

# Detection of Fish Antigens Aerosolized during Fish Processing Using Newly Developed Immunoassays

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## Key Words

Occupational exposure · Allergy · Allergen · Seafood · Fish · Air sampling · Immunological detection · Pilchard · Anchovy

## Abstract

**Background:** Aerosolization of fish proteins during seafood processing has been identified as a potential route for allergic sensitization and occupational asthma among workers involved in high-risk activities. The aim of this study was to develop immunological assays for the quantification of aerosolized fish antigens in a fish-processing factory. **Methods:** Polyclonal antibodies to the main fish species processed in the factory (anchovy and pilchard) were generated in rabbits and compared by ELISA inhibition assay and immunoblotting. These antisera were utilized to develop ELISA assays for the detection of fish antigens. The ELISA inhibition assays were evaluated by analyzing environmental air samples collected from three areas in a fish-processing factory: pilchard canning, fish meal production and lobster processing. **Results:** By immunoblotting, the rabbit polyclonal antibodies demonstrated IgG antibody binding patterns comparable with IgE antibodies of fish-sensitized pa-

tients, particularly in regard to the major fish allergens parvalbumins. The sensitivity of the fish-specific ELISA assays developed was 0.5 µg/ml. The ELISA inhibition assays were able to differentiate between the two different fish species of interest but did not recognize a crustacean species. Notable differences in exposure levels to canned pilchard and anchovy antigens were demonstrated in the three different working areas of the factory, with assays having a detection limit as low as 105 ng/m<sup>3</sup>. **Conclusion:** These ELISA-based assays are sensitive and specific to quantify differential exposure levels to fish antigens produced during fish processing, making it possible to investigate exposure-disease response relationships among workers in this industry.

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## Introduction

Aerosolization of seafood allergens during processing has been identified as a potential route for allergic sensitization and occupational asthma in the crustacean-processing industry [1]. Despite the known putative role of fish causing occupational allergies and asthma, the detailed detection and quantification of different bony fish

antigens during specific work processes in fish processing has not been previously undertaken [2]. This has possibly resulted in underdeveloped occupational hygiene surveillance programs of workers involved in high-risk jobs, accompanied by underdetection and underreporting of adverse health outcomes to fish [3].

Until recently, few exposure assessment techniques existed to directly measure protein allergens of plant or animal origin [4]. Traditional methods relied on total dust measurements, total protein concentrations or functional assays measuring enzyme activity. These approaches are unlikely to be sensitive and specific enough to investigate exposure-response relationships among sensitized workers. The recent introduction of highly sensitive immunoassays for the quantification of allergens in environmental air samples and their efficiency has contributed towards a better understanding of these relationships in population studies, including occupational settings [4, 5]. These studies provide clear evidence for the existence of exposure-response relationships for occupational allergens [6].

Most seafood allergens are very stable proteins that resist the effects of cooking, processing or even digestive processes [7]. Not only are fish allergens heat resistant, their antigenicity may also be enhanced by heat. This has been shown as early as 1921 when Kustner demonstrated that he could tolerate raw haddock but actually developed severe anaphylaxis after ingestion of boiled haddock [8]. In addition, it has been documented in some reports on seafood exposure that the heating process can produce additional allergens which increases the allergenicity of crustacean and fish allergens obtained from certain work processes [1, 9]. Therefore, measuring allergen exposure of seafood workers requires assays that can accurately detect and quantify specific allergens aerosolized in the workplace environment at various stages of the production process. Most previous attempts to measure airborne seafood allergens in the occupational context utilized human sera in radioimmunoassay procedures or monoclonal antibody-based assays [2]. However, these techniques have significant drawbacks, such as lack of specificity, since serum aliquots from different sensitized humans may demonstrate varying degrees of avidity for the different allergens. Monoclonal antibodies have the advantage of being highly specific [10]; however, they only detect particular allergens with identical epitopes, therefore possibly missing other uncharacterized allergens.

