

Research Note

Detection of Shrimp-Derived Components in Food by Real-Time Fluorescent PCR

JIJUAN CAO,^{1*} BING YU,² LIDAN MA,² QIUYUE ZHENG,¹ XIN ZHAO,¹ AND JUNYI XU¹¹Liaoning Entry-Exit Inspection and Quarantine Bureau, No. 60 Changjiang East Road, Dalian 116001, People's Republic of China; and²Dandong Entry-Exit Inspection and Quarantine Bureau, No. 1 Shiwei Road, Dandong 118000, People's Republic of China

MS 11-020: Received 16 January 2011/Accepted 5 June 2011

ABSTRACT

Crustaceans such as shrimp and crabs and their products are important allergens in food, and allergic reactions due to the consumption of shrimp and crabs are frequently reported. However, the chemical properties of shrimp-derived allergens, except for Pen a I, are still unclear. Therefore, it is important to establish a more sensitive and specific method for detecting the composition of foods containing shrimp. In the present study, we developed a real-time fluorescent PCR to identify the specific shrimp-derived components in food. The primers and TaqMan probes for real-time fluorescent PCR were designed based on 16S rRNA genes through comparing a large number of nucleic acid sequences from different species of shrimp that have been published by the National Center for Biotechnology Information. In total, 56 kinds of samples, including different kinds of shrimp, crab, fish, shellfish, and octopus, were subjected to detection by real-time PCR. The results indicated that real-time fluorescent PCR could successfully identify the shrimp-derived components. In order to explore the effect of food processing on detection sensitivity, fish powder containing shrimp powder was treated by heating at 133°C for 30 min. The limit of detection of shrimp-derived components in fish powder was 0.05% (wt/wt).

Shrimp is a popular food that is rich in proteins and other components with nutritional value. However, among eight categories of allergenic foods defined by the Food and Agriculture Organization of the United Nations, crustaceans, such as shrimp and crabs, and their products are important food allergens (3, 9, 13). The prevalence of shrimp and crab allergies in China is not known, but allergic reactions due to the consumption of shrimp and crabs are frequently reported (1, 15). Food allergy is an allergic reaction with clinical symptoms of rash, abdominal pain, vomiting, diarrhea, bronchial spasm, and low blood pressure due to the consumption of food. Serious allergic reactions can result in shock.

A glycoprotein antigen II with a molecular mass of 38 kDa was first extracted from shrimp, and this protein exhibited strong allergenicity (5). Later, a tropomyosin with a molecular mass of 36 kDa was also isolated from shrimp and identified as the major allergen Pen a I (6). Moreover, at least 13 kinds of shrimp-derived allergenic protein with a molecular mass range of 16 to 166 kDa have been demonstrated by Morgan et al. (10). Currently, although the chemical characterization of the shrimp-derived allergen Pen a I is clear, the chemical properties of other shrimp-derived allergens are still unknown (12).

A food allergen labeling system has been implemented in the United States, Europe, the United Kingdom, Hong

Kong, China, and other countries and regions. However, the complexity and diversity of the protein composition of shrimp species have hampered the development of methods for detecting shrimp-derived components in foods (4, 8, 11). Therefore, it is important to establish a PCR detection method for the composition of foods containing shrimp. In the present study, we compared a large number of nucleic acid sequences, published by National Center for Biotechnology Information (NCBI), from different shrimp species and designed primers and probes for real-time fluorescent PCR based on their 16S rRNA gene fragments to identify the specific shrimp-derived components. A real-time PCR method was developed to detect shrimp-derived components in food using TaqMan probes.

MATERIALS AND METHODS

Samples and DNA extraction. In total, samples of 14 kinds of shrimp, including prawns, *Crangon septemspinosa*, *Macrobrachium nipponense*, *Alpheus japonicus*, *Pandalus borealis*, and others; 5 kinds of crab, including *Orithyia sinica*, *Charybdis japonica*, *Eriocheir sinensis*, sea crabs, and others; 24 kinds of fish, including salmon, flounder, perch, herring, redfish, eel, and others; 12 kinds of clam, including oysters, scallops, red shell, and others; and one kind of octopus, the mollusc octopus, were used for specific experiments. All of these samples were collected from markets in Dalian, China. The samples were used for evaluating the detection sensitivity of real-time fluorescent PCR.

