IDENTIFICATION OF THE SPECIES USED IN PRODUCTION OF COMMERCIAL SAUSAGES IN SOUTH KOREA

Jin-Hyung Kim[4], Yong-Jun Kang[2], Yoo-Kyung Kim[2,3], Hong-Shik Oh[3], In-Cheol Cho[2] and **Sang-Hyun Han[1*]**

[1]Educational Science Research Institute, Jeju National University, Jeju 63243, Republic of Korea; [2]Subtropical Livestock Research Institute, National Institute of Animal Science, RDA, Jeju 63242, Republic of Korea; [3]Faculty of Science Education, Jeju National University, Jeju 63243, Republic of Korea; [4]Animal Products Utilization Division, National Institute of Animal Science, Rural Development Administration, 1500 kongjwipatjwi-ro, Iseo-myeon, Wanju-gun, Jeollabuk-do, 565-851, Republic of Korea

Abstract – This study aimed to develop a rapid and simple method for identification of the origin species used in commercial sausages production by amplification patterns for mitochondrial DNA (mtDNA). Commercial sausages were purchased from retail markets and subjected to mtDNA analysis. To optimize the polymerase chain reaction (PCR) conditions, gradient PCR reactions were carried out to determine the primer annealing temperatures, and real-time PCR was done to check the minimal amount of DNA and to examine the cross-reaction. PCR products were observed on the gels suggesting that DNA molecules may be useful in the identification of the meat source in processed sausages. A similarity search of the DNA sequences showed that they were from pig, chicken, and fish, as described on the product labels. The real-time PCR results showed that the PCR products were observed above 10 fg (0.01 pg)/µl concentrations in pig, chicken, and processed fish meat DNA. No significant amplification was found in cross species. This PCR-based molecular method using mtDNA markers may provide useful information for food safety and traceability purposes by supplying molecular evidence for detecting and identifying the meat sources used in sausage production.

Key Words – meat origin, PCR, real-time PCR.

I. INTRODUCTION

Information on the species used in processed foods is very important to consumers. To supply credible evidence for food safety and traceability, meat product labels should contain information on all the components in the product, including the meat source. Recently, DNA is considered to be the most appropriate molecule for species identification in foods. DNA is relatively stable at high temperatures. Therefore, DNA can be analysed not only in fresh and frozen foods, but also in processed, degraded, or mixed conditions [1-3].

DNA-based methods have been developed for identifying mammalian species, and include PCR-RFLP (restriction fragment length polymorphism), species-specific PCR, probe hybridization, microarrays, and DNA sequencing [4-9]. The mtDNA sequence diversity of species is sufficient to discriminate between all species identified thus far [10-12].

DNA markers are considered powerful tools for determining the presence of source species after chemical and/or physical processing in industrial production and for supplying information on each species used in meat processing and production. The DNA technique is particularly useful for identifying the species in processed meat, which can often contain unidentifiable meat products from different species [13-16]. The illegal use of foreign meat sources and mislabelling of products have led to very serious social problems and decreased the consumer's choice of processed meat products. This study aimed to develop an mtDNA marker system using sequencing analysis,

origin-specific PCR (OS-PCR), and real-time PCR, and examine their potential to identify the source species used in the production of commercial sausages in Korea.

II. MATERIALS AND METHODS

A. Samples and DNA isolation

Forty commercial sausages (thirty-two of cooked emulsion type and eight of smoked type) were purchased from retail markets in Gyeonggi-do and Jeju-do, South Korea. Certified genomic DNA samples of species of pig, chicken, cattle, and horse were kindly provided by researchers at the National Institute of Animal Science, South Korea. DNA was extracted from sausage samples using standard technique [17], with a slight modification. To prevent human contamination during the DNA preparation, three different experimenters participated in the DNA isolation and carried out the experiments in three different laboratories.