The primary aim of this study was to develop sensitive and specific immunological assays to detect and quantify fish antigens in the fish-processing work environment. Polyclonal antibodies were generated in rabbits, since

they produce large amounts of sensitive antisera required for the immunological analysis in environmental surveys [4]. The use of these assays will enable the assessment of exposure-response relationships among jobs with different allergen load and health outcomes, including allergic sensitization and occupational asthma.

## Methods

### *Patient Sera*

Sera were collected from 5 patients with a known history of allergy to fish. All patients demonstrated specific IgE to all analysed fish species (pilchard, anchovy, hake and mackerel) using UniCAP RAST (Pharmacia and Upjohn, Uppsala, Sweden) and immunoblotting.

### *Preparation of Protein Extracts*

Protein extracts were prepared from two fish species, i.e. from canned pilchard (*Sardinops sagax*) and from fresh Cape anchovy (*Engraulis capensis*), from a crustacean species, namely Rock lobster (*Jasus lalandii*), and from fish meal, which consisted of about 80% anchovy and the remainder of the mixture of other fish species. About 10 g of tissue or fish meal was extracted with 100 ml phosphate-buffered saline overnight at 4°C. The extracts were sterile filtered (0.45 µm; Millipore), the protein concentration determined (BCA-protein assay; Pierce) and aliquots stored at -80°C until further use.

### *Production of Polyclonal Antibodies in Rabbits*

For the production of polyclonal antibodies, 500 µg (in 0.5 ml TBS) of each fish protein extract was used to immunize two New Zealand rabbits. The rabbits received subcutaneous injections at several sites, with the extracted proteins in 0.5 ml of complete Freund's adjuvant (Difco). Subsequent immunizations were performed with the same protein concentration in incomplete Freund's adjuvant at 3 and 6 weeks after the first immunization. Blood samples were taken for specific antibody titre analysis and the final bleed was performed at week 8. The Ethics Committee for Animal Experiments of the University of Cape Town approved this study and standard guidelines for laboratory animal care were followed (Institute of Laboratory Animal Resources Commission of Life Sciences NRC, 1996).

### *Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis and Immunoblotting*

To visualize antibody binding to different antigens in the seafood extracts produced in-house, proteins were separated according to their molecular weights using sodium dodecyl sulphate (SDS) gel electrophoresis on gradient gels (4–16%) under reducing conditions [11] and stained with Coomassie Blue. For immunoblotting, about 5 µg of proteins were transferred by electroblotting onto a polyvinylidene difluoride membrane (Hybond-PVDF; Amersham) employing the semidry method, and subsequently, the membranes were blocked with 2% powder skim milk in TBS-T (TBS with 0.05% Tween-20). To analyze patients' IgE reactivity, serum samples (1:30 in TBS) were incubated overnight with the immunoblot. The blots were incubated for 2 h with a mouse anti-human IgE antibody

with an alkaline phosphatase labelled mouse anti-human IgE antibody (1:1,000; Southern Biotechnology), washed and finally incubated using the NBT/BCIP (Roche) substrate.

For analysis of rabbit IgG-binding activity, membranes were incubated with different rabbit sera for 90 min, followed by incubation with goat anti-rabbit IgG antibody with an alkaline phosphatase labelled goat anti-rabbit IgG antibody (1:2,000; Southern Biotechnology), and the colour developed using the NBT/BCIP substrate (Roche). The method for the immunoblot analysis of patient and rabbit sera to recombinant parvalbumin is described in detail elsewhere [12].

#### *Development of ELISA for the Detection of Antigens*

Microtitre plates (Dynex flat bottom 2HB high binding plates) were coated with different concentrations (0.05–100 µg/ml) of the four seafood protein extracts diluted in 100 mM carbonate buffer. After blocking with 2% blocking reagent in TBS-T (powder skim milk in TBS-T), plates were incubated for 90 min at room temperature with the corresponding rabbit antisera diluted 1:8,000, 1:16,000, 1:32,000, 1:64,000, 1:128,000, 1:256,000 and 1:512,000 in TBS. After a wash with TBS-T, goat anti-rabbit IgG antibody (alkaline phosphatase labelled; Jackson Immuno Research Laboratories Inc.) was added (1:10,000 in TBS) for 90 min (room temperature), followed by a subsequent wash in TBS-T and incubation with the substrate (p-nitrophenyl phosphate; Sigma). Results were expressed as optical densities (optical density read at 410 nm). The background reading (negative rabbit serum) was subtracted from all the readings. Based on these results, the optimal antigen coating and corresponding antiserum concentrations were determined.

