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Development and Use of Quantitative Competitive PCR Assay for Detection of Poultry DNA in Sausage

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This study focused on the development and evaluation of a quantitative competitive polymerase chain reaction (QC-PCR) for detection and quantification of poultry DNA in sausage. PCR is well known to be quantitative if internal DNA standards are co-amplified together with the target DNA. A DNA competitor differing by 83 bp in length from the poultry target sequence was constructed and used for PCR together with the target DNA. Specificity of the new primers was evaluated with DNA from cattle and sheep. The results of QC-PCR showed that the percentage of contamination was in the range of 23.87–52.06%.

Key Words: contamination; poultry DNA; quantitative competitive PCR; sausage

INTRODUCTION

Food safety, quality, and composition have become the subjects of increasing public concern. Consumers have been given more choices with regard to food composition and dietary requirements via food labels. Various religious groups avoid specific meats such as beef or pork, and vegetarians choose not to consume any meat. Each constituency has an interest in ensuring the authenticity of the foods that they consume. Apart from the possible economic loss, correct species identification is important for consumers who may have specific food allergies (Lopez-Calleja et al., 2006). The health risks associated with potentially unsafe food and feedstock formulations, together with the

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increasing demand for information on the origin and composition of processed food products, have propelled the development of more accurate and powerful analysis methods (Lopez-Andreo et al., 2004). Proving conclusively that adulteration or contamination has occurred requires the detection and quantification of food constituents. This can be difficult because the materials replaced are often biochemically similar and food matrices are extremely complex and variable (Zhang et al., 2006). Lipid, protein and DNA based methods have been established for food identification. Lipid analysis is only applicable for gross measurement of animal-derived fats (Lumley, 1996; Saeed et al., 1989). Protein-based methods such as high performance liquid chromatography (HPLC) (Espinoza et al., 1996), enzyme-linked immunosorbent assays (ELISA) (Chen and Hsieh, 2000), and isoelectric focusing protein profiles (Skarpeid et al., 1998) are effective mainly for unprocessed food and are unable to differentiate species such as lamb and goat or chicken and turkey. Both require complicated procedures, and it has proved difficult to accurately quantify the analytes in a short time (Mayer, 2005). Methods based on protein analysis have been replaced by DNA-based methods because DNA has the advantage of being a relatively stable molecule and is more able to withstand heat processing (Behrens et al., 1999). Species-specific primers, alone or in combination with restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) technologies, have been designed for the identification of a large number of commercial mammals, birds, fish, and mollusks using mitochondrial DNA (Matsunaga et al., 1999; Wolf et al., 1999), ribosomal RNA (rRNA) (Rodrı´guez et al., 2001; Matsunaga et al., 1998), and nuclear genes (Hopwood et al., 1999; Lockley and Bardsley, 2000). Methods based on RFLP or RAPD can be used for the screening of a broader range of species, but such methods usually require the integrity of the DNA template and are more difficult to standardize for processed food samples. DNA-based methods developed for a quantitative detection are either based on quantitative competitive PCR (Wolf and Lu¨thy, 2001), densitometry (Calvo et al., 2002), or real-time PCR procedures (Dooley et al., 2004; Zhang et al., 2006). PCR analysis can be used as a quantitative method if internal standards (competitors) are co-amplified with the target DNA. Quantitative competitive PCR (QC-PCR) was first described in 1990 (Gilliland et al., 1990). The standard procedure for QC-PCR comprises four steps: (1) DNA extraction, (2) determination of DNA concentration, (3) QC-PCR with a defined internal DNA standard concentrations, and (4) separation of PCR products by gel electrophoresis (Hubner et al., 1999). QC-PCR can only be used for determination of relative amounts of target and standard if the regression coefficient r^2 is better than 0.99 and the slope of the regression line is very close to unity (Raeymaekers, 1993; Hayward-Lester et al., 1995). In Iran, due to the lower price of poultry meat compared to other types of meat products, some sausages factories try to add poultry meat to their products. Therefore, their

products show higher meat percentage without having extra expenditure. The objective of this study was to determine the percentage of the contamination of poultry DNA in sausage samples as a direct reflection of poultry meat contamination in the product.

