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Effects of Poor Sanitation Procedures on Cross-Contamination of Animal Species in Ground Meat Products

Sunjung Chung

Rosalee S. Hellberg

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Authors: Sunjung M. Chung[a] and Rosalee S. Hellberg[a]*

[a]Chapman University, Schmid College of Science and Technology, Food Science and Nutrition, One University Drive, Orange, CA 92866, USA

***Corresponding Author**:

Rosalee S. Hellberg, Ph.D.

Chapman University

One University Drive

Orange, CA 92866, USA

Ph: 714-628-2811

E-mail: hellberg@chapman.edu

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Abstract

The presence of <1% of an undeclared species in ground meat is generally thought to be indicative of cross-contamination as opposed to intentional mislabeling; however, this has not been experimentally tested. The objective of this study was to quantify the effects of poor sanitation on the cross-contamination of animal species in ground meat products, with the example of undeclared pork in ground beef. Cross-contamination was quantified using real-time polymerase chain reaction (PCR). Three different sanitation treatments were tested with a commercial grinder ("no cleaning", "partial cleaning", or "complete cleaning") in between grinding of pork and beef samples (13.6 kg each). A 100-g sample was collected for each 0.91 kg (2 lb) of beef processed with the grinder and each sanitation treatment was tested twice. For the "no cleaning" treatment, the first 100-g sample of ground beef run through the grinder contained 24.42 ± 10.41% pork, while subsequent samples (n = 14) contained <0.2% pork. With "partial cleaning," the first sample of ground beef contained 4.60 ± 0.3% pork and subsequent samples contained <0.2% pork. Pork was not detected in ground beef following "complete cleaning." These results indicate that incomplete cleaning of grinding equipment leads to species cross-contamination at levels of <1% in most cases. Proper sanitation procedures must be followed when grinding multiple species in order to prevent cross-contamination and product mislabeling.

Keywords: Species identification; cross-contamination; ground meat; pork; beef; real-time PCR

1. Introduction

Meat sold as whole cuts can often be visually identified to the species level; however, identification becomes more difficult following processing, such as grinding (Cawthorn, Steinman, & Hoffman, 2013). It can also be difficult to visually determine whether ground products contain a single species or multiple species. Because of this, higher-valued meat species are vulnerable to economically-motivated adulteration (EMA) through partial or complete substitution with a lower-valued species (Kane & Hellberg, 2016; Naaum et al., 2018) . For example, the average 2018 supermarket price of ground beef (90% lean or more) in the U.S. was $11.51/kg (USDA, 2019), compared to $6.51/kg for ground pork (USDA, 2019). When game meats are considered, the price differential can even be greater (Quinto, Tinoco, & Hellberg, 2016), with one U.S. study reporting a potential two-fold increase in profits as a result of the substitution of ground yak with ground beef (Kane & Hellberg, 2016). During the 2013 horse meat scandal in Europe, a variety of products labeled as containing beef had undeclared or improperly declared horse meat, a lower-valued species (O'Mahony, 2013). One of the beef burgers tested was found to contain equine DNA at a level of 29.1%, indicating intentional species substitution rather than cross-contamination from processing (O'Mahony, 2013).

Previous studies on processed meat products have reported the presence of undeclared species in approximately 14-35% of samples tested (Amaral, Santos, Oliveira, & Mafra, 2017; Calvo, Osta, & Zaragoza, 2002; Erwanto, Abidin, Muslim, Sugiyono, & Rohman, 2014; Flores-Mungia, Bermudez-Almada, & Vazquez-Moreno, 2000; Hsieh, Woodward, & Ho, 1995; Kane & Hellberg, 2016; Keyvan, İplikçioğlu Çil, Çınar Kul, Bilgen, & Tansel Şireli, 2017; Naaum et al., 2018; Okuma & Hellberg, 2015; Shehata et al., 2019). The presence of undeclared pork in meat products can infringe on religious practices, as consumption of pork is prohibited among Jewish

and Muslim communities (Erwanto et al., 2014). However, several studies have detected pork in ground meat products, including beef (Amaral et al., 2017; Erwanto et al., 2014; Naaum et al., 2018). In one study, 9 out of 39 beef meatballs purchased from local markets in a predominantly Muslim region of Indonesia were found to contain undeclared pork (Erwanto et al., 2014). In Canada, undeclared pork was detected in 6 out of 15 Halal sausages and 16 out of 26 non-Halal products containing ground meat obtained from retail markets (Amaral et al., 2017). Additionally in Canada, 14 of 100 samples of sausages tested contained undeclared species, including a "beef" sausage with detectable levels (> 1%) of sheep and four "beef" sausages with >1% of pork (Shehata et al., 2019). Shehata et al. (2019) concluded that the presence of 1% or more of an undeclared species was more likely due to the addition of the secondary species during production than a result of trace contamination.

