

Detection of fish allergen by droplet digital PCR

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Abstract

Fish is one of fourteen allergens that must be highlighted on the label within the ingredients list. The European regulation is very restrictive to allergens with zero tolerance. Therefore, it is important to establish sensitive and specific methods for detecting fish allergen. Applicability to detect and quantify fish allergen by droplet digital polymerase chain reaction (ddPCR) has been evaluated in this work. Genomic DNA of three species belonging to the most common fish families were analyzed. PCR primers were designed to amplify a 166 bp region of the 18S rRNA gene. Comparative studies were performed to establish the optimal primer and probe concentrations. Annealing temperature was determined by using thermal gradient. The results have shown good applicability of the optimized 18S rRNA gene-method to detect and quantify small amounts of the target in samples analyzed. However, validation studies are needed in order to apply ddPCR technology for routine allergens analysis.

Introduction

In human diet, fish is a valuable source of essential amino acids, polyunsaturated fatty acids, and lipid-soluble vitamins.

Although its nutritional benefit, fish is also one of the most important triggers of severe food-allergic reactions. Fish allergy is a pathophysiological, IgE-mediated immune response to specific fish proteins. The major fish allergens are the parvalbumins, that share the identity of sequences between 61-93% among the various species. There are numerous documented cases of allergy to one specific or all species of fish. The great diversity of species consumed, often regional and the great heterogeneity of the methods of diagnosis, makes difficult to understand which is the threshold limit over which to protect the consumer.

Numerous studies have estimated the prevalence of fish allergy, usually ranging from 0.3-0.5% (Koppelman and Hefle Sue, 2006).

The symptoms of this allergy appear within 60 minutes of exposure and include acute and generalized urticaria, nausea, vomiting, abdominal cramps, diarrhea, wheezing and asthma. In the most severe cases, anaphylaxis shocks can potentially life threatening.

Small amounts such as milligrams of protein can immediately lead to allergic reactions. The only proven and effective treatment is to conduct a diet free of fish and their derivatives. To protect allergic people, European legislation demands the obligatory labelling of 14 allergenic ingredients, including fish, by Annex II of Regulation (EU) N°1169/2011 (Regulation (EU) N°1169/2011). Current European legislation does not define allergen threshold values also known as levels action. However, some products on the market could contain traces of allergens due to cross-contaminations during the food manufacturing processes. Issues related to extractability and matrix effects remain a permanent challenge. Therefore, accurate, sensitive and fast detection methods that permit the direct recognition of allergens in food samples are highly recommendable.

For detecting fish allergen, the most common techniques used are protein-based and DNA-based methods. The first as the enzyme-linked immunosorbent assay (ELISA) is a system based on the detection of the major fish allergenic protein, parvalbumin (Houhoula *et al.*, 2015). The second such as Real-Time quantitative PCR (qPCR), is a specific, sensitive approach for detecting and for quantification of DNA. The main advantage of the PCR methods is that they are not sensitive to factors such as pressure and heat, which can produce changes in the protein conformation and can inhibit its detection.

Some of the Real-Time PCR limitation, including certified standard and inhibition factor, have been proposed to overcome using digital PCR.

An approach, named droplet digital PCR (ddPCR), combines partitioning of the PCR mix test into several thousands or millions of individual droplets, in a water-oil emulsion. After end-point PCR amplification, each partition is scrutinized and defined as positive (presence of PCR product) or negative (absence of PCR product), and it is then calculated directly from the ratio of positive to total partitions, using binomial Poisson statistics (Morrisette *et al.*, 2013). Use of ddPCR provides higher sensitivity, more accurate data in low quantities of target DNA and more tolerance to inhibitors. ddPCR has already been applied for monitoring transgenic presence in complex food and feed matrices. The higher

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Key words: Food allergy reaction, Fish allergen, Droplet digital PCR.

Contributions: the authors contributed equally.

Conflict of interest: the authors declare no potential conflict of interest.

Funding: none.

Received for publication: 28 December 2017.

Revision received: 21 May 2018.

Accepted for publication: 30 August 2018.

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Italian Journal of Food Safety 2018; 7:7264

doi:10.4081/ijfs.2018.7264

sensitivity of ddPCR has been reported for examining population dynamics of bacteria in soil and waters (Kim *et al.*, 2014; Cao *et al.*, 2015), in medicine to quantify HIV virus (Strain *et al.*, 2013), to detect tumor (Shoda *et al.*, 2017), environment (Hyun-Gwan *et al.*, 2017) and traceability of products (Scollo *et al.*, 2016; Junan *et al.*, 2017).

