Method of Detecting Genetically Modified Chicken Containing Human Erythropoietin Gene

Osamu Nakajima,*a Kosuke Nakamura,a Kazunari Kondo,a Hiroshi Akiyama,b and Reiko Teshima*

a Division of Novel Foods and Immunochemistry; National Institute of Health Sciences; and b Division of Foods Additives, National Institute of Health Sciences; 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan.

Received March 5, 2013; accepted July 3, 2013

Genetically modified (GM) chickens carrying the human erythropoietin (hEpo) gene have been developed to produce recombinant hEpo protein in eggs. However, such animals have not been approved as food sources in Japan. We developed a method for detecting the hEpo gene in chicken meat using a real-time polymerase chain reaction (real-time PCR). The hEpo gene was clearly detected in genomic DNA extracted from magnum and heart of a chimeric chicken containing the hEpo gene. A plasmid containing the hEpo gene was used as a standard reference molecule as well. The results clearly showed that our method was capable of detecting the hEpo gene contained in the plasmid in the presence of genomic DNA extracted from a raw chicken meat sample. We successfully used this method to test six samples of raw chicken meat and six samples of chicken meat in processed foods. This method will be useful for monitoring chicken meat that might have originated from GM chickens carrying the hEpo gene to assure food safety.

Key words genetically modified chicken; erythropoietin; real-time polymerase chain reaction

Materials and Methods

Materials Genomic DNA extracted from magnum and heart from a chimeric chicken containing the hEpo gene or from blood from a transgenic chicken containing green fluorescent protein (GFP) gene was kindly provided by Dr. Nishijima. Six samples of raw chicken meat (breast, sasami [white chicken meat], liver, leg, wing, and minced) and six samples of processed foods containing chicken meat (fried chicken, oyakodon [a bowl of rice topped with boiled chicken and eggs], yakitori with liver, chicken cutlet, teriyaki chicken, and chicken curry) as ingredients were purchased at a market in Tokyo. As a standard material for calibrating the standard plots for the real-time PCR results, a plasmid containing hEpo cDNA was purchased from Thermofisher Scientific (Cat. No. MHS1010–98053191; Lafayette, CO, U.S.A.).

Extraction of Chicken Genomic DNA Genomic DNA was extracted from 0.4–0.5 g of the chicken meat samples using a Blood & Cell Culture DNA Mini Kit (Qiagen, Hilden, Germany). The DNA concentration in the prepared DNA solution was determined by measuring the UV absorption at 260 nm using a GeneQuant pro spectrophotometer (GE Healthcare UK Ltd., Buckinghamshire, U.K.). The size of the DNA was analyzed using 0.8% (w/v) agarose gel electrophoresis.

Real-Time PCR Detection of hEpo Gene Real-time PCR was performed using the ABI 7900HT sequence detection system (Life Technologies, Carlsbad, CA, U.S.A.). All the reactions were run in triplicate using 96-well plates. A no-template control (NTC) was also prepared as a negative control for the analyses. The data were analyzed using data analysis software, version 2.1, included in the real-time PCR system. The baseline was set to cycles 3–15. A reaction with a Ct value less than 38 and exponential amplification, as judged by visual inspection of the respective normalized reporter signal (ΔRn) plot and multi-component plots, was scored as positive. If a Ct value could not be determined, the reaction was scored as negative. A reaction with a Ct value less than 38 without exponential amplification, as judged by visual inspection of the respective ΔRn plot and multi-component plots, was scored as negative. When the Ct value was more than 38,

The authors declare no conflict of interest.

* To whom correspondence should be addressed. e-mail: onakajim@nihs.go.jp © 2013 The Pharmaceutical Society of Japan
we considered the reaction to be negative because of the difficulty of confirming exponential amplification.

**Real-Time PCR Detection of hEpo Gene in Genomic DNA Extracted from Magnum and Heart of a Chimeric Chicken Containing hEpo Gene** Five nanograms of the genomic DNA was added in the PCR reaction mixture described in the real-time PCR detection of hEpo gene section. The presence of the hEpo gene in the sample was examined. Genomic DNA extracted from blood of a transgenic chicken containing GFP or from meat of a non-GM chicken was also measured as negative controls. The experiments were repeated three times.

