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Specific Detection of Potentially Allergenic Kiwifruit in Foods Using Polymerase Chain Reaction

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Kiwifruit (*Actinidia deliciosa* and *Actinidia chinensis*) is allergenic to sensitive patients, and, under Japanese regulations, it is one of the food items that are recommended to be declared on food labeling as much as possible. To develop PCR-based methods for the detection of trace amounts of kiwifruit in foods, two primer pairs targeting the ITS-1 region of the *Actinidia* spp. were designed using PCR simulation software. On the basis of the known distribution of a major kiwifruit allergen (actinidin) within the *Actinidia* spp., as well as of reports on clinical and immunological cross-reactivities, one of the primer pairs was designed to detect all *Actinidia* spp. and the other to detect commercially grown *Actinidia* spp. (i.e., kiwifruit, *Actinidia arguta*, and their interspecific hybrids) except for *Actinidia polygama*. The specificity of the developed methods using the designed primer pairs was verified by performing PCR experiments on 8 *Actinidia* spp. and 26 other plants including fruits. The methods were considered to be specific enough to yield target-size products only from the target *Actinidia* spp. and to detect no target-size products from nontarget species. The methods were sensitive enough to detect 5−50 fg of *Actinidia* spp. DNA spiked in 50 ng of salmon testis DNA used as a carrier (1−10 ppm of kiwifruit DNA) and 1700 ppm (w/w) of fresh kiwifruit puree spiked in a commercial plain yogurt (corresponding to ca. 10 ppm of kiwifruit protein). These methods would be expected to be useful in the detection of hidden kiwifruit and its related species in processed foods.

KEYWORDS: Food allergy; kiwifruit; *Actinidia* spp.; internal transcribed spacer; ITS; PCR

INTRODUCTION

Kiwifruit (*Actinidia deliciosa* cv. Hayward and *Actinidia chinensis* cv. Hort16A) is a major fruit that is cultivated extensively in New Zealand, Italy, Chile, France, Greece, and other subtropical areas including Japan. Kiwifruit allergy is one of the most important fruit allergies because of its serious symptoms and because many clinical cases have been reported worldwide (*1*−*3*). The general symptoms of the kiwifruit allergy are urticaria and oral allergy syndrome (OAS), the latter of which includes such symptoms as oral and pharyngeal itching, oral papules or blisters, lip irritation and swelling, labial edema, and glottis edema (*1*, *4*). Many clinical cases of kiwifruit allergy have been reported in Europe (*5*) and Japan (*6*, *7*), and the ingestion of a trace amount of kiwifruit induces the symptoms in sensitive patients (*8*).

In addition to kiwifruit, some other *Actinidia* spp. are also cultivated for food and distributed in the market (*9*). For example, a cultivar of *Actinidia arguta* (sarunashi), commercially known as baby kiwi, is grown and distributed in many countries. In addition, *Actinidia polygama* (matatabi) and interspecific hybrids of *Actinidia arguta* × *A. deliciosa* are also grown and consumed in Japan in the form of fresh fruit, juice, jam, and so on.

Some of the allergen molecules in kiwifruit have been reported (*10*). One of the major ones, actinidin (Act c 1), was also reported to be present in *A. arguta* (*11*−*13*). Although there have been few reports of the presence of kiwifruit allergen in *A. polygama*, the intake of *A. polygama* by patients with kiwifruit allergy might induce the allergy.

According to Japanese food labeling regulations, five food items (wheat, buckwheat, egg, milk, and peanut) must be declared on food labeling, and several detection methods for these items have been reported (*14*, *15*). In addition to these 5 items, a Japanese ordinance recommends that 20 food items, including kiwifruits, should be declared on food labeling as far as possible. In terms of these 20 food items, the detection

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methods remain to be developed. To assess the validity of food labeling and to ensure the safety of allergic patients, detection methods for these 20 items are indispensable.

Major techniques used in the detection of allergenic foods are ELISA and PCR. Both techniques have advantages and disadvantages. Whereas most of the ELISA methods target a specific allergenic protein and usually some cross-reactivity could occur, PCR targets a specific DNA sequence to detect the presence of the offending food. Therefore, PCR would be suitable for a final identification method of the presence of an allergenic ingredient for food labeling. In addition, it is generally thought that the damage done to DNA during food processing is relatively less compared to the damage done to proteins (*16*). In Japan, it is either mandatory or recommended to declare allergenic ingredients on food labeling when 10 ppm (μg/g or μg/mL) or more total protein of an allergenic food is present. As an allergenic food detection method, Japanese regulation specifies PCR for final identification of the presence of some allergenic ingredients after initial screening determination by ELISA. Both methods would be complementary to each other and should be necessary for an accurate allergenic ingredient testing.

The internal transcribed spacer (ITS) used for our PCR target is located between 18S (small subunit) and 26S (large subunit) nuclear ribosomal RNA genes, which include two spacers (ITS-1 and ITS-2) separated by the 5.8S rRNA gene. The ITS is known to be present in large copy numbers on genomic DNA and to be useful for congeneric or conspecific classifications (*17*−*19*). Use of these regions allows us to detect a target plant species with high sensitivity and specificity (*20*).

In the present study, we designed two primer pairs for PCR-based kiwifruit detection methods using ITS-1 as the target region; one was designed for detecting all of the *Actinidia* spp. and the other for detecting commercially grown *Actinidia* spp. but excluding *A. polygama*. The specificity, sensitivity, and analytical results from experiments using the developed methods to detect kiwifruit or sarunashi in several commercial products are reported.

MATERIALS AND METHODS

Samples Used in DNA Isolation. Two kiwifruits (*A. deliciosa* cv. Hayward and *A. chinensis* cv. Hort16A), two fruits of tara vine [sarunashi in Japanese (*A. arguta* cv. Issai, an unknown cultivar of *A. arguta* marketed as baby kiwi)], one interspecific hybrid of kiwifruit named Sanuki gold, two interspecific hybrids of *A. arguta* × *A. deliciosa* named Kosui and Shinzan, and one fruit of silver vine [matatabi in Japanese (*A. polygama*)] were purchased from local markets and farms in Japan. Apples (*Malus domestica*), aloe plants (*Aloe arborescens*), apricots (*Prunus armeniaca*), avocados (*Persea americana*), bananas (*Musa acuminata*), blueberries (*Vaccinium* spp.), cherries (*Prunus avium*), figs (*Ficus carica*), grapes (*Vitis* spp.), persimmons (*Diospyros kaki*), mangos (*Mangifera indica*), melons (*Cucumis melo*), oranges (*Citrus sinensis*), papayas (*Carica papaya*), peaches (*Prunus percica*), pears (*Pyrus communis*), pineapples (*Ananas comosus*), prunes (*Prunus salicina*), raspberries (*Rubus idaeus*), satsuma oranges (*Citrus unshu*), strawberries (*Fragaria* × *ananassa*), Japanese apricots (*Prunus mume*), corn (*Zea mays*), rice (*Oryza sativa*), soybean (*Glycine max*), and wheat (*Triticum aestivum*) were purchased at local supermarkets in Chiba and Tokyo, Japan. Some commercial products containing kiwifruit or sarunashi were also purchased, namely, a cereal with a dried fruit mix, a kiwifruit cookie, dried kiwifruits, gummy candies (assorted fruit flavors), a kiwifruit jam, a sarunashi jam, three kinds of juice or fruit drinks (100% kiwifruit juice, mixed fruits including kiwifruit, and 10% sarunashi juice), and two kinds of yogurt (one with pieces of mixed fruit and the other with pieces of kiwifruit only). Finally, some commercial products without kiwifruit or sarunashi in the list of ingredients, namely, a cereal with a dried fruit mix, a cookie with grapefruit jam, and fruit and vegetable drinks, were also purchased at local supermarkets in Chiba and Tokyo, Japan.

Primer Design. The DNA sequences of the ITS-1 region were used for the primer design. Twenty-eight sequences of the family Actinidiaceae (including 26 *Actinidia* spp.) and 29 sequences of plants used for food (including fruits) were obtained from GenBank. Because the sequences of banana (*Musa acuminata*), fig (*Ficus carica*), and persimmon (*Diospyros kaki*) were not found in GenBank, the sequences obtained from congeneric species of those fruits were used for the PCR simulations. When there was more than one ITS-1 sequence reported for an *Actinidia* species, the sequence most homologous to the *A. deliciosa* ITS-1 sequence was selected as the representative of that species. In addition, ITS-1 sequences of *A. deliciosa* cv. Hayward, *A. chinensis* cv. Hort16A, *A. arguta* cv. Issai, an unknown cultivar of *A. arguta* (baby kiwi), and *A. polygama* (matatabi) purchased for this study were determined by a direct sequencing method. Two sets of primer pairs (the F151 and R182 primer pair and the F123 and R178 primer pair) were designed on the basis of the highly homologous sequences among the target *Actinidia* spp. in the ITS-1 region. PCR simulations were performed with Amplify 1.0 software (Bill Engels, University of Wisconsin) to predict whether PCR products of the target size would be obtained from the DNA sequences of ITS-1 reported for 33 *Actinidia* spp., 2 species of the family Actinidiaceae other than *Actinidia* spp., and 29 other plants listed in **Table 1**. The primer pair of CP03-F (5′-CGG ACG AGA ATA AAG ATA GAG T-3′) and CP03-R (5′-TTT TGG GGA TAG AGG GAC TTG A-3′), designed to amplify a partial region of plant chloroplast DNA (*21*), was used to validate the quality of extracted DNA as templates. The primers were synthesized and purified with an oligonucleotide purification cartridge by Operon Biotechnologies, Inc.

DNA Extraction from Plants. Plant materials (the flesh of fruits, seeds, or leaves depending on the samples) were homogenized using an MM300 mixer mill (Retsch, Haan, Germany). DNA was extracted from 2 g of homogenized sample with 20 mL of buffer G2 (Qiagen, Hilden, Germany) and purified using Genomic-tip 20/G (Qiagen) according to the manufacturer's instructions. The DNA concentrations were determined by measuring the UV absorption at 260 nm. All DNA solutions were diluted to 20 ng/μL with TE (pH 8.0) and used for PCR templates. For the sensitivity studies, DNA solutions of *Actinidia* spp. were further diluted with 5 ng/μL salmon testis DNA (Sigma Chemical Co., St. Louis, MO) solution. In addition, *A. deliciosa* cv. Hayward DNA was diluted with 20 ng/μL of salmon testis DNA to obtain 0.1−10 ppm (wt/wt) of kiwifruit DNA in 50 ng of salmon testis DNA (the carrier DNA) and used for PCR templates.