Shrimp and fish samples were subjected to heat treatment at 133°C for 30 min and then ground into powder in the presence of liquid nitrogen. A total of 100 mg of sample powder was used for

* Author for correspondence. Tel: 0086-411-82583647; Fax: 0086-411-82583674; E-mail: cjj0909@163.com.

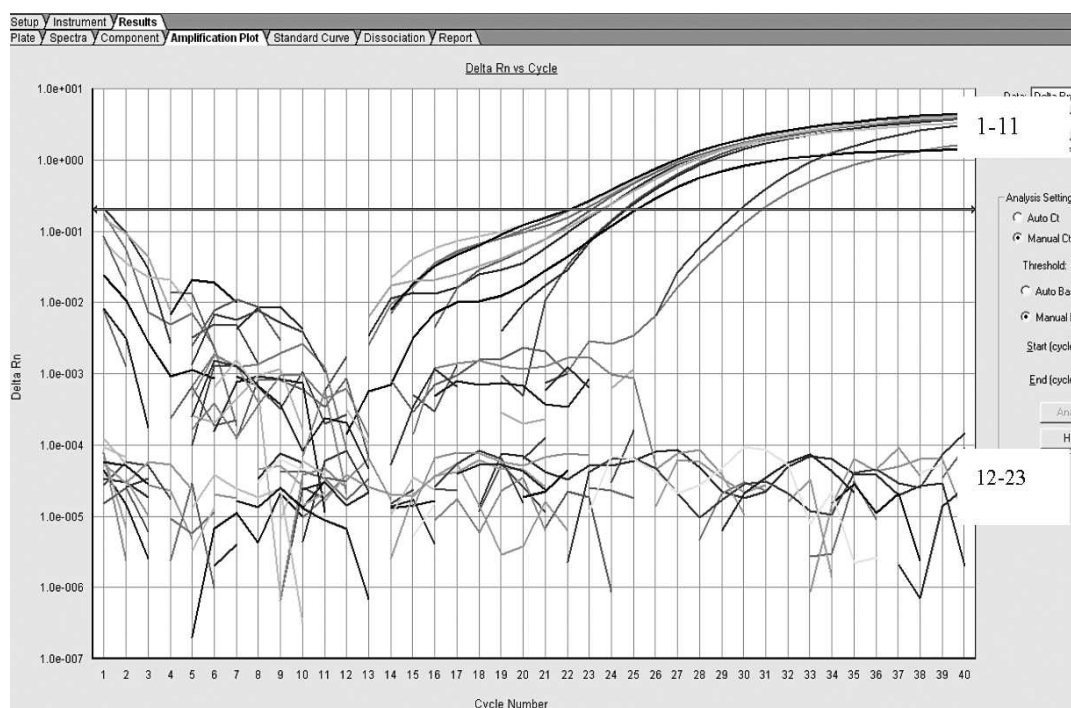


FIGURE 1. Real-time detection of shrimp-derived components using Shrimp 1 primers and probe. Curves 1 through 11 exhibit positive amplification for prawn, *Crangon septemspinosa*, *Macrobrachium nipponense*, *Alpheus japonicus*, *Metapenaeus ensis*, sea shrimp, *Brachium nipponense*, lobster, *Procambarus clarkii*, *Penaeus japonicus*, and sea crab, respectively. Curves 12 through 23 exhibit negative results for *Pandalus borealis*, dried peeled shrimp, *Acetes chinensis* Hansen, *Oratosquilla oratoria*, *Orithyia sinica*, *Charybdis japonica*, *Eriocheir sinensis*, *Chionoecetes bairdi*, fish, shellfish, oyster, and octopus, respectively.

DNA extraction through a Universal Genomic DNA extraction kit (version 3.0, TaKaRa, Dalian, China). Shrimp powder and fish powder were mixed at ratios of 10, 5, 0.5, 0.1, 0.05, 0.01, and 0%, and the DNA extracted from the mixture was used as the DNA template for real-time PCR. The series of sample mixtures were used for evaluating the detection sensitivity of real-time PCR.