**Calibration of Standard Plots for hEpo Gene in Real-Time PCR** The PCR reaction mixtures were placed in a final volume of 25 µL consisting of 12.5 µL of the universal PCR master mix (Life Technologies), 0.4 µL of the primer pair (25 µM each), 0.5 µL of the TaqMan probe (10 µM), and 2.5 µL of the control plasmid template DNA. The plasmid digested with Nco I was used as the template DNA. Standard plots were calibrated using five concentrations of the control plasmid template DNA, i.e., containing 20, 200, 2.0 k, 20 k, 200 k copies, respectively.

The primers and probes were designed using Primer Express 2.0 (Life Technologies). The nucleotide sequences of the sense and anti-sense primers were hEpo F (5’-AGGCCAGAGGAGGACCATCT-3’) and hEpo R (5’-GGAAGGTGTCAGCATGATTGTC-3’), respectively. The probe structure was hEpo Pro, 6-carboxy-fluorescein (FAM)-CAT TCC TAA CACTAGCCCTATAMRA, which was modified in the quencher from the previous report.10 The amplicon length was 106 bp. These primers and probe were used instead of those for hEpo gene detection. Singleplex PCR was adopted. The PCR reaction conditions were the same as described in the section above. The ΔRn threshold for plotting the Ct values was set at 0.10 during exponential amplification.

**RESULTS**

**Extraction of DNA from Chicken Meat Samples** The characteristics of the genomic DNA extracted from the various chicken meat samples are shown in Table 2 and Fig. 1. The genomic DNA yields varied. The genomic DNA samples appeared to be appropriate for use in real-time PCR tests based on the ratios of the absorbances at 260 nm to those at 280 nm and the ratios of the absorbances at 260 nm to those at 230 nm. For the raw meat samples, a larger amount of shorter DNA was obtained from the liver sample, compared with the other meat samples, based on the absorbance at 280 nm and 0.8% agarose gel electrophoresis. Shorter DNA was also obtained from the samples of chicken meat in the processed foods, compared with the samples of the raw chicken meats, based on the results of 0.8% agarose gel electrophoresis. In particular, the genomic DNA extracted from the chicken curry was relatively short. This result indicated that the genomic DNA in the chicken meat had been degraded during food processing. We successfully amplified endogenous cytochrome b gene from all the genomic DNA.

**Design of Primers and Probe for Real-Time PCR** Detailed information regarding the hEpo cDNA inserted into the genomes of GM chickens was not available. First, we ran hEpo cDNA through a database, and two types were found: one with and one without Lys at codon 143. Both cDNAs consisted of nucleotide sequences encoding a signal peptide and the mature protein. When we attempted to design specific primers and probes based on the cDNA sequence to detect the hEpo gene sequence inserted into the chicken genome, two candidates were found within the nucleotide sequence encoding the mature protein. One primer contained different sequences between the two types of hEpo cDNAs. The set that did not contain this primer was used, since it should be
capable of detecting both types of hEpo genes simultaneously.

**Real-Time PCR Detection of hEpo Gene in Genomic DNA Extracted from Magnum and Heart of a Chimeric Chicken Containing hEpo Gene** The hEpo gene was clearly detected in genomic DNA extracted from magnum and heart of a chimeric chicken containing the hEpo gene using real-time PCR. However, it was not detected in genomic DNA extracted from blood of a transgenic chicken containing GFP gene or from meat of a non-GM chicken. The Ct values of the hEpo gene are shown in Table 1.

**Establishment of Calibration Plots** A commercially available plasmid containing hEpo cDNA was purchased and used as a standard. We confirmed that amplification plots could be obtained and that the hEpo gene in the plasmid could be detected using real-time PCR. The amplification plot and the standard plot are shown in Fig. 2. The results were thought to be reproducible based on the five formulae of the standard plots described in the figure legend.

