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# Comparison of Real-Time PCR and ELISA-Based Methods for the Detection of Beef and Pork in Processed Meat Products


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1 **Comparison of Real-time PCR and ELISA-Based Methods for the Detection of Beef and**  
2 **Pork in Processed Meat Products**

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24 **Abstract**

25 Two commonly used methodologies for species detection within processed meat products  
26 are real-time polymerase chain reaction (PCR), a DNA-based method, and enzyme-linked  
27 immunosorbent assay (ELISA), a protein-based method. In this study, a real-time PCR assay was  
28 compared to a commercial ELISA kit based on sensitivity, specificity, agreement among  
29 duplicate samples, cost, time, and ease of use. Fifteen reference samples containing known  
30 percentages (0.1-99.9%, w/w) of pork and beef were analyzed in duplicate using both methods.  
31 Thirty commercial products, including sausages, pet treats, and canned meats, were also tested in  
32 duplicate with each method. Reference sample analysis showed real-time PCR was able to detect  
33 pork in duplicate samples at 0.10% and beef at 0.50% in the binary mixtures. ELISA detected  
34 pork in duplicate samples at 10.0% and beef at 1.00% in the binary mixtures. When the results of  
35 reference and commercial samples were combined, real-time PCR demonstrated the greatest  
36 agreement among duplicate samples, at 96.7%, compared to 95.6% agreement for ELISA. The  
37 real-time PCR assay used in this study was found to be less expensive, while ELISA was less  
38 time-consuming and easier to perform. Both methods were successful at identifying species in  
39 ground meats, sausage, and deli meat samples; however, pet treats and canned meats proved  
40 more challenging. Overall, it was determined that the real-time PCR assay was optimal for  
41 species identification in processed meat products when a low detection limit is required;  
42 however, the ELISA kit may be advantageous in other situations due to its ease of use.

43 **Keywords:** Real-time PCR, ELISA, Mislabeling, Species Identification, Beef, Pork

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## 47 **1. Introduction**

48 Meat and meat-based products make up a significant percentage of the American diet,  
49 with beef and pork being the top two red meats consumed (USDA 2013). On a per capita basis,  
50 consumption levels in the U.S. for 2016 have been projected to be 24.5 kg for beef and 21.3 kg  
51 for pork (USDA 2013). These species are also significant contributors to the global food trade,  
52 with 59.0 million tons of beef and veal and 109.3 million tons of pork expected to be produced  
53 globally in 2016 (USDA 2016). Meat species are often identifiable when sold as whole cuts;  
54 however, processing conditions and techniques may change the texture, flavor, and color of  
55 meat, making it difficult to authenticate species in food products containing processed meats  
56 (Cawthorn, Steinman, & Hoffman, 2013). The inability to readily identify meat species in  
57 processed products gives rise to the potential for species mislabeling, in which one species is  
58 substituted either partially or completely for another species.

59 In many cases, species mislabeling is a form of economically motivated adulteration  
60 (EMA), in which a product is intentionally mislabeled for reasons of economic gain (FDA 2009).  
61 Due to price differences among meat species, there are economic incentives associated with meat  
62 species mislabeling. For example, the average retail value of beef in 2015 was US\$13.31/kg  
63 while the average retail value of pork for the same year was US\$8.49 (USDA 2016), resulting in  
64 an economic incentive for the substitution of pork for beef in a processed product. Substitution  
65 can occur at any point in the supply chain, from the slaughterhouse up until the point of sale  
66 (Premanandh, 2013). Meat products may also become mislabeled due to cross contamination  
67 when processing different types of meat on the same equipment, as has been suggested in  
68 previous studies (Hsieh, Woodward, & Ho, 1995; Kane & Hellberg, 2016; Okuma & Hellberg,  
69 2015).

70 Mislabeled meat species in processed foods and pet foods has a number of potentially  
71 detrimental effects such as the exposure of consumers and pets to meat allergen risks,  
72 infringement of religious practices, and economic deception (Ballin, 2010). A number of studies  
73 have detected undeclared species in processed meat products such as deli meats, minced meats,  
74 burger patties, sausage, pet foods, and canned meats (Ayaz, Ayaz, & Erol, 2006; Cawthorn et al.,  
75 2013; Di Pinto et al., 2015; Flores-Munguia, Bermudez-Almada, & Vazquez-Moreno, 2000;  
76 Hsieh et al., 1995; Okuma & Hellberg, 2015). Some of the most commonly undeclared species  
77 within these products were beef, pork, poultry, and sheep. These findings are concerning from a  
78 religious point of view, as some religions prohibit the consumption of beef and/or pork (Sattar et  
79 al., 2004). In addition to the studies mentioned above, horsemeat was detected as an undeclared  
80 ingredient in numerous beef products in the 2013 horsemeat scandal in Europe (National Audit  
81 Office 2013).

82 Adulteration and misbranding of meat products is prohibited under the United States  
83 Code (USC) Meat Inspection Act (21 USC § 610) (United States Code [USC] 2011).

