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Publisher: Taylor & Francis

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Food Additives & Contaminants: Part A

Publication details, including instructions for authors and subscription information:

<http://www.tandfonline.com/loi/tfac20>

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Published online: 26 Jul 2013.

To cite this article: Food Additives & Contaminants: Part A (2013): Detection of allergenic parvalbumin of Atlantic and Pacific herrings in fish products by PCR, Food Additives & Contaminants: Part A, DOI: 10.1080/19440049.2013.817024

To link to this article: <http://dx.doi.org/10.1080/19440049.2013.817024>

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Detection of allergenic parvalbumin of Atlantic and Pacific herrings in fish products by PCR

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(Received 7 March 2013; final version received 14 June 2013)

The conventional polymerase chain reaction (PCR) method to detect the major allergenic protein parvalbumin beta 2 of Atlantic herring (*Clupea harengus*) and Pacific herring (*Clupea pallasii*) was developed. The specific set of primers for the amplification of the partial genomic sequence of the *pvalb 2* gene encoding the main fish allergen of both herrings was designed and applied to the investigation of 24 commercial fish products. The targeted amplicon size was 189 bp of *pvalb 2* gene of Atlantic herring and Pacific herring. As the internal amplification control, the DNA of *18S rRNA* gene for eukaryotes (141 bp) was successfully used. The specificity of designed primer pair using 26 various fish species was assessed. The intrinsic detection limit was 10 pg μl^{-1} of the present specific DNA. Atlantic herring or Pacific herring allergenic parvalbumins were detected in 22 investigated fish products in conformity with the package declaration. Two fish products were negative in spite of the declaration. The proposed PCR method is specific enough and can be used for the detection of Atlantic and Pacific herrings' major allergen parvalbumin beta 2 in fish food products.

Keywords: fish allergens; DNA; food allergens; canned products; PCR

Introduction

Food allergies have become a serious health problem all over the world. In industrialised countries 1–3% of adults and up to 8% of children and adolescents are affected by food allergies (Ehlert et al. 2008). Fish is the third most frequent allergen after eggs and cow's milk in parts of Europe (Pascual et al. 2008). The major fish allergens are parvalbumin proteins (Lim et al. 2008). There are two distinct phylogenetic lineages of parvalbumins, alpha and beta. Most allergic reactions to fish are caused by beta-parvalbumins (Ma et al. 2008). According to Kobayashi et al. (2006) parvalbumins are located in white fish muscle primarily with its content from four to eight times higher than in dark fish muscle. It is estimated that 50% of individuals allergic to some type of fish are at risk of reacting to second species; those allergic to some type of shellfish carry a risk of 75% because of greater similarity among tropomyosins than parvalbumins (Tsabouri et al. 2012).

Accurate and complete food labelling is important for conscious and informed nutrition to prevent the health risks as allergies are considered. Allergens' labelling legislation can improve a consumer's protection because the only possibility of preventing an allergic reaction is the strict avoidance of the consumption of such allergenic food. European Union (EU) Directives 2000/13/EC and 2003/89/EC require the declaration of the exact allergenic food as celery, mustard, sesame seed, tree nuts, peanuts, milk, eggs, fish, crustaceans, soybeans and sulphites. Later, Commission Directive 2006/142/EC announced, moreover, the inclusion

of lupine and molluscs to the previous list of allergens and they are officially recognised as allergens by Directive 2007/68/EC. Consequently, accurate and reliable labelling practices are required to enable the allergic consumer to prevent the health difficulties of retail food products. Several methods have been published for the detection of food allergens such as enzyme-linked immunoabsorbant assay (ELISA), PCR, real-time PCR, lateral-flow immunochromatographic assay (LFA), dipsticks, microarrays or biosensors or DNA application of multi-analyte profiling (Poms et al. 2004; Schubert-Ulrich et al. 2009; Hildebrandt 2010). Choi and Hong (2007) reported on the possibility of the quick detection of parvalbumin allergen of Pacific mackerel (*Scomber japonicus*) in food by PCR. Rehbein and Lopata (2011) focused on reverse-transcription PCR using specific primers to detect the presence of parvalbumin in different tissues of three sturgeon species (*Acipenser baeri*, *A. gueldenstaedtii*, *A. ruthenus*). Mislabelling has also been detected in fish-based dietetic complements by DNA-based molecular methods (Ardura et al. 2011).

The purpose of this work was to develop a quick and cheap conventional PCR method for the detection of the major allergenic protein parvalbumin beta 2 of Atlantic and Pacific herrings (*Clupea harengus* and *Clupea pallasii*) via targeting segments of genes encoding the protein markers in fish food products to avoid health risks of allergic consumers. The method was verified by the monitoring of these allergens in total of 24 food products. Twenty-two products were declared as Atlantic herring and two fish products as Pacific herring.