MATERIALS AND METHODS

Sausage Samples

Five samples of sausage from different supermarkets were analyzed to check the poultry DNA in each sample. Each sample in four replicates was analyzed. Authentic samples of sausage were also obtained.

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DNA Extraction

Sausages samples (120 mg) were transferred into a 1.5 m l-microtube separately. One thousand microliters lysis reagent, 50 μL of 0.5 M EDTA, 5 μL of 10% sodium dodecyl sulphate (SDS), and 25 μL of 20 mg/mL proteinase K were added. The mixture was incubated at 65ºC overnight on a thermomixer, and 5 μL RNase (10 mg/mL) was added to the mixture for another 4 h. After digestion, samples were centrifuged at 5000g for 20 s. Supernatant (500 μL) transferred into a new 1.5 mL-microtube. Then DNA was extracted from sausage samples according to the guanidium thiocyanate-silicagel method (Boom et al., 1990). Quality and quantity of DNA were measured by spectrophotometerically taking the optical density at wave lengths of 260 and 280 nm, respectively.

Simplex PCR

A amplification of a 183 bp fragment from poultry DNA was carried out in a total volume of 25 μL in 0.5 mL tubes containing 1 unit of *taq* DNA polymerase, 200 μM of each dNTPs, 2.5 mM $MgCl_2$, 2.5 μL PCR product (10 X), 14 μL ddH_2O, 3 μL DNA, and 1.5 μL of each primer (5 pM) (Table 1). The thermal cycling was as follows: 10 min at 94ºC for initial denaturation, 35 cycles of

Table 1: Primers that used for amplification of competitor and 12s rRNA.

Name	Sequence 5´-3´
Pou F[a]	TGA GAA CTA CGA GCA CAA AC
Pou R[a]	GGG CTA TTG AGC TCA CTG TT
PouLAM F[b]	tgagaactacgagcacaaacCATTCGCTAATCAGTGGTGG
PouLAM R[b]	gggctattgagctcactgttGTCGTTCGAGTGCTATCTTGG

[a]Used for mt-DNA 12s rRNA amplification (Dalmasso et al., 2003).
[b]Used for competitor amplification.

amplification (30 s at 94ºC, 45 s at 60ºC, 45 s at 72ºC), and final extension for 5 min at 72ºC. PCR products were analyzed by gel electrophoresis in a 1.5% agarose gel and stained by ethidium bromide.

Construction of the Poultry DNA Competitor

Competitor was constructed similar to strategies described earlier (Grassi et al., 1994; Schanke et al., 1994; Studer et al., 1998). Competitor primers were designed by using primer premier5 software (Premier Biosoft International, USA) based on lambda phage genome sequence in NCBI Gen Bank with code NC_001416 (Table 1). PCR for poultry DNA competitor was performed to amplify a 100 bp fragment. DNA competitor differing by 83 bp in length from the poultry target sequence was constructed and used for PCR together with the target DNA. Competitor amplification was carried out in a total volume of 25 μL in 0.5 ml tubes containing 1 unit of *taq* DNA polymerase, 200 μM of each dNTPs, 2.5 mM $MgCl_2$, 2.5 μL PCR product (10 X), 17.5 μL ddH_2O, 0.5 μL lambda phage DNA, and 0.75 μL of each primer (5 pM). The thermal cycling was as follows: 4 min at 94ºC for initial denaturation, 35 cycles of amplification (30 s at 94ºC, 30 s at 56ºC, 60 s at 72ºC), and final extension for 5 min at 72ºC. PCR products were analyzed by gel electrophoresis in a 1.5 % agarose gel and stained by ethidium bromide. To provide competitor and different dilutions of competitor, the 100 bp PCR product from an agarose gel was purified by using of kit a Diatom DNA Elution kit (Moscow, Russia). Different dilutions of 10^{-1} to 10^{-10} were constructed from competitor.