B. Conventional PCR and real-time PCR

To amplify the mtDNA fragments, universal primers and origin-specific primers were designed using mitochondrial genome sequences (DQ356938 from Atlantic cod, NC_000845 from domestic pig, and AP003317 from chicken). Information on the primers and standard sequences is shown in Table 1.

Table 1. Primer information used in this study

Species	Primer name	Nucleotide sequence (5'->3')
Pig	3sp_COI_F	ATYACTATACTACTRACAGACCG
Chicken	3sp_COI_R	AAKGGTTCTTTTTTACCTGAATAGTA
Fish meat		
Pig	Pig_CYTB_F	CAACAACGCATTCATTGACC
	Pig_CYTB_R	AATATGGATGCTCCGTTTGC
Chicken	Gal_CYTB_F	TCTATTCGCCTATGCCATCC
	Gal_CYTB_R	TACTGGTTGGCTTCCGATTC
Fish meat	Fish_mtF	TCTTACCCGGGGTTGGAACTTTA
	Fish_mtR	AGGGGGATTAGATGAAGGGCTA

In order to determine the optimal annealing temperature of each primer pair, gradient PCR was carried out using Mastercycler Gradient (Eppendorf, Germany). The DNA concentration for PCR amplification was also checked by serial dilution samples from the 100 ng ($1x10^{-7}$ g)/µl to 10 ag ($1x10^{-17}$ g)/µl of genomic DNA solutions by real-time PCR using Rotor-Gene Q (Qiagen, USA) with Rotor-Gene SYBR Green PCR Kit (Qiagen, USA) followed by manufacturer's guide. Cross-reaction test was conducted by conventional PCR, gradient PCR, and real-time PCR for other species (horse, cattle, sheep, turkey, duck, quail, and pheasant). The PCR reaction was carried out in 20 µl volumes with Maxime *i*-Star Taq Premix (Intron Biotechnology, South Korea). PCR amplification was performed under the following conditions: an initial 2 min denaturation at 94° C, followed by 35 cycles of 30 sec at 94° C, 30 sec at 62° C, and 30 sec at 72° C.

C. DNA sequencing and data analysis

The purified PCR product amplified using a pair of universal primers was inserted into a plasmid vector using the TOPO™ TA Cloning Kit (Invitrogen, USA) according to the manufacturer's instructions. Fifteen colonies were chosen from each transformation reaction plate, and plasmid DNA was purified using the QIAprep Spin Miniprep Kit (Qiagen, USA). Nucleotide sequencing was carried out using the DYEnamic ET Dye Terminator Kit (Amersham Biosciences, USA). Clustal W was used for multiple alignments of the obtained sequences and DnaSP was used to determine the nucleotide diversity. A BLAST search of the NCBI's nucleotide database was conducted, and the results were compared with the unique *COI* sequences.

III. RESULTS AND DISCUSSION

To identify the meat sources used for the processed meat products, we examined the PCR-mediated molecular identification using universal primers and OS primer sets. PCR products, approximately 190-bp length, were found on the agarose gels from all the sausage DNA samples when amplifying with a universal primer set.

Figure 1 shows the PCR amplification patterns using universal primer set (Figure 1(A)) and OS primer sets (Figure 1(B)-(D)). PCR products were observed in all sausage DNA samples in PCR assay using a pair of universal primers (Figure 1(A)). The pig-specific PCR reaction produced 228-bp PCR bands on the gels only from the pig DNA samples but not from those of chicken DNA and fish meat DNA (Figure 2(B)). In addition, the chicken- and fish meat-specific PCR reactions also produced the origin-specific amplicons (208-bp in

chicken and 150-bp in fish), respectively (Figure 1(C) and 1(D)).

