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

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**Effects of poor sanitation procedures on cross-contamination of animal species in ground meat products**

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1 **Abstract**

2 The presence of <1% of an undeclared species in ground meat is generally thought to be  
3 indicative of cross-contamination as opposed to intentional mislabeling; however, this has not  
4 been experimentally tested. The objective of this study was to quantify the effects of poor  
5 sanitation on the cross-contamination of animal species in ground meat products, with the  
6 example of undeclared pork in ground beef. Cross-contamination was quantified using real-time  
7 polymerase chain reaction (PCR). Three different sanitation treatments were tested with a  
8 commercial grinder (“no cleaning”, “partial cleaning”, or “complete cleaning”) in between  
9 grinding of pork and beef samples (13.6 kg each). A 100-g sample was collected for each 0.91 kg  
10 (2 lb) of beef processed with the grinder and each sanitation treatment was tested twice. For the  
11 “no cleaning” treatment, the first 100-g sample of ground beef run through the grinder contained  
12  $24.42 \pm 10.41\%$  pork, while subsequent samples (n = 14) contained <0.2% pork. With “partial  
13 cleaning,” the first sample of ground beef contained  $4.60 \pm 0.3\%$  pork and subsequent samples  
14 contained <0.2% pork. Pork was not detected in ground beef following “complete cleaning.”  
15 These results indicate that incomplete cleaning of grinding equipment leads to species cross-  
16 contamination at levels of <1% in most cases. Proper sanitation procedures must be followed  
17 when grinding multiple species in order to prevent cross-contamination and product mislabeling.

18

19

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

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## 24 **1. Introduction**

25 Meat sold as whole cuts can often be visually identified to the species level; however,  
26 identification becomes more difficult following processing, such as grinding (Cawthorn,  
27 Steinman, & Hoffman, 2013). It can also be difficult to visually determine whether ground  
28 products contain a single species or multiple species. Because of this, higher-valued meat species  
29 are vulnerable to economically-motivated adulteration (EMA) through partial or complete  
30 substitution with a lower-valued species (Kane & Hellberg, 2016; Naaum et al., 2018) . For  
31 example, the average 2018 supermarket price of ground beef (90% lean or more) in the U.S. was  
32 \$11.51/kg (USDA, 2019), compared to \$6.51/kg for ground pork (USDA, 2019). When game  
33 meats are considered, the price differential can even be greater (Quinto, Tinoco, & Hellberg,  
34 2016), with one U.S. study reporting a potential two-fold increase in profits as a result of the  
35 substitution of ground yak with ground beef (Kane & Hellberg, 2016). During the 2013 horse  
36 meat scandal in Europe, a variety of products labeled as containing beef had undeclared or  
37 improperly declared horse meat, a lower-valued species (O'Mahony, 2013). One of the beef  
38 burgers tested was found to contain equine DNA at a level of 29.1%, indicating intentional  
39 species substitution rather than cross-contamination from processing (O'Mahony, 2013).

40 Previous studies on processed meat products have reported the presence of undeclared  
41 species in approximately 14-35% of samples tested (Amaral, Santos, Oliveira, & Mafra, 2017;  
42 Calvo, Osta, & Zaragoza, 2002; Erwanto, Abidin, Muslim, Sugiyono, & Rohman, 2014; Flores-  
43 Mungia, Bermudez-Almada, & Vazquez-Moreno, 2000; Hsieh, Woodward, & Ho, 1995; Kane &  
44 Hellberg, 2016; Keyvan, İplikçioğlu Çil, Çınar Kul, Bilgen, & Tansel Şireli, 2017; Naaum et al.,  
45 2018; Okuma & Hellberg, 2015; Shehata et al., 2019). The presence of undeclared pork in meat  
46 products can infringe on religious practices, as consumption of pork is prohibited among Jewish

47 and Muslim communities (Erwanto et al., 2014). However, several studies have detected pork in  
48 ground meat products, including beef (Amaral et al., 2017; Erwanto et al., 2014; Naaum et al.,  
49 2018). In one study, 9 out of 39 beef meatballs purchased from local markets in a predominantly  
50 Muslim region of Indonesia were found to contain undeclared pork (Erwanto et al., 2014). In  
51 Canada, undeclared pork was detected in 6 out of 15 Halal sausages and 16 out of 26 non-Halal  
52 products containing ground meat obtained from retail markets (Amaral et al., 2017). Additionally  
53 in Canada, 14 of 100 samples of sausages tested contained undeclared species, including a “beef”  
54 sausage with detectable levels (> 1%) of sheep and four “beef” sausages with >1% of pork  
55 (Shehata et al., 2019). Shehata et al. (2019) concluded that the presence of 1% or more of an  
56 undeclared species was more likely due to the addition of the secondary species during  
57 production than a result of trace contamination.