In addition to religious concerns, the presence of undeclared pork in ground beef can pose food safety risks when the meat is not cooked properly (USDA, 2013a). According to FSIS, only 6% of people check the internal temperature when cooking hamburgers at home and one in four hamburgers does not reach the recommended temperature of 71.1 °C (USDA, 2013a). Exposure to an undercooked hamburger containing undeclared pork could potentially lead to illness from pathogens associated with pork, such as *Yersinia enterocolitica* or the parasite *Trichinella spiralis* (USDA, 2013b). An outbreak investigation involving undeclared pork in ground beef would likely be complicated because these pathogens are not typically associated with ground beef.

The presence of undeclared meat species in processed products is also a health concern for individuals with allergies to certain red meats (Wolver, Sun, Commins, & Schwartz, 2013). These allergies can be naturally derived or developed as a result of a bite by the lone star tick.

Patients with this condition are advised to avoid mammalian meats like beef, pork, lamb, and venison due to the possibility of a life-threatening allergic reaction. However, previous studies have reported the presence of undeclared red meats in ground poultry products (Calvo et al., 2002; Flores-Mungia et al., 2000; Kane & Hellberg, 2016; Naaum et al., 2018; Okuma & Hellberg, 2015).

According to the United States Code (USC) 21 U.S.C. § 601(n) (1-4), § 607(d) and § 453(h) (1-4), meat and poultry products are considered misbranded when the product is mislabeled, intended to be sold under a different name, an imitation that is not labeled as such on the packaging, or when the label of the product is misleading. The sales of meat products that are misbranded and/or misleading in text, container shape, or other forms of misrepresenting the actual content of meat products are prohibited in 21 U.S.C. § 601(n) (1-4), § 607(d) and § 453(h) (1-4). As discussed above, the presence of undeclared meat species in processed products is sometimes a result of EMA, in which the product is intentionally mislabeled for the purpose of economic gain (FDA, 2009). However, in cases where a higher-value meat species is detected as an adulterant in a lower-value meat product, the motive of adulteration is unknown. Previous studies have suggested that this may be due to the use of by-products from the higher-value species (Naaum et al., 2018) or cross-contamination of equipment used to process multiple meat species (Hsieh et al., 1995; Kane & Hellberg, 2016; Okuma & Hellberg, 2015). For example, Kane and Hellberg (2016) found that ground chicken obtained from a local supermarket in the U.S. tested positive for higher-value species (i.e., beef, turkey, and lamb), and Naaum et al. (2018) detected beef in chicken and pork sausages.

In order to differentiate between intentional adulteration and cross-contamination, Premanandh, Sabbagh, and Maruthamuthu (2013) referred to a proposal by the European

Commission (FSA, 2010) on the issue of low-level detection of unauthorized genetically modified organisms in products. The proposal suggested that the detection of unauthorized genetically modified materials at levels of <0.1% in feed should be considered equivalent to zero for the purpose of enforcement. Of note, EU laws on labeling requirements for genetically modified material in food or feed products do not apply if the material is present at levels of ≤0.9%, provided that the material is adventitious or technically unavoidable (Regulation (EC) No 1829/2003 and No 1830/2003). However, some Member States have adopted a stricter threshold of <0.1% (EC, 2015). Along these lines, Premanandh et al. (2013) suggested that ≥1% of an undeclared meat species should be considered substantial enough to investigate the possibility of intentional adulteration or gross negligence, and subsequent studies have used this cut-off value to distinguish deliberate adulteration from cross-contamination (Kang & Tanaka, 2018; Naaum et al., 2018). However, the actual percentage of meat species that is carried over as a result of cross-contamination of equipment has not been experimentally determined. Therefore, the objective of this study was to quantify the effect of poor sanitation procedures on the cross-contamination of animal species in ground meat products. The example of undeclared pork in ground beef was utilized to test a range of sanitation procedures.