DNA Extraction from Commercial Products and Kiwifruit-Spiked Sample. An entire pack of each commercial product was homogenized using a mixer mill (IMF-300; Iwatani International Corp., Tokyo, Japan). Then, DNA was isolated from the homogeneous mixture according to the same method using Genomic-tip 20/G (Qiagen) as described above (×1 DNA extraction scale). Because the DNA samples extracted from kiwifruit jam, kiwifruit juice, and gummy candies did not consistently yield the expected PCR products, the amount of the sample and buffer G2 was increased 10 times, and the extracted DNA was purified using Genomic-tip 100/G (×10 DNA extraction scale). To obtain amplifiable DNA from dried kiwifruit, it was first necessary to wash off the surface coating with distilled water. For the sensitivity studies, DNA was also isolated from a commercial plain yogurt spiked with 1700 ppm (w/w) of fresh kiwifruit (Hayward) puree (0.6% protein content measured with a 2-D Quant kit; GE Healthcare U.K. Ltd., Little Chalfont, U.K.). The DNA concentrations were determined by measuring the UV absorption at 260 nm and adjusted to 20 ng/μL with TE (pH 8.0) for PCR. In most cases, however, the DNA samples extracted from the commercial products were less than 20 ng/μL and were directly subjected to PCR without dilution.

PCR. PCR was carried out in a 25 μL reaction volume containing 0.2 mM of each dNTP, 1× buffer (PCR buffer II), 1.5 mM $MgCl_2$, 0.625 unit of AmpliTaq Gold (Applied Biosystems, Foster City, CA), 0.5 μM of each primer pair (the F151 and R182 primer pair or the F123 and R178 primer pair), and 5 fg−50 ng of template DNA. The amplifications were performed in a GeneAmp PCR system 9600

Table 1. Specificity Prediction of Primer Pairs (**A**, F151 and R 182; **B**, F123 and R178) with PCR Simulation Software

species (common name)	GenBank Accession no.	weight no.[a] (amplicon size)[b] (**A**) F151 and R182 primer pair	(**B**) F123 and R178 primer pair
family Actinidiaceae			
genus *Actinidia*			
A. deliciosa (Hayward)	AB253775	6*[c]	6*
A. chinensis (Hort16A)	AB253776	5*	6*
A. deliciosa	AF323830	6*	6*
A. arguta (Issai)	AB253777	5*	5*
A. arguta (baby kiwi)	AB253778	5*	6*
A. arguta	AY216736	5*	6*
A. arguta	AF323836	5*	6*
A. arguta	AF323835	5*	6*
A. polygama (matatabi)	AB253779	5*	–
A. polygama	AF323796	5*	–
A. callosa	AF323829	6*	6
A. chrysantha	AF323797	5*	6
A. cylindrica	AF323807	5*	6
A. eriantha	AF323801	5*	6
A. fulvicoma	AF323799	5*	6
A. glaucophylla	AF323798	5*	6
A. hemsleyana	AF323802	4*	–
A. henanesis	AF323841	6*	6
A. indochinensis	AF323810	5*	6
A. kolomikta	AF323837	5*(75 bp)	–
A. latifolia	AF323825	5*	6
A. macrosperma	AF323834	5*	6
A. melanandra	AF443211	5*	6
A. melliana	AF323821	5*	5
A. persicina	AF323814	5*	–
A. rufa	AF323838	5*	6 (93 bp)
A. rufa	AF323839	5*	5 (93 bp)
A. sabiifolia	AF323813	5*	6
A. styracifolia	AF323822	5*	6
A. valvata	AF323842	5*	6
A. zhejiangensis	AF323819	5*	–
other genera			
Clematoclethra lasioclada	AF323805	–	–
Saurauia zahlbruckneri	AF396452	–	–
plants used for food (containing major fruits)			
Aloe vera (aloe)	AF234345	–	–
Carica papaya (papaya)	AY461547	–	–
Cucumis melo (melon)	CME488233	2 (49 bp)	–
Diospyros whyteana (relative of persimmon)	AF396234	–	–
Ficus tonduzii (relative of fig)	AY730140	–	–
Fragaria × *ananassa* (strawberry)	AF163494	–	–
Malus domestica (apple)	MDU16195	–	–
Mangifera indica (mango)	AB071674	–	–
Musa beccarii (relative of banana)	AF434900	–	–
Persea americana (avocado)	AF272322	2 (39 bp)	–
Prunus armeniaca (apricot)	AF318756	–	–
Prunus avium (cherry)	AF318737	–	–
Prunus domestica (plum)	AF318713	–	–
Prunus mume (Japanese apricot)	AF318728	–	–
Prunus persica (peach)	AF318741	–	–
Pyrus calleryana (pear)	PCU16202	–	–
Pyrus pyrifolia (pear)	AF287247	–	–
Pyrus salicifolia (relative of pear)	AF186532	–	–
Rubus idaeus (raspberry)	AF055757	–	–
Vaccinium corymbosum (blueberry)	AF419778	–	–
Vaccinium uliginosum (blueberry)	DQ217769	–	–
Vitis rotundifolia (grape)	AY037922	–	–
Vitis vinifera (grape)	AF365988	–	–
Arachis hypogaea (peanut)	AF156675	–	–
Fagopyrum esculentum (soba)	AB000330	–	–
Glycine max (soybean)	AF144654	–	–
Oryza sativa (rice)	AF169230	–	–
Triticum aestivum (wheat)	AM040486	–	–
Zea mays (corn)	U46648	–	–

[a] An approximate guide to the quality of the matches and the strength of the amplifications. The larger the weight number (1–6), the higher the probability of amplification. A dash (–) indicates no amplicon was predicted. [b] The predicted size of the amplicon, which is different from the target size. The amplicon sizes reported here are 2 bp shorter than those predicted by Amplify 1.0, which takes into account the terminal transferase activity of DNA polymerase (F151 and R182, 74 bp; F123 and R178, 92 bp). [c] An asterisk (*) indicates the target *Actinidia* spp. in each PCR primer pair.

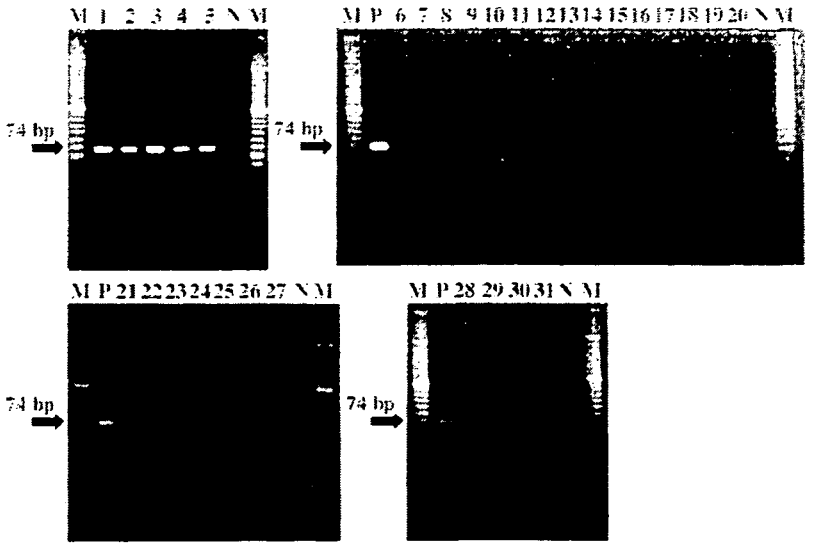

Figure 1. Specificity of the F151 and R182 primer pair for all *Actinidia* spp. The arrowhead indicates the expected PCR product. M, DNA marker (20 bp ladder, Takara Bio Inc.); P, amplification of 500 pg of Hayward genomic DNA as a positive control; N, negative control (no template). (Lanes 1–31) Amplification of 50 ng of Hayward (1), Hort16A (2), Issai (3), baby kiwi (4), matatabi (5), aloe (6), pineapple (7), papaya (8), orange (9), satsuma orange (10), melon (11), persimmon (12), fig (13), strawberry (14), apple (15), mango (16), banana (17), avocado (18), apricot (19), cherry (20), Japanese apricot (21), peach (22), prune (23), pear (24), raspberry (25), blueberry (26), grape (27), wheat (28), rice (29), soybean (30), and corn (31) genomic DNA.

(Applied Biosystems) as follows: preincubation at 95 °C for 10 min; 50 cycles consisting of denaturation at 95 °C for 0.5 min, annealing at 60 °C for 0.5 min, and extension at 72 °C for 0.5 min; and a final extension at 72 °C for 7 min. When the F123 and R178 primer pair was used, the number of PCR cycles was reduced to 40. The PCR products (5 μL) were electrophoresed on a 3% agarose gel containing ethidium bromide and analyzed with a ChemiDoc XRS illuminator (Bio-Rad Laboratories, Inc., Hercules, CA). Sensitivity studies for each primer pair were performed in eight replicate runs. All of the DNA samples used for the specificity and sensitivity studies gave the expected PCR products with the CP03-F and CP03-R primer pair used for the quality validation of the DNA (*21*).

RESULTS

Primer Design for Detection of Kiwifruit. Two sets of primer pairs were designed. One primer pair was designed to detect all of the *Actinidia* spp. including kiwifruit, *A. arguta*, and *A. polygama*. The other was designed to detect kiwifruit and *A. arguta* but not *A. polygama* in commercially grown *Actinidia* spp. Each primer was carefully designed so that the nucleotides at the position corresponding to the 3′ end of the primer would be the same in all of the sequences of the target species and would differ from those of the nontarget species. Consequently, the F151 (5′-GTG ACA CTC TCA TTC CCC G-3′) and R182 (5′-TTG CAT TCT TGT TCA AGT TCC TTG A-3′) primer pair was designed for the detection of all the *Actinidia* spp., and the F123 (5′-CGG GTG TGC TCG TGT-(C) TG-3′, 5′-CGG GTG TGC TCG TGC CG-3′) and R178 (5′-CTT GTT CAA GTT CCT TGA CGC G-3′) primer pair was designed for the detection of kiwifruit and *A. arguta*.

Specificity Analysis of Both Primer Pairs Predicted Using PCR Simulation Software. The specificity of the primer pairs was predicted with PCR simulation software. With both primer pairs, PCR products of the expected sizes (F151 and R182, 74 bp; F123 and R178, 92 bp) were predicted from the ITS-1 sequences of the target *Actinidia* spp., which are indicated with an asterisk (*) in **Table 1**. Although products of the target size were predicted from the nontarget wild *Actinidia* spp. when the F123 and R178 primer pair was used, the primer pair successfully differentiated *A. polygama* (matatabi) from other commercially distributed *Actinidia* spp., that is, kiwifruits and sarunashi (**Table 1B**). We considered that amplification products of the target size predicted from the wild species of *Actinidia* would not cause significant problems in developing kiwifruit detection methods, because those wild species were presumed unlikely to be mixed in foods. No products were predicted for the other plants used for food (including fruits), except for some products of nontarget size predicted from melon and avocado when the primer pair of F151 and R182 was used (**Table 1A**).