Design of primers and probes. The 16S rRNA genes of the various kinds of shrimp were differentiated by comparing the nucleic acid sequences published by NCBI. Different gene loci were selected to design specific primers and probes for amplifying the 16S rRNA gene fragments of shrimps as follows: Shrimp 1 Forward, 5'-GAT TAA GTT ACT TTA GGG ATA ACA GCG-3'; Shrimp 1 Reverse, 5'-TAA AGG TCG AAC AGA CCT TCT CAC T-3'; and Shrimp 1 Probe, 5'-FAM-CTT CCT TGA AAG TTC ATA TCG ACA GGA AGG G-Eclipse-3' (amplified fragment length of 135 bp); and Shrimp 2 Forward, 5'-AAG TCT AGC CTG CCC ACT G-3'; Shrimp 2 Reverse, 5'-GTC CAA CCA TTC ATA CAA GCC-3'; and Shrimp 2 Probe, 5'-FAM-AGA CTA ATG ATT ATG CTA CCT TCG CAC GGT C-Eclipse-3' (amplified fragment length of 119 bp). The design of specific primers and probes for amplification of the 16S rRNA gene of Arctic shrimp is as follows: *Pandalus borealis* 3 Forward, 5'-ATT AAG TTA CTT TAG GGA TAA CAG C-3'; *Pandalus borealis* 3 Reverse 1, 5'-ACC TTA ATT CAA CAT CGA GGT C-3'; *Pandalus borealis* 3 Reverse 2, 5'-CTT AAT TCA ACA TCG AGG TCG C-3'; and *Pandalus borealis* 3 Probe, 5'-FAM-ACT CCC GTC GAT AAG AAC TCT CAG GG-Eclipse-3' (amplified fragment length of 85 bp).

DNAMAN 8.0 software was used to design primers and probes according to the consistency of annealing temperatures between primers and probes, the similarity of GC content, and other factors. The primers and probes were synthesized and

purified by TaKaRa Biotechnology Co. Ltd. (Dalian, China) through polyacrylamide gel electrophoresis. The purified primers and probes were stored at -20°C for future use.

Real-time fluorescent PCR amplification. A 25- μl volume of the reaction mixture was composed of 5 μl of $10\times$ PCR buffer containing Mg^{2+} , 0.5 μl of forward and reverse primers (10 $\mu\text{mol/liter}$), 1 μl of probe (10 $\mu\text{mol/liter}$), 2 μl of deoxynucleoside triphosphates (10 $\mu\text{mol/liter}$), 0.2 μl of *Taq* DNA polymerase (DRR030A, 5 U/ μl ; TaKaRa), 2 μl of template DNA (0.1 to 2 μg), and an additional 13.8 μl of water.

The real-time fluorescent PCR conditions were as follows: 1 cycle of 95°C for 10 s, and 40 cycles of 95°C for 5 s and 60°C for 34 s. The fluorescence signal was determined at the annealing stage during each cycle. PCR amplification was conducted in a real-time fluorescent PCR instrument (ABI 7500, Applied Biosystems, Foster City, CA).

RESULTS

DNA fragment amplification for specific genes of shrimp-derived components. The DNA from 56 kinds of samples was subjected to real-time detection using Shrimp 1 primers and probes. The DNA fragments from 11 kinds of samples, including prawn, *C. septemspinosa*, *M. nipponense*, *A. japonicus*, *Metapenaeus ensis*, sea shrimp, *Brachium nipponense*, lobster, *Procambarus clarkii*, *Penaeus japonicus*, and sea crab, were successfully amplified with strong fluorescence signals, as shown in Figure 1. The sea crab samples exhibited a fluorescence signal with nonspecific amplification. However, no fluorescence signal was observed for Arctic shrimp, dried peeled shrimp, *Acetes*