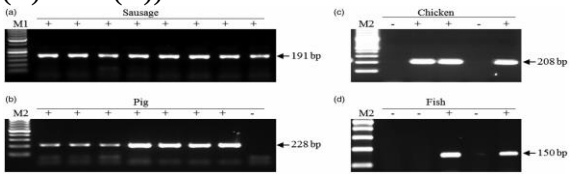

Figure 1. PCR amplification patterns. (A) an universal primer set 3sp_COI_F and 3sp_COI_R; (B) pig-specific Pig_CYTB_F and CYTB_R; (C) chicken specific Gal_CYTB_F and Gal_CYTB_R; (D) fish-specific Fish_mtF and Fish_mtR. + and − indicate the presence and absence of the meat on the labels, respectively. M1 and M2 are 50-bp and 100-bp ladder, respectively.

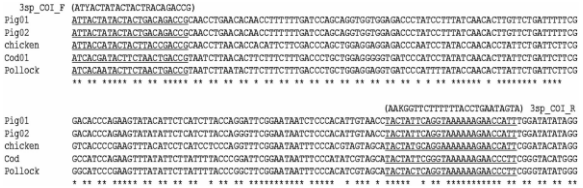

Figure 2. Multiple sequence alignments for *COI* sequences determined in this study. Majority of pigs have two types of DNA sequences (Pig01 and Pig02), but most chickens showed an identical sequence (chicken). Two types of fish meat sources were found from cod and pollock. The underlined sequences indicate the sequences of the universal primer set.

The DNA sequencing results revealed no variation in the size of the PCR products amplified from the sausage DNA. The sequence identities of our *COI* sequences were very similar (98.1-100%) to those deposited in the NCBI's database. Figure 2 shows the multiple sequence alignments for the *COI* sequences of pig, chicken, cod, and pollock mostly found in the sausage DNA.

Specificity was evaluated by real-time PCR reactions, including DNA from pig, chicken, horse, cattle, sheep, turkey, duck and pheasant, processed fish meat and water (negative control). All cross-test reactions were amplified over a Ct value of 35 or had no detectable signals, indicating no PCR products were produced. Significant amplification signals were just observed from reactions with pig, chicken, and processed fish meat, while no amplification signals were detected with DNA from cattle, horse, sheep, goat, cattle, duck, and pheasant. Therefore, the newly developed OS primers had good specificity for pig, chicken, and fish meats.

Sensitivity of OS primers was also evaluated by real-time PCR (Figure 3).

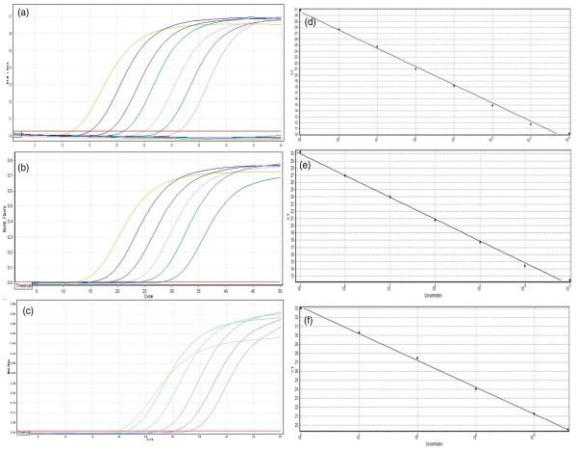

Figure 3. Real-time PCR amplification patterns of pig, chicken, and fish meat. (A–C) show amplification patterns for serial dilutions of pig (A), chicken (B), and fish meat (C). (D–F) show the standard curves for (A–C).

Table 2. Ct values according to the DNA concentrations obtained by real-time PCR

DNA conc. (/ul)	Pig	Chicken	Fish meat
100 ng	8.3	9.1	14.7
10 ng	11.1	12.8	17.4
1 ng	14.4	14.7	20.2
100 pg	17.9	18.0	21.9
10 pg	20.6	21.1	24.8
1 pg	23.4	24.3	27.6
100 fg	26.2	27.1	30.5

When the DNA amount of pig, chicken, and processed fish meat was 1 pg, the Ct values for each species were about 23.4, 24.3, and 27.6, respectively (Table 2). DNA samples from the three target meat sources (pig, chicken, and processed fish meat) were 10-fold serially diluted from 100 ng/µl to 10 ag/µl and used as templates for real-time PCR to construct standard curves. The significant amplification signals could be observed above 10 fg/µl (0.01 pg/µl) concentrations in pig, chicken, and fish meat. Sausage DNA samples containing different percentages of pig, chicken, and fish were tested by the real-time PCR system. All meat mixtures yielded positive results in the real-time PCR, showing the same results from conventional OS-PCR reactions (Table 3).