To date the absence of fish allergens certified reference materials and the presence of inhibitors in food complex matrix suggest that ddPCR could be used as suitable technologies for allergens analysis. The aim of this work was to study the applicability of ddPCR on fish samples.

Materials and Methods

DNA from species: *Salmo salar*, *Gadus morhua*, *Scomber scombrus*, *Todarodes sagittatus* and *Penaeus kerathurus* was extracted from 200 mg of fresh or frozen tissue of whole-animals collected from local market. Species identification was based on labelling standards of regulation (CE) 11 december 2013, n. 1379. A modified cetyltrimmonium bromide (CTAB) protocol according to the report of Tezlaff *et al.* (2017) was used. Quantity and quality DNA were estimated at $\lambda = 260$ nm and by the ratio DO 260/DO280 using Synergy multi-mode microplate reader (BioTeK Instrument, Inc., Vermont, USA). DNA solution was frozen at

-20°C until use it for downstream reactions.

A ddPCR protocol targeting 18S rDNA was used to develop detection of fish allergen according to the report of Herrero *et al.* (2014). AJ 427629.1 *Salmo salar* 18S rRNA gene sequence was used as target for the design of primers P forw: GTACACACG GCCGGTACAGT and P rev: CATGGGTT TTGGGTCTGGATAA 18.S rDNA sequences alignment was performed to identify highly conserved common region to the species of fish, more often used in fish food using Bioedit Software (<http://www.mbio.ncsu.edu/BioEdit/BioEdit.html> v7.0.5). Primer were drawn using Primer 3 plus software (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus> v.0.4.0). The probe was the same used in the study of Herrero *et al.* (2014) with labelling the 5'-terminal nucleotides with 6- carboxy- fluoresceine (FAM) reporter dye and the 3'terminal nucleotide with non-fluorescent quencher (Sigma-Aldrich).

Primers and probe were first tested in Real-time PCR (10 min at 95°C, followed by 35 cycles of 15 s at 95°C, and 1 min at 60°C). The reactions were carried out in 20 µL containing: 10 µL of 1×TaqMan Universal PCR Master MixII (Thermo Fisher scientific), 900 nM of each primer, 250 nM of probe, 6'-carboxyfluorescein (6-FAM) and on 3'-end with a not fluorescent quencher; DNA and RNA free water. qPCR reactions were performed in triplicate and were carried out by using ABI PRISM 7900 HT Sequence Detection system.

The reference material SureFood® QUANTARD 40 (R-biopharm) containing fish and all potentially allergenic food ingredients (except for mollusks, lactose and sulphur dioxide) in a concentration of 40 ppm as standard reference fish material for all qPCR runs was also used. All ddPCR experiments were carried out by using QX200 Droplet Digital PCR (ddPCR™) System – Bio-Rad. The method was optimized using different concentrations of primers (900nM and 600 nM) and probe (250nM and 160nM). The annealing temperature for each assay was determined by using thermal gradient from 55°C to 65°C and from 58 to 60°C. Then, 600 nM primers, 160nM probe concentrations and a 58°C annealing temperature were used. ddPCR reaction mixtures included 10 µL of 2X ddPCR master mix and optimized concentrations of primer and probe in a 20 µL final volume. Ten-fold serial dilutions of DNA from the three fish species were tested in qPCR and ddPCR runs. For ddPCR technology, the absolute quantification of DNA per sample (copies/µL) was processed using QuantaSoft (v.1.7.4.091).

The linear relationship was produced by

plotting respectively log DNA per reaction against the quantitation cycle (Cq) and by plotting quantity of pg DNA against copy number concentration.

Results

DNA from three fish species was amplified in Real-Time. qPCR with strong fluorescence and only a specific 166 bp target located in 18S region was obtained. Conversely, no amplification of DNA from the mollusk *Todarodes sagittatus* and from the crustacean *Penaeus kerathurus*, was obtained in both methods as expected.

Discrimination between positive and negative droplets was increased in ddPCR by using different concentrations of probe and primers (data not shown).

The best annealing temperature for the three fish species was in the range 58°C to 60°C and the optimum annealing temperature was 58°C (Figure 1).

PCR additional population of droplets (green droplet) with fluorescence values between the true positive (blue droplet) and negatives (grey droplet) in 2-D fluorescence plot were highlighted (Figure 2) and it could

be due to variability inside species.

Fish DNA close to 0.18 pg were clearly detected by ddPCR compared to qPCR analysis. For the same amount of DNA, a Cq value of 37.98 was obtained with the last method (data not shown). ddPCR data have shown a linear relationship ($R^2 = 0.9998$) between pg DNA against copy number concentration (Figure 3).