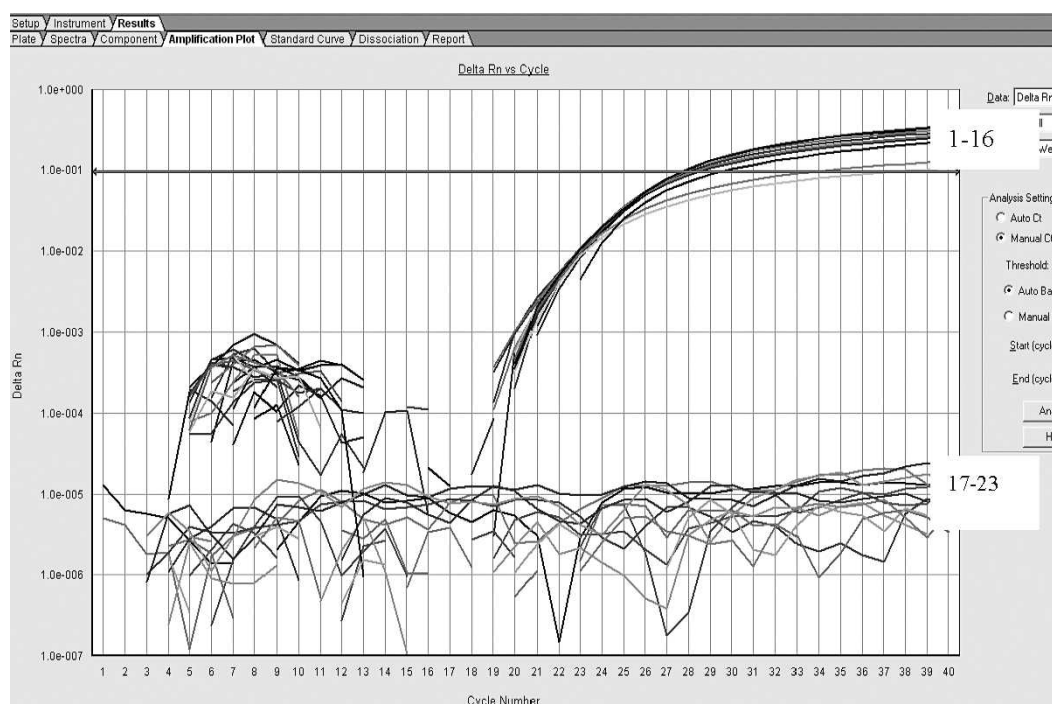


FIGURE 2. Real-time detection of shrimp-derived components using Shrimp 2 primers and probe. Curves 1 through 16 exhibit positive amplification for prawn, *Crangon septemspinosa*, *Macrobrachium nipponense*, *Alpheus japonicus*, *Pandalus borealis*, *Metapenaeus ensis*, sea shrimp, *Brachium nipponense*, lobster, *Procambarus clarkii*, *Penaeus japonicus*, dried peeled shrimp, *Acetes chinensis* Hansen, *Oratosquilla oratoria*, *Orithya sinica*, and *Charybdis japonica*, respectively. Curves 17 through 23 exhibit negative results for *Eriocheir sinensis*, *Portunus trituberculatus*, *Chionoecetes bairdi*, fish, shellfish, oyster, and octopus, respectively.

chinensis Hansen, and *Oratosquilla oratoria* samples. Similarly, 4 other kinds of crab, 24 kinds of fish, and 12 kinds of shellfish and octopus did not exhibit any fluorescence signal. While using Shrimp 2 primers and probes, 16 samples, including prawn, *C. septemspinosa*, *M. nipponense*, *A. japonicus*, *P. borealis*, *Metapenaeus ensis*, sea shrimp, *B. nipponense*, lobster, *P. clarkii*, *P. japonicus*, dried peeled shrimp, dried mall shrimp, *O. oratoria*, *O. sinica*, and *C. japonica*, were also successfully amplified to reveal strong fluorescence signals, as shown in Figure 2. However, *O. sinica* and *C. japonica* revealed weaker, nonspecific amplification, and other samples did not exhibit any fluorescence signal.

DNA fragment amplification of a specific gene from Arctic shrimp components. The shrimp components in *P. borealis* were not detected by using Shrimp 1 primers and probes during real-time PCR detection. Therefore, a specific primer and probe set was designed for real-time fluorescent PCR detection of *P. borealis*. Only the DNA fragment from Arctic shrimp samples was amplified to reveal a strong fluorescence signal using this set, while 13 other kinds of shrimp, 5 kinds of crab, 24 kinds of fish, 12 kinds of shellfish, and octopus did not exhibit any fluorescence signal, as shown in Figure 3. These results suggested that the primers and probes designed for Arctic shrimp samples had excellent specificity and were suitable to identify the shrimp-derived components from *P. borealis*.