Table 3. Species identification of pig, chicken and fish meat by DNA sequencing and PCR assays

Species	No. of records	DNA sequencing	OS-PCR	Real-time PCR
Pig	38	38	38	38
Chicken	13	13	13	13
Fish*	6	8	8	8

* Fish-derived PCR amplicons were found in eight sausage DNA samples, of which six samples listed meat on the labels.

As shown in Figure 3, the standard curves showed a linear correlation between Ct values and log-DNA concentrations. The correlation coefficients (R^2) were 0.99970 (pig-specific primer set), 0.99939 (chicken-specific primer set), and 0.99827 (fish-specific primer set) (Figure 3(D)-(F)), indicating significant linear relationships. Amplification efficiencies all exceeded 90%. Moreover, further electrophoresis analysis of real-time PCR products showed that there was no PCR product unexpected found from the sausage DNA samples (Figure 4), and from artificial mixed DNA solutions of different species sources.

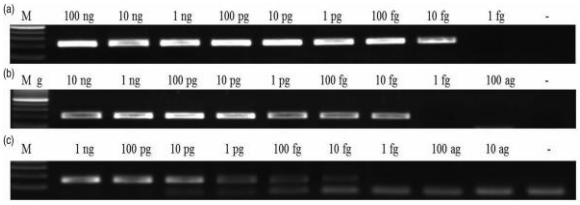

Figure 4. Analysis for real-time PCR products. (A) pig; (B) chicken; (C) fish meat. M is 100-bp ladder. Numbers on top of the lanes indicate DNA concentrations in 1 µl solution used for PCR amplification.

Table 4 presents the species information listed on the sausage product labels and those identified by the similarity comparison of the *COI* sequences in this study. The sausage labels listed three different animal sources: pig, chicken, and fish meat. The information on the source animal on the product label of most of the sausages matched the source identified with the molecular data. The DNA sequencing results revealed no DNA from foreign mammalian species. In two DNA samples, the *COI* sequences corresponded to that of an unlabelled animal species. It is likely that the unlisted species was introduced unintentionally by cross-contamination during the sausage production. The unlabelled product was identified as a fish (Atlantic cod) by DNA sequencing.