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Quantitative Competitive PCR

Competitive PCR by using DNA-template and competitor (different dilutions) was carried out in a total volume of 25 μL in 0.5 mL tubes containing 1 unit of taq DNA polymerase, 200 μM of each dNTPs, 2.5 mM $MgCl_2$, 2.5 μL PCR product (10 X), 12.5 μL ddH_2O, 2 μL of DNA-template, 1 μL (5 pM) of competitor, and 2 μL (5 pM) of each primer (Table 1). The cycling condition was as follow: 10 min at 94ºC for initial denaturation, 35 cycles of amplification (30 s at 94ºC, 45 s at 60ºC, 45 s at 72ºC), and final extension for 5 min at 72ºC. PCR products were analyzed by gel electrophoresis in a 1.5% agarose gel and stained by ethidium bromide.

Optimizing the Suitable Dilutions of Competitor

For determination of suitable dilutions, many separate competitive PCR reactions were performed with all different dilutions from 10^{-1} to 10^{-10}. Electrophoresis analysis denoted that suitable dilutions for competitor fragment were 10^{-1}, $10^{-1.7}$ and 10^{-2} (Fig. 1).

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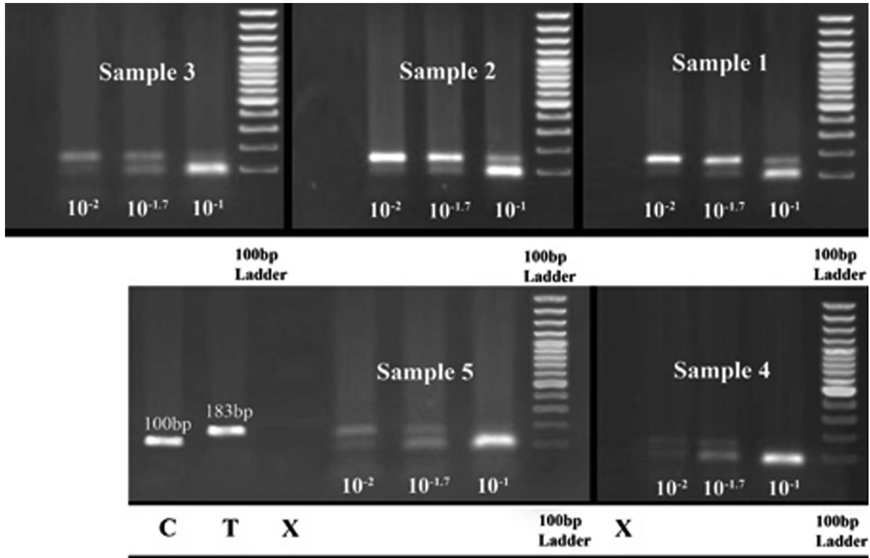

Figure 1: QC_PCR for five different samples of sausage in three different dilutions (10^{-1}, $10^{-1.7}$ and 10^{-2}) of competitor. One sample from each replicate for five samples had been showed. T = Target DNA, C = Competitor DNA. X = PCR negative control/mastermix without DNA.

Determination of Standard Equation

To determine the percent of contamination in samples, a standard equation by use of authentic samples was obtained. After DNA extraction, authentic samples were contaminated at five different level of percent, containing 40%, 30%, 20%, 10%, and 1% by use of pure poultry pure DNA. Competitive PCR for artificial contaminated samples was performed (Fig. 2). A standard equation was obtained by using the log (target/competitor) from artificial contaminant samples was obtained by using JMP software (ver 7.0, SAS Institute Inc, USA) (Table 2):

$$Y = -237.3 - 4975.07 X_1 + 7066.62 X_2 - 648.84 X_3;\ r^2 = 0.99$$

where Y, X_1, $X_{2,}$ and X3 are the density of contamination in sample, the log (target/competitor) for 10^{-1}, $10^{-1.7}$ and 10^{-2} dilutions of competitor, respectively.