Specificity and Sensitivity of the Detection Method for All *Actinidia* Species. The specificity of the proposed detection method using the F151 and R182 primer pair was confirmed by the PCR experiments described under Materials and Methods. As shown in **Figure 1**, a PCR product of the target size (74 bp) was amplified from the genomic DNAs extracted from *A. deliciosa* cv. Hayward, *A. chinensis* cv. Hort16A, *A. arguta* cv. Issai, *A. arguta* (baby kiwi), and *A. polygama* (matatabi). The nucleotide sequence analyses of the PCR products confirmed that the target sequences of the *Actinidia* spp. had been amplified using the designed primer pairs (data not shown). Although some nonspecific products were sporadically amplified from satsuma orange, persimmon, and orange, all of them were different in size from the target products (data not shown). Furthermore, the sensitivity of the proposed detection method was confirmed using PCR. As shown in **Figure 2**, the PCR products of the target size were detected from 50 fg of DNA of all the target *Actinidia* spp., including three interspecific hybrids in all eight replicate runs. The target products were also detected in 50 ng of salmon testis DNA spiked with 1 ppm (w/w) of Hayward DNA and in a yogurt sample spiked with 1700 ppm (w/w) of fresh kiwifruit puree, which corresponded to ca. 10 ppm of kiwifruit protein (data not shown).

Specificity and Sensitivity of the Detection Method for Kiwifruit and *A. arguta*. The specificity of the proposed detection method using the F123 and R178 primer pair was

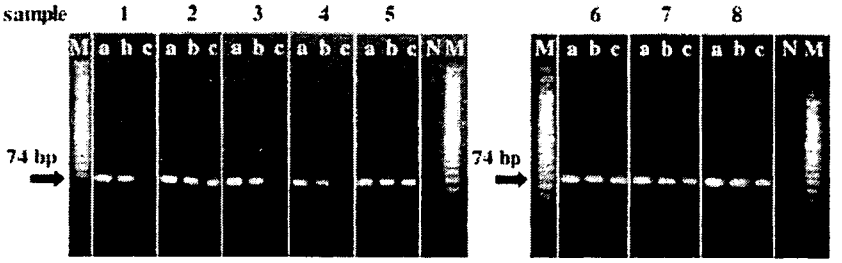

Figure 2. Sensitivity of the F151 and R182 primer pair for all *Actinidia* spp. The arrowhead indicates the expected PCR product. M, DNA marker (20 bp ladder, Takara Bio Inc.); N, negative control (no template). (Samples 1–8) Genomic DNA of Hayward (1), Hort16A (2), Issai (3), baby kiwi (4), matatabi (5), Kosui (6), Shinzan (7), and Sanuki gold (8). (Lanes a–c) Amplification of 500 fg (a), 50 fg (b), and 5 fg (c) of sample genomic DNA.

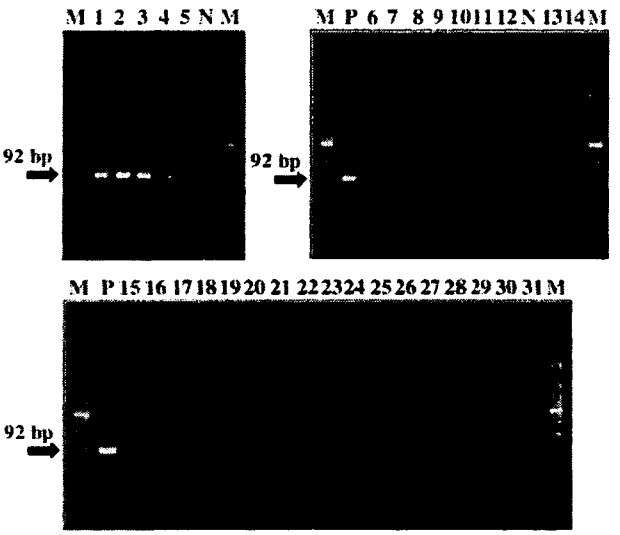

Figure 3. Specificity of the F123 and R178 primer pair for kiwifruit and *A. arguta* in commercially grown *Actinidia* spp. The arrowhead indicates the expected PCR product. M, DNA marker (20 bp ladder, Takara Bio Inc.); P, amplification of 500 pg of Hayward genomic DNA as a positive control; N, negative control (no template). (Lanes 1–31) Amplification of 50 ng of Hayward (1), Hort16A (2), Issai (3), baby kiwi (4), matatabi (5), corn (6), soybean (7), pear (8), strawberry (9), persimmon (10), grape (11), blueberry (12), banana (13), orange (14), peach (15), apricot (16), cherry (17), Japanese apricot (18), raspberry (19), prune (20), apple (21), papaya (22), mango (23), avocado (24), fig (25), pineapple (26), aloe (27), melon (28), satsuma orange (29), rice (30), and wheat (31) genomic DNA.

confirmed using PCR analysis. As shown in **Figure 3**, a PCR product of the target size (92 bp) was detected in the genomic DNAs extracted from *A. deliciosa* cv. Hayward, *A. chinensis* cv. Hort16A, *A. arguta* cv. Issai, and *A. arguta* (baby kiwi), but was not detected in the genomic DNA extracted from *A. polygama* (matatabi). The nucleotide sequence analyses of the PCR products confirmed that the expected sequences of the *Actinidia* spp. had been amplified (data not shown). Although nonspecific products were often amplified from some of the fruits and grains tested, all of them were different in size from the target (**Figure 3**). The sensitivity of the proposed method was also examined using PCR analysis. As shown in **Figure 4**, PCR products of the target size were amplified from 500 fg of DNA of all the target *Actinidia* spp., including three interspecific hybrids, in all eight replicate runs. The target products were also detected in 50 ng of salmon testis DNA spiked with 10 ppm (w/w) of Hayward DNA and in a yogurt sample spiked with 1700 ppm (w/w) of fresh kiwifruit puree, which corresponded to ca. 10 ppm of kiwifruit protein (data not shown).

Analysis of Commercial Products. A sufficient amount (20 ng/μL) of DNA for PCR was obtained only from the yogurts, the cereal products, and the cookies using Genomic-tip 20/G (×1 DNA extraction scale). Less than 20 ng/μL of DNA could be obtained from the other processed food samples using the same DNA extraction method. From the samples containing kiwifruit or sarunashi (samples 1−11), amplification products could be detected using both primer pairs, except in the case of the DNAs extracted from kiwifruit jam (5a), dried kiwifruit (7a), and gummy candies (11a), as shown in **Table 2**. By increasing the size of the sample and the buffer scale (×10 extraction scale), amplification products were obtained from the kiwifruit jam (5b) and the gummy candies (11b). As for the dried kiwifruit (7b), amplification products could be detected only when the samples for the DNA extraction were washed with distilled water prior to homogenization. From the samples that do not contain kiwifruit or sarunashi (samples 12−14), amplification products could not be detected using either set of primer pairs. To assess the template DNA quality, the CP03-F and CP03-R primer pair for detecting a partial region of plant chloroplast DNA was used for an internal control PCR, and the expected amplification products (124 bp) were detected in all samples tested with the exception of kiwifruit juice (9a and 9b), which nonetheless yielded a positive amplicon (74 and 92 bp) by kiwifruit PCR. On the basis of the electrophoresis analyses of the DNA extracted from the kiwifruit juice, we suggested that DNA fragmentation into approximately 100 bp (which resulted in a smear pattern) had occurred due to the processing (data not shown) and that this could have caused the negative result in the validation primer pair (CP03-F and CP03-R), which gave a longer amplicon than kiwifruit primer pairs. A validation primer pair that would give a shorter amplicon should be employed when the samples containing highly fragmentated DNA are analyzed.

DISCUSSION

Kiwifruit is known to cause serious allergic reactions. Nishiyama et al. (*13*, *14*). reported that actinidin, the major allergenic protein, was present not only in kiwifruit (*A. deliciosa* cv. Hayward) but also in Issai (*A. arguta*) and Shinzan (an interspecific hybrid of *A. arguta* × *A. deliciosa*). In fact, IgE antibody cross-reactivity between *A. arguta* and kiwifruit (*A. deliciosa*) has also been reported (*22*). In addition, some studies have suggested that certain other kinds of kiwifruit proteins can induce allergic symptoms. Lucas et al. reported that *A. chinensis*, which contains a small amount of actinidin, exhibited cross-reactivity with the serum IgE of a patient sensitive to *A. deliciosa* and provoked allergic symptoms (*23*). Therefore, it would be

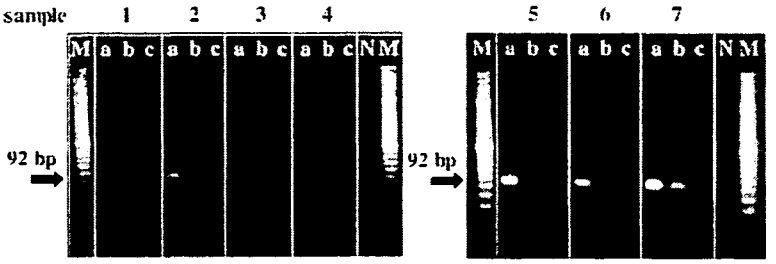

Figure 4. Sensitivity of the F123 and R178 primer pair for kiwifruit and *A. arguta* in commercially grown *Actinidia* spp. The arrowhead indicates the expected PCR product. M, DNA marker (20 bp ladder, Takara Bio Inc.); N, negative control (no template). (Samples 1–7) Genomic DNA of Hayward (1), Hort16A (2), Issai (3), baby kiwi (4), Kosui (5), Shinzan (6), and Sanuki gold (7). (Lanes a–c) Amplification of 500 fg (a), 50 fg (b), and 5 fg (c) of sample genomic DNA.

Table 2. Investigation of Commercial Products with PCR

no.	sample	concentration of template DNA (ng/μL)	PCR results[a] CP03-F and CP03-R primer pair	F151 and R182 primer pair	F123 and R178 primer pair
commercial products containing kiwifruit or sarunashi					
1	yogurt with mixed fruit pieces	20	+	+	+
2	yogurt with kiwifruit pieces only	20	+	+	+
3	cereal with dried fruit mix	20	+	+	+
4	kiwifruit cookie	20	+	+	+
5a	kiwifruit jam (×1)	<10	+	−	−
5b	kiwifruit jam (×10)	20	+	+	+
6	sarunashi jam	<10	+	+	+
7a	dried kiwifruit	<10	+	−	−
7b	dried kiwifruit (washing)	<10	+	+	+
8	fruit drink mixed fruits including kiwifruit	11	+	+	+
9a	100% kiwifruit juice (×1)	<10	±	+	+
9b	100% kiwifruit juice (×10)	20	−	+	−
10	10% sarunashi juice	<10	+	+	+
11a	gummy candies (×1)	<10	+	−	−
11b	gummy candies (×10)	<10	+	±	−
commercial products whthout kiwifruit or sarunashi in the list of ingredients					
12	cereal with dried fruit mix (not including kiwifruit)	20	+	−	−
13	cookie with grapefruit jam (not including kiwifruit)	20	+	−	−
14	fruit and vegetable drink (not including kiwifruit)	15	+	−	−

[a] Two independent PCR on a DNA preparation from each sample: +, 2/2; ±, 1/2; −, 0/2.

desirable for patients with kiwifruit allergy to avoid consuming such kiwifruits and their relatives. Although cross-reactivity between *A. polygama* and kiwifruit has not been reported because of the limited consumption of *A. polygama*, all *Actinidia* spp. may to some extent be potentially allergenic. Therefore, we first designed a primer pair to detect all *Actinidia* spp. On the other hand, among the edible *Actinidia* spp. distributed in the Japanese market, the fruit of *A. polygama* is easily distinguishable from the others by its appearance. Moreover, as mentioned above, the allergenicity or cross-reactivity of *A. polygama* cannot be excluded entirely. Therefore, we designed another primer pair that detects both kiwifruit and *A. arguta* but not *A. polygama* among commercially distributed *Actinidia* spp.