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Materials and methods

Sample preparation and DNA isolation

The samples of canned fish meat (muscle pieces) were first immersed in water for 1 h. Then DNA isolation from the muscles (two isolations from different parts of washed fish product) was performed using commercial DNeasy Mericon Food Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

PCR reagents and protocol

For the detection of Atlantic and Pacific herrings' parvalbumin one set of primers amplifying partial sequences of *pvalb* 2 gene encoding parvalbumin beta 2 protein was designed (GenBank Accession Number FN545288).

A PCR system for the detection of the *pvalb* 2 gene (189 bp) and *18S rRNA* gene for eukaryotes (141 bp), according to Fajardo et al. (2008) as the amplification control in canned products, was constructed (Figures 2(a) and (b)). The primers CluHaPaF5'-ccgctgatgatgtgaagaag-3' CluHaPaR and 5'-gcaggaacagcctgagagag-3' (product size = 189 bp) were designed using program Primer3. The reaction mix contained 2 × 25 µl of Multiplex PCR Master Mix (Qiagen), 5 µl primer mix (two primer pairs of final 1 µmol concentration – for the amplification of *pvalb* 2 gene and *18S rRNA* gene for eukaryotes DNA), 15 µl RNase-free water and 5 µl DNA template. The total volume of the reaction mix was 50 µl. The negative control was composed of 5 µl RNase-free water instead of DNA, 5 µl of defined DNA were used as the positive control. The PCR program was modified according to the experimental studies and consisted of an initial denaturation step at 95°C for 9 min, followed by 35 cycles composed of denaturation step at 95°C for 30 s, annealing at 66°C for 30 s and extension at 72°C for 60 s, and an final extension step at 72°C for 5 min.

Separation and visualisation of PCR products

Separation and visualisation of the PCR amplicons from canned products were carried out using horizontal electrophoresis in 2.5% agarose gel on a 0.5 × TBE containing 0.1 µl ml⁻¹ GelGreen stain. A 50 bp and 100 bp ladder (Qiagen) were used as the markers. The amplification fragments were analysed under ultraviolet (UV) light using an Ultraviolet Transluminator (Spectroline, Westbury, NY, USA).

Specificity of designed primers

The potential cross-reactions were tested on 26 samples of muscles of various whole fresh fish species morphologically confirmed as: *Oncorhynchus gorbuscha*, *Salmo salar*, *Pangasius hypophthalmus*, *Salmo gairdneri*, *Mullus surmuletus*, *Argentina silus*, *Scomber scombrus*, *Oreochromis niloticus*, *Sparus auratus*, *Platichthys flesus*, *Sarda sarda*, *Thunnus albacares*, *Thunnus thynnus* 1, *Thunnus*

alalunga, *Thunnus pelamis*, *Thunnus thynnus* 2, *Gadus morhua*, *Merluccius merluccius*, *Gadus merlangus*, *Theragra chalcogramma*, *Pollachius virens*, *Sardina pilchardus*, *Sardinops sagax*, *Sardinella gibbosa*, *Sprattus sprattus*, and *Clupea pallasii* (Figures 1(a) and (b)).