RESULTS

Simplex PCR for five different samples of sausage denoted that each sample was contaminated with poultry residuals. For these samples, competitive PCR reactions with three suitable dilutions (10^{-1}, $10^{-1.7}$ and 10^{-2}) were performed. Quantitative competitive PCR for each sample in four replicates was performed.

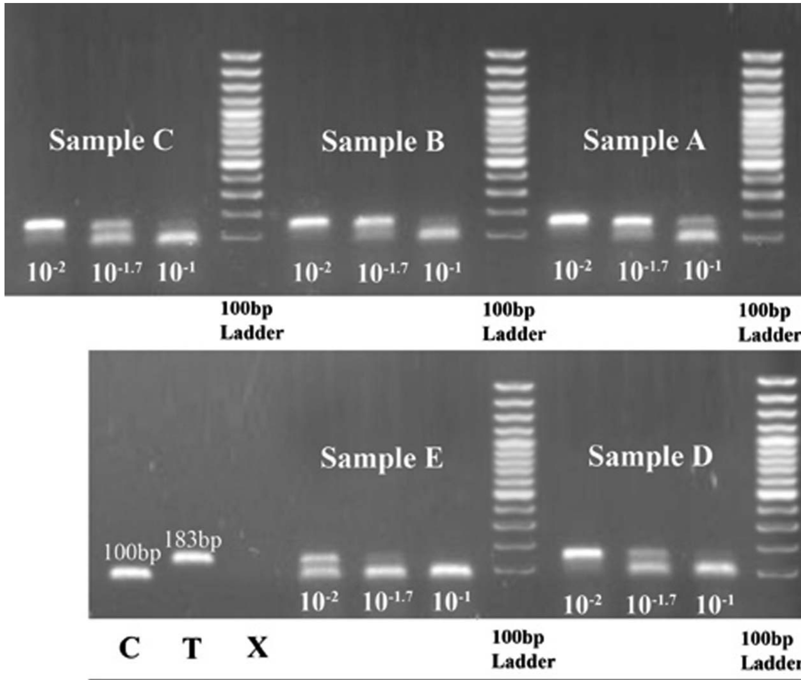

Figure 2: QC_PCR for artificial contaminant samples in three different dilutions (10^{-1}, $10^{-1.7}$ and 10^{-2}) of competitor. A, B, C, D and E, 40%, 30%, 20%, 10% and 1% contaminated samples, respectively. T = Target DNA, C = Competitor DNA. X = PCR negative control/mastermix without DNA.

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Table 2: Estimation standard equation by using log (target/competitor) from artificial contaminant samples.

Y	X_1	X_2	X_3
860	–0.038	0.147	0.204
654	–0.133	0.041	0.083
430	–0.151	–0.012	0.07
215	–0.156	–0.036	0.05
21.5	–0.179	–0.101	–0.12

Y: 860, 645, 430, 215 and 21.5, is densities for 40%, 30%, 20%, 10% and 1% contaminated samples, respectively.
X_1, X_2 and X_3 are log (target/competitor) for 10^{-1}, $10^{-1.7}$ and 10^{-2} dilutions of competitor, respectively.

The relative intensities of the PCR signals originating from the competitor and from the target were measured by imageJ processing software (ver 1.6, National Institutes of Health, USA). Then log (target/competitor) for dilutions of each sample was calculated, and standard graphs for each sample were designed (Fig. 3). For each sample in four replicates, log (target/competitor)

