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Analytical Methods

A group-specific, quantitative real-time PCR assay for detection of crab, a crustacean shellfish allergen, in complex food matrices

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ABSTRACT

A real-time PCR assay was developed for detection of crab, a crustacean allergen, in food products. Group-specific primers and probes were developed to detect numerous species of crab. Method validation included tests of detection in complex food matrices, evaluation of commercial food products, and cross-reactivity testing on a wide variety of crustaceans. The method was able to detect several species of crab spiked into complex food matrices at levels ranging from 0.1 to 10^5 parts per million (weight/weight), worked equally well on different platforms, exhibited high specificity for crab over other types of crustaceans, and yielded much higher signals from commercial food products listing crab as an ingredient than from those containing other crustaceans.

1. Introduction

Food allergies affect approximately 15 million Americans, with allergy to crustacean shellfish affecting approximately 6 million, or 2% of the American population (Sicherer, Munoz-Furlong, & Sampson, 2004; Wild & Lehrer, 2005). There is currently no cure or treatment, so affected individuals must avoid the foods to which they are allergic. As a result, consumers with food allergies must rely on proper food labeling to identify the presence of food allergens. To protect allergic consumers, U.S. Congress passed the Food Allergen Labeling and Consumer Protection Act of 2004 (Public Law 108–282, Title II) (FALCPA), requiring the presence of any of the eight major food allergens, including crustacean, be declared on the label.

To enforce FALCPA, accurate and highly sensitive detection assays for allergens in food products are needed, and PCR-based assays represent one of the major methods for detecting food allergens (Sharma, Khuda, Parker, Eischeid, & Pereira, 2017). Conventional end-point PCR has been shown to successfully detect crab at trace levels in several complex food matrices, and performed well in a large multi-laboratory validation (Sakai, Adachi, Akiyama, & Teshima, 2013; Taguchi et al., 2011). Real-time quantitative PCR (qPCR) has proven especially useful for both detection and quantitation of trace levels of allergenic foods with high specificity. Our laboratory has previously reported group-specific qPCR based detection assays for numerous species of shrimp and lobster, as well as one designed for blue crab species (Eischeid, 2016; Eischeid, Kim, & Kasko, 2013).

This report describes the development and evaluation of a group-specific qPCR based assay for detection of trace amounts of crab in food products. The method development and validation reported herein includes previously unpublished qPCR primers and probes which were designed specifically for this work. In this work, the assay was 1) evaluated in complex food matrices using 7 different species of crab from 6 different genera, 2) employed in the analysis of commercial food products, and 3) tested for cross-reactivity using nearly 100 samples and 30 species of crustaceans. Performance of the assay was also compared on 3 different real time PCR systems to confirm the performance was consistent between platforms.

2. Materials and methods

2.1. Primer and probe design

Primers and probes for crab species were designed as previously described for shrimp and lobster (Eischeid, 2016; Eischeid et al., 2013). Full mitochondrial genome sequences were obtained from Genbank for a variety of species including king crabs, mud crabs, blue crabs, other swimming crabs, and a rock crab. Sequences for the 12S rRNA gene were manually extracted from the full mitochondrial genomes. Accession numbers and species used in primer and probe design are as follows: NC_024202 (*Lithodes nintokuae*), NC_020029 (*Paralithodes camtschaticus*), NC_021458 (*Paralithodes brevipes*), NC_012567 (*Scylla tranquebarica*), NC_012572 (*Scylla paramamosain*), NC_012565 (*Scylla*

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Table 1
Evaluation in Complex Food Matrices.

	Linear Range	12S Efficiency	12S R^2	IAC C_T avg.	IAC C_T SD (RSD)
Detection of Crab in a Complex Food Matrix-Macaroni Salad (all PCR on Mx3005P)					
[a]Blue Crab	0.1–10^6 ppm	112%	1.00	26.85	0.19 (0.71%)
[a]Snow Crab	1–10^5 ppm	101%	1.00	24.64	0.27 (1.1%)
[a]Rock Crab	1–10^5 ppm	114%	0.98	34.77	0.32 (0.92%)
[ab]Red King Crab-Expt 1	0.1–10^6 ppm	102%	0.99	31.75	0.38 (1.2%)
[d]Red King Crab-Expt 2	0.1–10^4 ppm	102%	0.96		
[a]Blue King Crab	1–10^6 ppm	120%	0.99	30.74	0.31 (1.0%)
[a]Red Crab	1–10^6 ppm	130%	0.95	26.76	0.15 (0.56%)
[a]Dungeness Crab	0.1–10^6 ppm	96%	0.98	26.78	0.17 (0.63%)
Platform Comparison- Red King Crab in Pork Dumplings					
[bc]Mx3005P	0.1–10^6 ppm	111%	0.99	28.74	0.16 (0.56%)
[c]CFX96-Expt 1	0.1–10^6 ppm	104%	0.98	29.11	0.29 (0.99%)
[d]CFX96-Expt 2	0.1–10^4 ppm	98%	0.98		
[c]7500 Fast	0.1–10^6 ppm	100.50%	0.97	29.50	0.32 (1.1%)
Platform Comparison-Red King Crab in Clam Juice					
[b]Mx3005P	0.1–10^6 ppm	110%	1.00	28.87	0.19 (0.66%)
CFX96-Expt 1	0.1–10^6 ppm	105%	1.00	28.28	0.36 (1.3%)
[d]CFX96-Expt 2	0.1–10^4 ppm	106%	0.99		
7500 Fast	0.1–10^6 ppm	99.40%	0.99	30.63	0.23 (0.75%)