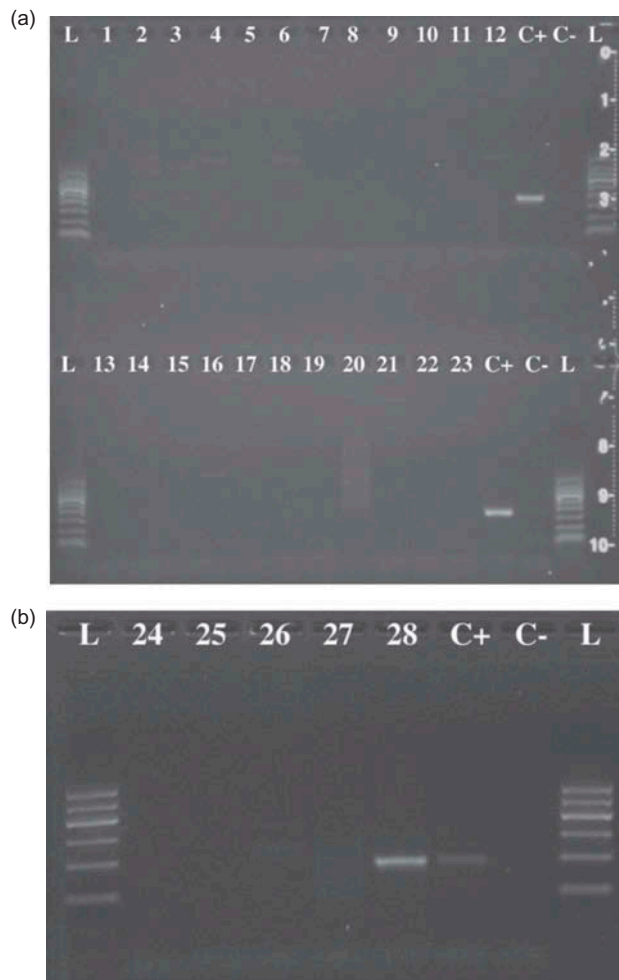


Figure 1. (a) Specificity of the method (189 bp) – all samples were prepared from morphologically confirmed fresh whole fish. Lane L, ladder/marker 50 bp; lane 1, *Oncorhynchus gorbuscha*; lane 2, *Salmo salar*; lane 3, *Pangasius hypophthalmus*; lane 4, *Salmo gairdneri*; lane 5, *Mullus surmuletus*; lane 6, *Argentina silus*; lane 7, *Scomber scombrus*; lane 8, *Oreochromis niloticus*; lane 9, *Sparus auratus*; lane 10, *Platichthys flesus*; lane 11, *Sarda sarda*; lane 12, *Thunnus albacares*; lane 13, *Thunnus thynnus*; lane 14, *Thunnus alalunga*; lane 15, *Thunnus pelamis*; lane 16, *Thunnus thynnus* 2; lane 17, *Gadus morhua*; lane 18, *Merluccius merluccius*; lane 19, *Gadus merlangus*; lane 20, *Theragra chalcogramma*; lane 21, *Pollachius virens*; lane 22, *Sardina pilchardus*; lane 23, *Sardinops sagax*; C+, positive control (fresh whole fish of *Clupea harengus* confirmed by barcoding); and C–, negative control. (b) Specificity of the method (189 bp). Closely related fish species. Lane L, ladder/marker 100 bp; lane 24, *Sardinops sagax*; lane 25, *Sardina pilchardus*; lane 26, *Sprattus sprattus*; lane 27, *Sardinella gibbosa*; lane 28, *Clupea pallasii*; C+, positive control (fresh whole fish of *Clupea harengus* confirmed by barcoding); and C–, negative control.

Amplification control

To control the efficiency of the DNA isolation, a primer pair for universal detection of the DNA derived from eukaryotes was used. The primer pair detects the DNA of *18S rRNA* gene for eukaryotes (141 bp) and enables the detection of the eukaryotes' DNA in the investigated sample, and controls the correct DNA isolation from the targeted sample (Fajardo et al. 2008).

PCR product sequencing

The primers for *Clupea harengus* and *Clupea pallasii* were designed based on the sequences AP009133 and AP009134. F primer: CAACTAATCACAAAGATATTGGTACC, R primer: AAGAATTGGGTCTCCCCCT – product size 665 bp (Lavoue et al. 2007). The reaction mixture (20 µl) contained 10 µl PPP Master Mix (Top-Bio, s.r.o., Prague, Czech Republic), 2 µl solution of primers (final concentration of each primer was 400 nM), 6 µl of water and 2 µl of sample. The amplifications were run in the T100 Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using the following programme: initial denaturation step at 94°C for 1 min followed by 35 cycles at 94°C for 15 s, 58°C for 15 s, and 72°C for 60 s, and closed by the extension step at 72°C for 7 min. The amplified DNA was determined by electrophoresis in 1% agarose gel. The obtained product was cleaned on the NucleoSpin Extract II column (Macherey-Nagel GmbH, Düren, Germany) and send to Generi Biotech s.r.o. (Hradec Kralove, Czech Republic) for both strained sequencing. Both species (*C. harengus* and *C. pallasii*) were confirmed.

Results and discussion

In this study the conventional PCR for the amplification of specific sequence of *parvalbumin beta 2* gene (189 bp) encoding the parvalbumin beta 2 protein, the major allergen of Atlantic herring (*C. harengus*) was used. While testing the specificity of designed primer pair for *parvalbumin beta 2* gene on a spectrum of 26 fish species a strong cross-reaction to closely related Pacific herring (*C. pallasii*) was detected (Figures 1(a) and (b)). Other taxonomically close species as *Sprattus sprattus*, *Sardina pilchardus*, *Sardinella gibbosa* and *Sardinops sagax* were negative. This PCR method was applied to the analysis of 24 commercial fish products from the retail market, 22 of them with the declaration of Atlantic herring and two products with the declaration of Pacific herring. In Central Europe Atlantic herring is most often used in fish food products in the retail market.