Next, the control plasmid was spiked into one of the genomic DNA samples extracted from the raw chicken meat samples. We then confirmed that the amplification plots could be obtained with the plasmid and that the hEpo gene in the plasmid could be detected in the presence of genomic DNA extracted from the raw chicken meat sample. The amplification plot and the standard plot in the presence of the chicken genomic DNA are shown in Fig. 3. The results were thought to be reproducible based on the five formulae of the standard plots described in the figure legend.

When the control plasmid was added to the PCR reaction mixture containing each genomic DNA extracted from the six samples of chicken meat in the processed foods, the hEpo gene was detected and the standard plots could be calibrated.

**Survey of Twelve Samples of Chicken Meats** To assess the applicability of our method, we tested twelve samples of chicken meats (consisting of six samples of raw chicken meats and six samples of chicken meat in processed foods). The standard plots were used to determine whether the hEpo gene was detected in these samples of chicken meats. The hEpo gene was not detected in any of the samples examined in the survey.

An analysis of the endogenous chicken cytochrome b gene yielded similar amplification plots and Ct values for all the samples. The Ct values of the chicken cytochrome b gene and the hEpo gene are shown for the various chicken meat samples in Table 1. Additionally, all the genomic DNA samples were confirmed to have been extracted from chicken meat, not from pork, beef, mutton or horseflesh.12

**DISCUSSION**

In this study we developed a real-time PCR method for detecting the hEpo gene in the chicken genome as a means of testing chicken meat for contamination with GM chicken meat carrying the hEpo gene.

We confirmed that the hEpo gene was clearly detected in genomic DNA extracted from magnum and heart of a chimeric chicken containing the hEpo gene using real-time PCR. However, meat of a chimeric chicken containing the hEpo gene was not available. Therefore, we used a plasmid containing hEpo cDNA as an alternative standard reference to develop the detection method as well. Our model experiment, in which the control plasmid was spiked into genomic DNA from a chicken meat sample and then detected, was thought to be acceptable because plasmids containing cloned transgenic sequences have been previously used to analyze GM materials in food.5

When we searched databases for chicken erythropoietin or a similar gene, nothing was found. So, we experimentally...
Fig. 2. Real-Time PCR Analysis of hEpo Gene in the Absence of Chicken Genomic DNA

The experiments were repeated five times. The formulas for the standard plots are described below: $y=-3.29x+42.7$ ($R^2 0.985$), $y=-3.21x+42.4$ ($R^2 0.971$), $y=-3.07x+42.8$ ($R^2 0.975$), $y=-3.21x+42.5$ ($R^2 0.978$), $y=-3.21x+43.3$ ($R^2 0.970$). The results of the first experiment are shown in the figure.

Fig. 3. Real-Time PCR Analysis of hEpo Gene in the Presence of Chicken Genomic DNA

The experiments were repeated five times. The formulas for the standard plots are described below: $y=-3.24x+43.4$ ($R^2 0.964$), $y=-3.39x+43.5$ ($R^2 0.978$), $y=-3.15x+43.1$ ($R^2 0.965$), $y=-3.29x+43.4$ ($R^2 0.986$), $y=-3.02x+42.1$ ($R^2 0.967$). The results of the first experiment are shown in the figure.
examined the chicken genome using real-time PCR. The hEpo gene was not detected in the chicken genome without spiking with the control plasmid. This result, combined with the results that the spiked hEpo cDNA in the control plasmid was detected and that the endogenous chicken cytochrome b gene was detected, indicated that the sequences detected by the primers and probe did not exist in the chicken genome and that the samples examined in our survey were derived from non-GM chickens.

As described above, the hEpo gene was detected in the presence of genomic DNA extracted from one of the raw chicken meat samples when the control plasmid had been spiked into the PCR reaction mixture. These results strongly suggested that our detection method could be used to detect meat from GM chickens carrying the hEpo gene.