#### *ELISA Inhibition Assay*

To assess the sensitivity of the rabbit antibodies in detecting antigens, ELISA inhibition assays were performed. Extracts of the two fish species analysed, pilchard and anchovy, were coated on the ELISA plates at the optimized coating concentration, incubated with the optimized antibody dilution and one dilution above and treated as described above. For the analysis of the environmental samples, inhibition curves using known standard concentrations of the inhibitor were used to determine the limit of detection for each specific ELISA plate. After coating and blocking of the ELISA plates, 50 µl of respective extracts were added to the wells in a 1:2 dilution series, followed by 50 µl of rabbit serum. The lowest concentration of antigen detected was calculated from the slope of the inhibition curve. In the environmental samples, the antigen concentration obtained (in micrograms of antigen per millilitres of extract) was adjusted to the mean volume of air flowing through the personal air-sampling pump to gain a concentration (in micrograms of antigen per cubic metres of sampled air).

To evaluate the selectivity of the generated antisera, each ELISA plate was coated with the four generated protein extracts at their optimal antigen concentrations and incubated with the two utilized antisera, anti-pilchard and anti-anchovy, at their optimal dilutions. Antibody reactivity of the rabbit antisera to the extracts against which they were raised as 100% binding and the other reactivities were calculated accordingly.

#### *Collection of Environmental Samples from Different Departments of a Fish-Processing Factory*

The cannery department (handling pilchard), fish meal production (handling mainly anchovy) and the rock lobster department of

a fish-processing factory were included in a preliminary environmental hygiene survey. SKC Aircheck samplers (Model 224-PC-CXR) and Alpha-1 battery-operated air-sampling pumps (Du Pont and Gill Air) running at an average flow rate of 3 litres per minute were used to collect samples over the 8-hour work shift. The aerosols were collected on polytetrafluoroethylene membrane-laminated filters (0.3 µm; QuanTec-Air Inc.) and housed in a Personal Environmental Monitor (PEM<sub>10</sub>; manufactured by MSP Corp., Minneapolis, Minn., USA) attached to the sampling device. The preselector in the PEM<sub>10</sub> has an upper median cut diameter of 10 µm, resulting in the collection of the 'thoracic' fraction (i.e. reaching the tracheo-bronchial and alveolar regions of the respiratory tract), of particulate on the filter, since occupational asthma was the main health outcome of interest. Filters were weighed before and after sampling to determine the amount of aerosols deposited and blanks were collected as controls. The filter pieces were extracted into 0.5 ml phosphate-buffered saline 0.05% Tween-20 overnight on a shaker at 4°C, the supernatants removed and stored at -20°C until further use.

## **Results**

### *Production and Evaluation of Rabbit Antisera Directed Against Fish Protein Extracts*

All immunized rabbits produced high titres of specific antibodies to the respective fish extracts by week 8. The electrophoretic separation of the fish protein extracts demonstrated bands ranging from approximately 10 to over 250 kDa for both the pilchard and anchovy extracts (fig. 1a). Subsequent immunoblotting of the pilchard (canned) and raw anchovy extract was performed to identify antigens with similar molecular weights detected by the different antisera (fig. 1b). The anti-pilchard (canned) serum in lanes a of figure 1b demonstrates strong antibody binding to pilchard proteins with molecular weights of about 12 and 20 kDa, as well as weaker bands in the range of 35–55 kDa. The same serum detected only a 12-kDa band in the anchovy protein extract and no other bands. However, the anti-anchovy serum detected proteins with similar molecular weights in the pilchard extract, but as expected, demonstrated much stronger binding to the corresponding anchovy extract (fig. 1b, lanes b). The anchovy proteins with strong antibody binding have molecular weights of about 12 kDa, as well as weights in the range of 25–45 kDa. None of the two control sera of non-immunized rabbits demonstrated any antigen binding to the pilchard protein extract (fig. 1b, lanes c, d). Subsequently, the binding reactivity of the produced rabbit antisera to fish parvalbumin (using recombinant protein) was compared with sensitized individuals (fig. 2). These immunoblots (IgE binding of patient sera and IgG binding of rabbit sera) are shown in order to identify the major fish allergen recognized by the rabbit sera. All five

