti-tropomyosin capture antibody; dAb- α TM, detection antibody.

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Introduction

Occupational allergy and asthma has become a serious health concern, especially for workers in the seafood industry. Increased global consumption and changing dietary habits have greatly facilitated seafood production (Lopata and Jeebhay, 2013; Lopata and Lehrer, 2009). This in turn, has caused more workers to be exposed to seafood allergens on a daily basis. According to a report by the Food and Agriculture Organization (FAO) in 2010, nearly 45 million people are involved in seafood and aquaculture production. Several studies have shown that the prevalence of occupational asthma among workers exposed to shellfish is between 4% and 36% (Bonlokke et al., 2012; Granslo et al., 2009; Howse et al., 2006). Moreover, workers with occupational asthma to shellfish

were shown to develop ingestion-related food allergies to the same shellfish species (Jeebhay et al., 2001). Occupational exposure to shellfish allergens can elicit upper and lower respiratory tract symptoms such as asthma, rhinitis and can even cause skin symptoms (Aasmoe et al., 2005; Bang et al., 2005; Lopata and Jeebhay, 2013).

In the seafood industry, workers are constantly exposed to air-borne shellfish particulate matter arising from the different processing techniques. Several studies have shown the presence of allergenic proteins in air-borne particulate matter. These allergens are responsible for causing allergic sensitization among affected workers (Abdel Rahman et al., 2011, 2013; Taylor et al., 2000). Serum IgE antibody reactivity to crab proteins among snow crab processing workers due to occupational exposure of crab matter has been reported previously (Cartier et al., 1984, 1986; Gill et al., 2009; Malo et al., 1997; Weytjens et al., 1999). Aerosolization of shellfish allergens occurs due to processes such as filleting, freezing, cooking, smoking, drying and techniques using high pressure water or air (Jeebhay et al., 2001; Lopata and Jeebhay, 2013). Processes such as butchering, de-gilling and particularly boiling, have been shown to cause excessive bioaerosol formation. The wet or dry air-borne particles may then be inhaled by exposed workers. Abdel Rahman et al. demonstrated elevated levels of air-borne crab allergens in specific work stations such as butchering and cooking as compared to cleaning, packing and storage (Abdel Rahman et al., 2012).

The commonly consumed shellfish can be divided in two groups; crustaceans (shrimps, crabs, lobsters) and mollusks (oyster, mussels, octopus, squid) (Lopata and Kamath, 2012; Lopata et al., 2010). The major shellfish allergen is a 33–36 kDa muscle protein called tropomyosin (TM). Over 80% of shellfish sensitized patients are known to react to this major allergen. As a muscle protein, tropomyosin exists in a dimeric confirmation which complexes with troponin to cover the actin-binding sites during muscle contraction. Due to this role, tropomyosin is a highly expressed protein found mainly in the edible meat. Tropomyosin displays a remarkable stability to heating and is able to retain its allergenicity even in heat-processed shellfish products (Abramovitch et al., 2013; Johnston et al., 2014; Kamath et al., 2013).

Tropomyosin displays high amino acid sequence (primary structure) identity within crustaceans, ranging from 95% to 100% (Kamath et al., 2013). Interestingly, crustacean tropomyosin shares a certain degree of amino acid homology of 75–83% sequence identity with house dust-mite and insect tropomyosins. A variety of mite and cockroach allergens have been implicated in air-borne exposure and sensitization, however, tropomyosin is only a minor allergen in both allergens sources. This is most likely due to low immunological cross-reactivity as well as low relative abundance of this protein compared to consumed shellfish (Arlian et al., 2009; Pomes et al., 2007).

Several studies have shown that antibody reactivity to tropomyosin is a good predictor of shellfish allergy (Gámez et al., 2011; Kamath et al., 2013). Due to its excellent structural stability and detailed characterization, tropomyosin was chosen as an ideal molecular marker for detecting air-borne shellfish allergens in this study.

We report the development and validation of a highly sensitive immunoassay to detect and quantify aerosolized tropomyosin in air samples collected from crab processing factories. Using this immunoassay, we were able to quantify air-borne tropomyosin in a worker- and activity-specific manner.

The approach of using a recombinant protein as standard and purified natural allergen to generate the capture antibody for increased sensitivity and specificity has not been employed previously.

This methodology can be modified for the quantification of other major food allergens and would be an important tool in monitoring air-borne allergen levels in different work environments. This can subsequently assist in establishing safety paradigms to control the unintentional generation of aerosolized allergens and accidental sensitization of exposed workers.

Methods

Allergen standard: expression and purification of recombinant tropomyosin

Recombinant tropomyosin was expressed and purified as described previously (Kamath et al., 2013). Briefly, total RNA was extracted from fresh specimens of black tiger prawn (*Penaeus monodon*) using the RNAeasy mini extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Complementary DNA (cDNA) was reverse transcribed from the total RNA using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Basel, Switzerland), following the manufacturer's instructions. The coding region for tropomyosin was amplified by PCR using forward 5'-GCGGATCCGACGCCATCAAGAAGAAGATGC-3' and reverse 5'-GCGAATTCTTAGTACCCAGACAGTTCGCTG-3' primers. The PCR conditions were set as follows, 94 °C for 2 min, 30 cycles of 94 °C for 20 s, 55 °C for 20 s, 72 °C for 30 s and a final elongation step, 72 °C for 7 min. The 860 bp amplified product was cloned into the expression vector pRSET-A using the *Bam*H1 and *Eco*R1 restriction sites. The recombinant expression vector, pRSET-A-TM was transformed into BL21 *Escherichia coli* strain and expression of the recombinant tropomyosin with a HIS-tag, induced using 1 mM Isopropyl β-D-1-thiogalactopyranoside (Amresco, USA). The bacterial cells were washed with extraction buffer (25 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1 mM imidazole) and lysed using a French pressure cell. After centrifugation at 6000 × g for 15 min, the recombinant tropomyosin was purified using HIS-Trap FF Affinity Column (GE Healthcare, USA). The fraction containing the recombinant protein was further purified using a Superdex G75 16/600 size exclusion column (GE Healthcare, USA) on a Biologic Duoflow FPLC (BioRad, Hercules, CA, USA). The purified recombinant tropomyosin was labeled "rTm" and stored in aliquots at –80 °C until further use.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed as described earlier (Abdel Rahman et al., 2010) to confirm the purity of the tropomyosin standard and analyze the binding characteristics of the purified antibodies.

Twelve micrograms of the protein samples was heated in Laemmli buffer containing dithiothreitol and loaded onto a 12% bis-acrylamide gel. Protein separation was performed at 180 V using a Mini-Protean Tetra Cell electrophoresis system (BioRad, Hercules, CA, USA). The separated proteins were visualized by staining with Coomassie brilliant blue R250 (BioRad, Hercules, CA, USA).