84 Adulteration and misbranding are considered to be separate terms, but both include forms of  
85 mislabeling. For example, adulteration can occur when an ingredient has been completely or  
86 partially omitted, and/or if any ingredient has been substituted within a meat product. A  
87 misbranded product includes one whose labeling is false or misleading or if it is offered for sale  
88 under the name of another food.

89 In order to determine if meat species have been partially or completely substituted for  
90 cheaper alternatives in processed food products, DNA or protein-based methods are often used  
91 (Ballin, 2010). Two of the most commonly used methods for this purpose are enzyme-linked  
92 immunosorbent assay (ELISA), a protein-based method, and real-time polymerase chain reaction

93 (PCR), a DNA-based assay. The USDA Food Safety and Inspection Service (FSIS)  
94 Microbiology Laboratory Guidebook relies on a sandwich ELISA method for identifying animal  
95 species in cooked and canned meat and poultry products (USDA 2005). Sandwich ELISA is  
96 recognized as being sensitive and robust and it has been used in several studies for detecting low  
97 levels of a target species within mixtures (Jones & Patterson, 1985; Liu, Chen, Dorsey, & Hsieh,  
98 2006; Yamamoto, Kato, Endo, Kotoura, & Takeda, 2015). On the other hand, real-time PCR is a  
99 commonly used method for detection of meat species in processed products (Camma, Di  
100 Domenico, & Monaco, 2012; Kesmen, Gulluce, Sahin, & Yetim, 2009; Lahiff et al., 2002) and it  
101 has been used in a number of previous studies to reveal instances of product mislabeling (Kane  
102 & Hellberg, 2016; Okuma & Hellberg, 2015). A real-time PCR assay developed by Camma et al.  
103 (2012) was reported to demonstrate high sensitivity, specificity, and repeatability for meat  
104 species identification within meat mixtures. The assay was based on TaqMan™ chemistry with  
105 minor groove binder (MGB) probes and targeted regions of the cytochrome *b* and 16S rRNA  
106 genes. The authors reported the ability to identify turkey, chicken, beef, pork and sheep species  
107 in raw and cooked meat mixtures using this assay.

108         While both ELISA and real-time PCR are commonly used methods for species detection  
109 in foods, a direct comparison of the two methodologies for the purpose of detecting meat species  
110 in processed products has not been carried out. The results of such a comparison would help to  
111 facilitate the detection of food fraud by indicating which methodology is more appropriate for  
112 use with processed meat products. Therefore, the objective of this study was to compare the  
113 commercial ELISA kit described in the USDA/FSIS Microbiology Laboratory Guidebook to a  
114 published real-time PCR assay for the detection of beef and pork species in processed meat  
115 samples. Real-time PCR primers and probes developed by Camma et al. (2012) were chosen for



116 use in this study because they have been extensively tested and validated for detection of the  
117 target meat species in both raw and cooked samples. The methods were evaluated on multiple  
118 factors, including sensitivity, specificity, agreement among duplicate samples, hands-on  
119 technician time required per sample, total time required per sample, cost per sample, and ease of  
120 use.

## 121 **2. Materials and Methods**

### 122 *2.1. Reference sample collection and preparation*

123 Lean, raw cuts of beef and pork were purchased from a local supermarket and were used  
124 in this study to create reference tissue mixtures. Prior to mixing, a 25 mg portion of raw meat  
125 from each species was collected to undergo DNA extraction and serve as a positive control for  
126 real-time PCR. The positive controls for ELISA were provided by the manufacturer. Reference  
127 tissue mixtures were prepared using 0.1-99.9% w/w of one meat species mixed with the second  
128 species, with a total weight of 50 g per sample (Table 1). Each meat sample was homogenized  
129 with 50 mL of sterile deionized water in a 12-speed Oster blender (Neosho, MO, USA) for 1 min  
130 at speed 6. Blender parts were cleaned and sterilized in between each sample. Blended samples  
131 were refrigerated overnight.

132 Next, blended samples were heat-treated following the USDA protocol (USDA 2005) for  
133 the identification of animal species in meat and poultry products, with some modifications. A  
134 portion ( $20 \pm 2$  g) of the blended sample was combined with  $40 \pm 0.5$  mL of sterile deionized  
135 water in a sterile jar. The sample jar was covered with a screw-top sterile lid and placed in a 95  
136 °C water bath for  $15 \pm 1$  min, then cooled to room temperature. The contents were then poured  
137 into a sterile 24-oz Whirl-pak bag (Nasco, Salida, CA) and mixed in a Stomacher® 400  
138 Circulator (Seward, Davie, FL, USA) for 60 s at 230 rpm. The bag was removed from the

139 Stomacher® and left undisturbed for 1 h at room temperature. Next, approximately 50 mg of  
140 tissue from each sample was transferred into two separate 1.5 ml Safe-Lock microcentrifuge  
141 tubes (Eppendorf, Hauppauge, NY, USA) and stored at -20 °C until DNA extraction. Two 1.5-  
142 mL aliquots of the liquid portion of the mixture were transferred to two separate 1.5 mL Safe-  
143 Lock microcentrifuge tubes and centrifuged at 10,300 rpm for 10 min. Next, 1.0 mL of each  
144 supernatant was transferred into a 1.5 mL Safe-Lock microcentrifuge tube and stored at -20 °C  
145 until ELISA.