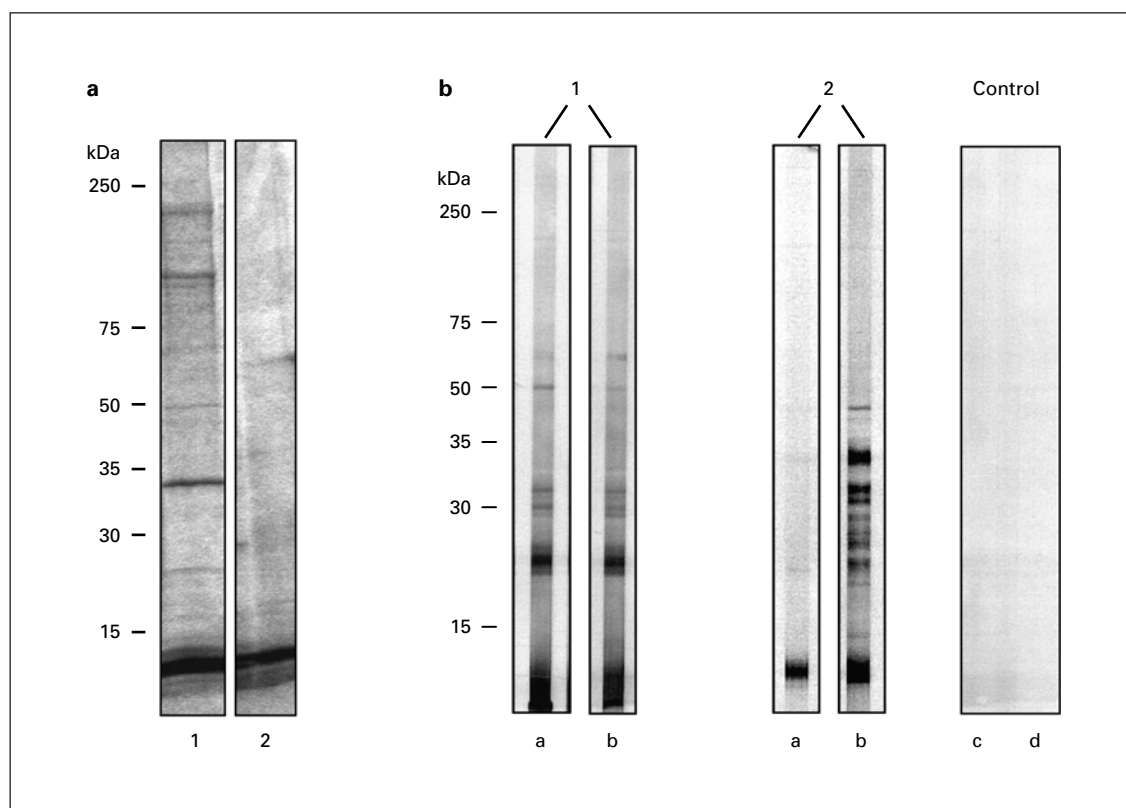


**Fig. 1.** **a** SDS gel electrophoresis of pilchard (1) and anchovy (2) protein extracts, stained with Coomassie Blue. **b** Immunoblotting of antibody reactivity of anti-pilchard rabbit sera (lanes a) and anti-anchovy rabbit sera (lanes b) to the pilchard extract (1) and anchovy extract (2). Binding to the pilchard protein extract of pre-immune rabbit sera is indicated as lanes c and d.

sera of fish-sensitized individuals demonstrated strong IgE antibody binding to the 6.5-kDa band of recombinant fish parvalbumin (fig. 2a). This band was also recognized by the anti-pilchard and anti-anchovy rabbit sera (fig. 2b), but not by the control negative sera (lanes 3, 4). It is of note that the 12-kDa parvalbumin in SDS gel electrophoresis usually appears with a molecular weight of about 6.5 kDa.