CD spectroscopy of allergen standard

Circular dichroism spectroscopy was performed to analyze the alpha helical content of rTM and compare it to purified natural prawn tropomyosin. Natural and recombinant tropomyosin samples were prepared in PBS, pH 7.2 and adjusted to a final concentration of 3 μM. CD spectroscopy was performed on a J715 Spectropolarimeter (Jasco, USA) with continuous nitrogen flushing at 25 °C. All measurements were performed using a 10 mm quartz cuvette over a wavelength range of 190–260 nm. For wavelength analysis, the tropomyosin samples were scanned with a step width of 0.2 nm and bandwidth of 1 nm at 100 nm/min averaging over

eight scans. Final data was expressed as mean residual ellipticity (θ) after subtracting PBS blank spectrum.

Production of polyclonal anti-tropomyosin antibodies

Protein extraction and estimation

To prepare the antigen mixture to generate antibodies in rabbits, protein extracts were generated from four crustacean species; Black tiger prawn (*Penaeus monodon*), Vannamei prawn (*Litopenaeus vannamei*), Banana prawn (*Fenneropenaeus merguensis*) and School prawn (*Metapenaeus macleayi*) as described previously (Kamath et al., 2013). The complete shellfish specimen, in its outer shell, was heated in liquid (PBS) at 100 °C for 20 min. The outer shell of the specimen was then removed and the edible tissue cut into small pieces. Fifty grams of the muscle mass was homogenized in 150 mL of phosphate buffered saline (PBS) for 10 min using an Ultra turrax blender (IKA, Staufen, Germany), agitated for 3 h at 4 °C followed by centrifugation at 14,000 rpm for 15 min. The supernatant was clarified through a glass fiber filter, followed by filtration through a 0.45 μ m membrane filter (Millipore, Billerica, MA, USA) and stored at –80 °C until further use.

To characterize the generated polyclonal antibody, unheated protein extracts were prepared from prawn (*Penaeus monodon*), crab (*Portunus pelagicus*), lobster (*Jasus edwardsii*), house dust mite (*Dermatophagoides pteronyssinus*), fish (*Lates calcarifer*), and pork (*Sus scrofa*) as described elsewhere (Abramovitch et al., 2013).

Tropomyosin–antigen mix preparation

The protein extracts produced in Section “Protein extraction and estimation” were used as starting material to purify tropomyosin. Ten milligrams of the protein extract was loaded on a mini Macro-prep High Q strong anion exchange column, (BioRad, Hercules, CA, USA) in 30 mM Tris–HCl, pH 6.5. Tropomyosin was eluted from the column using a linear gradient with increasing sodium chloride salt concentration in Tris–HCl buffer from 0.4 M to 0.6 M, over 20 column volumes. The purified TM fractions were pooled and a buffer exchange with phosphate buffered saline (PBS) performed using Amikon spin filters with a 3 kDa molecular weight cut-off (Millipore, Billerica, MA, USA). The tropomyosin fractions from the four crustacean species were mixed and adjusted to a final concentration of 1 mg/mL in sterile PBS.

Rabbit immunization

The immunization of rabbits was performed at Institute of Medical and Veterinary Science (IMVS), Adelaide, Australia. New Zealand rabbits were injected with the prepared tropomyosin immunogen along with Freund’s adjuvant in four doses at two-week intervals. The pre-bleed was collected at week 0 to serve as a negative control. A test bleed was collected at week 7 to test the production of the antibodies and the final bleed conducted at week 9. The collected serum was stored at –80 °C until further processing.

Enrichment and purification of polyclonal IgG anti-tropomyosin antibodies (capture antibodies)

For the development of the sandwich ELISA, IgG antibodies were enriched from the rabbit serum using sodium sulfate precipitation. Sodium sulfate (Sigma–Aldrich, USA) was added to 10 mL of serum in small quantities at a time, to a final concentration of 18% (w/v). The serum was then centrifuged at 1500 \times g for 10 min at ambient temperature, the pellet resuspended in 18% (w/v) sodium sulfate solution, re-centrifuged and finally re-dissolved in 5 mL of sterile PBS, pH 7.2. The IgG enriched serum fraction was subsequently dialyzed against PBS overnight and then stored at –80 °C until further use.

The Aminolink Plus Immobilization kit (Thermo Scientific, Melbourne, Australia) was used to prepare a tropomyosin affinity

column for the isolation and purification of tropomyosin specific IgG antibodies from the IgG enriched rabbit serum fraction. The procedure was followed according to the manufacturer’s instructions. Briefly, rTm was covalently bound to the column, the remaining active sites blocked and washed, and equilibrated in PBS, pH 7.2. The IgG enriched rabbit serum fraction was loaded onto the tropomyosin affinity column and incubated for 30 min. The anti-tropomyosin IgG antibody fraction was subsequently eluted using 0.2 M glycine hydrochloride, pH 2.5 and neutralized with 1 M Tris–HCl pH 8.5. The purified antibody fraction was then dialyzed against PBS, pH 7.2 and stored in aliquots with 0.05% sodium azide at –20 °C for further use. This fraction was labeled cAb- α TM.

Biotinylation of detection antibodies

A fraction of the anti-tropomyosin IgG antibodies were biotinylated using the EZ-Link[®] Sulfo-NHS-LC-Biotin (Thermo Fischer Scientific, Melbourne, Australia) following the manufacturer’s instructions. A 100 fold molar excess of biotin was used to biotinylate the antibodies from a freshly prepared 10 mM biotin stock solution. After biotinylation, the antibody solution was dialyzed against PBS, pH 7.4 to remove the excess reactants. Sodium azide was added to the antibody solution to a final concentration of 0.05% (w/v) and stored at 4 °C in amber colored tubes and labeled dAb- α TM.

Immunoblotting

Serum IgE immunoblotting was performed to test patient IgE reactivity to rTm as described previously (Abramovitch et al., 2013; Johnston et al., 2014). Sera were pooled from four subjects with a convincing clinical history of allergic reactivity to ingested shellfish and confirmed IgE binding to natural purified prawn tropomyosin. The subjects were recruited by The Alfred Hospital, Allergy Clinic, Melbourne, Victoria, Australia.

To analyze the binding characteristics of the capture antibody to various antigenic sources, the separated proteins on an SDS-PAGE gel were transferred to a polyvinylidene difluoride (PVDF) membrane using the Semi-dry TransBlot Apparatus (BioRad, Hercules, CA, USA). After blocking with 1% (w/v) bovine serum albumin (BSA) in PBS-T, the membrane was subsequently incubated with the capture antibody, cAb- α TM, diluted 1:10,000 in 0.5% BSA, PBS-T and goat anti-rabbit IgG antibody conjugated with HRP (Promega, USA) diluted 1:40,000. The membrane was visualized using the enhanced chemiluminescent technique as previously reported (Abdel Rahman et al., 2011).

Tropomyosin amino acid sequence alignment

To predict the capture antibody binding characteristics to shellfish tropomyosin, a protein sequence alignment was performed to compare vertebrate and invertebrate tropomyosin. cDNA based protein sequences for tropomyosin were collected from the NCBI database with the following accession numbers; Black tiger prawn, accession number (AAX37288.1), Blue swimmer crab (AGE44125.1), Rock lobster (AFY98827.1), House dust mite (ACI32128.1), German cockroach (AAF72534.1), Cod fish (BAC44994.1) and Pig tropomyosin (NP_001090952.1). Sequence alignment, matrix identity scores and percent similarity were calculated using Clustal O algorithm in Jalview program (Waterhouse et al., 2009).