Table 4. List of species found in sausage labels and those in this study

Sample no.	Label species			DNA sequencing			OS-PCR			Real-time PCR		
	Pig	Chicken	Fish meat	Pig	Chicken	Fish meat	Pig	Chicken	Fish meat	Pig	Chicken	Fish meat
SS01	+	-	-	+	-	-	+	-	-	+	-	-
SS02	+	-	-	+	-	-	+	-	-	+	-	-
SS03	+	-	-	+	-	-	+	-	-	+	-	-
SS04	+	-	-	+	-	-	+	-	-	+	-	-
SS05	+	-	+	+	-	+	+	-	+	+	-	+
SS06	+	-	-	+	-	-	+	-	-	+	-	-
SS07	+	-	-	+	-	-	+	-	-	+	-	-
SS08	+	-	+	+	-	+	+	-	+	+	-	+
SS09	+	-	-	+	-	-	+	-	-	+	-	-
SS10	+	-	-	+	-	-	+	-	-	+	-	-
SS11	+	-	-	+	-	-	+	-	-	+	-	-
SS12	+	+	-	+	+	-	+	+	-	+	+	-
SS13	+	+	-	+	+	-	+	+	-	+	+	-
SS14	+	-	-	+	-	-	+	-	-	+	-	-
SS15	+	+	-	+	+	-	+	+	-	+	+	-
SS16	+	-	+	+	-	+	+	-	+	+	-	+
SS17	+	+	-	+	+	-	+	+	-	+	+	-
SS18	+	+	-	+	+	-	+	+	-	+	+	-
SS19	+	-	-	+	-	-	+	-	-	+	-	-
SS20	+	+	-	+	+	-	+	+	-	+	+	-
SS21	+	+	-	+	+	-	+	+	-	+	+	-
SS22	+	-	-	+	-	-	+	-	-	+	-	-
SS23	+	-	-	+	-	-	+	-	-	+	-	-
SS24	+	-	-	+	-	+	+	-	+	+	-	+
SS25	+	+	-	+	+	-	+	+	-	+	+	-
SS26	-	-	+	-	-	+	-	-	+	-	-	+
SS27	+	+	-	+	+	-	+	+	-	+	+	-
SS28	+	-	-	+	-	-	+	-	-	+	-	-
SS29	+	+	-	+	+	+	+	+	+	+	+	+
SS30	+	+	-	+	+	-	+	+	-	+	+	-
SS31	+	-	-	+	-	-	+	-	-	+	-	-
SS32	+	-	-	+	-	-	+	-	-	+	-	-
SS33	+	-	-	+	-	-	+	-	-	+	-	-
SS34	-	-	+	-	-	+	-	-	+	-	-	+
SS35	+	-	-	+	-	-	+	-	-	+	-	-
SS36	+	+	-	+	+	-	+	+	-	+	+	-
SS37	+	-	+	+	-	+	+	-	+	+	-	+
SS38	+	-	-	+	-	-	+	-	-	+	-	-
SS39	+	+	-	+	+	-	+	+	-	+	+	-
SS40	+	-	-	+	-	-	+	-	-	+	-	-

*, SS24 and SS29 have no fish meat on the labels, but we found the fish-derived DNA in this study.

From the conventional OS-PCR and real-time PCR assays, we obtained molecular data similar to those from DNA sequencing and the records on the labels of the commercial sausages. PCR products derived from pigs and chickens were detected in 38 and 13 sausage DNA samples, respectively, which indicated the use of each animal source in the sausage production. We found PCR products derived from fish meat in eight sausage DNA samples. Six sausage samples listed the inclusion of fish on their labels. The other two did not contain any information on the inclusion of fish in the sausage production. However, the PCR bands from the fish-specific PCR assay confirmed the existence of fish-derived DNA in the sausages. Further analyses of DNA sequencing and a BLAST search showed 100% sequence identities to the sequences previously reported from Atlantic cod. As the presence of the PCR products points to the presence of DNA, we concluded that these two sausage samples contained fish-derived DNA molecules.

IV. CONCLUSION

Species information in processed meats and processed foods is increasingly important for consumers around the world who wish to know the species of origin in processed meat products. Food

manufacturers should record the meat origin on their labels of processed foods. For religion purposes, meat species identification is especially important for Muslim and Hindu religious Halal authentication. Many molecular methods have been developed to better perform this task, including DNA extracted from various sample sources. In this study, we obtained good results with regard to the molecular identification of the species of origin in commercial sausages using a two-step approach based on universal and OS-PCR primers in conventional PCR and real-time PCR. Both the PCR results confirmed the DNA of the source animal and the species of origin in commercial sausages. We detected meat sources from animals not listed on the product labels in two samples. In addition, we could determine the minimal amounts of above 0.01 gp/ul concentrations by real-time PCR analysis for sausage DNA. Consequently, we have developed a specific and sensitive molecular technique for detection and identification of meat components in processed food as well as raw meats. This PCR-based molecular method could be offered rapid and precise information on the origins of source animals used in the production of commercial sausages. These results suggest that this method may play an important role in quality control of meat products as providing the scientific evidence of the source species.

