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Quantitative PCR Detection of Pork in Raw and Heated Ground Beef and Pâté

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Quantitative estimates are important to establish whether pork adulteration in ground beef and pâté is accidental or intentional. A PCR procedure has been developed and evaluated to quantify pork in heated and nonheated meat and pâtés by densitometry using a specific and sensitive repetitive DNA element. Thirty, twenty-five, and twenty PCR cycles were carried out to find the best standard curve and correlation between pork content and band intensity. Twenty cycles showed the best results, quantifying degree contamination up to 1% pork in beef (heated and nonheated) and pork in duck pâté with a minimum error. Finally, fraud was found in commercial pâtés.

KEYWORDS: Pig; species identification; contamination; fraud; quantification

INTRODUCTION

Meat products sold for public consumption must be accurately labeled as to what meat species they contain. However, fraudulent or unintentional mislabeling still exits which may not be detected, resulting in poor quality of the product. One frequently encountered form of adulteration for economic gain is the addition of pork to comminuted meats. Undeclared pork is an undesirable contaminant for vegetarians, for religious reasons as well as because of the potential introduction of allergens, bacteria, and parasites.

Numerous analytical methods which rely on proteins analysis have been developed for qualitative pork identification (*1*−*3*). Morales et al. (*4*) developed an indirect enzyme-linked immunosorbent assay (ELISA) to quantitatively determine raw pork adulteration in beef and chicken. In the same way, Martin et al. (*5*) developed a radial immunodiffusion test and ELISA to quantify pork adulteration in raw ground beef. Another semiquantitative ELISA was developed by Berger et al. (*6*). However, proteins lose their biological activity after animal death, and their presence and characteristics depend on the cell types. Furthermore, most of them are heat-labile. Thus, for species identification and quantification in processed food, a DNA method rather than protein analysis would be preferable.

Numerous DNA procedures have been developed for qualitative species identification such as the dot-blot technique (*7*) and polymerase chain reaction (PCR) (*8*, *9*). Several analytical methods using PCR technology have been developed to quantify genetically modified organisms (GMOs) (*10*), bacterial pathogens (*11*), and others.

In this paper, we describe the use of a specific PCR amplification of a repetitive DNA element, for the quantification by densitometry of pork in processed and unprocessed food, because of its simplicity, specificity, and sensitivity.

MATERIALS AND METHODS

Preparation of Samples. Meat samples were taken and minced with a pair of scissors and placed into a 1.5 mL tube, to avoid contamination. Samples containing 0%, 0.001%, 0.005%, 0.01%, 0.1%, 1%, 2%, 5%, 10%, 25%, 50%, 75%, and 100% pork in beef were taken, each with a total weight of 0.5 g. Meat samples were heated, autoclaving at 50, 80, and 120°C respectively, using a holding period of 30 min. Raw samples were also analyzed. In the same way, pork and duck pâté mixtures were made containing 0%, 0.001%, 0.005%, 0.01%, 0.1%, 1%, 2%, 5%, 10%, 25%, 50%, 75%, and 100% pork, each weighing 0.5 g. Genomic DNA was extracted according to a previously described procedure (*12*). Five hundred milligrams were incubated, adding 0.5 mL of extraction buffer (10 mM Tris (pH 8.0), 100 mM EDTA, 0.5% (w/v) sodium dodecyl sulfate (SDS)) and 100 μg of proteinase K. The sample was incubated at 52 °C for 2h. Protein was precipitated by addition of 200 μL of satured NaCl (0.5 M), followed by agitation and centrifugation (7000*g*) for 15 min at 4 °C. The supernatant was subjected to phenol-chloroform extraction. Two volumes of cold ethanol were added, and the solution was kept at −80 °C for 1 h. The resulting DNA precipitated was collected by centrifugation (7000*g*) for 30 min at 4 °C and then washed in 70% ethanol, vacuum-dried, and resuspended in 30 μL of buffer [Tris (10 mM)−EDTA (1.0 mM), pH 7.5].

PCR Amplification of Specific Fragment Of Pig DNA. Specific pig DNA fragment was a SINE (Short Interspersed Nuclear Element) repetitive element (*13*). Specific pork PCR amplification was carried with primers designed as follows: 5′-GGATCCGGCATTGCCGTTAG-3′ (forward primer) and 5′-GTCTTTTTTTGCCATTTCTTGG-3′ (reverse primer).

Double-stranded amplifications were carried out in a final volume of 25 μL, containing 10 mM Tris-HCl, pH 8.8,1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.2 mM each of dATP, dTTP, dGTP, and dCTP, 20 pmol of each primer, 20 ng of template DNA, and 2 U of Taq polymerase (Promega, Promega Corporation, Madison, WI 53711-

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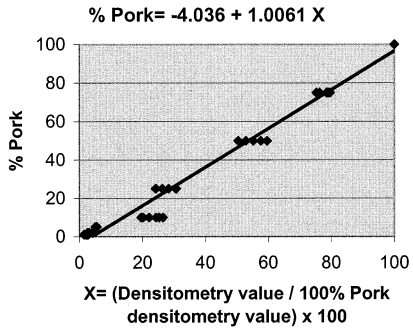

Figure 1. Pork PCR detection. Standard curve showing the equation and fit of the line.