The evolutionary history was inferred using the Minimum Evolution method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The ME tree was

searched using the Close-Neighbor-Interchange (CNI) algorithm at a search level of 1. The analysis involved 7 amino acid sequences. There were a total of 284 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

Air sampling and elution of aerosolized allergen, tropomyosin

Collection of aerosolized tropomyosin was performed using polytetrafluoroethylene (PTFE) filters with a pore size of 1.0 μm (Millipore, Billerica, USA). The filter cassette apparatus was attached to a pump (SKC Ltd., UK) through a tube and the airflow adjusted to 2.5 L/min. The average airflow rate through the filter, was calculated as the mean of initial and final airflow at the start and stop of the pump, respectively. Where a change in the airflow from start to finish was more than 10%, the samples were discarded.

The air-collection apparatus was setup in a backpack which was carried by each worker during their normal shift. The air collection inlet was placed in the workers personal breathing zone (PBZ) so as to sample the air available for breathing.

Elution of the collected allergens was performed using Nunc-ImmunoMinisorp tubes (Nunc, USA) to minimize the loss of allergen content due to adsorption on the tube walls. The PTFE filters were removed from the cassettes and placed in tubes containing 1 mL of Phosphate Buffered Saline (PBS) with 0.5% Tween 20 and 0.2 mM Sodium azide. The extraction was performed, by placing the tubes on a rotation tilter for 2 h at room temperature. The eluate was transferred to a new Minisorp tube and Bovine Serum Albumin (BSA) added to a final concentration of 1 mg/mL. This eluate was then aliquoted in 200 μL volume and stored in mini Eppendorf tubes at -80°C until further analysis. A clean unused filter was eluted simultaneously using the extraction procedure above and regarded as a blank sample.

Assay procedure

All incubations were performed with 100 μL /well at room temperature for 1 h unless otherwise stated. All washing steps were performed using PBS with 0.05% (v/v) Tween 20, pH 7.2 (PBS-T) and repeated three times on an EL405 Autoplate washer (BioTek Instruments, Winooski, USA) unless otherwise stated. A 96 well high binding Costar microtitre plate (Sigma-Aldrich, USA) was coated with cAb- αTM diluted 1:500 in carbonate buffer, pH 9.6 and incubated overnight at 4°C . After washing, the wells were blocked with 270 μL of Pierce Superblock buffer (Thermo Fischer Scientific, Melbourne, Australia). Next either the standards, serially diluted from 10 to 0.02 ng/mL, or the test air samples or test blanks were added to the wells and incubated for 3 h. After washing, the detection antibody dAb- αTM diluted 1:500 in dilution buffer (PBS-T containing 1 mg/mL BSA) was added to the wells. Subsequently, the streptavidin-horse radish peroxidase conjugate (Sigma-Aldrich, USA) diluted 1:10,000 in dilution buffer was added to the wells and incubated for 30 min. The wells were then washed five times and patted dry. To visualize antibody binding, 100 μL of 3,3',5,5'-tetramethylbenzidine substrate (Becton Dickinson, USA) was added to the wells until a blue coloration started forming in the blank wells and the reaction stopped using 1 N hydrochloric acid. The color development in the wells was measured at 450 nm using a Versamax Microplate Reader (Molecular Devices, California, USA).

Validation of sandwich ELISA

Linearity of the standard curve, calculation of limit of detection (LOD) and limit of quantitation (LOQ) and non-specificity

The goodness of linearity of the rTm standard curve was assessed using the R^2 value based on a four-parameter logistic curve calculated using SoftMax Pro software v5.2 (Molecular Devices,

California, USA). Non-specific binding of the assay was analyzed by omitting the capture antibody, cAb- αTM or the detection antibody, dAb- αTM . TM levels in the air collection samples were derived by interpolation of the absorbance readings of the rTm standard curve using four parametric logistic algorithm. The air collection samples were diluted in the range of 1:2–1:80 to obtain an absorbance value within the linear range of the standard curve. The allergen standard curve was included in every 96 well plate used to analyze the test samples.

To test the sensitivity of this assay, blank samples were run in 12 separate experiments in triplicates. The limit of detection (LOD) was calculated as the mean of the blank samples plus three times the standard deviation. The limit of quantitation (LOQ) was calculated as the mean of the blank samples plus ten times the standard deviation (Armbruster et al., 1994).

Spike recovery assays

Spiking tests were performed to test the matrix interference effects of the extraction buffer and other extraneous air-borne entities in the actual air sample. Seventeen random air-collection samples were selected with either low levels of tropomyosin or with levels below the LOQ, and spiked with rTm. An equal volume of test samples and rTm (spike) were mixed to a final concentration of 1 ng/mL. These spiked samples were then frozen at -80°C overnight to simulate the air collection sample preparation process. The next day, the spiked samples were thawed to room temperature and tested using the immunoassay as described in Section "Assay procedure". Percent recovery (%) was derived by dividing the measured TM concentration of the spiked sample by the sum of TM concentration of un-spiked sample and the spike concentration; [spiked sample (ng/mL)]/(un-spiked sample (ng/mL) + Spike, 1 ng/mL). The recovery rates of the sample had to fall within 70–110% for the assay to pass (Lexmond et al., 2011).

Precision of the ELISA

The precision of this sandwich ELISA was tested on the basis of intra-assay and inter-assay variability. Air-collection test samples with tropomyosin levels below the LOQ were pooled and diluted 1:2 in extraction buffer to be spiked. Three spiked samples were prepared at low (0.2 ng/mL), medium (0.5 ng/mL) and high (1.0 ng/mL) concentrations of rTm. For intra-assay variability tests, 9 replicates were tested for each spike concentrations (low, medium and high) in one single 96 well plate which included a standard curve. To test the inter-assay variability, the 3 spiked samples were tested in six different experiments over three days by two independent operators. Each test sample was tested in triplicates and each experiment included a standard curve. For the assay to pass, the co-efficient of variation (%CV) of the replicates had to fall within 20%, for both intra-assay and inter-assay tests. In addition, the percent recovery had to fall within 20% of the theoretical concentration (Kelley and DeSilva, 2007).

Statistical analysis

The Mann-Whitney U Test was used to compare the allergen exposure levels in each category of work-tasks. A p value of less than 0.05 was considered significant. All statistical analysis was performed using GraphPad Prism version 6.02 (GraphPad, USA).