The results for real-time fluorescent PCR detection using the primers and probes for Shrimp 1, Shrimp 2, and *Pandalus borealis* 3 are summarized in Table 1. As for the

unknown samples, all of the ones with shrimp-derived components could be detected by using the three sets of primers and probes simultaneously, due to their complementary roles. When using the Shrimp 1 and Shrimp 2 primers and probes for real-time PCR detection, DNA from *O. sinica*, *C. japonica*, and sea crab samples could be amplified, which suggests that crabs and shrimps have a relatively close genetic relationship and belong to Arthropoda: Decapoda crustaceans. Both crab and shrimp are important food allergens among the crustaceans (9, 13). When using the Shrimp 1, Shrimp 2, and *Pandalus borealis* 3 primers and probes for real-time PCR detection, if there are two positive results, we can decide directly that there are shrimp components in the food; if there is only one positive result, the false-positive results can be excluded through further sequencing and BLAST analysis of the DNA amplified from PCR by using the Shrimp 1 and Shrimp 2 primers.

Sensitivity of detection methods. The prawn samples were used to determine the detection limits of the real-time PCR method using the Shrimp 1 primers and probe. The prawns as raw materials were subjected to heat treatment, which could result in DNA breakage and loss and affect detection sensitivity. In our study, the shrimp and fish samples were heated at 133°C for 30 min according to the previously reported method (14). The real-time PCR detection limit is shown in Figure 4. The DNA from samples containing 10, 5, 0.5, 0.1, and 0.05% shrimp powder revealed fluorescence signals with amplification; in contrast, samples with 0.01% shrimp powder or without