Discussion

EC No 1169/2011 establishes the obligation to label for 14 allergens, including fish, even if the threshold values are not defined.

Any fish ingredient of food may be called *fish*, provided that the name and presentation of such food does not refer to a specific species. For this reason, 18S rRNA sequences of fish species were selected and aligned to find DNA homologies and to design primers to locate a short amplicon as a target of fish presence. ddPCR was tested to detect a sensitive screening method for fish allergen in foods to prevent the allergy reaction in consumers.

Low levels of nucleic acids and PCR

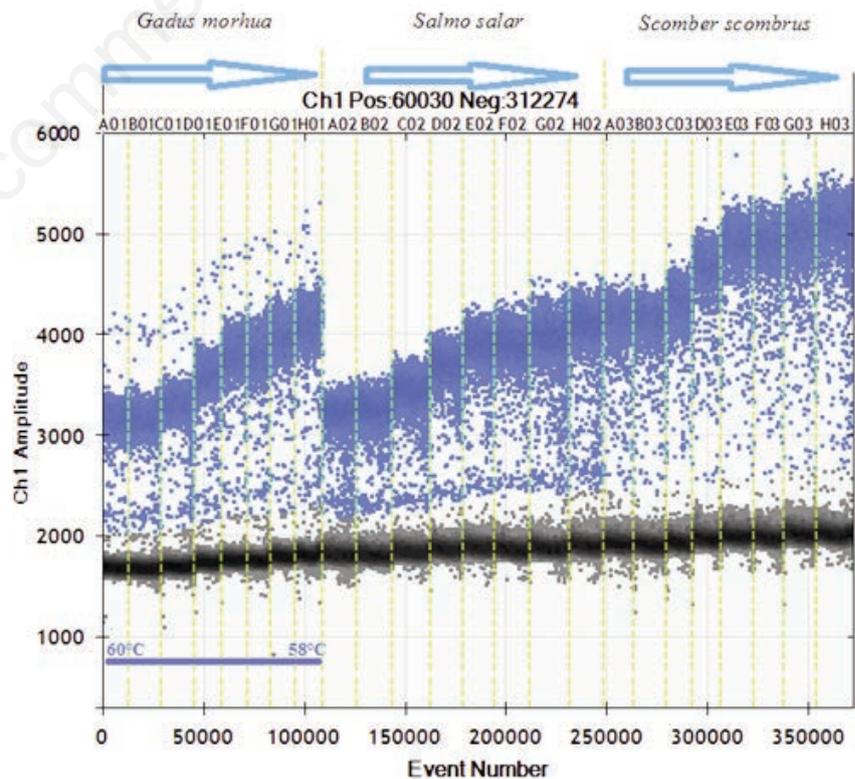


Figure 1. Three fish species *Gadus morhua*, *Salmo salar*, *Scomber scombrus* gradient annealing from 58 to 60°C in ddPCR.

inhibitors represent a major issue affecting performance in qPCR over ddPCR. Moreover, certified standards are necessary. ddPCR method overcomes these issues.

Additional population of droplets with fluorescence values between the true positive and negatives were highlighted in 2-

D fluorescence plot (Figure 2). The possible sources of such intermediate droplets including suboptimal PCR amplification are due to sequence variances, nonspecific amplification and so on (Witte *et al.*, 2016). This additional amplification was not detected in a standard real time PCR reaction

because the measured fluorescence is the sum of all amplification processes in the reaction mixture. We can assert that DNA allergen quantification by ddPCR is more accurate compared to qPCR.

In our work the droplet *rain* was reduced by making gradient annealing and changes of the PCR cycling program, probe and primers concentration with the purpose of increasing the specificity and to improve the assay.

Low concentration of fish detected by ddPCR makes it a promising method to test fish allergen in food.

Conclusions

To date this is the first study using ddPCR assay to quantify fish allergen. The use of ddPCR demonstrated detection of fish allergen small quantity. Presence of additional reaction products was also observed during ddPCR reactions. These could be result for example of intraspecies variability, SNPs, or interspecies. Further validation studies are needed in order to apply ddPCR technology for specific fish species and for routine allergens analysis.

Furthermore, regulatory guidance on thresholds for allergen in foods it would be desirable with the aim to improve the quality of life for food-allergic consumers.