#### *Development of ELISA for Quantification of Fish Antigens*

The optimal antigen concentrations established by checkerboard titration for pilchard (canned) and raw anchovy were 1.56 and 3.13  $\mu\text{g/ml}$ , respectively. To assess the sensitivity of the rabbit antibodies generated, ELISA inhibition assays were performed at two antiserum dilutions. The inhibition curve of canned pilchard and raw anchovy are shown in figures 3a and b and demonstrated

very similar slopes for the two antiserum concentrations, suggesting that ELISAs were optimized and the specifications chosen were appropriate. The curves indicated that an antigen concentration as low as 0.5  $\mu\text{g/ml}$  caused inhibition and could be detected by the assay. The standard curves of the inhibition assays demonstrated good consistency with coefficient variation of 7 and 12.7% for pilchard and anchovy, respectively.

Cross-reactions of the rabbit anti-sera were analyzed by ELISA against the two fish extracts as well as fishmeal and rock lobster, thereby demonstrating the specificity for detecting fish antigens (table 1). The interaction of coating antigens, i.e. the pilchard (canned) extract and corresponding rabbit anti-pilchard (canned) serum, was set to 100%, and all other reactivities were calculated accordingly. Anti-pilchard serum demonstrated limited cross-reactivity to anchovy and minimal cross-reactivity to fish meal and rock lobster.

**Fig. 2.** Immunoblotting of antiserum binding to recombinant carp parvalbumin. The known fish allergic patient antisera are labelled from A to E (**a**). The rabbit antisera are labelled from 1 to 4 (**b**); 1 Anti-pilchard (canned), 2 Anti-anchovy (raw), 3, 4 Serum control of two non-immunised rabbits.

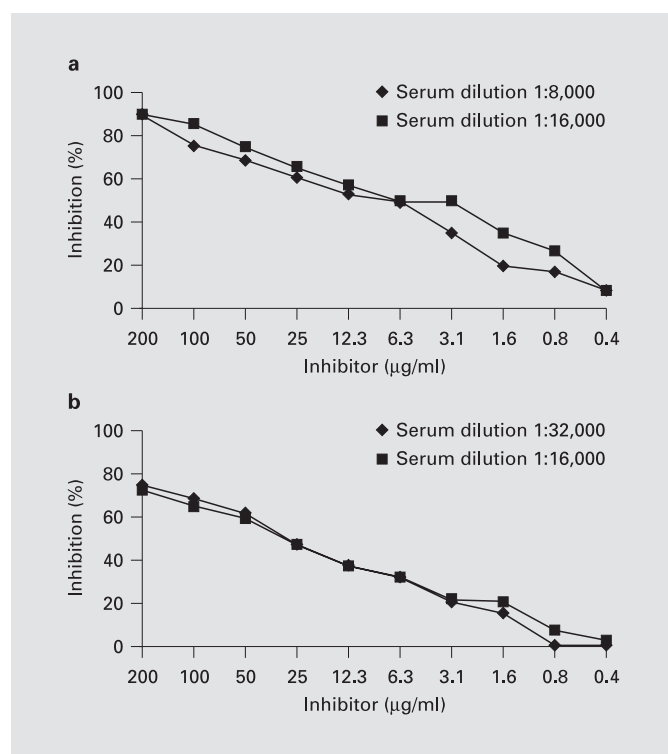
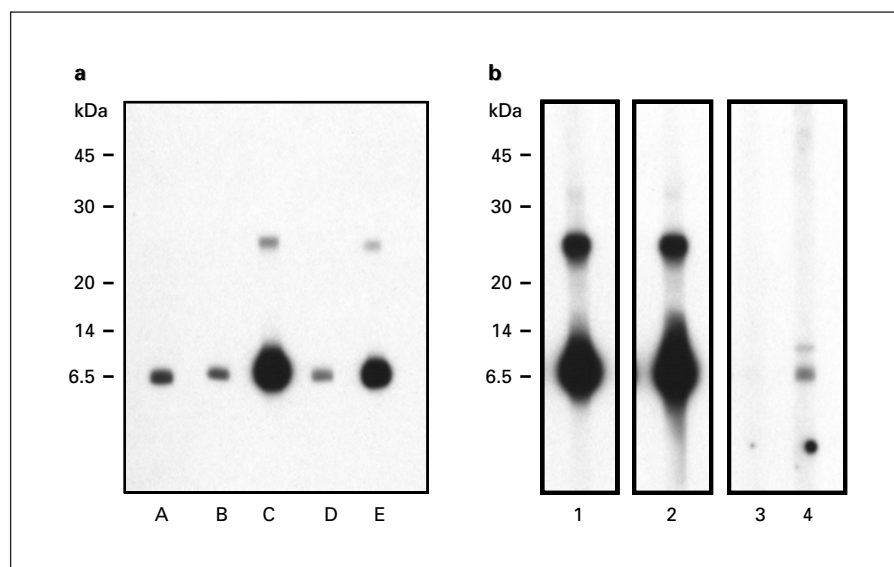