The ITS regions used in this study should be useful sequences not only for the genus-specific detection of all the *Actinidia* spp. but also for the detection of certain selected species from among the *Actinidia* spp. The results of PCR simulation with the designed primer pairs showed that products of the target sizes were predicted from the target species.

The specificities of the PCR methods examined with the materials purchased from the market were consistent with those predicted by the PCR simulations. Although some unexpected PCR products were amplified from some fruits and grains, their sizes were clearly different from that of the target. Although these simulation results are not definitive, they should give us a good indication of the specificity of the designed primer pairs. The results of the PCR simulation and the actual PCR suggested that these methods were specific enough to detect the genomic DNA extracted from kiwifruit and the target *Actinidia* spp.

Sensitivity studies showed that, with both primer pairs, the expected PCR products were detectable from a salmon testis DNA spiked with 50−500 fg of DNA of *Actinidia* spp., as low as 1−10 ppm (w/w) of kiwifruit DNA, or a yogurt sample spiked with 1700 ppm (w/w) of fresh kiwifruit puree (corresponding to ca. 10 ppm of kiwifruit protein). In terms of sensitivity, this level was comparable to that of the buckwheat detection method developed previously (*20*). Thus, the primer pairs designed in this work could provide reliable, specific, and sensitive detection of the presence of kiwifruit and other potentially allergenic kiwifruit-related plant species. Quantification of the kiwifruit amount might also be possible when a proper internal standard is used in real-time PCR (*24*).

The applicability of the proposed methods was assessed by analyzing several commercial products containing processed kiwifruit. All tested samples except for the gummy candies gave products of the target size with both primer pairs. The gummy candies were fruit-flavored gummies that included kiwifruit flavor. The ingredient list stated that the product contained kiwifruit juice as a colorant. Therefore, we concluded that the

absence of amplified PCR products from the candies was probably due to the limited amount of kiwifruit DNA in the gummy candies.

Only the yogurts, cereals, and cookies yielded more than 20 ng/μL DNA. Because these products would contain a large quantity of the DNA originally present in the respective raw materials (milk, lactic acid bacteria, cereal grains, and so forth), most of the DNA yielded from these products was mainly attributable to the matrix materials. On the other hand, the amounts of DNA extracted from jams and juices containing either kiwifruit or sarunashi (*A. arguta*) and from dried kiwifruit were lower than expected, even if large-scale DNA extractions were performed. Some improvements were made in the detection of kiwifruit from the kiwifruit jam and the gummy candies by scaling up the sample size of DNA extraction or washing the dried kiwifruit with distilled water prior to the homogenization for DNA extraction. Further studies are underway to improve the DNA extraction and the interlaboratory validation of the methods using processed food models that contain a known amount of kiwifruit.

In conclusion, we designed two kiwifruit detection primer pairs and developed a highly sensitive and specific PCR method of kiwifruit detection using these primer pairs. These methods would be expected to be useful for detecting kiwifruit in processed foods to confirm the validity of food labeling and to ensure the safety of allergic patients.

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PCR Method of Detecting Pork in Foods for Verifying Allergen Labeling and for Identifying Hidden Pork Ingredients in Processed Foods

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A PCR method to detect porcine DNA was developed for verifying the allergen labeling of foods and for identifying hidden pork ingredients in processed foods. The primer pair, F2/R1, was designed to detect the gene encoding porcine cytochrome b for the specific detection of pork with high sensitivity. The amplified DNA fragment (130 bp) was specifically detected from porcine DNA, while no amplification occurred with other species such as cattle, chicken, sheep, and horse. When the developed PCR method was used for investigating commercial food products, porcine DNA was clearly detected in those containing pork in the list of ingredients. In addition, 100 ppb of pork in heated *gyoza* (pork and vegetable dumpling) could be detected by this method. This method is rapid, specific and sensitive, making it applicable for detecting trace amounts of pork in processed foods.

Key words: food allergy; PCR; pork; cytochrome b; detection of allergen

Epidemiological studies suggest that between 10% and 20% of the world population exhibits some form of IgE-mediated hypersensitivity which is manifested as asthma, atopic dermatitis, or allergic rhinitis.[1] Among these, allergic reactions to foods are important worldwide health problems. Hen's egg,[2] cow's milk,[3] wheat[4] and peanuts[5] are generally known allergens for food-allergic patients. In contrast, meat is generally less allergenic than common allergy-inducing foods. Thus, a quarter century ago, children with food allergies were advised to be placed on an elimination diet that included meat.[6]

However, there is increasing evidence that even meat can provoke allergic reactions in sensitized patients. The prevalence of beef, pork, and chicken allergy has been reported to be 73%, 58%, and 41%, respectively, among 57 subjects with suspected meat allergies in USA.[7] The frequency of sensitization in skin prick test to pork has been reported to be 2.0% in Germany.[8] The cross-reactivity of pork and cat epithelia/dander has been reported and called "pork-cat syndrome."[9–11] Nearly all patients with IgE antibodies to pork also have IgE antibodies to cat epithelia/dander. However, among patients with IgE antibodies to cat epithelia/dander, only about 20% had IgE antibodies to pork.[9] Hilger *et al.*[11] have performed immunoblotting and cross-inhibition assays, and found porcine serum albumin (PSA) and cat serum albumin (*Fel d* 2) to be jointly recognized molecules. Inhibition assays showed that the spectrum of IgE reactivity to cat serum albumin completely contained IgE reactivity to PSA, suggesting that sensitization to cats was the primary event.[11]

Labeling for food allergens is required in many countries to prevent health hazards caused by foods containing allergens. In Japan, labeling is divided into the two stages, mandatory and recommended, according to the number of cases of actual illness and degree of seriousness.[12] The Ministry of Health, Labour and Welfare has made it mandatory to declare five food items (eggs, milk, wheat, buckwheat, and peanuts) and notified their recommendation to declare another 20 items (abalone, squid, salmon roe, shrimp/prawn, oranges, crab, kiwi fruit, beef, tree nuts, salmon, mackerel, soybeans, chicken (poultry), pork, mushrooms, peaches, yams, apples, gelatin, and bananas).[12] As already mentioned, beef, chicken and pork are recommended to be separately labeled in Japan. Pork has also become one of the labeled foods among 11 items in Korea.

To verify allergen labeling of foods and in order to identify hidden allergens in processed foods, it is important to provide sensitive and specific detection methods. In general, these methods are based on the detection of species-specific proteins by enzyme-linked immuno-

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 1 atgaccaaca tccgaaaatc acacccacta ataaaaatta tcaacaacgc attcattgac

61 ctcccagccc cctcaaacat ctcatcatga tgaaacttcg gttccctctt aggcatctgc

121 ctaatcttgc aaatcctaac aggcctgttc ttagcaatac attacacatc agacacaaca
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                                                <------------

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301 ggtctatact acggatccta tatattccta gaaacatgaa acattggagt agtcctacta

361 tttaccgtta tagcaacagc cttcataggc tacgtcctgc cctgaggaca aatatcattc
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(to be continued)

Fig. 1. Part of the Nucleotide Sequence of the Primers and Target Region on the Cytochrome b Gene. ——▶ and ◀—— indicate the forward (F2) and reverse (R1) primers, respectively.

sorbent assay (ELISA) and of species-specific DNA molecules by the polymerase chain reaction (PCR).[13] PCR leads to *in vitro* amplification of specific target DNA sequences by using the appropriate oligonucleotide primer pairs. This technique offers a high level of sensitivity, and an optimized PCR procedure amplifies the specific target sequence even in a very complex pool of genomic sequences.

We developed in this study a specific PCR method to detect porcine DNA in foods. This method enabled a trace amount (100 ppb) of pork contained in the model processed food to be successfully detected. The advantages of the method over such other methods as ELISA are also discussed in this paper.

Materials and Methods

Samples and DNA extraction. All meat (pork, beef, chicken, mutton, boar meat, and horseflesh) samples, the pork liver sample and processed foods (pork liver, meat ball, meat loaf, salami sausage, Wiener sausage, bacon, and smoked pork tongue) were from commercial sources. The other 53 food items (adzuki bean, almond, apple, banana, basil, black bean, blueberry, bonito, broccoli, buckwheat, cabbage, caraway, carrot, cashew nut, cherry bean, cinnamon, clam, clove, cucumber, flatfish, garlic, grape, green chive, green pepper, horse mackerel, japanese pepper, kidney bean, kiwi fruit, lettuce, mackerel, nutmeg, octopus, oregano, parsley, peach, peanut, pepper, porgy, rice, red kidney bean, rosemary, salmon, sardine, saury, scallop, shrimp, soybean, spinach, squid, surf smelt, thyme, tuna, and wheat flour) were also obtained from commercial sources. DNA was prepared by using an mtDNA Extractor CT kit (Wako Pure Chemicals) based on the method described by Ishizawa *et al.*[14] and Sambrook and Russell[15] according to the manufacturer's instructions. Briefly, 200–250 mg of the milled food sample was homogenized with the homogenization buffer by a bead shaker. The mitochondria-rich fraction was obtained by centrifugation of the homogenate. DNA was extracted with the extraction solutions and centrifuged. To the resulting supernatant, a sodium iodide solution and isopropanol were added. DNA was obtained as a precipitate after centrifugation. After drying, DNA was dissolved in TE buffer, and the DNA concentration was measured by absorbance at 260 nm.

Oligonucleotide primers. Six primer sequences were designed from the published DNA sequence of pork cytochrome b (Fig. 1). The three forward primers were as follows: F1 primer (5′-TCTTAGGCATCTGCCTAATCTTG-3′), F2 primer (5′-TCTTGCAAATCCTAACAGGCCTG-3′), and F3 primer (5′-TCGAGACGTAAATTACGGATGAG-3′). The three reverse primers were as follows: R1 primer (5′-TTTGCATGTAGATAGCGAATAAC-3′), R2 primer (5′-GGATCCGTAGTATAGACCTCGG-3′), and R3 primer (5′-GCTATAACGGTAAATAGTAGGAC-3′). Each primer was synthesized and purified by a reversed-phase cartridge before being used for detection.

PCR and agarose gel electrophoresis. PCR was carried out in a final reaction volume of 20 µl containing the DNA sample (1 µl), forward and reverse primers (1 µl each), 10× PCR buffer (2 µl), 50 mM of $MgCl_2$ (0.6 µl), 2.5 mM of dNTP (0.4 µl), Taq DNA polymerase (0.1 µl), and distilled water (13.9 µl). Amplification was carried out with recombinant Taq DNA polymerase (Invitrogen).