2. Materials and Methods

2.1 Pure pork DNA standards

Pork lean meat (300 g) was purchased from a local supermarket and transported on ice to the laboratory for immediate processing. The exterior layer of the meat was removed as described in Amaral et al. (2017). A 25-mg sample of the pork was collected in 1.5 mL safe lock tubes and stored at -20 °C. DNA was extracted as described in section 2.5. After DNA extraction, the pure pork DNA standard samples were serially diluted in molecular-grade water

to obtain extracts with the following concentrations: 50 ng/µL, 5 ng/µL, 0.5 ng/µL, 0.05 ng/µL, 0.005 ng/µL, 0.0005 ng/µL, and 0.00005 ng/µL (Amaral et al., 2017). The entire process was repeated three times and each sample underwent real-time PCR as described in section 2.6.

2.2 Reference binary species mixture samples

The ability of the real-time PCR assay to accurately quantify pork in beef was assessed using reference binary species mixtures prior to performing the meat grinding experiments described in section 2.3. Samples (100-500 g) of pork butt roast and beef boneless chuck roast were purchased from a local supermarket and transported on ice to the laboratory. Samples were processed immediately upon arrival at the laboratory. The exterior layer of the meat samples was removed as described in Amaral et al. (2017). Reference species mixtures (50 g) were made using the following proportions of pork/beef: 0%/100% (beef control), 0.0001%/99.9999%, 0.0005%/99.9995%, 0.001%/99.999%, 0.01%/99.99%, 0.1%/99.9%, 1%/99%, 5%/95%, 10%/90%, and 100%/0% (pork control) using the procedure described in Amaral et al. (2017). The mixtures were homogenized with 50 mL of sterile deionized water using a 12 speed Oster® blender (Neosho, MO, USA) for 1 min at speed 6, as described in Perestam, Fujisaki, Nava, and Hellberg (2017). The blender parts were cleaned and autoclaved after each use. This process was repeated twice, resulting in three separate sets of reference samples. Following homogenization, each reference sample mixture underwent DNA extraction followed by real-time PCR as described in sections 2.5-2.6.

2.3 Treatment sample collection and grinding

Beef boneless chuck roast and pork butt roast were purchased from a local supermarket and transported to the laboratory on ice for immediate processing. A total of 13.6 kg (30 lb) per species was used for each grinding treatment, based on grinding practices reported for

139 independent retail establishments (Gould et al., 2011). Upon arrival at the laboratory, the meat
140 samples were cut into 1-inch cubes with autoclaved knives to facilitate grinding. Meat from each
141 species was prepared using separate cutting boards, gloves, and knives to avoid cross-
142 contamination. A subsample (50 g) of each species was collected and homogenized with 50 mL
143 sterile deionized water using a 12 speed Oster® blender for 1 min at speed 6. A portion (~25 mg)
144 of each homogenized subsample underwent DNA extraction and real-time PCR (described in
145 sections 2.5-2.6) to verify the presence or absence of pork DNA in the meat samples prior to
146 grinding. Meat samples were ground using a Kitchener #8 Commercial Grade Electric Stainless-
147 Steel Meat Grinder .5 HP 370W (Shanghai, China). Each grinding session began with 13.6 kg of
148 pork, followed by one of the cleaning treatments described in section 2.4, then 13.6 kg of beef. A
149 100-g sample of ground beef was collected at the beginning of each 0.91 kg (2 lb) of meat
150 exiting the grinder for a total of 15 samples. Each 100-g subsample was homogenized with 100
151 mL of sterile deionized water using a 12 speed Oster® blender for 1 min at speed 6. The blender
152 parts were cleaned and autoclaved after each use. Following homogenization, the samples
153 underwent DNA extraction and real-time PCR as described in sections 2.5-2.6.

154 2.4 Cleaning treatments

155 The cleaning treatments were divided into three categories: “no cleaning”, “partial
156 cleaning”, and “complete cleaning”. These categories were determined based on personal
157 communications with and observations of local butcher shops and grocery store delis. For the
158 “no cleaning” category, beef was ground immediately after pork with no cleaning step in
159 between. For the “partial cleaning” category, the grinder’s outer blade and hopper tray were
160 wiped with paper towels in between meat species, with no additional cleaning. In the “complete
161 cleaning” category, all parts of the grinder were disassembled in between meat species, and the

grinder's manufacturer recommended cleaning procedure of washing all parts in warm soapy water was followed using brushes provided with the grinder. Each cleaning treatment was tested in a series of two trials.