The specific DNA was detected in 22 samples. Two fish product samples (numbers 21 and 22) were negative in spite of the Atlantic herring declaration (Figure 2(a)). Probably another fish species can be present in these two fish products because DNA is present in these samples – the internal amplification control that used the *18S rRNA*

gene for eukaryotes by Fajardo et al. (2008) was positive in all 24 samples (Figures 2(a) and (b)). This method cannot differentiate between these two taxonomically closely related herring species. The species specificity of *C. harengus* and *C. pallasii* was confirmed by sequencing. In the region of mitochondrial *cytochrome c oxidase subunit I* gene (COI) were designed primers for fish DNA barcoding based on the sequences of AP009133 and AP009134 (Ward et al. 2005).

The sensitivity of the method was tested by preparing model samples with the various DNA concentration as: 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg and 1 fg. The obtained detection limit was as low as 10 pg µl⁻¹ of both *C. harengus* and *C. pallasii* DNA (Figure 3). Choi and Hong (2007) developed the conventional PCR test to detect the allergenic parvalbumin of *Scomber japonicus* with the level of sensitivity of 5 ng of mackerel DNA in 50 ng of surimi DNA. Catanese et al. (2010) developed the multiplex PCR for the species authentication of mackerels of genus *Scomber* in processed food (*S. japonicus*, *S. scombrus*, *S. australasicus*, *S. colias*). They concentrated on the species-specific identification similarly like Espineira et al. (2009) who used the forensically informative nucleotide sequencing (FINS) technique to prevent the species substitution in seafood products based on the use of the *cytochrome b* gene as a molecular marker. This method is suitable to assess the correct seafood traceability. Kuehn et al. (2012) compared the quantitative ELISA with the conventional PCR method and its suitability to detect fish allergens. They concluded that both the protein (from 1 to 15 ppm in fresh fish and from 30 to 170 ppm in processed fish) and the DNA-based method have sufficient sensitivity (0.2–10 ng of fish DNA) to protect fish proteins-allergic consumers. Rehbein et al. (2013) studied the allergenic proteins parvalbumins of *C. harengus* by HPLC-MS and obtained three cross-reactive isoforms which were stable against heat or acid treatment. Parvalbumins share various degrees of amino acid homologies ranging from 60% to 80%, which probably explains variable degrees of clinical cross-reactivity in children with fish allergy (Tsabouri et al. 2012). And these homologies are probably the reason why we were not able to distinguish between two closely related herrings. In spite of the fact that we were not able to differentiate Atlantic and Pacific herrings, the gender-specific procedure can be applied to the authorities responsible for food control of fish food products labelling and package declaration and can help the better protection of fish-allergic consumers.

Conclusion

The proposed PCR method is specific and sensitive enough and can be used as a fast, simple, 1-day procedure for the detection of *pvalb 2* gene marker encoding the parvalbumin beta 2 protein of two closely related herring