After an initial denaturation step at 94 °C for 240 sec, the PCR conditions were optimized as follows: 37 cycles at 94 °C, 30 sec; 50 °C, 30 sec; and 72 °C, 60 sec. In order to confirm the amplification of the target sequence and the pork specificity of the oligonucleotide pair, the PCR product was electrophoresed on 2% agarose gel,

stained with ethidium bromide, and analyzed with Printgraph (Atto, AE-6932GXCF).

Cloning and sequencing of the amplified fragment. The amplified fragment generated with primer pair F2/R1 was subcloned into pCR®4-TOPO® vector with TOPO TA-cloning® kit (Invitrogen), and the recombinant plasmids were transformed into the *Escherichia coli* strain DH5α™-T1^R competent cell. The sequence of the clones was determined by using the BigDye® Terminators v1.1 cycle sequencing kit (Applied Biosystems) and ABI PRISM® 3100 genetic analyzer (Applied Biosystems).

Detection of porcine DNA in heat-treated pork mince. Pork mince (10 g, 5 mm thickness) was put into a bag, boiled for 10 min or autoclaved at 120 °C for 10 min. DNA was extracted from the heat-treated mince and PCR (37 cycles) was performed as already described.

Detection of porcine DNA in a baked or fried pork slice. Both sides of a pork loin slice (4 mm thickness) was baked on a heat plate (at 160 °C) for 3 min each side. Separately, a pork loin slice (4 mm thickness) was fried in oil (170 °C) for 1.5 min. DNA was extracted from the center portion of the resulting baked or fried slice and PCR (37 cycles) was performed as already described.

Sensitivity of the method. Eight mixing levels of the wheat mitochondrial DNA sample (10 ng/μl) containing 0, 10 fg/μl, 100 fg/μl, 1 pg/μl, 10 pg/μl, 100 pg/μl, 1 ng/μl, and 10 ng/μl of porcine mitochondrial DNA were prepared with serial dilution. PCR (37 cycles) was performed as already described to investigate the sensitivity of the method.

Detection of porcine DNA in a pork and vegetable dumpling (gyoza). A pork sample, after removing the fat, was minced, and 9-fold volume of water was added prior to homogenization (10% homogenate). The 10% homogenate was sequentially diluted with distilled water and designated as the 1%, 1000 ppm, 100 ppm, 10 ppm, 1 ppm, 100 ppb, 10 ppb, and 1 ppb homogenates. Separately, the ingredients for the dumpling (cabbage, green chive, and garlic) were also minced with a food processor.

These vegetables (18 g) were mixed with the minced pork or each homogenate (2 g), wrapped with gyoza wrapping made from wheat flour, heated by microwave for 2 min, and then minced with a food processor. DNA was extracted from the heat-processed dumpling and PCR (37 cycles) was performed as already described.

Results and Discussion

Some PCR methods for detecting allergenic foods such as buckwheat (*Fagopyrum spp.*)[16] and peanut (*Arachis hypogaea*)[17] have been reported. The choice of the target gene and the design of the primers have a great impact on the sensitivity and specificity of a detection system. It is well-known that very sensitive PCR assays can be established when the primer target is a multicopy gene such as a mitochondrial gene.[13] We chose in this study the porcine cytochrome b region of mitochondrial DNA as the target to detect pork.

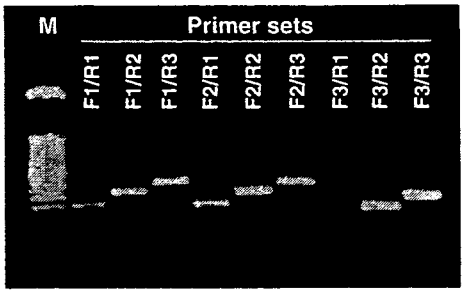

Fig. 2. Agarose Gel Electrophoresis of the PCR Products Amplified from Porcine Mitochondrial DNA by the Nine Primer Pairs.
The amplification of 10 ng of porcine DNA is shown. M, 50 bp ladder size standard.

Comparing the cytochrome b DNA sequence of pig, cattle, chicken, sheep, and horse according to the NCBI database, three forward (F1, F2 and F3) and reverse (R1, R2 and R3) primers were designed for the porcine-specific regions (Fig. 1). Figure 2 shows agarose gel electrophoresis of PCR products amplified from the nine primer pairs. The sizes of the PCR products amplified by primer pairs F1/R1, F1/R2, F1/R3, F2/R1, F2/R2, F2/R3, F3/R1, F3/R2, and F3/R3 were 148, 212, 268, 130, 194, 250, 45, 109, and 165 bp, respectively.

Since DNA is often degraded in processed foods, the amplified DNA fragment needs to have a size of 60–150 bp for PCR to detect hidden allergens.[13] PCR products amplified by primer pairs F1/R1, F2/R1 and F3/R2 met this criterion among the primer pairs tested. Among these three, F3/R2 was initially thought to be most desirable since its PCR product was shortest. However, our preliminary experiment revealed that the PCR product band was false-positively detected when DNA was extracted from carrot and PCR was performed when using F3/R2 (data not shown). The F1/R1 and F2/R1 primer pairs gave almost the same sensitivity. However, since F2/R1 gave the second shortest PCR product among the three primer pairs, we used this for further analyses. When using F2/R1, no such false-positive detection was apparent among all 53 food items tested. Furthermore, the nucleotide sequence analysis of the PCR product obtained by using the F2/R1 primer pair confirmed the intended sequence of porcine cytochrome b.

To investigate the feasibility of using this method for commercial food products, we obtained 7 food products (pork liver, meat ball, meat loaf, salami sausage, Wiener sausage, bacon, and smoked pork tongue) from a market

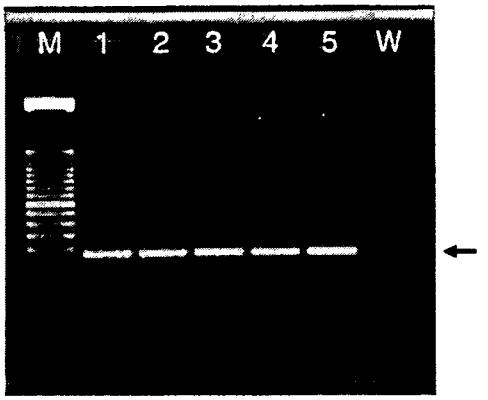

Fig. 3. Detection of Porcine DNA in the Processed Food Models.
The arrow indicates the expected PCR product (130 bp). Lanes 1, boiled; 2, autoclaved; 3, baked; 4, fried; 5, PCR product from porcine mitochondrial DNA (positive control). M, 50 bp ladder size standard. W, water (negative control).

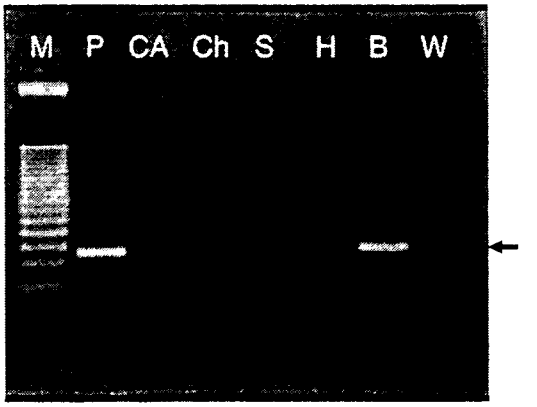

Fig. 4. Specificity of the PCR Method Using the F2/R1 Primer Pair.
The arrow indicates the expected PCR product (130 bp). The amplification of 10 ng of DNA from pig (P), cattle (Ca), chicken (Ch), sheep (S), horse (H), and boar (B) is shown. M, 50 bp ladder size standard. W, water (negative control).

and evaluated them for the presence of porcine DNA. According to the ingredients list on the label of each these products, all contained pork. Although we have omitted the figure showing agarose gel electrophoresis of the PCR products, porcine DNA was clearly detected with the method.

We examined the applicability of the method with boiled, autoclaved, baked and fried heated pork samples (Fig. 3). It was confirmed that heat processing hardly affected the sensitivity of the method.

Next, the specificity and sensitivity of the method were evaluated. As shown in Fig. 4, an amplified DNA fragment (130 bp) was specifically detected from porcine DNA, while no amplification, except for boar meat, of the 130 bp product occurred in such other species as cattle, chicken, sheep, and horse. It was revealed that DNA from boar meat was amplified by the primer used with our method. Matsunaga *et al.*[18] have already reported that the identification of six types of meat (cattle, pig, chicken, sheep, goat and horse) was possible by using PCR primers designed according to the DNA sequence of the cytochrome b gene. The primers in their study could give different length fragments from the six types of meat and hence distinguish among them. In respect of pork, the size of the PCR products amplified by the primers was 398 bp. Although their method is certainly useful, the size (398 bp) is presumably too long to detect porcine DNA in processed food according to the above-mentioned criterion about the amplified DNA fragment, since DNA is often degraded in processed food.[13]

Next, the sensitivity of the developed method was confirmed in the presence of wheat DNA (Fig. 5). Although non-specific products from wheat DNA were observed, even 1 pg of a porcine DNA sample was clearly detected in 10 ng of matrix wheat DNA.

Since actual food samples are made from many kinds of organic material of animal and plant origin and from inorganics, there would be the possibility of the ingredient(s) interfering with DNA extraction or inhibiting PCR. Thus, we investigated the sensitivity of the developed method when applied to the processed food models. For this purpose, we made *gyoza* (pork and vegetable dumpling) which contained several amounts (10% to 1 ppb) of pork, and extracted DNA for PCR. As shown in Fig. 6, porcine DNA could be detected in *gyoza* at over the 100 ppb level, although non-specific products from other DNA were faintly observed. The Japanese labeling system for allergenic food material stated that if more than 10 ppm of a specified allergenic protein is contained in a food, labeling of that food material is necessary. The sensitivity of the developed method was thus judged to be sufficient to verify the allergen labeling of foods.

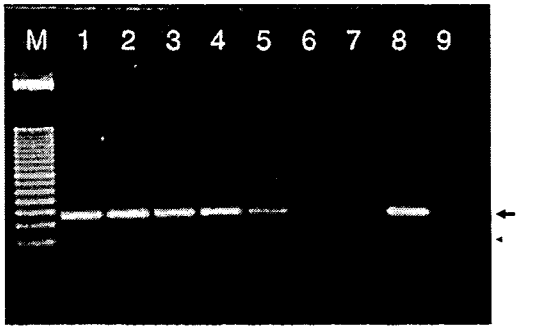

Fig. 5. Sensitivity of the PCR Method Using the F2/R1 Primer Pair.
The arrow indicates the expected PCR product (130 bp). The amplification of 10 ng (lane 1), 1 ng (lane 2), 100 pg (lane 3), 10 pg (lane 4), 1 pg (lane 5), 100 fg (lane 6), 10 fg (lane 7), and 0 fg (lane 9) of porcine DNA is shown. lane 8, PCR product from porcine mitochondrial DNA (positive control). The small arrowhead indicates the non-specific product from wheat DNA. M, 50 bp ladder size standard.