We applied the detection method to twelve test samples, and the hEpo gene was not detected in any of the samples. When the genomic DNA was analyzed using 0.8% agarose gel electrophoresis, the genomic DNA extracted from the raw chicken liver sample and the samples of chicken meat in the processed foods were found to be shorter than those from the raw chicken meat samples. However, we considered that all the tested genomic DNAs were measurable in our real-time PCR detection method because the amplification plots for the endogenous chicken cytochrome b reactions were clearly and similarly detected when testing all the genomic DNAs, as shown in Table 2. Furthermore, when the control plasmid was spiked into a PCR reaction mixture containing the extracted genomic DNA, the hEpo gene was detected. This result confirmed that real-time PCR could detect the hEpo gene in the presence of the chicken genomic DNA. These results suggested that meat from a GM chicken carrying the hEpo gene would be detected if it were subjected to our method. We concluded that the samples tested in our study did not contain meat from a GM chicken carrying the hEpo gene.

The C-value for chicken is 1.25 according to the Animal Genome Size Database, meaning that one chicken haploid cell contains a genome weighing 1.25 pg. In our study, we used 130 ng of genomic DNA extracted from each sample of chicken meat per reaction. This amount of chicken genome corresponds to that contained in 52000 cells. If one assumes that one copy of the hEpo gene was inserted into the chicken genome contained in one cell and that the contamination ratio of meat from a GM chicken was 100%, 52000 copies of the hEpo gene should exist per putative GM sample. Based on this copy number of the hEpo gene in the reaction and the standard plot shown in Fig. 3, the target gene would be stably detected using our method if a GM sample were to be examined.

As described above, we think that the six samples of chicken meat in the processed foods were successfully examined using our detection method. This outcome was probably due to the use of a short target sequence for the real-time PCR detection, and possible impurities in the genomic DNA preparations did not inhibit the PCR reaction. However, further study will be necessary to extend the applications of this detection method to more complex processed food products. For example, appropriate methods of preparing genomic DNA from chicken meat in vacuum-packed foods or canned chicken meat and the application of these methods to the testing of genomic DNA should be examined.

Many kinds of GM chickens have been developed recently. Among them, we selected GM chickens carrying the hEpo gene as the subject of this study, and the method was developed to detect the GM meat. The same strategy described here could be used to detect chicken meat from other kinds of GM chickens.

In conclusion, a rapid detection method for the hEpo gene inserted in a GM chicken genome has been established. This method could be used to monitor chicken meat for contamination from meat originating from GM chickens carrying the hEpo gene to assure food safety.

Acknowledgments We thank Dr. Ken-ichi Nishijima of Nagoya University for providing genomic DNA extracted from the chimeric chicken containing the hEpo gene and from the transgenic chicken containing the GFP gene. We thank Professor Hironori Horiuchi of Hiroshima University for valuable advice. This study was supported by a Grant from the Ministry of Health, Labour and Welfare of Japan.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
Samples* & Genomic DNAs & Ct values for chicken cytochrome b gene & Ct values for hEpo gene* \\
& & & \\
Yield (µg/g) & $A_{260}/A_{230}$ & $A_{260}/A_{280}$ & & \\
\hline
Raw chicken & Breast & 58 & 1.85 & 2.33 & 16.5±0.2 & u.d. (9) \\
& Sasami & 110 & 1.91 & 2.36 & 18.2±0.6 & u.d. (9) \\
& Liver & 1000 & 1.90 & 2.36 & 17.2±0.9 & u.d. (9) \\
& Leg & 190 & 1.91 & 2.35 & 16.8±0.3 & 39.1 (1), u.d. (8) \\
& Wing & 170 & 1.90 & 2.41 & 16.3±0.3 & u.d. (9) \\
& Minced & 150 & 1.93 & 2.36 & 17.3±0.6 & u.d. (9) \\
\hline
Processed chicken & Fried chicken (Fried) & 290 & 1.96 & 2.34 & 17.0±1.1 & u.d. (9) \\
& Oyakodon (Boiled) & 430 & 1.95 & 2.30 & 18.2±0.4 & 39.5 (1), u.d. (8) \\
& Yakitori with liver (Baked) & 770 & 1.96 & 2.31 & 16.1±0.3 & 39.4 (1), u.d. (8) \\
& Chicken cutlet (Fried) & 100 & 1.75 & 2.44 & 17.5±0.8 & u.d. (9) \\
& Teryaki chicken (Baked) & 69 & 1.73 & 2.41 & 16.8±1.0 & 39.8 (1), u.d. (8) \\
& Chicken curry (Boiled and autoclaved) & 300 & 1.84 & 2.42 & 17.5±0.2 & u.d. (9) \\
\hline
\end{tabular}
\caption{Characterization of Chicken Genomic DNAs and Ct Values of Chicken Cytochrome b Gene and hEpo Gene}
\end{table}
REFERENCES