2.5 DNA extraction

DNA extraction was performed using the DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD), according to the manufacturer's instructions. Lysis was carried out using an Eppendorf ®Thermomixer C set at 56 °C with shaking at 300 rpm for 2 h. DNA was eluted in 100 µL AE buffer pre-heated to 37 °C. Each set of extractions included a reagent blank with no tissue added as a negative control. The concentration of each DNA extract was measured with an Eppendorf BioPhotometer (Hauppauge, NY). DNA extracts were stored at -20°C until real-time PCR.

2.6 Real-time PCR quantification

Reaction mixtures were prepared as described in Amaral et al. (2017), with 2 µL DNA extract (≤ 50 ng/µL), 10.0 µL of 2X SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA), 200 nM of each primer (Prk-F/Prk-R or 18SRG-F/18SRG-R), and 4.0 µL of molecular grade water for a total reaction volume of 20 µL. The pork-specific Prk-F/Prk-R primers (Prk-F: CTG CCC TGA GGA CAA ATA TCA TTC and Prk-R: AAG CCC CCT CAG ATT CAT TCT ACG) targeted a region of the cytochrome *b* gene (Amaral et al., 2017). The 18SRG-F/18SRG-R primers (18SRG-F: CTC CCC TAT CAA CTT TCG ATG GTA and 18SRG-R: TTG GAT GTG GTA GCC GTT TCT CA) targeted a universal region of eukaryotic 18S ribosomal DNA (Costa, Oliveira, & Mafra, 2013). Parallel reactions with both primer sets were carried out for each sample (Amaral et al., 2017). Thermal cycling was carried out using a Qiagen Rotor-Gene® Q Real-time PCR Cycler using the settings described in Kang and Tanaka (2018): 95 °C for 5 min,

followed by 30 cycles of 95 °C for 15 s and 66 °C for 45 s. For the melting curve, the temperature was ramped from 65 °C to 95 °C, and raised 0.2 °C every 10 s (Amaral et al., 2017). Each real-time PCR run included the following controls: pure pork DNA positive controls (0.5 ng/µL, 5 ng/µL, and 50 ng/µL), reagent blank from DNA extraction, and no-template control (NTC). The threshold was set automatically by the Rotor-Gene Q software (upper limit 0.43; lower limit 0.07) and melting point values were taken from the highest peak temperature.

2.7 Pork quantification and statistical analysis

The amount of pork in each sample was quantified as described in Amaral et al. (2017). ΔCq was calculated using the following formula: ΔCq = Cq(pork) – Cq (endogenous gene), where Cq(pork) refers to the Cq value obtained for the pork-specific *cytb* assay and Cq (endogenous gene) refers to the Cq value obtained for the universal 18S rRNA assay. A standard curve was created using the reference pork/beef species mixtures described above. The average ΔCq for each reference sample was calculated based on the results of real-time PCR on triplicate DNA extracts. This value was plotted on the y-axis and logarithm of pork meat percentage on the x-axis. The linear equation obtained from the standard curve was used to determine the average percent of pork in each treatment sample based on the ΔCq obtained for that sample. The linear equation was also used to determine the estimated percentage of pork in each reference sample and the standard deviation, coefficient of variation (CV), and bias were calculated for each reference sample. All calculations were performed in Microsoft Excel 2016 (Redmond, WA, USA).

3. Results and Discussion

3.1 Pure pork DNA standards

The real-time PCR assay used in this study was able to detect pure pork DNA with the pork-specific primer down to levels of 0.001 ng of pork DNA (Fig. 1). In comparison, Amaral et al. (2017), detected pork DNA at levels as low as 0.01 pg using the same primers and reaction mixture. The difference in results is most likely due to the number of pPCR cycles run: Amaral et al. (2017) used 44 cycles compared to 30 cycles in the current study. However, the results of linearity testing were very similar between the two studies: $R^2 = 0.995$ for the current study experiment and $R^2 = 0.996$ for Amaral et al. (2017).