**Fig. 3.** ELISA-inhibition assay for the detection of pilchard (canned) (**a**) and anchovy (**b**) antigens.

**Table 1.** Assessment of reactions of rabbit anti-anchovy and anti-pilchard sera to four different protein extracts

| ELISA coating antigen | Anti-anchovy serum | Anti-pilchard (canned) serum |
|-----------------------|--------------------|------------------------------|
| 1 Anchovy             | <b>100</b>         | 45                           |
| 2 Pilchard (canned)   | 59                 | <b>100</b>                   |
| 3 Fish meal           | 24                 | 19                           |
| 4 Rock lobster        | 1                  | 0                            |

Antibody reaction of the rabbit antisera to the homologue protein extracts were set as 100% binding (bold). Other reactions were calculated in percentage accordingly.

#### *Analysis of Environmental Samples Obtained from the Fish-Processing Factory*

Environmental air samples were collected over an 8-hour shift in a fish factory from the three main departments. To evaluate the newly developed ELISA assay in detail, all 27 environmental samples were analyzed with both pilchard and anchovy fish antisera (table 2). The limits of detection of the assay ranged between 105 and 136 ng/m<sup>3</sup> for batches analyzed on different days. Quantification of antigens in aerosols produced in different exposure settings in the factory demonstrated different exposure levels. As expected, high concentrations of pilchard were detected mainly in the canning department (exhaust box), ranging from 442 to 556 ng/m<sup>3</sup>, whereas high concentrations of anchovy, 422–2,662 ng/m<sup>3</sup>, were

**Table 2.** Analysis of ambient environmental samples in a fish-processing factory for the presence of fish antigens using the ELISA inhibition assay

| Department           | Location      | Sample | Pilchard antigen concentration ng/m <sup>3</sup> | Anchovy antigen concentration ng/m <sup>3</sup> |
|----------------------|---------------|--------|--------------------------------------------------|-------------------------------------------------|
| Fish canning         | autopacker    | 1      | <LOD                                             | <LOD                                            |
|                      | autopacker    | 2      | 148                                              | <LOD                                            |
|                      | autopacker    | 3      | <LOD                                             | <LOD                                            |
|                      | control       | 4      | <LOD                                             | <LOD                                            |
|                      | control       | 5      | <LOD                                             | <LOD                                            |
|                      | control       | 6      | <LOD                                             | <LOD                                            |
|                      | exhaust box   | 7      | 442                                              | 105                                             |
|                      | exhaust box   | 8      | 488                                              | 488                                             |
|                      | exhaust box   | 9      | 556                                              | 554                                             |
|                      | sorting table | 10     | <LOD                                             | <LOD                                            |
|                      | sorting table | 11     | 138                                              | <LOD                                            |
|                      | sorting table | 12     | 156                                              | <LOD                                            |
|                      | control       | 13     | <LOD                                             | <LOD                                            |
| Fish meal processing | bagging       | 14     | 312                                              | 2,662                                           |
|                      | bagging       | 15     | 190                                              | 2,390                                           |
|                      | bagging       | 16     | 136                                              | 422                                             |
|                      | control       | 17     | <LOD                                             | <LOD                                            |
| Lobster processing   | cooker        | 18     | <LOD                                             | <LOD                                            |
|                      | control       | 19     | <LOD                                             | <LOD                                            |
|                      | control       | 20     | <LOD                                             | <LOD                                            |
|                      | cooker        | 21     | <LOD                                             | <LOD                                            |
|                      | packaging     | 22     | <LOD                                             | <LOD                                            |
|                      | washing       | 23     | <LOD                                             | <LOD                                            |
|                      | packaging     | 24     | <LOD                                             | <LOD                                            |
|                      | control       | 25     | <LOD                                             | <LOD                                            |
|                      | packaging     | 26     | <LOD                                             | <LOD                                            |
|                      | cooker        | 27     | <LOD                                             | <LOD                                            |