Results

Characterization of the allergen standard (rTm)

The allergen standard, rTm was successfully expressed in a BL21 *E. coli* bacterial expression system as represented in Fig. 1. rTm

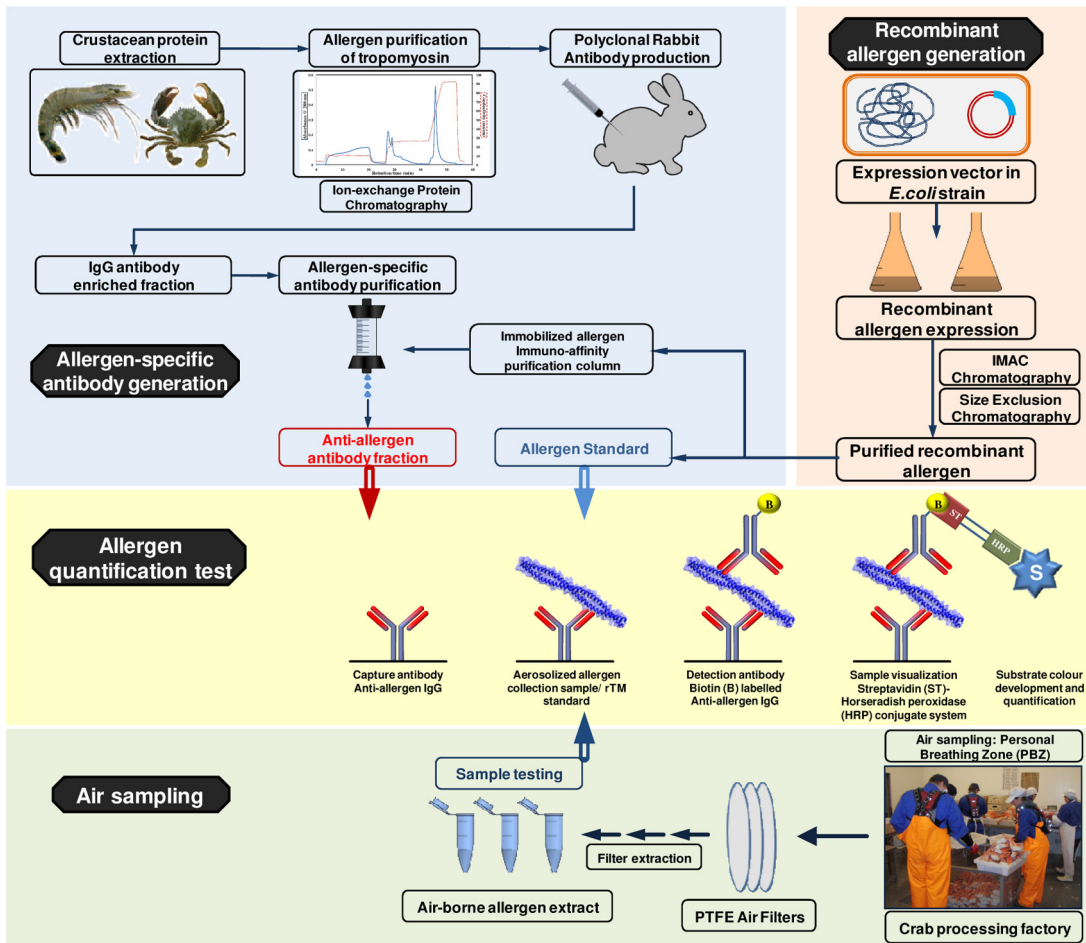


Fig. 1. Schematic representation of the methodology and setup used for the detection and quantification of the air-borne shellfish allergen tropomyosin.

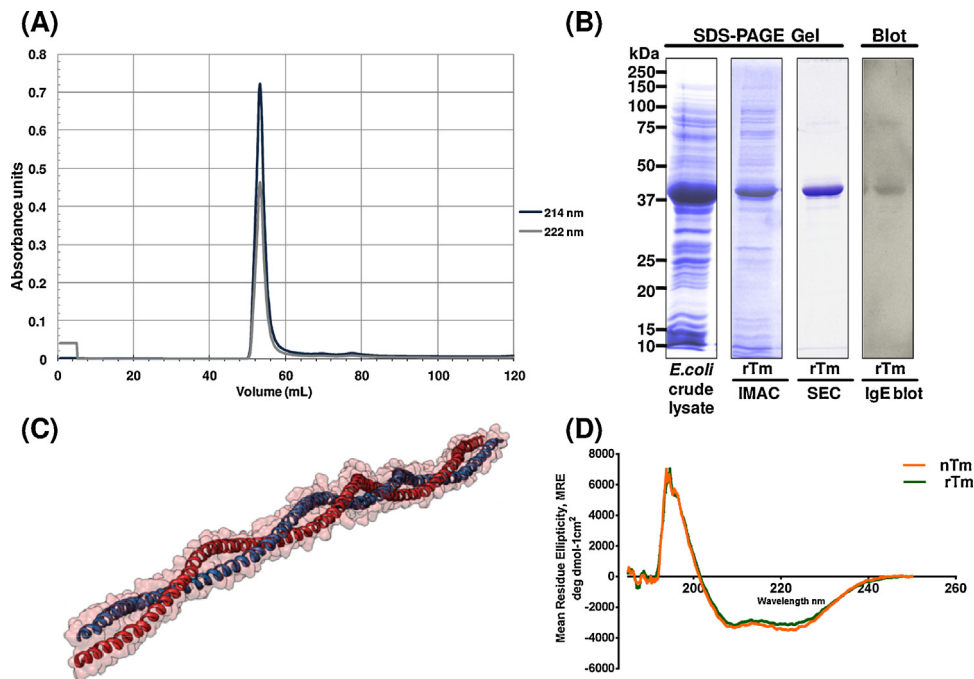


Fig. 2. Characterization of the immunoassay allergen standard, recombinant tropomyosin, rTm. (A) Protein purification profile of rTm using size exclusion chromatography. (B) SDS-PAGE analysis of the various purification stages of the allergen standard using nickel affinity (IMAC), size exclusion chromatography (SEC) and IgE immunoblotting analysis of rTm using pooled patient sera. (C) Three dimensional homology model of crustacean tropomyosin in dimeric form. (D) Comparison of alpha-helical content of allergen standard, rTm (green) and natural tropomyosin, nTm (orange) using CD spectroscopy. (For interpretation of the references to color in text, the reader is referred to the web version of this article.)

was expressed as a fusion protein with a 6X Histidine-tag at the N-terminal end of the protein to facilitate purification. Affinity chromatography was performed to purify rTm from the crude bacterial lysate. However, several additional bands could be observed in the affinity purified fraction (Fig. 2A). Therefore, size exclusion chromatography (SEC) was subsequently performed as an additional purification step. The final product was visible as a single band of approximately 40 kDa. To demonstrate immunological reactivity, immunoblotting experiments were performed (Fig. 2B). Immunoblotting with patient sera confirmed IgE antibody reactivity and thus the allergenicity of rTm. Protein homology modeling of the allergen tropomyosin represents its highly stable alpha-helical structure and the favorable formation of a homo-dimeric state (Fig. 2C).

CD spectroscopic analysis of rTm and natural tropomyosin was performed, to confirm appropriate protein folding and structure of the recombinant protein and its subsequent use as an allergen standard (Fig. 2D). A distinct negative signal at 208 and 222 nm is typical for an alpha helical protein. This was also observed for the rTm as compared to the purified natural tropomyosin. The CD spectrum for rTm was almost identical to that of natural tropomyosin.