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

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

70 Patients with this condition are advised to avoid mammalian meats like beef, pork, lamb, and  
71 venison due to the possibility of a life-threatening allergic reaction. However, previous studies  
72 have reported the presence of undeclared red meats in ground poultry products (Calvo et al.,  
73 2002; Flores-Mungia et al., 2000; Kane & Hellberg, 2016; Naaum et al., 2018; Okuma &  
74 Hellberg, 2015).

75 According to the United States Code (USC) 21 U.S.C. § 601(n) (1-4), § 607(d) and § 453(h)  
76 (1-4), meat and poultry products are considered misbranded when the product is mislabeled,  
77 intended to be sold under a different name, an imitation that is not labeled as such on the  
78 packaging, or when the label of the product is misleading. The sales of meat products that are  
79 misbranded and/or misleading in text, container shape, or other forms of misrepresenting the  
80 actual content of meat products are prohibited in 21 U.S.C. § 601(n) (1-4), § 607(d) and § 453(h)  
81 (1-4). As discussed above, the presence of undeclared meat species in processed products is  
82 sometimes a result of EMA, in which the product is intentionally mislabeled for the purpose of  
83 economic gain (FDA, 2009). However, in cases where a higher-value meat species is detected as  
84 an adulterant in a lower-value meat product, the motive of adulteration is unknown. Previous  
85 studies have suggested that this may be due to the use of by-products from the higher-value  
86 species (Naaum et al., 2018) or cross-contamination of equipment used to process multiple meat  
87 species (Hsieh et al., 1995; Kane & Hellberg, 2016; Okuma & Hellberg, 2015). For example,  
88 Kane and Hellberg (2016) found that ground chicken obtained from a local supermarket in the  
89 U.S. tested positive for higher-value species (i.e., beef, turkey, and lamb), and Naaum et al.  
90 (2018) detected beef in chicken and pork sausages.

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

93 Commission (FSA, 2010) on the issue of low-level detection of unauthorized genetically  
94 modified organisms in products. The proposal suggested that the detection of unauthorized  
95 genetically modified materials at levels of <0.1% in feed should be considered equivalent to zero  
96 for the purpose of enforcement. Of note, EU laws on labeling requirements for genetically  
97 modified material in food or feed products do not apply if the material is present at levels of  
98  $\leq 0.9\%$ , provided that the material is adventitious or technically unavoidable (Regulation (EC) No  
99 1829/2003 and No 1830/2003). However, some Member States have adopted a stricter threshold  
100 of <0.1% (EC, 2015). Along these lines, Premanandh et al. (2013) suggested that  $\geq 1\%$  of an  
101 undeclared meat species should be considered substantial enough to investigate the possibility of  
102 intentional adulteration or gross negligence, and subsequent studies have used this cut-off value  
103 to distinguish deliberate adulteration from cross-contamination (Kang & Tanaka, 2018; Naaum  
104 et al., 2018). However, the actual percentage of meat species that is carried over as a result of  
105 cross-contamination of equipment has not been experimentally determined. Therefore, the  
106 objective of this study was to quantify the effect of poor sanitation procedures on the cross-  
107 contamination of animal species in ground meat products. The example of undeclared pork in  
108 ground beef was utilized to test a range of sanitation procedures.

## 109 **2. Materials and Methods**

### 110 **2.1 Pure pork DNA standards**

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

116 to obtain extracts with the following concentrations: 50 ng/ $\mu$ L, 5 ng/ $\mu$ L, 0.5 ng/ $\mu$ L, 0.05 ng/ $\mu$ L,  
117 0.005 ng/ $\mu$ L, 0.0005 ng/ $\mu$ L, and 0.00005 ng/ $\mu$ L (Amaral et al., 2017). The entire process was  
118 repeated three times and each sample underwent real-time PCR as described in section 2.6.

## 119 2.2 Reference binary species mixture samples

120 The ability of the real-time PCR assay to accurately quantify pork in beef was assessed  
121 using reference binary species mixtures prior to performing the meat grinding experiments  
122 described in section 2.3. Samples (100-500 g) of pork butt roast and beef boneless chuck roast  
123 were purchased from a local supermarket and transported on ice to the laboratory. Samples were  
124 processed immediately upon arrival at the laboratory. The exterior layer of the meat samples was  
125 removed as described in Amaral et al. (2017). Reference species mixtures (50 g) were made  
126 using the following proportions of pork/beef: 0%/100% (beef control), 0.0001%/99.9999%,  
127 0.0005%/99.9995%, 0.001%/99.999%, 0.01%/99.99%, 0.1%/99.9%, 1%/99%, 5%/95%,  
128 10%/90%, and 100%/0% (pork control) using the procedure described in Amaral et al. (2017).  
129 The mixtures were homogenized with 50 mL of sterile deionized water using a 12 speed Oster®  
130 blender (Neosho, MO, USA) for 1 min at speed 6, as described in Perestam, Fujisaki, Nava, and  
131 Hellberg (2017). The blender parts were cleaned and autoclaved after each use. This process was  
132 repeated twice, resulting in three separate sets of reference samples. Following homogenization,  
133 each reference sample mixture underwent DNA extraction followed by real-time PCR as  
134 described in sections 2.5-2.6.