5399). The DNA was amplified in a Biometra Thermal cycler (Biometra Ltd., Whatman House, St. Leonard's Road, 20/20 Maidstone, Kent, ME 16 OLS, UK). Thirty, twenty-five, and twenty cycles were performed with the following step-cycle profile: strand denaturation at 94 °C for 30 s, primer annealing at 50 °C for 30 s, and primer extension at 72 °C for 30s. The last extension step was 5 min longer. An initial denaturation at 94 °C for 4 min was performed to improve the final result. Electrophoresis of a 10 μL portion of the amplification was carried out for 45 min at 100 V in a 2% agarose gel, containing ethidium bromide (1 μg/mL) in TBE buffer. Bands intensity were quantified by densitometry using Molecular Analyst software and Gel Doc 1000 machine (Bio-Rad Laboratories Ltd. Bergueda 1, Parc de Negocis Mas Blau, 08820 El Plat de Llobregat, Barcelona. Spain).

Standard curves were calibrated with the mixtures described in Preparation of samples, using 10 measurements for each mixture (two raw, two heated at 50 °C, two heated at 80 °C, two at 120 °C, and two pork and duck pâtés).

Analysis of Unknown Samples from Commercial Pâtés. Nine pure duck pâtés and one duck pâté with a 3% lard were bought in a store in order to verify the pork content. After PCR amplification, the pork content was calculated using the standards curve.

Statistical Analysis. Densitometric measurements of the bands were used for obtaining standard curves and correlation coefficient by regression analysis for 30, 25, and 20 PCR cycles. The coefficient of variation (CV) was used to compare the variation among the replicates from the different standards and sample means. The Student *t* test was used to measure statistical significance of differences between percentage of pork in known sample and the mean recovery from the samples.

RESULTS AND DISCUSSION

The SINE repetitive element used was chosen for its specificity and sensitivity for pork detection. In this way, using 30, 25, and 20 PCR cycles with this fragment, you can detect up to 0.005%, 0.1%, and 1% pork in beef (raw and heated) and pork in duck pâté, respectively (*13*).

Correlation coefficient between the band intensity and pork content was calculated for 30, 25, and 20 PCR cycles. Twenty cycles obtain the best standard curve. The correlation coefficients were 0.75, 0.85, and 0.99 for 30, 25, and 20 cycles, respectively. This is because using more than 20 cycles, you get to the saturation PCR limit, and the quantification is not linear. The pork content of unknown samples can be easily quantified using the standard curve generated with a scale of pork in beef (raw, heated, and pâtés) (**Figure 1**). PCR amplification provided good estimates of extracts containing 1−75% pork in ground beef (raw and heated) or duck pâté (**Table 1**). The mean *t* value was 1.66, indicating that the predicted pork content mean from pork percentage in known samples was not significantly different ($P > 0.05$). It is important to remark that around 10% pork content, you are overestimating the actual pork amount present (**Table 1**). **Figure 2** shows the different band intensities from 11−100% after PCR amplification.

Table 1. Determination by Specific Pig DNA Amplification of Percent Pork Recovered in Beef (raw and heated) and Duck pâté Samples

% pork in known sample	% pork[a], mean[b]	SD	CV[c]
1	2.4	0.819	34.25
2	3.68	1.053	28.6
5	5.46	0.268	4.9
10	23.4	7.349	31.4
25	27.75	2.571	9.26
50	56.73	4.237	7.46
75	77.92	1.808	2.32

[a] Calculation based on the regression line. [b] Values are means for replicate samples with $n = 10$. [c] Coefficient of variation [CV = 100 × (SD/X)]; mean CV = 16.88.

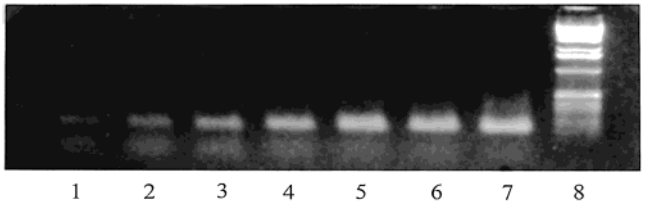

Figure 2. PCR profile using 20 cycles in heated meat. Line 1, 1% pork; line 2, 2% pork; line 3, 10% pork; line 4, 25% pork; line 5, 50% pork; line 6, 75% pork; line 7, 100% pork; line 8, 1 Kb BRL marker (Gibco).

It is important to remark that we always related each densitometric analysis to a 100% pork sample, which is analyzed in all quantification experiments. So we are avoiding differences in the band intensity among different PCR or electrophoresis.

Finally, we also analyzed commercial pâtés at random, both canned and bought by weight. Six good-quality high-priced duck pâtés did not present pork as a component. However, low-quality duck pâtés included pork which had not been specified in the label. Three of four low-priced quality pâté showed pork contamination, one with approximately 50% pork and the other two with 5%. One pâté labeled as 97% duck with 3% lard showed 26% pork contamination.

One of the goals of this standard curve is that you can use it with raw and heated meat and pâtés because the area obtained in this research was minimal.

Since this specific pig DNA fragment gives reasonably accurate and reproducible estimates, it may be used in this way to determine whether the adulteration was accidental or economically motivated.

With respect to pork quantification, the cause of a positive result should be clarified according to whether it is due to adulteration of the product or inadequate handling during manufacture in exceptional cases. Meyer et al. (*14*) do not consider it desirable to have a detection limit below 0.1% pork. However, Jewish and Arabic populations consider it desirable to have a detection limit as low as possible.

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