[a] used in determining performance across species (Fig.1a)
[b] used in determining performance across food matrices (Fig.1b)
[c] used in determining performance across platforms (Fig.1c)
[d] duplicate independent experiments were carried out to conform to the method validation guidelines set forth by the U.S. FDA (2015). The highest level tested in the duplicate experiments was 10^4 ppm. Macaroni salad, pork dumplings, and clam juice were purchased from a local market; full ingredient lists are provided in Supplementary Material. Method details are provided in Section 2.2.

serrata), NC_012569 (*Scylla olivacea*), NC_006281 (*Callinectes sapidus*), NC_005037 (*Portunus trituberculatus*), and KJ132446(*Metacarcinus magister*). All 12S rRNA gene sequences were aligned, and primers and probe were designed from the alignment using Allele ID software (Premier BioSoft, Palo Alto, CA, USA). Primers and probes were designed specifically to bind crab sequences and not to bind 12S rRNA gene sequences from other crustaceans, such as shrimp and lobster. This method of assay design yielded a combination which bound to all species used in design[1]: 6 different forward primers (5′-3′ sequences: TCTTTCCAGGCTTACTTTCCAG, GGCACATCTACTTTGTTACG, TAAACCTACTATGTTACGACTTATCTC, CACCTACTTTGTTACGACTTATTTC, CCTACTATGTTACGACTTATCTC, *GCTTATAAATAAGTTAAAGTGGGTTAC*), 8 different reverse primers (5′-3′ sequences: CTTAGTTAGATATAAGCTCTAAATCATG, GCTAATTAGATATAAGCTCTAAAGCATG, AATTACTTGATATAGGCTCTAAAATATGTAC, AATTTAATATGTTTAGTTAGATATAAGC, AAATTTAATATGTTTAATTAGATATAAGC, ATTAATTTTAATTACTTGATATAGGC, AATAGTTTAGTATGGTATTTAGATATAGGC, *TGTACATGATTTAGAACTTATATCTC*), and a single probe (5′-FAM-ACATATCGC-ZEN-CCGTCGCTTTC-3′-IowaBlack FQ). Species-specific information regarding individual primer and probe sequences is provided as Supplementary Material. All PCR products were approximately 100 bp in size, with minor variations depending on species. An exogenous internal control developed by Deer, Lampel, and Gonzalez-Escalona (2010) was used as previously described (Eischeid, 2016; Eischeid et al., 2013). Primers, probes, and ultramer DNA template for the internal control were purchased from Integrated DNA Technologies (Coralville, IA).

2.2. Evaluation in complex food matrices

Sample preparation, DNA extraction, and PCR were carried out as previously described with minor modifications (Eischeid, 2016; Eischeid et al., 2013). For method evaluation in foods, crab meat was spiked at levels of 0.1, 1, 10, 100, 1000, 10^4, and 10^5 ppm (w/w) into three commercial food products obtained from a local market: pork dumplings, macaroni salad, and clam juice (Table 1; full ingredient lists are provided in Supplementary Material). In order to ensure accurate spiking at the very low levels used in this work, homogenates containing known amounts of crab meat were prepared and these homogenates were added to foods. All experiments included a matrix blank consisting of food matrix with no crab, a homogenization blank consisting of only buffer with no food matrix and no crab, and an extraction blank consisting only of buffer and introduced at the DNA extraction step. In most experiments, a sample consisting entirely of crab DNA (10^6 ppm, w/w) was included as a positive control not subject to the effects of the food matrix. Samples were homogenized using 5 g sample in 20 ml of 1–2% SDS buffer at 10,000–12,000 rpm for 30–40 s using an IKA Tube Mill (IKA, Wilmington, NC). As some of the milling tubes leaked, homogenization tests were carried out to confirm that leakage did not cause contamination of subsequent samples (data not shown). DNA was extracted from 200 µl of homogenate using a combined rapid salt extraction (Aljanabi & Martinez, 1997) and the DNeasy kit (catalog No. 69506, Qiagen, Valencia, CA) as previously described (Eischeid, 2016; Eischeid et al., 2013). DNA was eluted in 60 µL Buffer AE and concentration was determined using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). For macaroni salad and pork dumplings, template DNA samples were diluted to 10 ng/µL in water and 5 µL (50 ng total DNA) was used per reaction in PCR. For clam juice, template DNA was used undiluted at 5 µL per reaction because DNA concentrations were below 10 ng/µL in this food matrix. Real time PCR was carried out as described in Section 2.5.

2.3. Cross-reactivity testing

Cross-reactivity testing was carried out using nearly 100 samples from 30 species of crustaceans (Table 2). For these tests, DNA was extracted directly from crustacean tissue using the DNeasy kit (Qiagen, Valencia, CA) as per manufacturer's instructions. All DNA samples were diluted to 100 pg/µL in water and 5 µL (500 pg total DNA) was used per reaction; this is equivalent to the amount of crab DNA present in a 10^4 ppm-spiked sample. PCR was carried out as described in Section 2.5 on the Mx3005P platform, and data were analyzed for C_T value and

[1] Primer sequences in italics represent primers which were inadvertently included in PCR. These two primers target a different part of the 12S rRNA gene from that which the probe targets and likely did not affect assay performance.