Binding properties of the capture antibody

The polyclonal anti-tropomyosin antibody, cAb- α TM was successfully isolated from the IgG enriched fraction of the immunized rabbit serum as shown in Fig. 1. Immunoblotting demonstrated specific binding of cAb- α TM to a 37 kDa band from prawn crude extract (Fig. 3A). Specific antibody reactivity to tropomyosin was confirmed with strong binding to rTm (Fig. 3B). Moreover, antibody reactivity was observed to the dimeric form of tropomyosin formed at 75 kDa. The antibody binding characteristics of cAb- α TM was analyzed against various antigenic sources. cAb- α TM showed strong binding to tropomyosin from the crustaceans analyzed; crab, prawn and lobster. On the other hand, no binding was observed to extracts from fish, chicken and *E. coli* (Fig. 3C). Interestingly, antibody binding was observed to tropomyosin from house dust mite extract at 37 kDa region. Thus, antibody specificity was demonstrated to invertebrate tropomyosin.

Selective antibody binding to crustacean tropomyosin may be attributed to molecular differences in the primary structure of tropomyosin among vertebrates and invertebrates (Fig. 4A). The compared crustacean tropomyosin was at least 94% identical among each other. However, when compared to vertebrate tropomyosin, the maximum percent identity was only 58% (Fig. 4B). Cockroach and house dust mite tropomyosin, both of which have been characterized as allergens, were closely related to crustacean tropomyosin with 79–82% sequence identity (Fig. 4B and C).

Linearity of allergen standard curve

A ten point serial dilution curve of rTm was used in the range of 0.02–10 ng/mL concentration, diluted using air sample extraction buffer. A standard curve was generated using the absolute allergen concentrations in ng/mL and its corresponding absorbance values measured at 450 nm using a four-parameter logistic regression algorithm. Error bars represent the standard deviation. The goodness of fit (R^2) was 0.998 averaged from six individual experiments ($n=6$) (Fig. 5A). The linear region of the standard curve, 0.02–1.25 ng/mL was used for the quantification of tropomyosin in the air collection samples.

To quantify tropomyosin in the air collection samples they had to be diluted from at least 1/2 to 1/80 to bring the absorbance values within the linear range of the standard curve. This allowed for accurate measurement of samples with very high or low allergen content. Use of higher concentrations of rTm, beyond 2.5 ng/mL,

Table 1

Matrix interference and spike recovery analysis of the Tm sandwich ELISA. Spike recovery assay was performed on 17 random air collection samples by spiking with 0.5 ng/mL of rTm standard. % recovery was calculated by comparing the theoretical concentration and detected concentration of rTm in ng/mL. Mean recovery was calculated as $78.8 \pm 6.5\%$.

Sample no.	Unspiked air-collection samples Tm (ng/mL)	Spiked samples with rTm (0.5 ng/mL) Tm (ng/mL)	Recovery of spike Tm Percent (%)
1	<LOQ	0.36	71.0
2	<LOQ	0.39	78.0
3	<LOQ	0.40	80.0
4	<LOQ	0.34	67.6
5	<LOQ	0.43	86.0
6	<LOQ	0.41	81.2
7	<LOQ	0.36	71.6
8	<LOQ	0.40	79.0
9	<LOQ	0.34	68.4
10	<LOQ	0.46	91.8
11	<LOQ	0.40	79.4
12	0.69	0.94	78.9
13	0.31	0.64	79.6
14	0.36	0.71	83.2
15	0.89	1.24	89.2
16	0.29	0.60	76.2
17	0.49	0.78	79.0

did not result in a proportional increase in the absorbance values (Fig. 5B). The limit of detection (LOD) was calculated to be 60 pg/mL and the limit of quantitation (LOQ) was determined to be 100 pg/mL.

Assay specificity

The accuracy of an immunoassay depends on the absence of non-specific antibody binding. This can be attributed to specific non-specific binding and non-specific, non-specific binding (Kelley and DeSilva, 2007). We tested cAb- α TM for specific, non-specific binding and as shown in Fig. 3 no binding was observed to any other protein but tropomyosin. Subsequently, we performed the assay with the entire standard curve by omitting the capture antibody, cAb- α TM or the detection antibody. This confirmed the specificity of this assay to rTm with absence of non-specific binding to other reagents, since a complete loss of signal was observed (Fig. 5A). Finally, we performed the spike recovery test to analyze the interference from non-related matrix agents present in the air-borne particulate matter collected in the air samples (Table 1). Results are shown as percent recovery of the rTm spike (0.5 ng/mL) in blank samples and test samples with tropomyosin content below the assay LOQ. The mean recovery of the spike was calculated to be $78.8 \pm 6.5\%$ which passed our pre-set criteria (Kelley and DeSilva, 2007; Lee et al., 2006; Lee and Hall, 2009). Moreover, there were no cases of positive interference among control samples. Two of 17 samples had a recovery below 70% suggesting possible interference with the sample matrix.

Intra-assay and inter-assay variability

Intra- and inter-assay variability tests were conducted to test the precision and accuracy of the assay (Table 2). Variability was tested for low, medium and high concentration of tropomyosin (Fig. 5C). The mean recoveries of the samples were over 70% and the coefficient of variation was <20% for all three concentrations of rTm tested. This data are in concordance with the acceptance criteria for assay validation (Kelley and DeSilva, 2007; Lee et al., 2006; Lee and Hall, 2009).

