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Original Research Article

A new real-time PCR quantitative approach for the detection of shrimp crustaceans as potential allergens

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ABSTRACT

Allergy to crustaceans is an increasingly important food safety issue. To protect people from experiencing adverse allergic reactions, reliable methodologies are necessary to verify the labelling of processed seafood. In the present work, two new DNA-based approaches targeting the 16S rRNA mitochondrial gene are proposed to detect crustaceans in foods using a qualitative PCR assay specific for crustaceans (shrimps, lobsters and crabs) and a quantitative real-time PCR assay specific for shrimp crustaceans. The real-time PCR system allowed the detection and quantification down to 0.1 pg and 0.0001% (*w/w*) of shrimp DNA and shrimp in model mixtures, respectively. The method exhibited high performance for quantitative analysis in the range of 0.0001% to 50% as inferred by the calibration curve parameters being effectively validated with blind mixtures. The qualitative PCR assay can provide a simple, fast and high throughput tool for screening the presence of crustaceans in processed foods, while the proposed real-time PCR method proved to be a useful tool for the accurate detection and quantification of shrimp in foods at trace levels.

1. Introduction

The international trade of seafood species and products has been growing over the years, mainly due to the high nutritive value and popularity of these foods across many countries. However, the increased consumption of seafood has been followed by more frequent reports of allergic reactions among consumers (Lopata et al., 2010). Recent data suggest that shellfish allergy can affect up to 2.5% of the general population (especially among adults) (Woo and Bahna, 2011). Crustaceans, such as shrimps, crabs or lobsters, represent a major food resource with high commercial value, being also responsible for eliciting the majority of the allergic reactions related to shellfish (Pedrosa et al., 2015; Thalayasingam and Lee, 2015; Khora, 2016). About 60% of all crustaceans (13.9 million tonnes), based on global capture and production, belong to shrimps/prawns, from which *Litopenaeus vannamei* is the main crustacean species, with a production of 3.9 million tonnes in 2015 (FAO, 2017).

Crustacean-allergic individuals can experience moderate to strong adverse immunological reactions, including anaphylaxis. Accordingly, for the protection of at least 95% of this population group, a reference dose of 10 mg of shrimp protein (corresponding to 44 mg of shrimp) has been recently proposed using, in the case of shrimp, threshold data of $n < 80$ individuals (Allen et al., 2014). The establishment of reference doses for allergenic food residues, including shrimps, is part of the VITAL (Voluntary Incidental Trace Allergen Labelling) program of The Allergen Bureau of Australia & New Zealand (ABA), in order to guide advisory labelling decisions to use on food labels (Allen et al., 2014; Taylor et al., 2014). Therefore, allergic individuals should rely on the labelling information to avoid the consumption of any offending food. Besides, cross-contamination occurrences during production, storage or transport may lead to the inadvertent presence of allergens in foods (Herrero et al., 2012; Fernandes et al., 2017). Hence, in order to protect sensitised persons, the labelling of fourteen allergenic ingredients (including crustaceans) is demanded by the European Union legislation (Directive 2007/68/EC, Regulation (EU) No 1169/2011). To comply with it, several producers use frequently the precautionary labelling "may contain traces of…", which often causes some indecision or rejection by food allergic consumers. Therefore, sensitive analytical methods aimed at detecting trace levels of the allergenic ingredients in complex matrices are needed to verify the labelling of crustaceans.

According to the Regulation (EU) No 1169/2011, the detection of the allergenic food is demanded, but the target analyte does not necessarily have to be the allergenic protein itself. In this sense, both protein- and DNA-based methods have been widely used for allergen detection (Zhenxing et al., 2010; Eischeid et al., 2013; Prado et al., 2016). For protein analysis, the enzyme-linked immunosorbent assay (ELISA) is by far the most widely used immunochemical technique to identify and quantify allergens in food (Lopata et al., 2010; Zhenxing