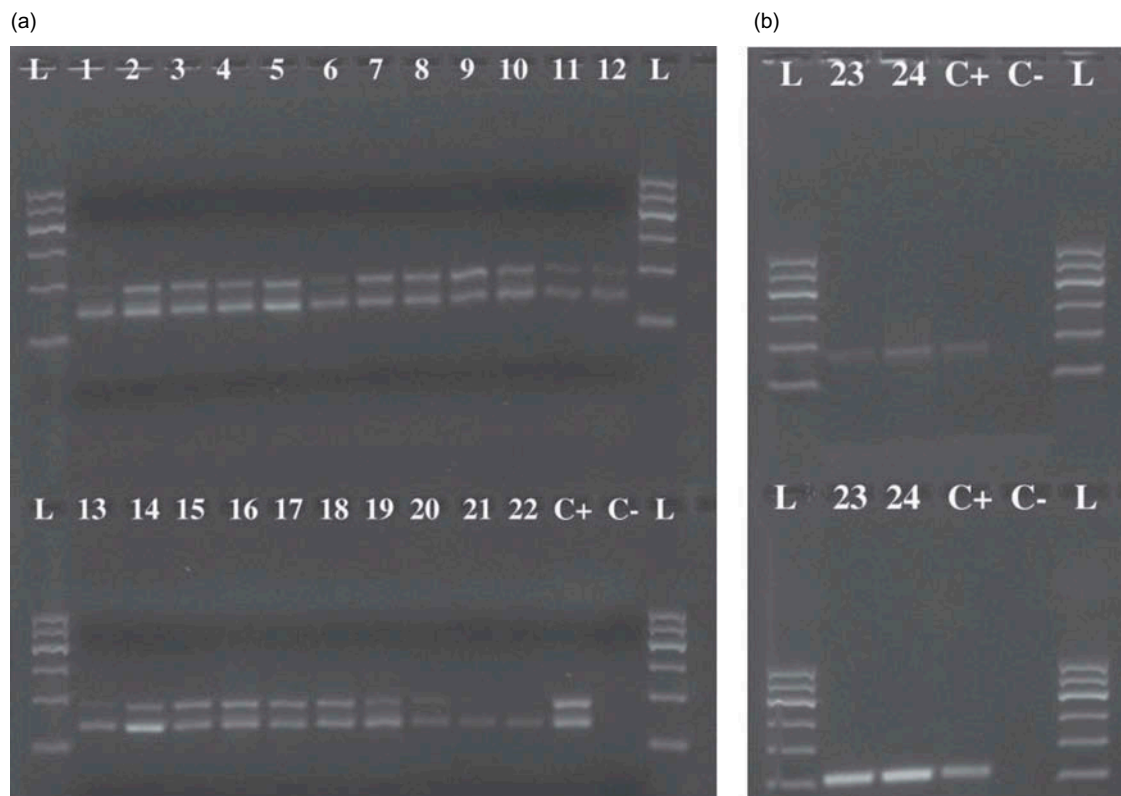


Figure 2. (a) Fish food products with the Atlantic herring declaration. Lane L, ladder/marker 100 bp; lane 1, herring fillets in tomato sauce; lane 2, smoked herring in oil; lane 3, herring in oil (slices); lane 4, herring fillets in gourmet sauce with vegetables; lane 5, herring fillets in mustard sauce; lane 6, herring with pepper; lane 7, herring fillets in mushroom cream; lane 8, herring fillets in pepper cream; lane 9, herring fillets in mustard cream 1; lane 10, herring fillets in tomato sauce 1; lane 11, herring fillets in mustard and egg cream; lane 12, herring fillets in tomato cream; lane 13, Gdansk herring; lane 14, herring fillets in vegetable oil; lane 15, herring fillets in dill and herbal cream; lane 16, herring in tomato sauce (slices); lane 17, herring fillets in gourmet sauce with vegetables; lane 18, herring fillets in mustard cream 2; lane 19, herring fillets in spicy vegetable oil; lane 20, herring fillets in tomato sauce 2; lane 21, herring fillets in tomato sauce 3; lane 22, Rolled herring fillets with pepper and onion; C+, positive control (confirmed fresh whole fish of *Clupea harengus* by barcoding); C-, negative control; lines 1–22 and C+ always contain PCR product of *18S rRNA* eukaryotes gene – 141 bp (Fajardo et al. 2008). (b) Fish food products with Pacific herring declaration. Lane L, ladder/marker 100 bp; lane 23, Acana product 1; lane 24, Acana product 2; C+, positive control (confirmed fresh whole fish of *Clupea pallasii* by barcoding); C-, negative control; lower part of the picture – product of *18S rRNA* eukaryotes gene – 141 bp (Fajardo et al. 2008).

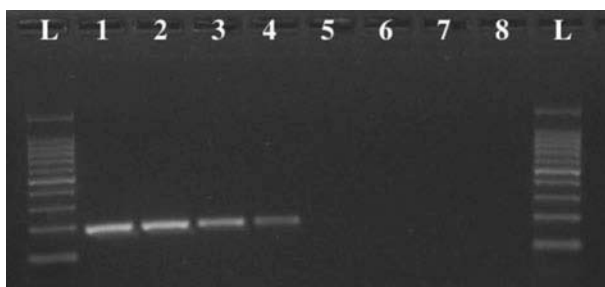


Figure 3. Limit of detection (LOD). Lane L, ladder/marker 100 bp; lane 1, DNA concentration, 10 ng μl^{-1} ; lane 2, 1 ng μl^{-1} ; lane 3, 100 pg μl^{-1} ; lane 4, 10 pg μl^{-1} ; lane 5, 1 pg μl^{-1} ; lane 6, 100 fg μl^{-1} ; lane 7, 10 fg μl^{-1} ; and lane 8, 1 fg μl^{-1} .

species (*C. harengus* and *C. pallasii*). The results demonstrate the applicability of this method. It can become a useful tool for the cheap routine food safety control of

declared allergens of Atlantic and Pacific herrings in food and fish products.

Acknowledgements

This research was financially supported by the Ministry of Agriculture of the Czech Republic (Grant Number MZe 00027 16202).

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