Taking together all the data obtained, the proposed method has at least three major advantages in its robustness, high sensitivity and specificity over the ELISA method for detecting pork in food. As already shown, the PCR method can even detect porcine DNA in

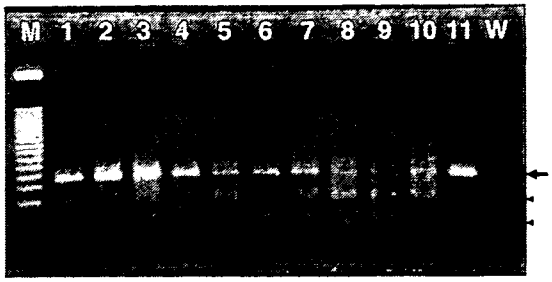

Fig. 6. Sensitivity for the Specific Detection of Porcine DNA from the Processed *gyoza* (pork and vegetable dumpling) Model.

The arrow indicates the expected PCR product (130 bp). The small arrowhead indicates the non-specific product from other DNA. Lanes 1, 10%; 2, 1%; 3, 1000 ppm; 4, 100 ppm; 5, 10 ppm; 6, 1 ppm; 7, 100 ppb; 8, 10 ppb; 9, 1 ppb; 10, 0 (without pork); 11, PCR product from porcine mitochondrial DNA (positive control). M, 50 bp ladder size standard. W, water (negative control).

heat-processed food. In contrast, the immunoreactivity of the detecting antibodies towards pork in food products is usually markedly decreased by heating. Moreover, it is well known that obtaining a high and specific antibody against skeletal muscle is very difficult. Indeed, we found, from our preliminary experiment, in which porcine protein was immunized to rabbits, that the titer and specificity of the antibodies obtained were both very low (data not shown). The developed PCR method can, however, distinguish pork from other meat, except for boar meat (Fig. 4), and can detect a 100 ppb level of pork contamination in the processed food models (Fig. 6).

In summary, a PCR method was developed for the specific detection of pork in food. Since this method is rapid, specific, and sensitive, it could be applicable for detecting trace amounts of pork in processed foods. This method would also benefit food manufacturers who must exclude pork contamination from their products for religious reasons.

Acknowledgment

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Biosci. Biotechnol. Biochem., **71** (12), 3131–3135, 2007

Communication

A Real-Time Quantitative PCR Detection Method for Pork, Chicken, Beef, Mutton, and Horseflesh in Foods

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A rapid real-time quantitative PCR method to detect trace amounts of pork, chicken, beef, mutton, and horseflesh in foods was developed. The primers and TaqMan MGB (minor groove binder) probes were designed on the gene encoding cytochrome b for the specific detection of each species. The limit of quantification of this method was found to be 100 fg/μl of each mitochondrial DNA in 10 ng/μl of the wheat mitochondrial DNA matrix. The calculated R^2 values of the standard curves for the five species ranged between 0.994 and 0.999. This method would be particularly useful in the detection of hidden meat mince in processed foods, which would verify food labeling and gain consumers' trust.

Key words: real-time PCR; cytochrome b; meat; mince; labeling

Since, in the modern diet, it is very common to make processed foods by adding minced meats, manufacturers are obligated to label raw materials properly. To verify food labeling is of great importance in order to ensure food safety (for example, to decrease the unexpected occurrence of food allergies) as well as to gain consumers' trust. Hence, it is necessary to establish a rapid method of detecting meat ingredients in processed foods to verify the labeling.

A number of methods are based on the detection of species-specific proteins by enzyme-linked immunosorbent assay (ELISA) and of species-specific DNA molecules by polymerase chain reaction (PCR).[1)] With regard to ELISA, to obtain a specific antibody against skeletal muscle is thought to be very difficult. Indeed, we found from our preliminary experiment, in which porcine meat protein was immunized to rabbits, that both the titer and the specificity of the antibodies obtained were low (unpublished data). On the other hand, an optimized PCR procedure could amplify the specific target sequence even in a very complex pool of genomic sequences. We reported recently specific and qualitative detection methods for pork[2)] and chicken[3)] in processed foods using conventional PCR. Another group has also reported a conventional PCR method of identifying six meats (cattle, pig, chicken, sheep, goat, and horse) as raw materials in meat products.[4)] These conventional PCR methods are simple and useful, but they appear to lack quantitative capabilities, sensitivity, and rapid analysis. Hence, in this study, we aimed to establish rapid real-time quantitative PCR detection methods for pork, chicken, beef, mutton, and horseflesh in foods.

It is well-known that sensitive PCR assays can be established when the primer target is a multicopy gene, such as a mitochondrial gene.[1)] We chose in this study the cytochrome b region of the mitochondrial DNA as the target to detect each meat. By aligning the cytochrome b DNA sequence of the pig, chicken, cattle, sheep, and horse according to the NCBI database, forward and reverse primers and TaqMan MGB probes were designed for the each meat-specific region (Fig. 1) after the PCR reactions were optimized. All meat (pork, chicken, beef, mutton, and horseflesh) samples were

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Abbreviations: FAM, 6-carboxy-fluorescein; MGB, minor groove binder; NFQ, non-fluorescent quencher; NTC, no-template control

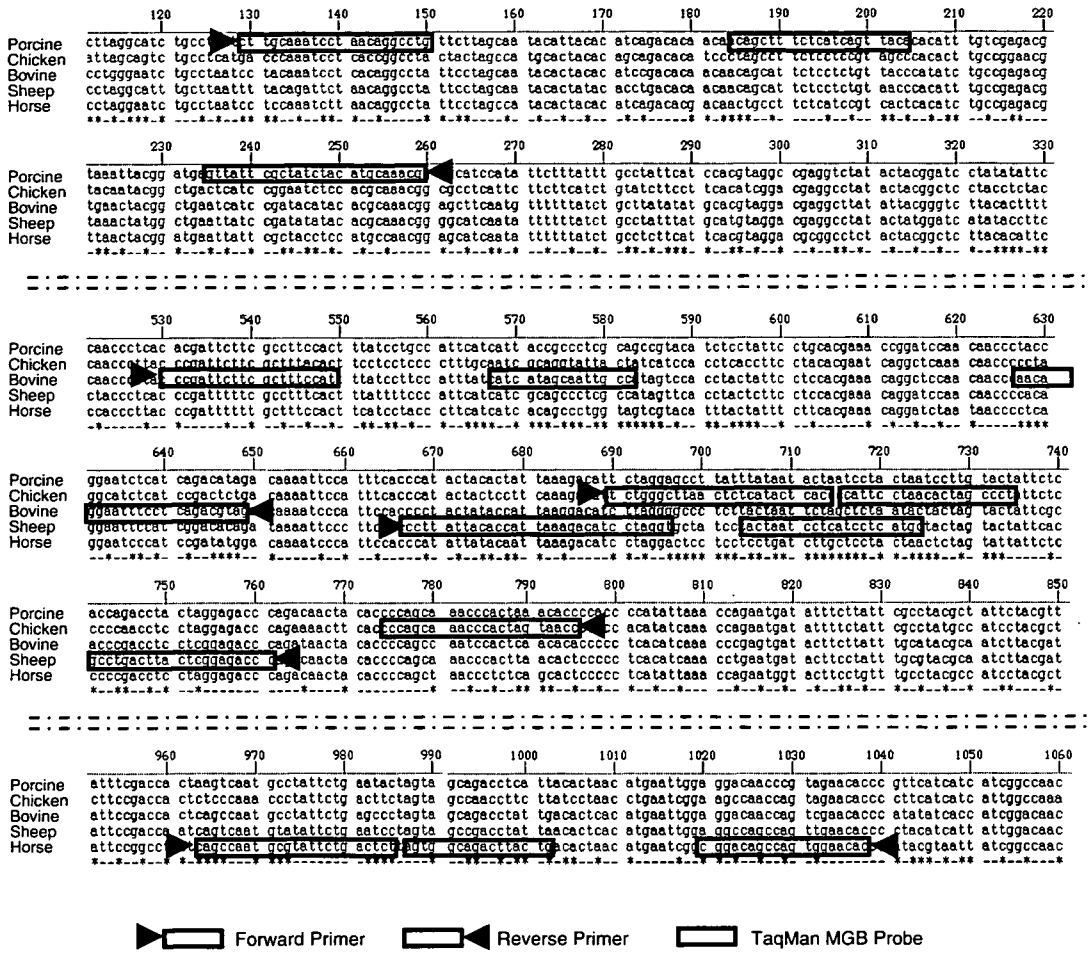

Fig. 1. Sequences of Primers and TaqMan MGB Probes Designed on Cytochrome b DNA for the Detection of Pork, Chicken, Beef, Mutton, and Horseflesh in Foods.

The list of these primers and probes is shown in Table 1.

Table 1. Sequences of Primers and Probes Used in This Study

		Sequence
Pork	Forward Primer	5′-CTTGCAAATCCTAACAGGCCTG-3′
(Porcine DNA)	Reverse Primer	5′-CGTTTGCATGTAGATAGCGAATAAC-3′
	TaqMan MGB Probe	5′-(FAM)-ACAGCTTTCTCATCAGTTAC-(NFQ)(MGB)-3′
Chicken	Forward Primer	5′-TCTGGGCTTAACTCTCATACTCACC-3′
(Chicken DNA)	Reverse Primer	5′-GGTTACTAGTGGGTTTGCTGGG-3′
	TaqMan MGB Probe	5′-(FAM)-CATTCCTAACACTAGCCCTA-(NFQ)(MGB)-3′
Beef	Forward Primer	5′-CCCGATTCTTCGCTTTCCAT-3′
(Bovine DNA)	Reverse Primer	5′-CTACGTCTGAGGAAATTCCTGTTG-3′
	TaqMan MGB Probe	5′-(FAM)-CATCATAGCAATTGCC-(NFQ)(MGB)-3′
Mutton	Forward Primer	5′-CCTTATTACACCATTAAAGACATCCTAGGT-3′
(Sheep DNA)	Reverse Primer	5′-GGGTCTCCGAGTAAGTCAGGC-3′
	TaqMan MGB Probe	5′-(FAM)-ACTAATCCTCATCCTCATGC-(NFQ)(MGB)-3′
Horseflesh	Forward Primer	5′-CAGCCAATGCGTATTCTGACTCT-3′
(Horse DNA)	Reverse Primer	5′-GTGTTCCACTGGCTGTCCG-3′
	TaqMan MGB Probe	5′-(FAM)-AGTGGCAGACTTACTG-(NFQ)(MGB)-3′

FAM, 6-carboxy-fluorescein; MGB, minor broove binder; NFQ, non-fluorescent quencher

from commercial sources, and their mitochondrial DNAs were prepared using an mtDNA Extractor CT kit (Wako Pure Chemicals, Tokyo) based on the method described by Ishizawa *et al.*[5] The endogenous 18S rRNA primers and probe (no. 4319413E, Applied Biosystems, Foster City, CA) for the internal control were labeled with fluorescent reporter dye VIC on the 5′ end and the meat probes were with FAM on the 5′ end.