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et al., 2010; Wang et al., 2011; Gomaa and Boye, 2015). In spite of the major advantages of this methodology associated with simplicity, speed, high specificity and sensitivity inherent to the antigen/antibody interaction, it also presents some drawbacks. Cross-reactivity phenomena can lead to the occurrence of false positive results, while conformational changes of proteins caused by food processing can lead to false negative results (Costa et al., 2014; Prado et al., 2016). On the other hand, DNA-based methods meet some advantages in comparison with immunochemical assays. DNA molecules are almost ubiquitous in any organic matter and more resistant to processing than proteins, which allows their analysis from difficult matrices, such as processed and complex foods (Herrero et al., 2012; Eischeid et al., 2013). For allergen analysis, real-time PCR-based methods have been widely applied (Mafra et al., 2008; Prado et al., 2016; Costa et al., 2017). Their specificity has relied mainly on primers and probes targeting sequences of genes encoding for allergenic proteins, but mitochondrial genes have also been demonstrated to be useful targets (Prado et al., 2016; Costa et al., 2017). In the case of crustacean detection and differentiation, the mitochondrial genes, such as 16S rRNA (Cao et al., 2011; Pascoal et al., 2011; Herrero et al., 2012; Eischeid et al., 2013; Mäde and Rohmberger, 2017; Zagon et al., 2017; Wilwet et al., 2018), 12S rRNA (Eischeid et al., 2013; Eischeid, 2016; Eischeid and Stadig, 2018) and cytochrome oxidase subunit I (COI) (Eischeid et al., 2013; Fernandes et al., 2017) have been used as specific markers, taking advantage of their high copy number that usually results in more sensitive assays. From the available reports on real-time PCR, several of them allow high sensitivity levels of detecting particular groups of crustacean species (Cao et al., 2011; Herrero et al., 2012; Eischeid et al., 2013; Eischeid, 2016; Mäde and Rohmberger, 2017; Zagon et al., 2017; Eischeid and Stadig, 2018). However, only a few reports are based on quantitative real-time PCR methods using model mixtures simulating low levels of spiked crustaceans (Eischeid et al., 2013; Eischeid, 2016; Eischeid and Stadig, 2018). Eischeid et al. (2013) describes two real-time PCR systems able to quantify DNA from penaeid shrimps and crabs. Eischeid (2016) reports a real-time PCR quantitative system specific for lobsters based on model mixtures as calibrants, followed by a similar approach for crabs (Eischeid and Stadig, 2018), both methods being effective for allergen quantification.

Considering the scarce methods for crustacean allergen quantification by DNA-based methods, the development of a quantitative real-time PCR system specific for a wide range of shrimp species is proposed, validated and applied to verify labelling compliance of foods. Additionally, the performance of quantitative analysis of the new method is compared with ELISA.

2. Materials and methods

2.1. Sample collection and preparation

Several crustacean specimens from different species ($n = 18$) (whiteleg shrimp – *Litopenaeus vannamei;* speckled prawn – *Metapenaeus monoceros*; Indian white prawn – *Fenneropenaeus indicus*; giant tiger prawn – *Penaeus monodon;* Jinga shrimp – *Metapenaeus affinis;* green tiger prawn – *Penaeus semisulcatus*; Argentine red shrimp – *Pleoticus muelleri;* razor mud shrimp – *Solenocera melantho;* Udang merah – *Solenocera crassicornis;* knife shrimp – *Haliporoides triarthrus;* common prawn – *Palaemon serratus*; scarlet shrimp – *Aristaeopsis edwardsiana,* Norway lobster – *Nephrops norvegicus;* European lobster – *Homarus gammarus*; Caribbean spiny lobster – *Panulirus argus;* crab – *Portunus validus;* velvet swimming crab – *Necora puber;* edible crab – *Cancer pagurus*) (Table 1) were purchased from local markets or provided by Marfresco (Loures, Portugal) and Brasmar Seafood Companies (Trofa, Portugal), who identified most of the species through morphological characteristics. The identity of *L. vannamei, M. Monoceros*, *F. indicus*, *P. monodon, M. affinis* was previously confirmed by sequencing (Fernandes et al., 2017). The selection of species was made regarding the relevance in terms of consumption and availability in different commercial areas of the Portuguese market.