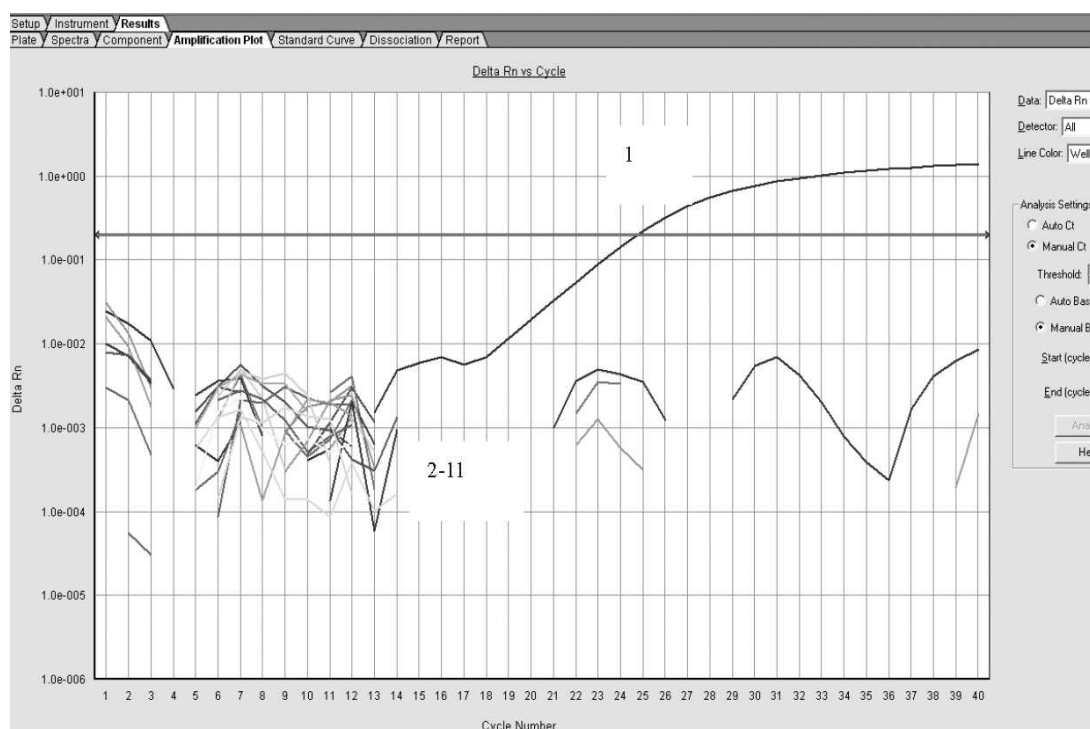


FIGURE 3. Real-time detection of shrimp-derived components using primers and probe specific for *Pandalus borealis*. Curve 1 exhibits positive amplification for *Pandalus borealis*. Curves 2 through 11 exhibit negative results for other shrimps, crabs, fish, shellfish, and octopus.

shrimp powder did not generate fluorescence signals. Experiments to determine detection sensitivity following heat treatment of samples at 133°C for 30 min, DNA extraction, and PCR amplification were repeated six times, and the established real-time fluorescent PCR provided a DNA detection limit of shrimp powder at 0.05%.

Results of detection method in actual food matrices.

In order to validate the method of detection of shrimp-derived components in food by real-time fluorescent PCR, DNA was extracted from food samples, such as shrimp dumpling prawn seasoning of instant noodles, shrimp paste, shrimp sauce, and shrimp soup, and detected using the

TABLE 1. Detection results for shrimp-derived components using three pairs of primers and probes

Sample no.	Sample type	Detection result using primer pair and probe set:		
		Shrimp 1	Shrimp 2	<i>Pandalus borealis</i> 3
1	Prawn	+	+	—
2	<i>Crangon septemspinosa</i>	+	+	—
3	<i>Macrobrachium nipponense</i>	+	+	—
4	<i>Alpheus japonicus</i>	+	+	—
5	<i>Pandalus borealis</i>	—	+	+
6	<i>Metapenaeus ensis</i>	+	+	—
7	Sea shrimp	+	+	—
8	<i>Brachium nipponense</i>	+	+	—
9	Lobster	+	+	—
10	<i>Procambarus clarkii</i>	+	+	—
11	<i>Penaeus japonicus</i>	+	+	—
12	Dried peeled shrimp	—	+	—
13	<i>Acetes chinensis</i> Hansen	—	+	—
14	<i>Oratosquilla oratoria</i>	—	+	—
15	<i>Orithya sinica</i>	—	+	—
16	<i>Charybdis japonica</i>	—	+	—
17	<i>Portunus trituberculatus</i>	+	—	—
18	<i>Eriocheir sinensis</i>	—	—	—
19	<i>Chionoecetes bairdi</i>	—	—	—
20	Octopus	—	—	—
21	Whitebait	—	—	—
22	Short-necked clam	—	—	—