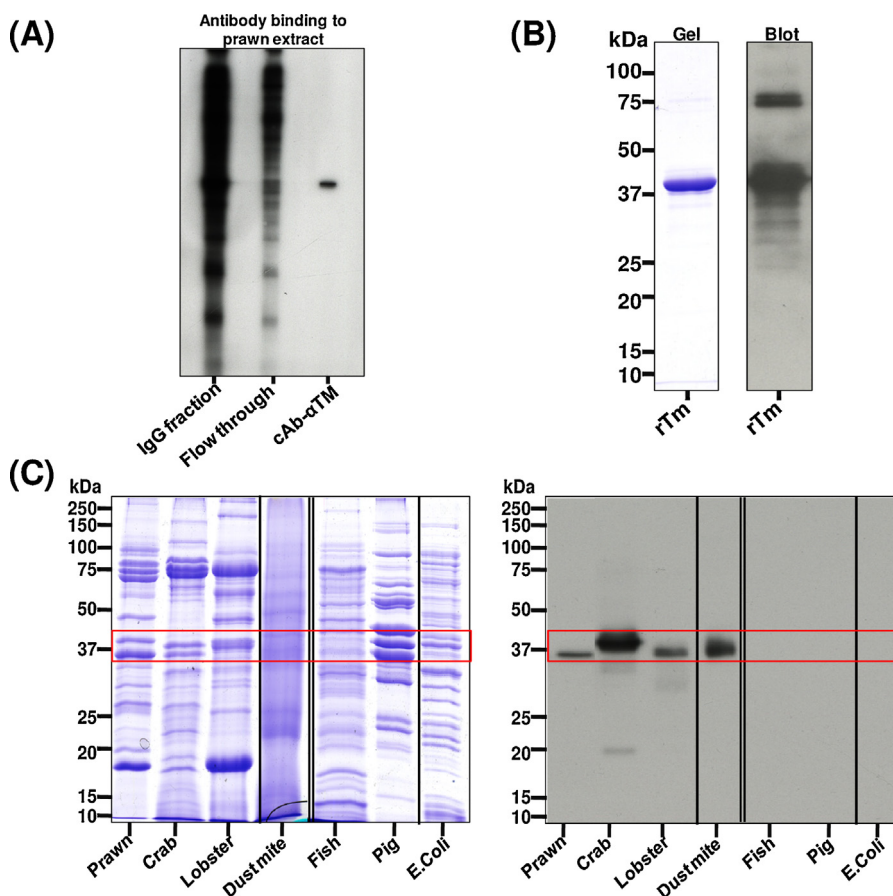


Fig. 3. Binding characteristics of polyclonal rabbit anti-tropomyosin IgG antibody. (A) Antibody binding patterns of various stages of rabbit antibody purification against prawn protein extract. (B) SDS-PAGE and immunoblotting of the allergen standard rTm using cAb-αTM antibody. (C) Specific antibody binding of cAb-αTM against different protein extracts of invertebrate and vertebrate species.

Analysis of air samples from crab processing factory

Air-samples were tested from the PBZs of workers from two different processing activities; king crab and edible crab (Table 3). The average air volume sampled was 1095 ± 118 L and 830 ± 371 L for edible crab and king crab processing, respectively. The amount of air-borne tropomyosin and exposure patterns differed among the two processing plants (Fig. 6). Tropomyosin in the king crab plant measured in the range of 0.15–75.89 ng/m³ whereas in the edible crab, it was 0.42–138 ng/m³.

In the edible crab processing, highest tropomyosin exposure was demonstrated for workers handling boiled meat and spin-celler (separator of meat from boiled crab). The lowest exposure

was in the scanning process, freezer and raw crab handling area. The tropomyosin exposure concentrations varied significantly among workers in the high exposure activities, handling boiled crab.

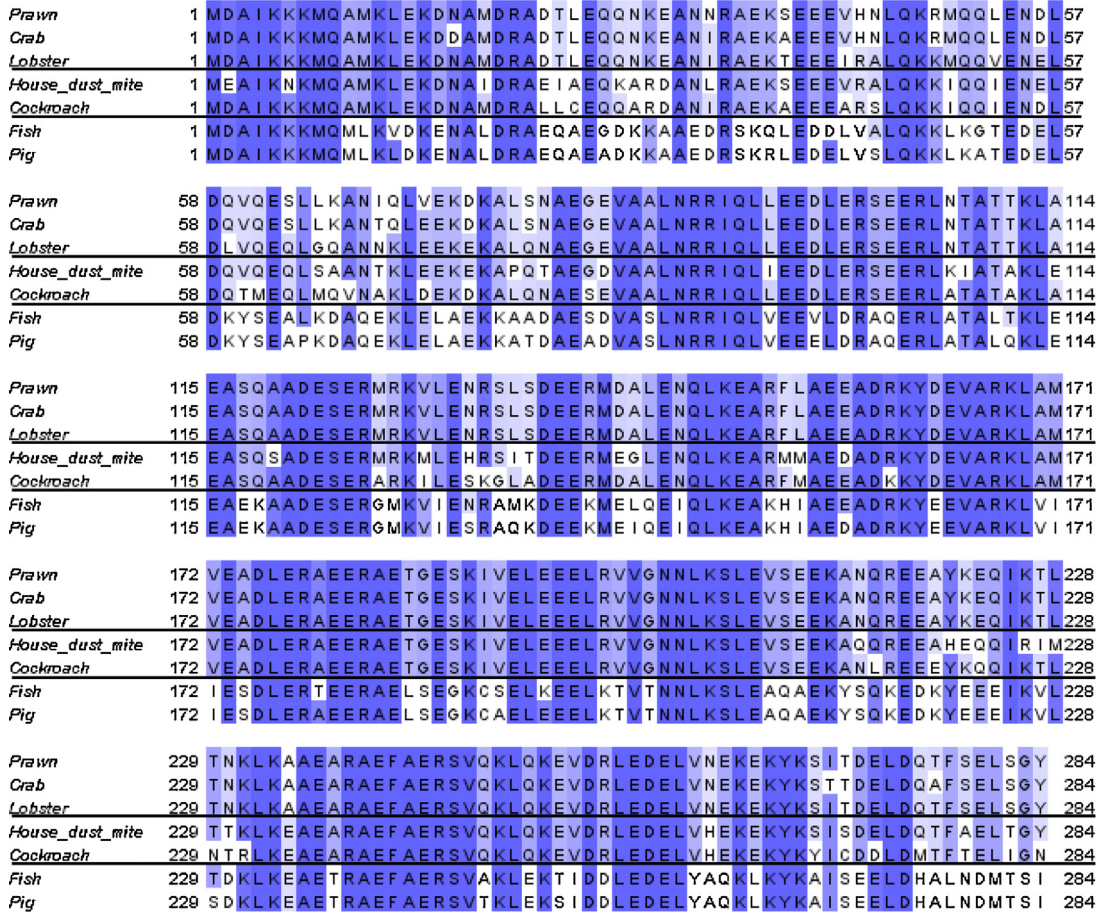
King crab processing was performed in batches interspersed with fish processing, due to the seasonal availability of the crab. The highest tropomyosin concentrations were identified for processes such as cleaning, cracking and crab degilling. Among workers sharing work tasks between crab and fish processing, moderate concentrations of tropomyosin exposure was observed. Locations and tasks such as truck driving, fish packing, receiving stations and fish gutting did not show any significant concentrations of air-borne tropomyosin.

Table 2

Assay precision of the immunoassay. Intra- and Inter-assay variability of the assay was tested using 0.2 ng/mL, 0.5 ng/mL and 1 ng/mL of rTm as low, medium and high antigen concentrations, respectively. The assay variability is shown in terms of the calculated mean concentration, standard deviation and percentage of the coefficient of variation. Recovery of the rTm concentrations were calculated as a percentage of the ratio of calculated versus theoretical concentrations. Number of replicates; n = 9 (intra-assay) and n = 6 (inter-assay).

rTm (ng/mL)	Mean (ng/mL)	Standard deviation, SD	Coefficient of variation, CV (%)	Recovery (%)
<i>Intra-assay variability</i>				
0.2	0.14	0.013	9	70.5
0.5	0.40	0.025	6.3	79.8
1.0	0.76	0.074	9.8	76.4
<i>Inter-assay variability</i>				
0.2	0.16	0.018	11.0	80.0
0.5	0.41	0.043	10.4	82.0
1.0	0.81	0.074	9.1	81.0

[A]



[B]

	Prawn	Crab	Lobster	Cockroach	House dust mite	Fish	Pig
Prawn	100						
Crab	97.5	100					
Lobster	94	93.7	100				
Cockroach	82.4	82	83.8	100			
House dust mite	80.3	79.6	82.4	79.6	100		
Fish	56	56	58.1	53.5	56.7	100	
Pig	55.6	55.6	57.4	54.6	57.4	92.6	100