3.2 Reference binary species mixtures

Pork was detected down to a level of 0.01% in the pork/beef reference binary species mixtures (Fig. 2). These results are consistent with those reported in Amaral et al. (2017), who also found 0.01% pork to be the minimum detectable amount in reference binary species mixtures. Kang and Tanaka (2018) tested reference binary mixtures of pork and beef with the same assay and were able to quantify pork in 20/20 samples with 0.01% pork but only 15/20 samples with 0.001% pork. Therefore, the authors determine the limit of quantification for pork in a binary mixture to be 0.01%.

As shown in Figure 4, a standard curve was constructed using the ΔCq values obtained for each sample and a linear equation was obtained. This equation was then used to estimate the percentage of pork in each reference binary species mixture (Table 1). The estimated values ranged from 0.01% to 16.1% pork, as compared to the actual values of 0.01% to 10% pork. The measured trueness or bias, which reflects the agreement between the estimated value and the actual value, ranged from -45.4% to 97.8% for the reference samples. The closest agreement was found for the sample with 0.1% pork, which had an estimated value of 0.14% pork (38.8% bias), while the greatest disagreement occurred for the sample with 1.0% pork, which had an estimated

value of 1.98% pork (97.8% bias). While the average estimated values were not an exact match to the actual values in most cases, they did provide an approximate value for the amount of pork in each sample. The CV, which is a measure of the variability of the data points around the mean, ranged from 6.65% to 53.0% for the reference binary species mixtures. The CV was highest (>27%) for samples containing lower amounts of pork (0.01-0.1%) and decreased to <20.0% in samples with greater amounts of pork (1.0-10.0%).

The CV and bias values obtained in the current study have a wider range than previous studies that used the same quantification method. Specifically, Kang and Tanaka (2018) reported bias values of -19.10% to 2.34% using the same quantification method as in the current study for replicate testing (n=3) of DNA extracts from binary mixtures of pork and beef containing 0.25-50% pork (CVs were not reported). Amaral et al. (2017) reported CVs ranging from 5.7 to 19.7% and bias of 5.6-10.1% for replicate testing (n=8) of DNA extracts from raw binary mixtures of pork and beef. Some of these differences are likely due to variability in the way that replicate testing was carried out. In the current study, three separate sets of binary mixtures were prepared and tested with real-time PCR. However, in previous studies, one set of binary mixtures was prepared and the DNA extracts were tested multiple times with real-time PCR. The additional variability introduced through repeated preparation of binary mixtures in the current study likely contributed to a wider range of CV values and is reflective of the overall method rather than the real-time PCR assay alone. This indicates a need to optimize the sample homogenization and DNA extraction steps in order to accurately capture the exact ratios of each species in the mixture. Additional reasons for differences in the results of the current study include variations in the number of replicates tested and/or the use of a different real-time instrument. Future research should be conducted to minimize the CV and bias values for replicates of binary

reference samples, either by optimizing the current sample preparation method or utilizing alternate techniques.

3.3 Ground meat treatment samples

The results of real-time quantification of samples that underwent different grinding treatments are shown in Table 3. For the “no cleaning” treatment category, the first 100 g of beef (Sample 1) contained the greatest amount of pork, at 24.42 ± 10.41% (range: 17.05-31.78%). However, the amount of pork in ground beef dropped down to 0.06 ± 0.08% (Sample 2) after the first 1.01 kg of beef was ground. Pork was detected at trace levels (≤0.01%) in the remaining samples within this treatment category. For “partial cleaning,” the first 100-g sample of ground beef contained 4.60 ± 0.30% of pork, which decreased to 0.086 ± 0.02% after 1.01 kg of beef was ground and then to 0.03 ± 0.03% after 1.92 kg was ground. The amount of pork in the remaining samples was ≤0.01%. Pork was not detected in any of the samples collected in the “complete cleaning” treatment category (Table 2). It is important to note that these results are based on two separate trials and it is possible that additional trials may have reduced the standard deviations associated with the percentage of pork in the samples.

Based on the results of all three treatment categories, it can be deduced that detection of pork at levels of ~25% in the first 100-g of ground beef exiting the grinder could be the result of cross-contamination of the grinding equipment. However, the likelihood of a 25% contamination event in a commercial sample is very low, considering that the first 100 g of meat exiting the grinder would likely be mixed with a larger sample of meat being processed with the grinder. For example, the grinder tray used in this study holds approximately 2.2 kg of meat, which could dilute the contaminant species in the original 100-g sample to ~1%. It should be noted that for both the “no cleaning” and “partial cleaning” treatments, the percent of pork decreased to < 1%

after just 1.01 kg of beef was ground. This is consistent with the assumption made in previous studies that cross-contamination is generally associated with the presence of <1% of an undeclared species (Kang & Tanaka, 2018; Naaum et al., 2018; Premanandh et al., 2013).