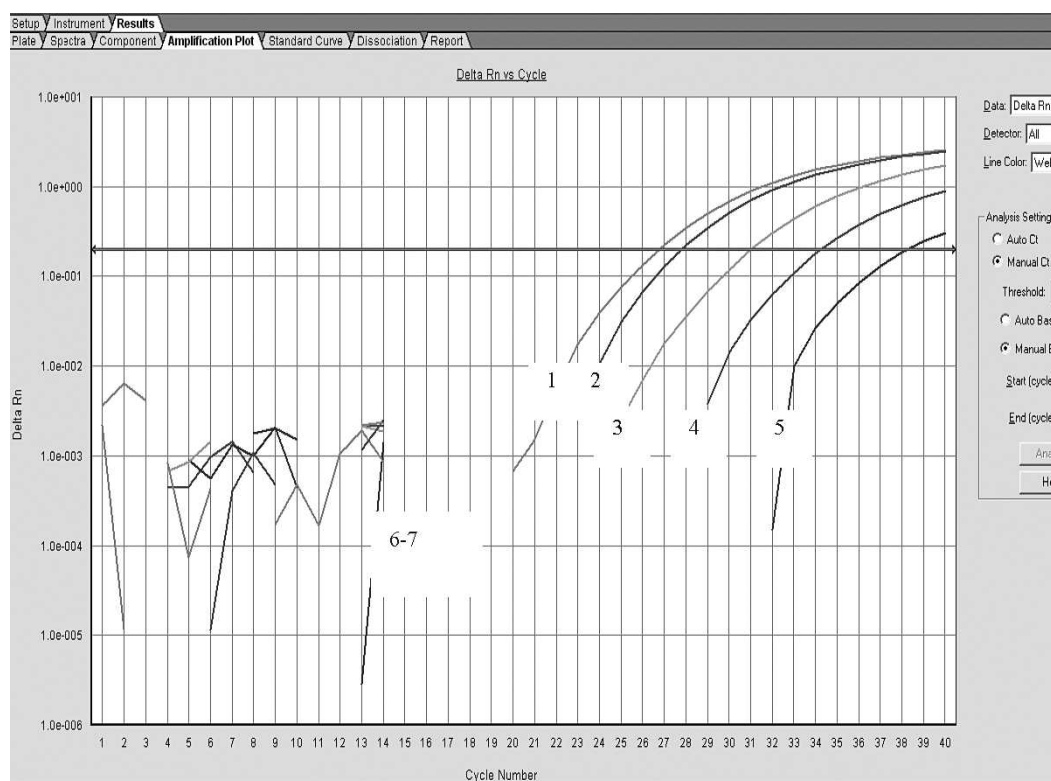


FIGURE 4. Detection sensitivity of shrimp powder samples using real-time fluorescent PCR. Curves 1 through 5 exhibit positive amplification of fish powder DNA samples containing shrimp powder DNA sample at proportions of 10, 5, 0.5, 0.1, and 0.05%, respectively. Curves 6 and 7 exhibit negative results for 0.01 and 0% of shrimp powder samples, respectively.

Shrimp 1 primers and probe for real-time PCR detection. Shrimp components were detected in the shrimp dumpling prawn seasoning of instant noodles and shrimp paste, but the shrimp components in shrimp sauce and shrimp soup could not be detected, suggesting that this method is not suitable for the detection of shrimp in sauce and soup. The major reason may be the difficulty in DNA extraction.

Shrimp powder was added to chicken, vegetables, and flour at 0.05%, and the extracted DNA from these mixtures was used as the DNA template of real-time PCR with Shrimp 1 primers and probe. The results showed that shrimp components could be detected in these sample mixtures. Therefore, real-time fluorescent PCR is a suitable method for detecting shrimp-derived components in foods, except for sauce and soup.

DISCUSSION

Currently, a variety of strategies are used to detect food allergens all over the world (2, 7). The majority of commercial and published allergen-specific assays are protein based, while only a few DNA-based assays have been developed so far. This is due to the quick performance and easy handling of enzyme-linked immunosorbent assays (ELISAs). However, ELISAs have some limitations. First, it is difficult to ensure consistency of human serum and achieve standardization with this method due to the dependence on human serum. Meanwhile, the quality and stability of animal antibodies also affect the detection results, and animal antibodies need a longer period of research and development. Moreover, some food processing

technologies, such as thermal processing, may destroy the allergens and result in reduced signal detection. Compared with ELISAs, which are based on monoclonal or polyclonal antibodies, another advantage of real-time PCR is that recombinant thermostable DNA polymerase for whole PCR can be synthesized and manufactured in unlimited amounts and constant quality. Furthermore, the specific reagents, such as primers and probes, that are needed for PCR can be designed to meet special requirements for specificity and sensitivity. Lastly, PCR offers unparalleled specificity in species identification, so it is a feasible method for the detection of allergenic food components from animals or plants with high homology. By the use of a single protocol, DNA from various species is extracted simultaneously from a complex food matrix. Therefore, a multianalyte detection that is based on the same sample preparation is possible. For this reason, PCR has great potential to become a screening method for allergens in food, because currently available ELISAs are still single-analyte tests and require optimized extraction conditions for each allergenic food that is to be detected. As for the wide range of protein allergens in shrimp, real-time PCR detection technology exhibits an advantage for fast screening of shrimp-derived components.