A total of 62 non-crustacean species was also used (pre-existing extracts or tissues) to evaluate the assay specificity: seafood species ($n = 28$) (Atlantic cod, Pacific cod, Alaska pollock, saithe, ling, Atlantic salmon, gilt-head bream, common sole, European pilchard, yellowfin tuna, European hake, North Pacific hake, Argentine hake, whiting, deepwater hake, ray, European seabass, whiting-pout, Atlantic horse mackerel, tadpole codling, rose fish, rock ling, Pacific mackerel, black scabbardfish, Nile perch, squid, mussel, undulated surf clam), meat species ($n = 15$) (wild boar, duck, partridge, hare, quail, pheasant, red deer, rabbit, chicken, turkey, lamb, ostrich, cow, horse, pig) and plant species ($n = 19$) (onion, garlic, parsley, pepper, bay leaf, sweet chili, tomato, maize, soybean, potato, manioc, lupin, chestnut, walnut, broad bean, rye, wheat, rice, pumpkin).

Several seafood products ($n = 18$), including shrimp patties, seafood broth/soups, surimi and pre-cooked dishes, were also acquired at local markets for assay applicability.

The species tissues and food samples were ground and homogenised separately in a laboratory knife mill Grindomix GM200 (Retsch, Haan, Germany) and stored at −20 °C until further analysis. All containers and grinding mill knives used during this procedure were previously treated with DNA decontamination solution (DNA-ExitusPlus™; AppliChem, Darmstadt, Germany).

2.2. Preparation of model mixtures

The preparation of binary model mixtures was intended to simulate a processed shrimp stuffing/filling (e.g., shrimp patty fill). Shrimp meat (*L. vannamei*) was boiled in water for 5 min, drained and minced in a laboratory knife mill Grindomix GM200 (Retsch, Haan, Germany). A mixture containing 50% (*w/w*) of ground-cooked shrimp kernel in béchamel sauce (milk, milk cream, wheat flour, maize starch and salt) was prepared by the addition of 200 g of shrimp to the same amount of sauce. Afterwards, successive additions of béchamel sauce to the 50% homogenised mixture enabled the preparation of the following binary proportions containing 10, 5, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005 and 0.0001% (*w/w*) of shrimp in béchamel. Identically, for method validation, blind mixtures were prepared to contain 8.0, 4.0, 2.5 and 0.25% (*w/w*) of shrimp in béchamel. The binary mixtures were stored at −20 °C until further analysis.

2.3. Primer and probe design

Sequences of the 16S rRNA gene were selected from NCBI database from a set of 17 different crustacean species (Fig. 1). However, it was not possible to find available sequences for the *P. validus* species. Sequence alignment was performed with BioEdit v.7.2.5 software (Ibis Biosciences, Canada) using ClustalW (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and examined for suitable regions for primer and probe design. The primers were designed to amplify as many species as possible within crustacean families; therefore regions of the 16S rRNA gene with very low intra- and interspecific variability, and also low intra- and intergenus variability were chosen (Eischeid et al., 2013; Mäde and Rohmberger, 2017). The primers (16SCrust2-F: TAA AGT CTG GCC TGC CCA CTG; 16SCrust1-R: AGC TTT ATA GGG TCT TAT CGT C) and probe (16SCrust1-P: FAM-TTA ATT GAA GGC TTG TAT GAA TGG TTG GAC-BHQ1) (fragment of 203 bp) were then submitted to a basic local alignment search tool BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), in order to identify regions of local similarity among homologue sequences of different species and to calculate the statistical significance of the matches. Primer properties, self-hybridisation and the absence of hairpins were evaluated using the software OligoCalc (http://www.basic.northwestern.edu./biotools/oligocalc.html). The oligonucleotides were synthesised by STABVIDA (Lisbon, Portugal).