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

Soichi Tanabe,¹ Eiji Miyauchi,¹ Akemi Muneshige,² Kazuhiro Mio,³ Chikara Sato,³ and Masahiko Sato²;
¹Graduate School of Biosphere Science, Hiroshima University, Higashi-hiroshima, Hiroshima 739-8528, Japan
²Central Research Institute, Itoham Foods Inc., Moriya, Ibaraki 302-0104, Japan
³Neuroscience Research Institute and Biological Information Research Center, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki 305-8566, Japan

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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 wheat⁴ and peanuts⁵ 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. 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-
sorbent assay (ELISA) and of species-specific DNA molecules by the polymerase chain reaction (PCR).\textsuperscript{13)} PCR leads to \textit{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, orangered, 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 \textit{et al.}\textsuperscript{14)} and Sambrook and Russell\textsuperscript{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’-TCTTAGGCATCTGCCTAA-TCTTG-3’), F2 primer (5’-TCTTGCAATATTCGAACCTG-3’), and F3 primer (5’-TCGAGACGTAAATCTACGGATGAG-3’). The three reverse primers were as follows: R1 primer (5’-TTTTGCATGTAGATAGCGAATAAC-3’), R2 primer (5’-GGATCCGTAGTATAGACCTCGG-3’), and R3 primer (5’-GCTATAACGGTAAATAGTAGTAGG-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\textsubscript{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, (to be continued)
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® 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.*) and peanut (*Arachis hypogaea*) 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. We chose in this study the porcine cytochrome b region of mitochondrial DNA as the target to detect pork.

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. 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.
and evaluated them for the presence of porcine DNA. According to the ingredients list on the label of each of 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 gyozas (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 gyozas 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.

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...
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.

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