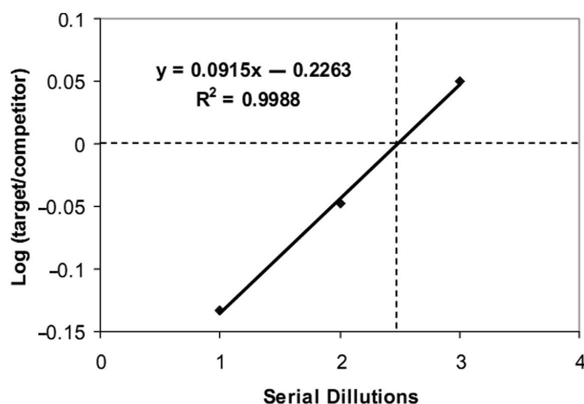

Figure 3: Quantitative application of QC_PCR. The relative band intensities for different samples were determined after gel electrophoresis and used to calculate the linear regression (Studer et al., 1998).

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Table 3: Average log (target/competitor) in each dilution for five samples.

Samples	X_1	X_2	X_3
1	–0.046	0.134	0.165
2	–0.095	0.146	0.227
3	–0.22	–0.025	0.025
4	–0.25	–0.068	0.0194
5	–0.17	0.0026	0.0764

X_1, X_2 and X_3 are log (target/competitor) for 10^{-1}, $10^{-1.7}$ and 10^{-2} dilutions of competitor, respectively.

average in each dilution to determine the percentage contamination percentage was calculated (Table 3).

The density of each sample was determined with input log (target/competitor) of dilutions $10^{-1}(X_1)$, $10^{-1.7}$ (X_2), and 10^{-2} (X_3) of each sample using the standard equation. It is important to note that the concentrations of pure DNA of poultry was 2150 µg/mL measured spectrophotometerically. With regard to the concentration of pure DNA of poultry, correction coefficient to determine contaminant percent of samples was equal to 0.0465. By using samples concentration and correction coefficient, contaminant percent of sausage samples was obtained (Table 4).

DISCUSSION

This study describes a possible solution of a common problem in food production: differentiation between technically unavoidable contamination and intentional admixture. Hubner et al. (1999) reported a QC-PCR system for detection of

Table 4: Densities and percentages for five different contamination samples of sausage.

Samples	Density ($\mu g\ ml^{-1}$)	Percent
1	831.41	38.66
2	1119.76	52.06
3	664.33	30.89
4	513.35	23.87
5	577.26	26.84

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genetically modified organism in food. Wolf and Lu¨thy (2001) used of QC-PCR for quantification of porcine DNA. A new poultry specific PCR system was developed showing high selectivity against other species frequently used such as cattle, sheep, and porcine. Quantitative competitive PCR (QC-PCR) offers a possibility of monitoring a certain limit of poultry DNA even in processed and heat-treated meat products. Some of the drawbacks found are inherent in any procedure based on DNA because DNA yields may depend on the source material, method of extraction, or fragmentation of DNA that takes place in highly processed food. The small amplicon length of 183 bp was chosen to enable amplification of DNA from sausage samples containing highly degraded nucleic acids and proved to be suitable for analysis of feedstuffs and meat products such as sausages and burgers. For transfer and application of the present results to different kinds of meat products, the variable ratios of nuclear and mitochondrial DNA as well as the variable amount of overall DNA per gram in different kinds of tissues such as muscle, liver, bacon fat, and rind must be taken into consideration. The content of mitochondria in liver cells and with it the content of mt-DNA is about three times higher compared to muscle cells (Altman and Katz, 1976). Therefore separate DNA mixtures should be performed for calibration of different products, for example, minced meat, liver pasty, or sausages. Beside quantitative analysis, QC-PCR allows us to monitor PCR inhibitors present in many feedstuffs and complex food products. The presence of inhibitors in the PCR mix will be noticed immediately, since both target DNA and competitor will be affected, and thus false negative results can be excluded. Furthermore it has to be pointed out that the present results solely refer to the DNA content, not to the content of fat or meat (Wolf and Lu¨thy, 2001). We conclude that the QC-PCR method presented enables a desirable and necessary monitoring of meat products and allows differentiation between contamination and admixture.

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