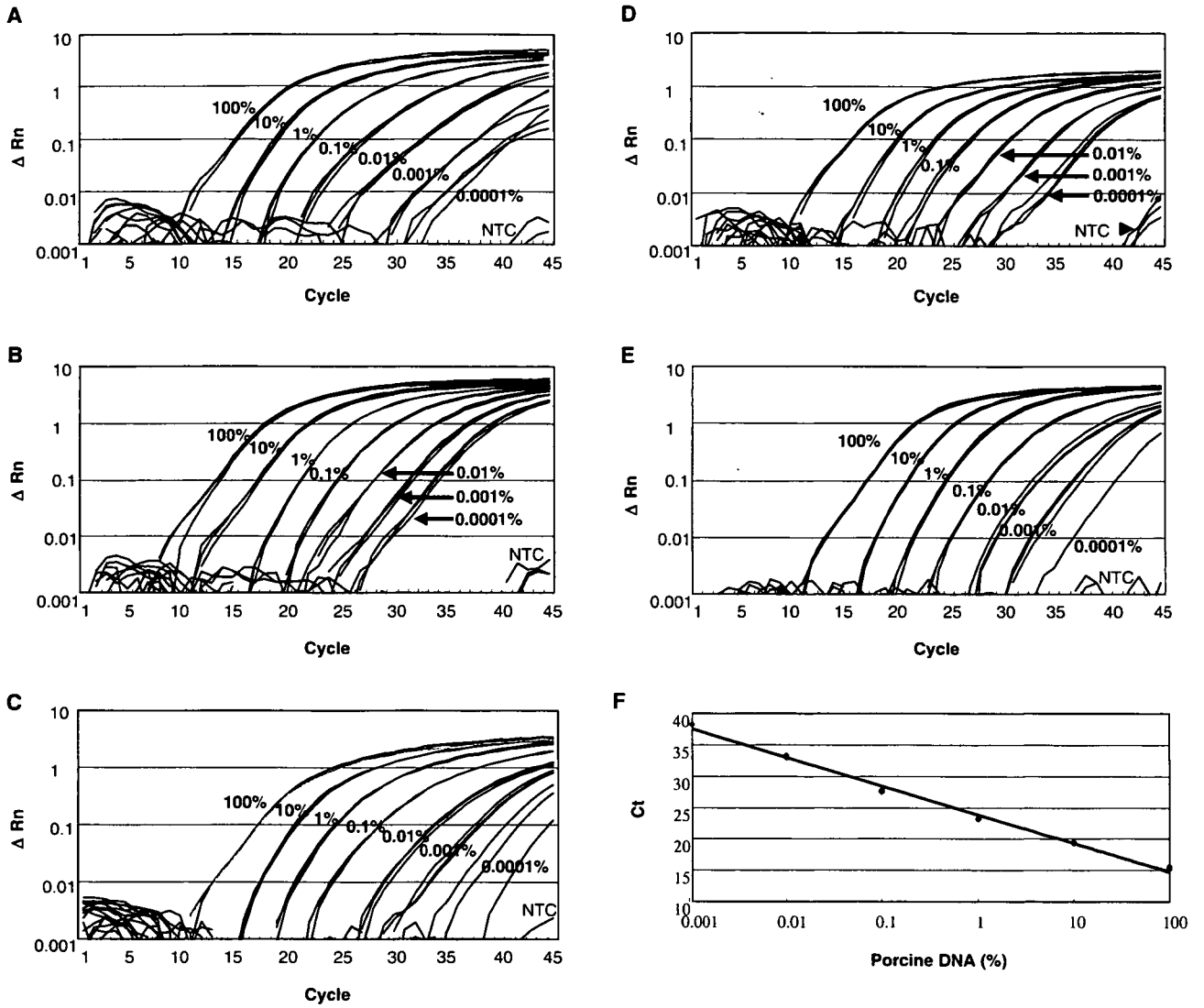

Fig. 2. Amplification Curves of Real-Time PCR Detection for Meat.
A, porcine; B, chicken; C, bovine; D, sheep; E, horse. All reactions were run in triplicate. NTC, no-template control. F, Standard curve for the porcine DNA generated from the amplification data given in (A) in the range between 0.001 and 100%.

A no-template control (NTC) was also prepared as the negative control for analysis.

Real-time PCR was performed using 7500 Real-Time PCR System (Applied Biosystems). All reactions were run in triplicate in 96-well plates. The PCR reaction mixtures were placed in a 25 µl final volume containing 2.5 µl of the template DNA, 12.5 µl of the universal PCR master mix (Applied Biosystems), 0.75 µl of the primer pair (10 µM each), and 0.5 µl of TaqMan MGB probes (10 µM), as listed in Table 1 (also shown in Fig. 1). The reaction conditions included the initiation step for 2 min at 50 °C, 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C, and 1 min at 60 °C. During PCR cycling, the software calculated the emissions of the reporter and passive reference dye (ROX) for each cycle. The real-time PCR system is supplied with Sequence Detection software able to determine the contributions of each component dye spectrum using a multicomponent algorithm. The ΔRn value (Fig. 2) expresses the magnitude of the normalized fluorescence signal generated by the reporter for each cycle during PCR amplification. The point at which the amplification plot crosses the threshold is defined as Ct (threshold cycle, Fig. 2).

The specificity of our real-time PCR method was evaluated using mitochondorial DNAs (10 ng/µl each) from pork, chicken, beef, mutton, and horseflesh, rabbit, and whale. Consequently, only the species-specific amplification was observed when using five sets of primers and probe (data not shown). Next, the mitochondrial DNAs extracted from the pork, chicken, beef, mutton, and horseflesh were diluted by TE buffer containing yeast RNA (50 µg/ml) to final concentrations of 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg/µl. Using 2.5 µl of each DNA solution, we evaluated the sensitivity of the real-time PCR system. Amplification curves

were stably obtained when the DNA template was lowered to 10 fg/μl. The calculated R^2 values of the standard curves were 1.000, 0.996, 0.998, 0.994, and 0.992 for porcine, chicken, bovine, sheep, and horse DNA respectively, in the range of 10 fg–100 pg/μl.

To mimic the conditions under which we measure the DNA from the meat (pork, chicken, beef, horseflesh, and mutton) in the processed foods, each species of mitochondrial DNA was mixed with wheat mitochondrial DNA at eight mixing levels (0, 0.0001, 0.001, 0.01, 0.1, 1, 10, and 100%). Wheat flour was obtained from a commercial source, and wheat DNA was prepared using the kit described above. Total DNA (wheat DNA plus DNA from each meat) concentration was adjusted to 10 ng/μl.

As shown in Fig. 2 A-E, the real-time quantitative PCR method proposed in this study allowed us to detect each DNA species over a very wide range. The amplification of each DNA species was clearly observed in a range between 0.001–100% in the wheat DNA matrix. In the case of 0.0001% of each DNA species, amplification was apparently detected, as compared to the no-template control (NTC), although the amplification curve lacked reproducibility. Hence, we concluded that the limits of quantification and those of detection were 0.001% (100 fg/μl) and 0.0001% (10 fg/μl), respectively. Since we found that the detection limit for porcine DNA by conventional PCR was 1 pg in our previous study,[2] the present results suggest that the real-time PCR method in this study is more sensitive.

The standard curve for porcine DNA was generated from the amplification data (Fig. 2 A), and the calculated R^2 value of the standard curve for the porcine DNA was 0.994 in the range of 0.001–100% (Fig. 2 F). Similarly, the calculated R^2 values of the standard curves for chicken, beef, sheep, and horse were 0.999, 0.994, 0.998, and 0.998, respectively (data not shown). Hence, the percentage of meat DNA in an unknown sample can be measured by interpolation from a standard curve of Ct values generated from known starting DNA concentrations.

In our previous study,[2,3] we found that the target sequence was successfully amplified in the heat processed food model and that the conventional PCR method is comparably applicable to processed foods if the amplified DNA fragments are 60–150 bp. At present, we preliminary confirm that meat DNA was detected in some processed food items by the real-time PCR method, used in this study, and are now undertaking feasibility analyses of the method developed using various kinds of processed food items. In the case of determination of meat content (W/W, %) in foods, it is necessary to convert from the determination of the copy numbers of meat target DNA sequences and from that of the copy numbers of the animal universal specific endogenous reference gene. Therefore, further study is needed to determine a way to convert the ratio of measured copy numbers to units of weight/weight.

Many real-time PCR systems have been developed to identify and quantify genetically modified (GM) maize, GM soybeans, and GM varieties of other agricultural commodities.[6–13] Since this method has been shown to be easily and reliably applied to various food products, the real-time PCR method is now widely utilized around the world. The detection targets for GM crops are chosen from transgenic species-specific genes. On the contrary, in the proposed system, the primers and TaqMan MGB probes are designed on the gene encoding cytochrome b for specific detection of each species, and a similar specificity and sensitivity were successfully obtained.

In Japan, in 2007, there occurred an incident that a certain meat processing company disguised pork mince as beef and this has focused attention on the labeling of mince in processed food. Also in Japan, the Ministry of Health, Labor, and Welfare has notified their recommendations to declare three meat items (beef, chicken (poultry), and pork) in the context of labeling systems for allergenic food materials. In the latter case, the system states that if greater than 10 μg/g (the corresponding allergnic food protein weight/food weight) is contained in a food material, labeling of such a material is mandatory. In both instances, the present real-time PCR detection method should meet such requirements.

In conclusion, we developed, for the first time, a rapid real-time quantitative PCR method to detect pork, chicken, beef, mutton, and horseflesh in foods. Since this method is rapid, specific, sensitive, and highly quantitative, it would be particularly useful in the detection of hidden meat mince in processed foods.

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FOOD COMPOSITION AND ADDITIVES

Interlaboratory Evaluation of Two Enzyme-Linked Immunosorbent Assay Kits for the Determination of Crustacean Protein in Processed Foods

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The labeling of foods containing material derived from crustaceans such as shrimp and crab is to become mandatory in Japan because of increases in the number of allergy patients. To ensure proper labeling, 2 novel sandwich enzyme-linked immunosorbent assay (ELISA) kits for the determination of crustacean protein in processed foods, the N kit (Nissui Pharmaceutical Co., Ltd, Ibaraki, Japan) and the M kit (Maruha Nichiro Holdings, Inc., Ibaraki, Japan), have been developed. Five types of model processed foods containing 10 and/or 11.9 μg/g crustacean soluble protein were prepared for interlaboratory evaluation of the performance of these kits. The N kit displayed a relatively high level of reproducibility relative standard deviation (interlaboratory precision; 4.0–8.4% RSD_R) and sufficient recovery (65–86%) for all the model processed foods. The M kit displayed sufficient reproducibility (17.6–20.5% RSD_R) and a reasonably high level of recovery (82–103%). The repeatability relative standard deviation (RSD_r) values regarding the detection of crustacean proteins in the 5 model foods were mostly <5.1% RSD_r for the N kit and 9.9% RSD_r for the M kit. In conclusion, the results of this interlaboratory evaluation suggest that both these ELISA kits would be very useful for detecting crustacean protein in processed foods.