[C]

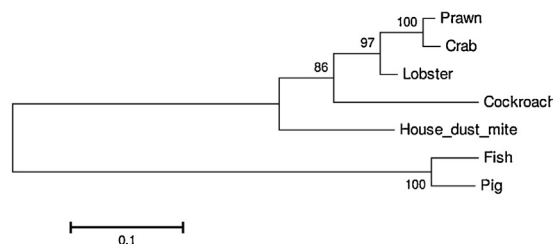


Fig. 4. Comparison of invertebrate tropomyosin primary structure. (A) Amino acid sequence alignment, for visual comparison of primary structure similarity of invertebrate and vertebrate tropomyosins. Accession number (Genbank nucleotide sequence database) Prawn, *Penaeus monodon*, accession number (AA37288.1); Crab, *Portunus pelagicus* (BAF47266.1); Lobster, *Jasus lalandii* (AFY98827.1); House dust mite, *Dermatophagoides pteronyssinus* (AC132128.1); Cockroach, *Blattella germanica*, (AAF72534.1); Fish, *Gadus chalcogrammus* (BAC44994.1); and Pig, *Sus scrofa* tropomyosin (NP.001090952.1). The colored shading intensity indicates percent identity. (B) Tropomyosin amino acid sequence identity table calculated using Clustal Omega. (C) Phylogenetic tree of invertebrate tropomyosin inferred using the minimum evolution method.

Discussion

Frequent occurrences of allergic reactions among seafood workers due to air-borne allergen exposure have been reported (Lopata and Jeebhay, 2013). Occupational asthma has been commonly associated with shellfish processing and previous studies have shown a prevalence of up to 36% (Jeebhay and Cartier, 2010). A strong correlation between the high air-borne allergen concentrations and development of allergenic sensitization and asthma has been suggested (Brisman, 2002; Jeebhay and Cartier, 2010). A number of tasks in the shellfish processing workplace put the workers at a greater risk of exposure and consequent sensitization to shellfish

allergens (Cartier, 2010; Lopata and Jeebhay, 2013). Currently, there is a lack of standardized, validated methods for monitoring the allergen concentrations in bioaerosols produced during shellfish processing.

The aim of this study was to develop and validate a sensitive antibody-based immunoassay for the detection and quantification of the shellfish allergen tropomyosin in bioaerosols produced during crab processing.

Traditionally, serum IgE antibodies from shellfish-sensitized individuals have been used to detect air-borne allergens, using an inhibition ELISA setup (Malo et al., 1997; Weytjens et al., 1999). This approach is useful for quantifying air-borne

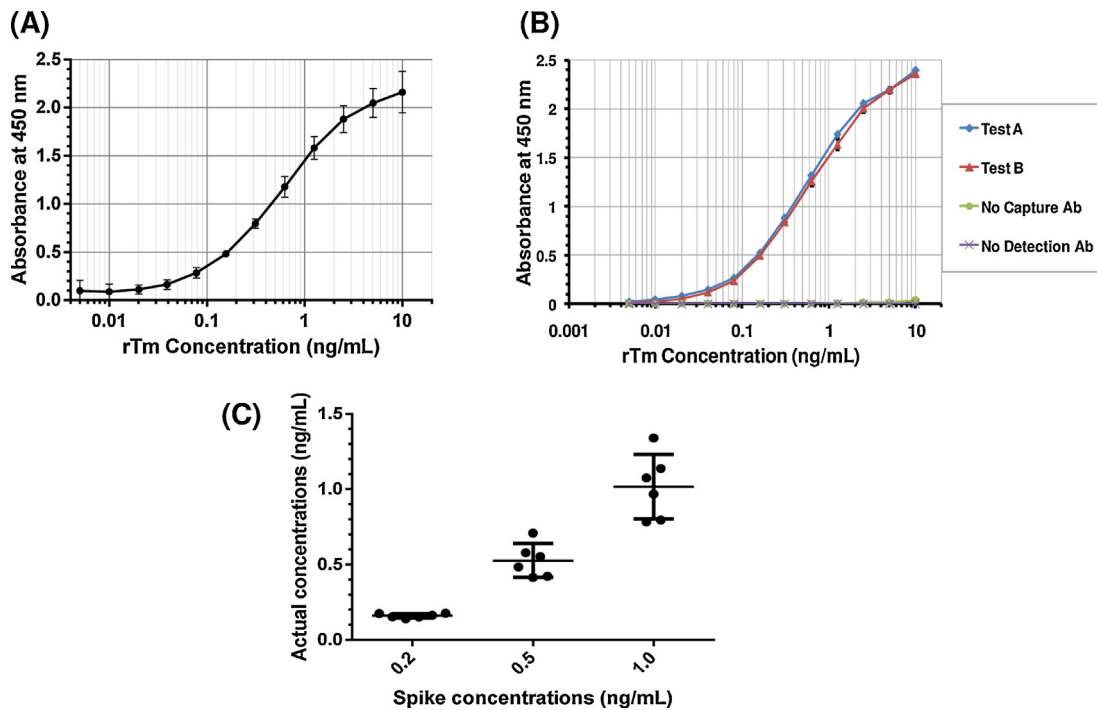


Fig. 5. Standard curve for the allergen standard, recombinant tropomyosin (rTm). (A) A 10 point serial dilution curve from 10 to 0.02 ng/mL, error bars indicate the standard deviation of each dilution over six individual experiments. (B) Assay reproducibility and specificity: Test A and B indicate the standard curves from two separate experiments. “●” and “X” indicate omission of the capture or detection antibodies respectively to analyze non-specific binding properties of the immunoassay. (C) Inter-assay variability test using rTm spiked samples at three different concentrations; 0.2, 0.5 and 1.0 ng/mL ($n = 6$).

IgE-reactive allergens, with the assay having a sensitive detection limit of 1 ng/mL. However, the major disadvantage of IgE antibodies is their low titer and difficulty in developing a standard assay model due to varied antibody reactivity in different patient sera.

ELISA based immunoassays have been previously applied for the detection of tropomyosin in food matrices, using monoclonal or polyclonal antibody based platforms (Jeoung et al., 1997; Seiki et al., 2007; Werner et al., 2007). However, the assay sensitivity, precision and matrix interference levels varied largely among the different assays. Monoclonal antibody based assays have the advantage of high specificity and lack of false-positive results in allergen detection (Lopata et al., 2002, 2005). Nevertheless, any modifications or changes in the conformation of the mAb-epitope on the allergenic protein may lead to loss of binding. Polyclonal antibody based assays on the other hand, can bind to the allergens at

multiple epitopes, thus minimizing the risk of protein conformational changes affecting the binding to the allergen.

In the present study, we have employed a modified approach for developing a highly sensitive immunoassay for the quantification of aerosolized crustacean allergens. One of the initial hurdles was to develop a polyclonal antibody based assay with high specificity and minimized unspecific binding. Natural tropomyosins purified from four different crustacean species were used for the generation of polyclonal rabbit antibodies. This increased the binding capacity of the antibody to tropomyosins with an amino acid sequence variation of over 95%. Moreover, a recombinant crustacean tropomyosin of high purity was used as the stationary phase for affinity purification of tropomyosin-specific IgG antibodies from the rabbit serum. The resultant final antibody fraction demonstrated high specificity to crustacean tropomyosin with no non-specific binding to other homologous proteins as shown in our validation tests.