To establish a reliable PCR identification method of shrimp-derived components, it is necessary to select an appropriate gene. The selected gene should have high specificity and relatively low variability among species. In order to select an appropriate gene fragment from shrimp, we used the nucleotide sequences published in the NCBI database. The 16S rRNA gene fragments of some candidate

genes were selected. Through comparison of the primer sequences with the NCBI database, BLAST analysis provided high matches of up to 100% in all shrimp primers. The prawns as the raw materials were subjected to heat treatment, which can result in DNA breakage and loss and affect detection sensitivity. The effect of heat treatment on the detection sensitivity of real-time PCR was explored, and a detection sensitivity of up to 0.05% roasted shrimp powder in fish powder could be achieved. The present study shows that real-time fluorescent PCR detection can provide an effective identification method, with high specificity and sensitivity, for shrimp-derived allergens in foods.

ACKNOWLEDGMENT

This study was supported by National nonprofit research entitled “quantitative identification method of food allergens” (project no. 2007GYJ036).

REFERENCES

1. An, A. Z., and Y. Q. Sun. 2003. One case of allergic shock caused by shrimp pit. *Chin. J. Med. Theory Pract.* 16:877.
2. Byun, M. W., J. H. Kim, J. W. Lee, J. W. Park, C. S. Hong, and I. J. Kang. 2000. Effects of gamma radiation on the conformational and antigenic properties of a heat-stable major allergen in brown shrimp. *J. Food Prot.* 63:940–944.
3. Daul, C. B., J. E. Morgan, and S. B. Lehrer. 1990. The natural history of shrimp hypersensitivity. *J. Allergy Clin. Immunol.* 86:88–93.
4. Gu, K. F., M. X. Gao, C. H. Li, and J. R. Pan. 2007. Allergic reactions and allergens of shrimps. *Chin. J. Food Sci. Technol.* 28: 239–240.
5. Hoffman, D. R., J. R. Day, and J. S. Miller. 1981. The major heat stable allergen of shrimp. *Ann. Allergy* 47:17–22.
6. Jeoung, B. J., G. Reese, P. Hauck, J. B. Oliver, C. B. Daul, and S. B. Lehrer. 1997. Quantification of the major brown shrimp allergen Pen a 1 (tropomyosin) by a monoclonal antibody-based sandwich ELISA. *J. Allergy Clin. Immunol.* 100:229–234.
7. Lai, H., Q. H. Zhao, and Y. P. Liu. 2004. Allergen compositions of Roche shrimps by Western blotting analysis. *Chin. J. Microbiol. Immunol.* 24:747.
8. Li, Z. X., H. Lin, and M. H. Li. 2005. Allergens in different shrimp species. *Fish. Sci.* 30:281–284.
9. Metcalfe, D. D., H. A. Sampson, and R. A. Simon. 1997. Food allergy: adverse reactions to foods and food additives, 2nd ed. Blackwell Science, Inc., Cambridge, MA.
10. Morgan, J. E., C. B. Daul, and S. B. Lehrer. 1990. The relationships among shrimp-specific IgG subclass antibodies and immediate adverse reactions to shrimp challenge. *J. Allergy Clin. Immunol.* 86:387–392.
11. Nagpal, S., L. Rajappa, D. D. Metcalfe, and P. V. S. Rao. 1989. Isolation and characterization of heat stable allergens from shrimp (*Penaeus indicus*). *J. Allergy Clin. Immunol.* 83:26–36.
12. Reese, G., R. Ayuso, T. Carle, and S. B. Lehrer. 1999. IgE-binding epitopes of shrimp tropomyosin, the major allergen Pen a 1. *Int. Arch. Allergy Immunol.* 118:300–301.
13. Schafer, T., E. Bohler, S. Ruhdorfer, L. Weigl, D. Wessner, J. Heinrich, B. Filipiak, H. E. Wichmann, and J. Ring. 2001. Epidemiology of food allergy/food intolerance in adults: associations with other manifestations of atopy. *Allergy* 56:1172–1179.
14. Tartaglia, M., E. Saulle, S. Pestalozza, L. Morelli, G. Antonucci, and P. A. Battaglia. 1998. Detection of bovine mitochondrial DNA in ruminant feeds: a molecular approach to test for the presence of bovine-derived materials. *J. Food Prot.* 61:513–518.
15. Xu, Y., and H. Xu. 2000. One case of allergic shock after eating Oratosquilla oratoria. *J. Shenyang Med. College* 2:106.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.