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In industrialized countries, food allergies have represented an important health problem in recent years, and it is estimated that approximately 8% of children and 2% of adults in these countries have some type of food allergy (1, 2). Burks et al. (3) estimated that approximately 120 deaths related to food allergies occur yearly in the United States. In Japan as well, the number of patients with food allergies, especially among young children, is increasing (4). To prevent possible life-threatening reactions, the only effective measure is to strictly avoid the consumption of allergenic foods because of the lack of effective medical treatment for food allergies. However, various studies have shown that severe allergenic reactions can be induced by the accidental intake of food products containing allergenic materials (5, 6). Therefore, sufficient information regarding potentially allergenic ingredients in food products is necessary.

The issue of a labeling system for allergenic ingredients in food products has been discussed by international organizations such as the Codex Alimentarius Commission of the World Health Organization (WHO) and the Food and Agriculture Organization (FAO). In 1999, the Joint FAO/WHO Codex Alimentary Commission Session agreed to label 8 kinds of foods that contain ingredients known to be allergens, including Crustacea (7). In Japan, the Ministry of Health, Labor and Welfare (MHLW) has enforced a labeling system for allergenic food materials since April 2002 in order to provide information about these foods to the allergic

consumer. According to the Japanese regulations, labeling for food products, including eggs, milk, wheat, buckwheat, and peanuts, is mandatory, and is recommended for 20 other food materials such as shrimp and crab in light of the number of patients and the degree of seriousness. In a ministry notification in 2002 (8), the MHLW announced the official Japanese methods for the detection of allergens for mandatory labeling and the threshold value for labeling [10 μg/g (the corresponding allergenic ingredient soluble protein weight/food weight)]. The MHLW also described the intervalidation protocol criteria in the official guidelines in 2006 (9). The outline of the intervalidation protocol criteria is as follows: number of laboratories, ≥8; number of incurred samples, ≥5; number of dose level, ≥1, including 10 μg/g (the corresponding allergen protein weight/food weight); recovery, 50–150%; reproducibility relative standard deviation (RSD_R), ≤25%. In the guideline, reference material, the initial extract solution, and the extraction procedure from allergen were also specified and standardized.

Epidemiological investigations in Japan have shown that the number of patients with a crustacean allergy such as to shrimp and crab has increased (10). In recommendations regarding labeling, Crustacea labeling would be particularly important because of the almost unlimited uses of Crustacea as an ingredient and because the number of patients with allergies to Crustacea has been increasing, although crustacean allergy is still less prevalent than, for example, milk allergy in the food-allergic population (11, 12). Crustacean allergic reactions can be elicited by the intake of trace amounts of crustacean proteins, and anaphylaxis caused by Crustacea has been reported (13, 14). Considering current conditions, the MHLW will have a plan for crustacean labeling to be mandatory in 2008. Therefore, a reliable detection method for crustacean protein monitoring is necessary to ensure accurate labeling. We have developed 2 enzyme-linked immunosorbent assay (ELISA) kits capable of highly sensitive crustacean protein detection. This paper describes the results of an interlaboratory evaluation of the performance of these ELISA kits.

Experimental

Materials and Methods

Preparation of crustacean powder.—Crustacean powder was prepared by homogenization followed by freeze-drying of the tail muscle of the shrimp (black tiger prawn, *Penaeus monodon*). The crustacean soluble protein (CP) from crustacean powder was extracted using the extraction buffer phosphate buffered saline (PBS), pH 7.4, containing 0.5% sodium dodecyl sulfate (SDS) and 2% β-mercaptoethanol. The CP content was then calculated using a 2-D Quant Kit (GE Healthcare, Buckinghamshire, UK). The amount of CP/1 g crustacean powder was approximately 684 mg.

Test Materials

Five model processed foods (fish sausage, freeze-dried egg soup, tomato sauce, creamy croquette, and chicken balls) containing CP were prepared and used as test materials. Crustacean powder was spiked at the ingredient stage before processing to establish a final level of 10 μg/g (CP weight/sample weight). In the case of freeze-dried egg soup, the CP content was calculated to be 11.9 μg/g (CP weight/sample weight). The prepared model processed foods were homogenized with a food processor (DLC-XG; Cuisinart, Stamford, CT) and sent to the participants as test materials.

Preparation of Model Processed Foods

All the model processed foods were prepared using procedures described by the manufacturers. To make a final protein concentration of 10 μg/g (CP weight/sample weight) in these foods, the amount of crustacean powder spiked in the foods at the ingredient stage was calculated, taking into account the protein content of the crustacean powder and the change in weight of the foods during preparation. Because the protein amount per 1 g crustacean powder was ca 684 mg, we spiked 14.6 mg crustacean powder to make 1 kg of each model processed food [the make-up of the final concentration is calculated for 10 μg/g (CP weight/sample weight)] because we estimated the ratio of CP weight to crustacean powder weight to be 68.4%.

The fish sausages were composed of minced fish flesh (yellow goatfish, atka mackerel, and walleye pollack), lard, sugar, salt, ice water, and crustacean powder. Lard, salt, sugar, ice water, and the crustacean powder were added to minced fish flesh and thoroughly mixed. The mixture was ground up using a small cutter, and the kneaded mixture was manually placed into fish sausage casings. These were then heated at 121°C for 15 min, cooled in flowing water for 5 min, and then placed in a refrigerator at 5°C overnight.

The freeze-dried egg soup was composed of egg, potato starch, milk sugar (lactose), salt, and crustacean powder. Eggs, potato starch, milk sugar, salt, and the crustacean powder were thoroughly mixed. The mixture was dissolved in a plastic tray, frozen in a deep-freezer at –80°C, and then lyophilized for 20 h.

Tomato sauce was composed of tomato, onion, olive oil, garlic, bay leaf, sugar, salt, black pepper, and crustacean powder. The measured raw materials were thoroughly mixed and placed in an aluminum pouch. The sauce was heated at 121°C for 1 min, cooled in flowing water for 5 min, and then placed in a refrigerator at 5°C overnight.

The creamy croquettes were composed of milk, butter, wheat, potato starch, salt, sugar, black pepper, flour, and crustacean powder. The raw materials were homogeneously mixed and kneaded for 20 min. The mixture was ground up using a small cutter, and the kneaded mixture was manually placed into casings, which were then preserved in a deep-freezer at –20°C until interlaboratory evaluation.

Table 1. Homogeneity test[a] results of processed food samples

Food	Mean, μg/g	RSD, %[b]	*n*	*F*-ratio	F_{crit}[c]
Fish sausage	6.5	4.5	6	1.6	4.4
Freeze-dried egg soup[d]	8.5	2.4	6	0.9	4.4
Tomato sauce	9.0	4.9	6	1.5	4.4
Creamy croquette	8.5	4.3	6	1.0	4.4
Chicken ball	7.1	5.5	6	3.8	4.4

[a] Homogeneity test was carried out with N kit.
[b] RSD% calculated from s_s (SD of sampling) and s_a (SD of analysis).
[c] F_{crit} = Critical *F*-value.
[d] CP was spiked at 11.9 μg/g.

The chicken balls were composed of white meat of chicken, lard, potato starch, sugar, and crustacean powder. Lard, potato starch, sugar, and the crustacean powder were added to ground white meat and thoroughly mixed. The mixture was ground up using a small cutter, and the kneaded mixture was manually placed into casings, which were then preserved in a deep-freezer at –20°C until interlaboratory evaluation.

Each type of model processed food was also prepared as a blank sample which did not include CP for the purpose of confirming potential contamination, determination of the limit of detection (LOD), the limit of quantification (LOQ), false positive, interference, and matrix effects. All the values determined in the blank samples of the model processed foods using both kits were less than respective LOD in in-house validation (data not shown).

Homogeneity Tests of the Test Materials

The homogeneity of the test materials was verified by the coordinator before distribution following the procedure described in the International Harmonized Protocol for Proficiency Testing of (Chemical) Analytical Laboratories (15), except that the number of test materials was 6. Twelve test portions of each test material were analyzed using the N kit. The obtained concentrations of CP were submitted to one-way analysis of variance (ANOVA). Table 1 shows the average concentration, the relative standard deviation (RSD) percentages calculated from s_s (SD of sampling) and s_a (SD of analysis) as well as the *F*-ratios. The *F*-ratios for all test materials were below the critical *F*-value.

ELISA Kits

Crustacean kit "Maruha" (M kit; 16).—The polyclonal and monoclonal antibodies to tropomyosin in the black tiger prawn (*Penaeus monodon*) are used in the M kit with the CP solution used as a calibrator. The outline of the procedure is as follows: Diluted standard solutions and sample solutions were added to a monoclonal antibody-coated module and incubated for 1 h at room temperature. After the module was washed, a solution of the polyclonal antibody, labeled with horseradish peroxidase, was added and allowed to stand for 1 h. After the second washing, a solution of 3,3′,5,5′-tetramethylbenzidine was added, and the module was allowed to stand at 25°C for exactly 20 min. The reaction was stopped by addition of 1 M sulfuric acid, and the absorbances were measured at 450 nm with 650 nm as the reference wavelength. The M kit was highly specific for the Decapoda group, apart from minor cross-reactivities to other Crustacea and Mollusks. We determined the LOD and LOQ according to International Standards Organization (ISO) and International Union of Pure and Applied Chemistry (IUPAC) guidelines (17). The LOD was calculated as 3 times the SD of the dilution buffer mean value after 25 experiments. The LOQ was calculated as 10 times the SD of the dilution buffer mean values after 25 experiments. The LOD of the M kit determined using the dilution buffer was 0.71 ng/mL, equivalent to 0.29 mg/kg samples, and the LOQ was 0.78 ng/mL, equivalent to a 0.31 mg/kg sample. Consequently, the practical determination range is between 0.78 and 50 ng/mL.

Food Allergen Test EIA Crustacean "Nissui" (N kit; 18).—The polyclonal and monoclonal antibodies to tropomyosin in the black tiger prawn (*Penaeus monodon*) are used in the N kit, with the CP solution used as a calibrator. The detection procedure was performed according to the manufacturer's instructions. Diluted standard solutions and sample solutions were added to a monoclonal antibody-coated module and incubated for 1 h at 25°C. After the module was washed, a solution of the polyclonal antibody, labeled with horseradish peroxidase, was added and allowed to stand for 1 h at 25°C. After the second washing, a solution of 3,3′,5,5′-tetramethylbenzidine was added and the module was allowed to stand at 25°C for exactly 20 min. The reaction was stopped by the addition of 0.5 M sulfuric acid, and the absorbances were measured at 450 nm with 650 nm as the reference wavelength. The N kit was also highly specific for the Decapoda group and showed the cross-reactivities to other Crustacea. We determined the LOD and LOQ according to ISO and IUPAC guidelines (17). The LOD was calculated as 3 times the SD of the dilution buffer mean value after 36 experiments. The LOQ was calculated as 10 times the SD of the dilution buffer mean values after 36 experiments. The LOD of the N kit determined using the dilution buffer was 0.4 ng/mL, equivalent to 0.16 mg/kg samples, and the LOQ