Table 3

Exposure to air-borne allergen tropomyosin among workers in crab-processing workplace according to specific tasks performed.

Designation	Task function	Number of samples	Allergen exposure		p Value*
			Median (ng/m ³)	Range (ng/m ³)	
A	Handling boiled meat	9	61.4	21.95–138.8	<0.0001
B	Crab cracking Gutting Raw meat handling Cutting Cleaning	22	23.48	<LOQ–75.89	<0.0001
C	Receiving station Scanning Sorting Packing	17	1.11	<LOQ–71.92	<0.0016
D	Transport/logistics Fish-related tasks	27	0.25	<LOQ–6.74	–

* P values were calculated using the Mann–Whitney U test in comparison to category D.

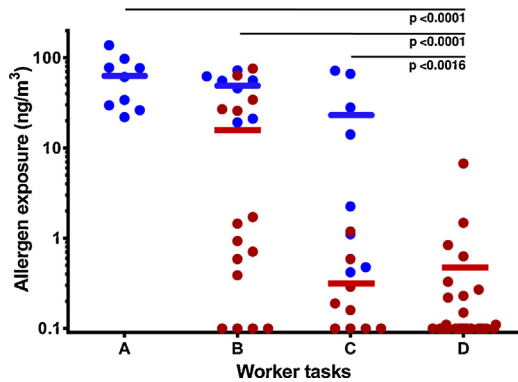


Fig. 6. Allergen exposure to crab processing workers involved in various tasks and locations. Allergen exposure to workers processing edible crabs is displayed as blue dots and king crabs in red dots. The mean exposure for each task category is shown as a horizontal line in blue and red for edible crab and king crab, respectively. Tasks: (A) Handling boiled meat, (B) crab cracking, gutting, raw meat, cutting and cleaning, (C) crab receiving station, handling, scanning, sorting and packing, and (D) logistics and fish-related tasks. (For interpretation of the references to color in text, the reader is referred to the web version of this article.)

Interestingly, the capture antibody was able to recognize house dust mite tropomyosin, due to the latter's high percent identity to crustacean tropomyosin. This however may not significantly affect the quantification of crab tropomyosin in a wet processing environment. Previous studies have shown that dust mites contain very low amounts of tropomyosin (Arlian et al., 2009). Moreover, it has been shown that tropomyosin may not be the main allergen involved in seafood-mite sensitization (Boquete et al., 2011).

To improve the assay specificity, recombinant tropomyosin was used as an allergen standard in the assay to quantify the amount of tropomyosin in the samples. The advantage of using a recombinant protein as a standard is the unlimited availability and consistent performance as opposed to the natural source which often demonstrates batch to batch variations. Previous studies on allergen detection have developed assays with sensitivities ranging from 1 ng/m³ to 105 ng/m³ (Lopata et al., 2005; Malo et al., 1997). The immunoassay developed in this study was able to achieve an allergen detection limit of 60 pg/m³. However, assays with higher sensitivity (10 pg/m³) have been developed for mouse or rat urinary allergens (Renstrom et al., 2002).

The main parameters of performance of the assay were established by our in-house validation. Spike recovery tests were performed to test the matrix interference effects and recorded as 79%. While the outcome fell within the acceptance criteria, it highlighted the effects of matrix components on allergen-antibody binding. This interference seems to result mainly from the extraction buffer used to elute the allergens from air sampling filters. The performance of the allergen standard curve was tested in the presence and absence of the buffer and showed a marked effect on the absorbance values of the allergen standard (data not shown). Therefore, to maintain similar levels of matrix effects from the buffer on the test samples as well as the standards, the later was diluted using the same extraction buffer as for the sample analysis. The accuracy and precision (reproducibility) of the assay was tested using inter-assay and intra-assay variability tests. Both the tests met the acceptance criteria of falling within 20% co-efficient of variation.

This developed immunoassay was utilized to assess the concentrations of aerosolized tropomyosin in two different processing activities; edible crab and king crab. In general, the levels of bioaerosols and subsequently the air-borne allergen concentrations are dependent on variables such as the kind of seafood being processed, amount of seafood being processed, size of the factory, layout of processing equipment and locations of ventilation

system. These variables vary with different locations and different seasons of seafood processing. For example, in this study, some king crab processing factories were mainly involved in fish processing and only temporarily converted to crab processing, depending on the availability and fishing season. Due to this, a small line of crab processing assembly was placed in relatively large production rooms where ventilation may not have been optimal near the processing activity. Some of the edible crab processing activities involved cooking the crabs. This was performed in defined locations or separate rooms with point ventilations. Although measures were taken to limit the exposure to the fumes from the cooking vats, many workers not involved in the cooking activities were stationed close by and were exposed to the fumes. The edible crab processing facility was designed specifically for crab production and it involved workers standing close together on a processing line. In the king crab factories, the larger rooms and fewer workers involved in processing and more manual labor might account for some of the differences in allergen concentrations in the air samples as compared to the edible crab factory.

Several shellfish allergens have been identified and characterized in commonly consumed shellfish species (Kamath et al., 2013, 2014; Lopata et al., 2010). Tropomyosin, however is the most abundant and heat-stable invertebrate allergen capable of inducing allergic sensitization. In this study, the highest exposure to tropomyosin was demonstrated during heating and boiling processes as shown previously (Malo et al., 1997). Recent studies have shown the ability of natural or heat generated tropomyosin fragments to elicit IgE reactivity. Moreover, enhanced IgE reactivity of tropomyosin after heating was demonstrated (Abramovitch et al., 2013; Kamath et al., 2013). This is of clinical importance since the heating processes do not only increase the aerosolization of allergens but can also exacerbate the IgE sensitization among affected workers. From the detection point-of-view, it is an advantage to employ a polyclonal antibody based assay, since monoclonal based assays may not be able to detect these allergen fragments aerosolized during the heating or boiling processes (Kamath et al., 2013). The importance of allergen fragments is particularly important in the case of tropomyosin, as eight IgE binding epitopes have been discovered spanning its entire length of the alpha-helical secondary structure (Kamath et al., 2013; Lopata et al., 2010; Reese et al., 2005). This may dramatically increase possible IgE cross-linking in sensitized individuals due to exposure to tropomyosin fragments.

In summary, we have developed and validated a highly sensitive assay for air-borne tropomyosin detection. Using this assay, we were able to quantify tropomyosin in the PBZ of workers performing different work activities in crab processing factories. High concentrations of tropomyosin were detected mainly in boiling, heating and de-gilling stations. The developed immunoassay is currently employed for monitoring allergen exposure in different crab processing factories as part of a bigger work-safety study in Norway. The methodological approach used for developing this assay offers opportunities to other food industries to monitor air-borne allergens, so as to improve work-safety and occupational health.

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