## 135 2.3 Treatment sample collection and grinding

136 Beef boneless chuck roast and pork butt roast were purchased from a local supermarket and  
137 transported to the laboratory on ice for immediate processing. A total of 13.6 kg (30 lb) per  
138 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

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

## 165 2.5 DNA extraction

166 DNA extraction was performed using the DNeasy Blood and Tissue Kit (Qiagen,  
167 Germantown, MD), according to the manufacturer's instructions. Lysis was carried out using an  
168 Eppendorf® Thermomixer C set at 56 °C with shaking at 300 rpm for 2 h. DNA was eluted in  
169 100 µL AE buffer pre-heated to 37 °C. Each set of extractions included a reagent blank with no  
170 tissue added as a negative control. The concentration of each DNA extract was measured with an  
171 Eppendorf BioPhotometer (Hauppauge, NY). DNA extracts were stored at -20°C until real-time  
172 PCR.

## 173 2.6 Real-time PCR quantification

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

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

## 191 2.7 Pork quantification and statistical analysis

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

## 205 3. Results and Discussion

### 206 3.1 Pure pork DNA standards

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

### 214 3.2 Reference binary species mixtures

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

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

230 value of 1.98% pork (97.8% bias). While the average estimated values were not an exact match  
231 to the actual values in most cases, they did provide an approximate value for the amount of pork  
232 in each sample. The CV, which is a measure of the variability of the data points around the  
233 mean, ranged from 6.65% to 53.0% for the reference binary species mixtures. The CV was  
234 highest (>27%) for samples containing lower amounts of pork (0.01-0.1%) and decreased to  
235 <20.0% in samples with greater amounts of pork (1.0-10.0%).

236         The CV and bias values obtained in the current study have a wider range than previous  
237 studies that used the same quantification method. Specifically, Kang and Tanaka (2018) reported  
238 bias values of -19.10% to 2.34% using the same quantification method as in the current study for  
239 replicate testing (n=3) of DNA extracts from binary mixtures of pork and beef containing 0.25-  
240 50% pork (CVs were not reported). Amaral et al. (2017) reported CVs ranging from 5.7 to 19.7%  
241 and bias of 5.6-10.1% for replicate testing (n=8) of DNA extracts from raw binary mixtures of  
242 pork and beef. Some of these differences are likely due to variability in the way that replicate  
243 testing was carried out. In the current study, three separate sets of binary mixtures were prepared  
244 and tested with real-time PCR. However, in previous studies, one set of binary mixtures was  
245 prepared and the DNA extracts were tested multiple times with real-time PCR. The additional  
246 variability introduced through repeated preparation of binary mixtures in the current study likely  
247 contributed to a wider range of CV values and is reflective of the overall method rather than the  
248 real-time PCR assay alone. This indicates a need to optimize the sample homogenization and  
249 DNA extraction steps in order to accurately capture the exact ratios of each species in the  
250 mixture. Additional reasons for differences in the results of the current study include variations  
251 in the number of replicates tested and/or the use of a different real-time instrument. Future  
252 research should be conducted to minimize the CV and bias values for replicates of binary

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

### 255 3.3 Ground meat treatment samples

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

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

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

#### 279 **4. Conclusions**

280 Quantification of undeclared species in ground meat products is important to help  
281 differentiate between intentional adulteration and cross-contamination (Amaral et al., 2017).  
282 Understanding the amount of an undeclared species that arises as a result of improper sanitation  
283 during grinding can help provide the basis for regulations and/or recommended cleaning  
284 practices within the industry. The results of this study indicate that cross-contamination of  
285 species is avoidable if equipment is thoroughly cleaned as instructed by the manufacturer, with  
286 all parts of the grinder being disassembled and washed with warm soapy water. However, when  
287 the equipment is not cleaned properly in between species, contamination of an undeclared  
288 species in the product will likely be observed, with most samples showing levels of <1% of the  
289 undeclared species. In cases where the grinding equipment is not completely cleaned in between  
290 species, the consumer should be informed of any additional species that may be present in the  
291 product, even at trace levels. Proper labeling of products is crucial to promote food safety,  
292 prevent allergen exposure, and avoid infringing on religious practices. Future studies should  
293 consider quantifying cross-contamination of animal species in a wider range of food products,  
294 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.

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

<sup>a</sup>Values are based on the average of three independent assays.

<sup>b</sup>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 <sup>a</sup>	St. Dev.	Average % pork <sup>a</sup>	St. Dev.	Average % pork <sup>a</sup>	St. Dev.
1	0.10	24.42	10.41	4.60	0.30	ND <sup>b</sup>	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

<sup>a</sup>Values are based on the results of two independent trials

<sup>a</sup>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  $C_q$  obtained with the universal eukaryotic primers was subtracted from the  $C_q$  for the pork-specific primers to obtain  $\Delta C_q$ . The average  $\Delta C_q$  is reported based on three independent assays, and error bars represent standard deviation.