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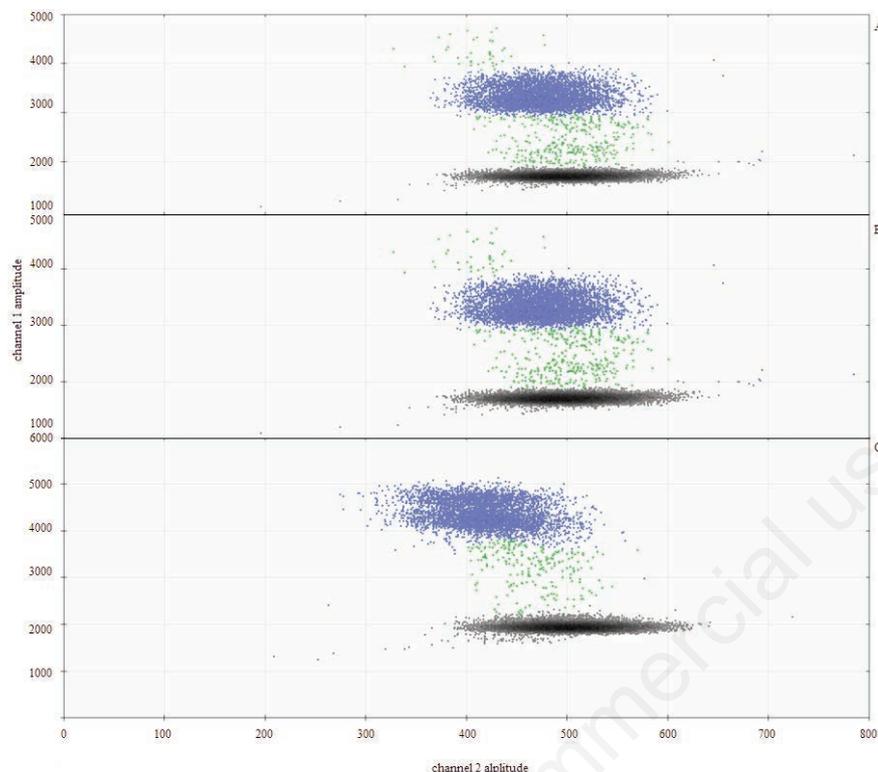


Figure 2. 2-D fluorescence plot in the order A (*Gadus morhua*), B (*Salmo salar*), C (*Scomber scombrus*).

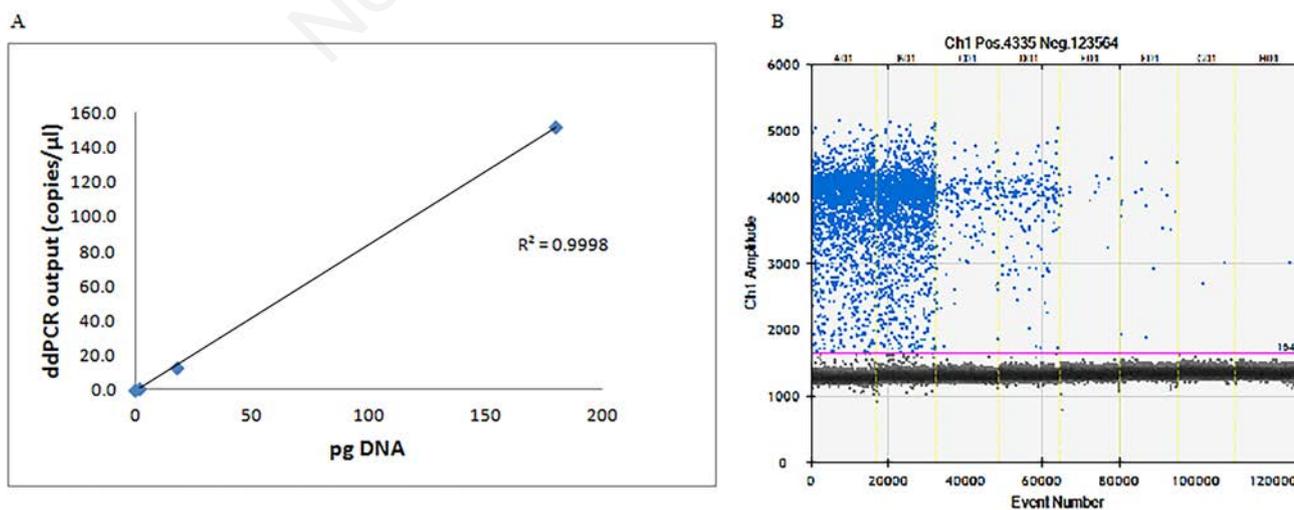


Figure 3. A) Linear relationship between pg DNA (axes X) and copy number concentration (axes Y); B) Ten-fold (from A01 to H01) serial dilutions of DNA from *Scomber scombrus* in ddPCR.

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