Limit of detection (LOD) was 105 ng/m<sup>3</sup>.

detected in samples obtained from fish meal bagging. Samples 7–9 showed unexpected elevated levels of anchovy, even though this fish species was not generally processed in the canning department. Fish antigen could not be detected within the detection limits of this assay in the lobster department, nor in all eight control blanks (unused filters).

## Discussion

IgE-mediated hypersensitivity to fish is a clinically significant problem in many countries where fishing plays a substantial role in the domestic and occupational life of

communities [13]. Although most allergic reactions to fish are caused by ingestion, occupational exposures to seafood allergens due to inhalation have also been positively correlated with allergic respiratory symptoms [1, 2]. A recent study conducted in South Africa among seafood-processing workplaces demonstrated that although fish was the most commonly processed seafood in the Western Cape province (76% of all workplaces), this was accompanied by a relatively low prevalence of asthmatic and other allergic symptoms reported by employers. It suggested a possible underdetection of adverse allergic outcomes attributed to the lack of appropriate monitoring systems for in vivo testing of exposed workers and quantification of environmental allergen exposure in the workplace environment [3].

Most studies that have addressed occupational exposure to seafood antigens have mainly concentrated on crustacean (crab and shrimp) and very scantily on fish (salmon, whiff megrim and hake) [10, 14–20]. Either pooled human sera from patients known to be allergic to the particular seafood agent or monoclonal antibodies to a major antigen previously identified were used for the detection of antigens. Monoclonal antibodies have the advantage of being highly specific [10] but only detect particular allergens with identical epitopes, therefore probably missing other uncharacterized allergens. Sera from fish-allergic patients were not considered in this study, since individual antigen-binding profiles can show marked differences and human serum has to be available in large quantities to enable detailed analyses of numerous environmental samples [4]. In this current study, polyclonal antibodies from rabbits were used for the development of a sensitive immunological assay to detect fish antigens in the occupational environment. The rabbit antisera generated in this study showed high titres of specific antibodies and detected pilchard and anchovy antigens at a very low concentration. To demonstrate the binding similarities of the generated polyclonal rabbit antisera with sera of fish-allergic patients, immunoblots were compared. Both serum sources demonstrated very similar and strong binding patterns of rabbit IgG and human IgE to the recombinant parvalbumin from carp (rCyp c 1.01) [12], increasing the confidence that the generated sera are able to detect the major fish allergen.

The cross-reactions of the produced rabbit antisera were further assessed by ELISA and showed significant degrees of specificity for the extracts against which they had been generated. To explain the limited degree of cross-reaction between the two generated anti-fish antisera, immunoblots of both protein extracts were com-

pared. Both rabbit antisera demonstrated the strongest binding to a protein with a molecular weight below 15 kDa, most probably representing parvalbumin. Additional bands at about 20, 30 and 50 kDa are observed, which appear to be detected by both antisera in the pilchard (canned) extract, resulting most probably from non-denatured parvalbumin dimers as previously documented [12]. However, also species-specific bands were recognized, in particular those of the anti-pilchard serum in the pilchard extract, ranging from 20 to 50 kDa. These bands were not detected in the anchovy extract. This indicates that the anti-pilchard serum is more specific than the anti-anchovy serum and that the cross-reaction observed against the anchovy extract (table 1) is mainly due to the cross-reaction to parvalbumin. Parvalbumins are small calcium-binding proteins which are the main allergens characterized in fish [12]. They are very heat stable and of the very first food allergens that have been identified in patients with fish allergy [21]. However, there was no antibody binding to the rock lobster extract, again supporting the specificity of the antisera produced.