4. Conclusions

Quantification of undeclared species in ground meat products is important to help differentiate between intentional adulteration and cross-contamination (Amaral et al., 2017). Understanding the amount of an undeclared species that arises as a result of improper sanitation during grinding can help provide the basis for regulations and/or recommended cleaning practices within the industry. The results of this study indicate that cross-contamination of species is avoidable if equipment is thoroughly cleaned as instructed by the manufacturer, with all parts of the grinder being disassembled and washed with warm soapy water. However, when the equipment is not cleaned properly in between species, contamination of an undeclared species in the product will likely be observed, with most samples showing levels of <1% of the undeclared species. In cases where the grinding equipment is not completely cleaned in between species, the consumer should be informed of any additional species that may be present in the product, even at trace levels. Proper labeling of products is crucial to promote food safety, prevent allergen exposure, and avoid infringing on religious practices. Future studies should consider quantifying cross-contamination of animal species in a wider range of food products, such as pet foods and animal feed.

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Table 1. Estimated percentages of pork in beef for reference binary species mixtures detected with real-time PCR.

Binary species mixture (pork/beef)	**Estimated % pork (Ave ± St.Dev.)[ab]**	**Coefficient of Variation (%)[b]**	**Bias[b]**
0.01%/99.99%	0.01 ± 0.00	28.0	-45.4
0.1%/99.90%	0.14 ± 0.07	53.0	38.8
1.0%/99.00%	1.98 ± 0.33	16.9	97.8
10%/90.00%	16.10 ± 1.07	6.65	61.0

[a]Values are based on the average of three independent assays.
[b]All values were calculated based on raw data and final answers were rounded.

Table 2. Percentage of pork in ground beef detected with real-time PCR for each cleaning treatment applied to the grinder. A 100-g sample of meat was collected for every 0.91 kg of beef ground; sample number refers to the order in which samples were collected following the treatment.

Sample number	Amount of beef ground (kg)	No cleaning		Partial cleaning		Complete cleaning	
		Average % pork[a]	St. Dev.	Average % pork[a]	St. Dev.	Average % pork[a]	St. Dev.
1	0.10	24.42	10.41	4.60	0.30	ND[b]	N/A
2	1.01	0.06	0.08	0.09	0.02	ND	N/A
3	1.92	0.01	0.00	0.03	0.03	ND	N/A
4	2.83	< 0.01	N/A	< 0.01	N/A	ND	N/A
5	3.74	< 0.01	N/A	0.01	0.01	ND	N/A
6	4.65	0.01	0.00	< 0.01	N/A	ND	N/A
7	5.56	0.01	0.01	< 0.01	N/A	ND	N/A
8	6.47	0.01	0.00	< 0.01	N/A	ND	N/A
9	7.38	<0.01	N/A	< 0.01	0.01	ND	N/A
10	8.29	0.01	0.00	0.01	0.01	ND	N/A
11	9.20	0.01	0.00	< 0.01	N/A	ND	N/A
12	10.11	0.01	0.00	0.01	< 0.01	ND	N/A
13	11.02	0.01	0.01	< 0.01	N/A	ND	N/A
14	11.93	<0.01	N/A	< 0.01	N/A	ND	N/A
15	12.84	< 0.01	N/A	< 0.01	< 0.01	ND	N/A

[a]Values are based on the results of two independent trials
[a]ND = Not detected

Figure Captions

Figure 1. Standard curve obtained for serially diluted pure pork DNA using real-time PCR with a pork-specific primer. Starting DNA quantity ranged from 0.001 ng to 100 ng. Error bars are based on the standard deviation.

Figure 2. Standard curve for reference binary species mixtures (0.01%, 0.1%, 1.0%, 10%, and 100% pork in beef) analyzed with real-time PCR. The Cq obtained with the universal eukaryotic primers was subtracted from the Cq for the pork-specific primers to obtain ΔCq. The average ΔCq is reported based on three independent assays, and error bars represent standard deviation.