Detection of Krill in Foods using Real Time PCR

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Abstract

FDA has received consumer complaints about food products, most notably sardines, containing krill as an undeclared crustacean shellfish allergen. The Food Allergen Labeling and Consumer Protection Act (FALCPA) requires that food products containing crustacean shellfish be labeled with the type of crustacean. Available antibody-based allergen detection assays, which detect proteins, do not distinguish crustacean type. Real time PCR assays, which detect DNA, can differentiate type of crustacean. The purpose of this work was to develop and evaluate a real-time PCR based method for detection of krill contamination in foods, with a focus on detection in sardines.

Introduction

Krill are small shrimp-like crustaceans which are found in all the world's oceans and serve as a primary food source for many larger marine predators. While krill is intentionally fished primarily for use in animal feeds, it may also be found as bycatch from other fisheries. FDA has received a number of consumer complaints about undeclared krill in foods, most commonly sardines. The Food Allergen Labeling and Consumer Protection Act (FALCPA) requires that foods containing any crustacean shellfish be labeled with crustacean type. PCR has been shown to successfully detect and distinguish crustacean shellfish allergens, though this work has been primarily conducted on shrimp, crab, and lobster. While shrimp, crab, and lobster are all crustaceans of the order Decapoda, krill are crustaceans of the order Euphausiacea. Both the allergenicity and taxonomy of krill with respect to the decapod crustaceans are unresolved. Analyses of krill allergenicity suggest that it is likely to cause reactions in crustacean-allergic individuals. Analyses of crustacean phylogeny yield differing results. Traditional phylogenetic analyses have held that Decapoda and Euphausiacea were sister groups. More recent work indicates that, rather than being a sister group to all decapod crustaceans, krill may have a common ancestor specifically with penaeid shrimp. Recent consumer complaints to FDA have highlighted the need for methods which can detect krill, while taxonomic differences suggest that an assay designed for shrimp may not necessarily work. In this work, we describe modification of a previously developed shrimp assay for specific detection of krill and suitable for the analysis of sardines.

Materials and Methods

A previously developed shrimp assay was adapted to detect krill through modification of primer and probe sequences targeting the 16S ribosomal RNA gene of mitochondria. Sequences of amplicon, primers, and probe from the shrimp assay were aligned with analogous sequences from krill using Geneious software (Biomatters Ltd, Auckland, NZ). Primers and probe for krill were designed manually based on visual inspection of krill and shrimp sequences. DNA was extracted using the DNeasy DNA extraction kit according to manufacturer's instructions (Qiagen, Valencia, CA). For testing in sardines, krill homogenate was first prepared in 1% SDS buffer to a final concentration of 0.25 g (250 mg) of krill meat/mL, and this was used to spike krill into sardines at levels of 0.1, 1, 10, 100, 1000, 10^4, and 10^5 mg krill meat/kg sardine meal (mg/kg, or ppm) prior to DNA extraction. Each PCR reaction contained 1x PCR buffer, 5 mM Mg, 1.2 mM dNTPs, 400 nM krill primers, 200 nM krill probe and 0.625 units Platinum Taq polymerase (Invitrogen/Life Technologies, Grand Island, NY) in a 12.5 µl reaction. Thermal cycling consisted of an initial cycle at 95°C for 3 minutes followed by 45 cycles of denaturation at 95°C for 15 seconds, annealing at 65°C for 30 seconds, and extension at 65°C for 2 minutes. PCR was carried out using the CFX 96 Real Time PCR system and CFX Manager software (BioRad, Hercules, CA).

Primer Set 1			
Primer Conc.	Linear range (pg/reaction)	R^2	Reaction Efficiency
200 nM	0.5 – 50,000	0.990	90.3 %
400 nM	0.05 – 50,000	0.995	103 %
800 nM	0.5 – 50,000	0.995	101 %

Primer Set 2			
Primer Conc.	Linear range (pg/reaction)	R^2	Reaction Efficiency
200 nM	0.5 – 50,000	0.982	98.7 %
400 nM	0.05 – 50,000	0.994	109 %
800 nM	0.05 – 50,000	0.993	116 %

Table 1: Optimization of krill primer concentrations.

Results and Discussion

Two different sets of modified primer and probe sequences were developed and tested, and both sets worked well for krill detection. Optimization of krill primer concentrations (Table 1) showed that both sets of primers yielded reaction efficiencies and statistical R^2 values in the optimal range, as well as linear ranges spanning 6-7 orders of magnitude, for nearly all conditions tested. A primer concentration of 400 nM was chosen for both primer sets. Both primer sets gave strong amplification from six different krill samples, frozen and freeze-dried, without nonspecific amplification of shrimp (Figure 2). One krill sample had weaker amplification than others using both primer sets but was still detectable: this was labeled "River Shrimp" and is likely a freshwater species, which differs from the marine species which were the focus of this assay.

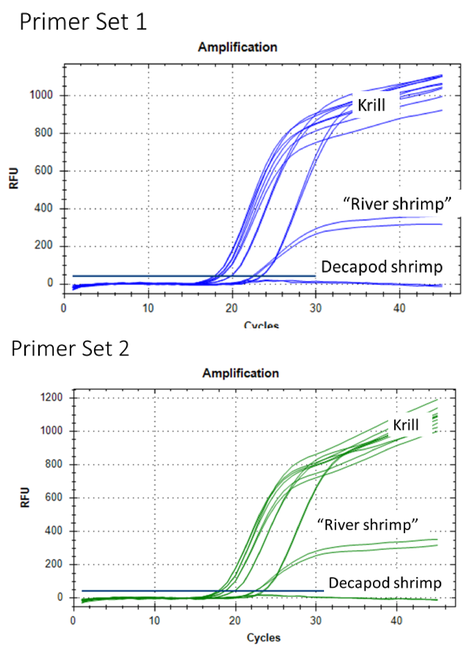

Figure 1. Krill assay results using six different krill samples and shrimp

Experiment	Primer Set	Linear Range	R2	Reaction Efficiency
Expt 1	Set 1	1-10^6 ppm	0.99	140%
Expt 1	Set 2	0.1-10^6 ppm	0.98	136%
Expt 2	Set 1	0.1-10^5 ppm	0.97	121%
Expt 2	Set 2	0.1 – 10^5 ppm	0.97	137%

Table 2. Krill assay results from testing in sardines

Tests of the assay in sardines were carried out using both primer sets in two different independent experiments (Table 2). Linear ranges spanned 7-8 orders of magnitude in all cases, and all statistical R^2 values except one were above the optimal value of 0.98. Reaction efficiencies for these experiments in sardines were higher than the reaction efficiencies in buffer (Table 1) for all conditions and were also outside the optimal accepted range of 90-110%. This appears to be the result of interference from the sardine matrix and may be investigated further in the future. It is not expected to interfere with accurate quantitation when sardines are used as standards. In real time PCR, accurate quantitation of unknowns requires that reaction efficiencies of standards and unknowns be equivalent, not that they be in the ideal range.

Conclusions

Consumer complaints to FDA about undeclared krill have highlighted the need for a detection assay specific to this crustacean. While krill may not be a close taxonomic relative of shrimp, this work has shown that minor changes in shrimp primers and probe were nonetheless able to yield an effective and specific krill assay. To the best of our knowledge, this is the first report of a real-time PCR assay for specific detection of allergenic krill in food products.

Questions?

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