Nevertheless, additional allergens have recently been described. Dory et al. [22] have demonstrated IgE-reactive proteins over a wide molecular weight range (13–130 kDa) depending on the storage conditions of codfish. In a recent study by Das Dores et al. [23], a novel 41-kDa allergen was identified in codfish. It has also been demonstrated that heat treatment of fish can produce novel allergens enhancing the allergenicity of the fish aerosols produced during cooking [8, 9]. The qualitatively different antigen binding profiles of the generated rabbit antisera, as compared with patient sera (sensitization mainly by ingestion), have important implications for the detection of aerosolized fish allergens, since additional allergens other than the well-described parvalbumin may be involved. Hence, utilization of rabbit antiserum, which detects the major fish allergens in addition to other fish antigens, is considered to be more advantageous for analysis of environmental samples than patient sera or even monoclonal antibodies, which mainly detect one major allergen. This should be viewed in the context of the molecular nature of aerosolized fish and crustacean allergens which predominate in the occupational environment, which is still unknown and has yet to be identified [2].

Using polyclonal rabbit antisera has demonstrated its usefulness in detecting fish antigens in aerosols generated in a factory processing pilchard and anchovy. The limit of detection was as low as 105 ng/m<sup>3</sup> for both fish types. The sensitivity for detection of pilchard and anchovy antigens in ambient environmental samples is within simi-

lar detection assay capabilities developed for salmon (100 ng/m<sup>3</sup>) [19]. However, this new assay seems somewhat less sensitive than previously reported assays for antigen detection of aerosolized whiff megrim/hake (2 ng/m<sup>3</sup>) and crustaceans such as the common crab (1 ng/m<sup>3</sup>) and snow-crab (53 ng/m<sup>3</sup>), but more sensitive than the recently reported assay for scampi (451 ng/m<sup>3</sup>) [10, 15, 17, 20].

These newly developed assays were able to detect different antigen exposure levels in various exposure settings (fish canning, fish meal production and lobster processing). Elevated levels of pilchard antigens were detected in the fish canning department, and much higher levels of anchovy than pilchard antigens were detected in the fish meal department. In addition, neither fish antigen could be detected in the lobster department. These findings correctly reflect the main seafood type being processed in these departments. The results also suggest that fish antigen exposure is very high in the fish meal production department and is an area for future detailed investigation to enable correlation with allergic health outcomes among exposed workers. These relationships could ultimately assist in the development of effective exposure prevention strategies in the fish-processing industry [24].

In conclusion, this study has illustrated the development of an immunochemical analytical technique for the determination of ambient fish (pilchard and anchovy) antigen concentrations and illustrated the good performance of the assay on environmental samples obtained from a fish-processing factory. This assay is currently being used in a survey of two major fish-processing plants to establish dose-response relationships between fish allergen exposure and allergic sensitization and occupational asthma.

## Acknowledgements

We would like to thank P.C. Potter of the Allergy Unit for providing the patient sera and B. Fenimore and A. Elliott for their technical support. The contribution of S. Mbuli, R. Beukes, E. Rusford, D. George and S. Hassan from the Department of Health Sciences at Peninsula Technikon in the collection of the environmental samples is also acknowledged. The analysis of recombinant parvalbumin was conducted in S. Spitzauer's and R. Valenta's laboratory who also provided financial support for I.S. This publication was supported by research grants from the Medical Research Council of South Africa, R01 Grant No. F002304 from NIOSH, CDC, USA, and grant F01804 from the Austrian Science Fund. Its contents are the sole responsibility of the authors and do not necessarily reflect the official views of these agencies.

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