REFERENCES

1. Lenstra, J. A. (2003). DNA methods for identifying plant and animal species in food. In M. Lees (ed.), Food Authenticity and Traceability. Cambridge: Woodhead Publishing Ltd; pp. 34-53.
2. Rodríguez, M. A., García, T., González, I., Asensio, L., Hernández, P. E. & Martín, R. (2004). PCR identification of beef, sheep, goat, and pork in raw and heat-treated meat mixtures. Journal of Food Protection 67: 172-177.
3. Singh, P. & Neelam, S. (2011). Meat species specifications to ensure the quality of meat: a review. International Journal of Meat Science 1: 15-26.
4. Matsunaga, T., Chikuni, K., Tanabe, R., Muroya, S., Shibata, K., Yamada, J. & Shinmura Y. (1999). A quick and simple method for the identification of meat species and meat products by PCR assay. Meat Science 51: 143-148.
5. López-Andreo, M., Lugo, L., Garrido-Pertierra, A., Prieto, M. I. & Puyet, A. (2005). Identification and quantitation of species in complex DNA mixtures by real-time polymerase chain reaction. Analytical Biochemistry 339: 73-82.
6. Kesmen, Z., Sahin, F. & Yetim, H. (2007). PCR assay for the identification of animal species in cooked sausages. Meat Science 77: 1-5.
7. Zhang, C., Fowler, M. R., Scott, N. W., Lawson, G. & Slater, A. (2007). A TaqMan real-time PCR system for the identification and quantification of bovine DNA in meals, milks and cheeses. Food Control 18:1149–1158.
8. Jonker KM, Tilburg JJ, Hagele GH, de Boer E. (2008). Species identification in meat products using real-time PCR. Food Additives & Contaminants 25: 527-533.
9. Koh BRD, Kim JY, Na HM, Park SD, Kim YH. (2011). Development of species-specific multiplex PCR assays of mitochondrial 12S rRNA and 16S rRNA for the identification of animal species. Korean Journal of Veterinary Service 34: 417-428.
10. Zehner, R., Zimmermann, S. & Mebs, D. (1998). RFLP and sequence analysis of the cytochrome b gene of selected animals and man: methodology and forensic application. International Journal of Legal Medicine 111: 323-327.
11. Cai, X., Chen, H., Lei, C., Wang, S., Xue, K. & Zhang, B. (2007). mtDNA diversity and genetic lineages of eighteen cattle breeds from *Bos taurus* and Bos indicus in China. Genetica 131: 175-183.
12. Chen, S. Y., Yao, Y. G. & Liu, Y. P. (2012). Species identification of ten common farm animals based on mitochondrial 12S rRNA gene polymorphisms. Animal Biotechnology 23: 213-220.
13. Bottero, M. T., Dalmasso, I. A., Nucera, D., Turi, R. M., Rosati, S., Squadrone, S., Goria, M. & Civera, T. (2003). Development of a PCR assay for the detection of animal tissues in ruminant feeds. Journal of Food Protection 66: 2307-2312.
14. Rao, Q. & Hsieh, Y. H. (2007). Evaluation of a commercial lateral flow feed test for rapid detection of beef and sheep content in raw and cooked meats. Meat Science 76: 489-494.
15. Yarmand, M. S. & Homayouni, A. (2010). Quality and microstructural changes in goat meat during heat treatment. Meat Science 86: 451-455.
16. Şakalar, E., Abasiyanik, M. F., Bektik, E. & Tayyrov, A. (2012). Effect of heat processing on DNA quantification of meat species. Journal of Food Science 77: N40-44.
17. Sambrook, J., Fritsch, E. F. & Manniatis, T. (1989). Isolation of high-molecular-weight DNA from mammalian cells. In: Molecular cloning: a laboratory manual, 2nd ed., New York: Cold Spring Harbor Laboratory Press. pp. 9.14-9.23.