1) Kodama D, Nishimiya D, Isvata K, Yamaguchi K, Yoshida K, Kawabe Y, Motono M, Watanabe H, Yamashita T, Nishijima K, Kamihira M, Iijima S. Production of human erythropoietin by chimeric chickens. Biochem. Biophys. Res. Commun., 367, 834–839 (2008).

2) Penno CA, Kawabe Y, Ito A, Kamihira M. Production of recombinant human erythropoietin / Fc fusion protein by genetically manipulated chickens. Transgenic Res., 19, 187–195 (2010).

3) Koo BC, Kwon MS, Lee H, Kim M, Kim D, Roh JY, Park YY, Cui XS, Kim NH, Byun SJ, Kim T. Tetracycline-dependent expression of the human erythropoietin gene in transgenic chickens. Transgenic Res., 19, 437–447 (2010).

4) Vaiitalingom M, Pijnenburg H, Gendre F, Brignon P. Real-time quantitative PCR detection of genetically modified Maximizer maize and Roundup Ready soybean in some representative foods. J. Agric. Food Chem., 47, 5261–5266 (1999).

5) Hübner P, Waiblinger HU, Pietsch K, Brodmann P. Validation of PCR methods for quantitation of genetically modified plants in food. J. AOAC Int., 84, 1855–1864 (2001).

6) Kuribara H, Shindo Y, Matsuoka T, Takubo K, Futo S, Aoki N, Hirao T, Akiyama H, Goda Y, Toyoda M, Hino A. Novel reference molecules for quantitation of genetically modified maize and soybean. J. AOAC Int., 85, 1077–1089 (2002).

7) Shindo Y, Kuribara H, Matsuoka T, Futo S, Sawada C, Shono J, Akiyama H, Goda Y, Toyoda M, Hino A. Validation of real-time PCR analyses for line-specific quantitation of genetically modified maize and soybean using new reference molecules. J. AOAC Int., 85, 1119–1126 (2002).

8) Huang HY, Pan TM. Detection of genetically modified maize MON 810 and NK603 by multiplex and real-time polymerase chain reaction methods. J. Agric. Food Chem., 52, 3264–3268 (2004).

9) Taverniers I, Windels P, Vaiitalingom M, Milemps A, Van Bockstaele E, Van den Eede G, De Loose M. Event-specific plasmid standards and real-time PCR methods for transgenic Bt11, Bt176, and GA21 maize and transgenic GT73 canola. J. Agric. Food Chem., 53, 3041–3052 (2005).

10) Toyota A, Akiyama H, Sugimura M, Watanabe T, Kikuchi H, Kanamori H, Hino A, Esaka M, Maitani T. Quantification of genetically modified soybeans using a combination of a capillary-type real-time PCR system and a plasmid reference standard. Biosci. Biotechnol. Biochem., 70, 821–827 (2006).

11) Rott ME, Lawrence TS, Wall EM, Green MJ. Detection and quantification of Roundup Ready soy in foods by conventional and real-time polymerase chain reaction. J. Agric. Food Chem., 52, 5223–5232 (2004).

12) Tanabe S, Hase M, Yano T, Sato M, Fujimura T, Akiyama H. A real-time quantitative PCR detection method for pork, chicken, beef, mutton, and horseflesh in foods. Biosci. Biotechnol. Biochem., 71, 3131–3135 (2007).

13) Kamihira M, Ono K, Esaka K, Nishijima K, Kigaku R, Komatsu H, Yamashita T, Kyogoku K, Iijima S. High-level expression of single-chain Fv-Fc fusion protein in serum and egg white of genetically manipulated chickens by using retroviral vector. J. Virol., 79, 10864–10874 (2005).

14) Animal Genome Size Database: <http://www.genomesize.com>, cited 1 August, 2012.