## 146 2.2. Commercial sample collection and preparation

147  
148 A total of 30 different commercial samples consisting of ground meats, cooked sausage,  
149 deli meats, pet treats, and canned meats were collected for beef and pork species identification  
150 using both real-time PCR and ELISA methods. A total of 11 samples listed beef or a beef  
151 derivative as an ingredient, 11 samples listed pork or a pork derivative as an ingredient, and 8  
152 samples listed both species or their derivatives as ingredients. Each commercial meat sample was  
153 prepared for DNA extraction and ELISA as described in section 2.1, with the exceptions that the  
154 cooked meat samples were not heat-treated and that  $60 \pm 0.5$  mL of sterile deionized water was  
155 added to  $20 \pm 2$  g of the pet treat samples prior to use of the Stomacher to allow for improved  
156 mixing of the low-moisture products.

## 157 2.3. DNA extraction and quantification

158  
159 DNA extraction was performed for all reference and commercial samples in duplicate  
160 using the DNeasy Blood and Tissue Kit, Purification of Total DNA from Animal Tissues, Spin-  
161 Column Protocol (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions.  
162 Lysis was carried out at 56 °C for 1-3 h or until tissue was completely lysed, with vortexing  
163 every 30 min. DNA was eluted in 50  $\mu$ L of pre-warmed (37 °C for 30 min) Buffer AE. The

164 extracted DNA was then quantified using a NanoDrop 2000 (Thermo Scientific, Waltham, MA,  
165 USA). Samples were diluted with AE Buffer to normalize the DNA concentration to 250 pg/ $\mu$ L  
166 for use in real-time PCR. Samples with less than 250 pg/ $\mu$ L after DNA extraction were not  
167 diluted. All DNA samples were stored at -20 °C until real-time PCR. A reagent blank with no  
168 sample added was included with each set of DNA extractions.

#### 169 2.4. Real-time PCR

170  
171 Real-time PCR was carried out on all duplicate DNA samples using a Rotor-Gene® Q  
172 Thermal Cycler (Qiagen) combined with species-specific primers and TaqMan™ probes  
173 developed previously for detection of pork and beef (Camma et al., 2012). Reactions were  
174 carried out in a singleplex format and targeted 166-bp (pork) and 183-bp (beef) regions of the  
175 gene coding for cytochrome *b*. Primers were synthesized by Integrated DNA Technologies  
176 (Coralville, IA, USA) and probes were obtained from Applied Biosystems (Austin, TX, USA).  
177 Each reaction tube contained 10  $\mu$ L of TaqMan Fast Universal PCR Master Mix (2X) (Applied  
178 Biosystems), 2.0  $\mu$ L of TaqMan MGB probe (2.5  $\mu$ M), 2.0  $\mu$ L of each oligonucleotide primer  
179 (3.0  $\mu$ M for beef, 9.0  $\mu$ M for pork), 2.0  $\mu$ L of molecular-grade water, and 2.0  $\mu$ L of DNA or  
180 negative control for a total reaction volume of 20.0  $\mu$ L. A non-template control (NTC)  
181 containing water instead of DNA was included alongside each set of samples tested with real-  
182 time PCR. Each PCR run also included three positive controls of the target meat species diluted  
183 to 250 pg/ $\mu$ L, 25 pg/ $\mu$ L, and 2.5 pg/ $\mu$ L. Cycling conditions were followed as described in  
184 Camma et al. (2012): 95 °C for 20 s, followed by 35 cycles of 95 °C for 1 s and 60 °C for 20 s.  
185 The results of each run were analyzed with the Rotor-Gene Q Software v.2.2.3 and a sample was  
186 determined to be positive if it had a Ct value for the meat species being tested (Okuma &  
187 Hellberg, 2015). Results obtained were qualitative and reported as presence or absence of the

188 target species.

## 189 2.5. ELISA

190  
191 ELISA was carried out for all samples in duplicate using the supernatants prepared as  
192 described in section 2.1. Each duplicate sample was tested with both the ELISA-TEK Cooked  
193 Beef Species Kit and the ELISA-TEK Cooked Pork Species Kit (ELISA Technologies,  
194 Gainesville, FL, USA) following the USDA protocol for identification of animal species in meat  
195 products (USDA 2005). A Thermo Scientific AccuWash microplate washer was used to  
196 complete all wash steps. Each test run included four positive controls and four negative controls  
197 supplied by the kit. The results were read with a BMG Labtech FLUOstar Omega microplate  
198 reader (Cary, NC, USA). Samples were determined to be if positive if the raw OD value of one  
199 or both of the replicate wells was greater than 0.250 and if the results of all controls were in  
200 accordance with USDA guidelines (USDA 2005).

## 201 3. Results and Discussion

### 202 3.1. Specificity and sensitivity

203 As shown in Table 1, both ELISA and real-time PCR showed 100% specificity during  
204 reference sample testing, with no cross-reactivity detected for the non-target species in the  
205 pork/beef binary mixtures. In terms of sensitivity, ELISA was able to consistently detect pork in  
206 the binary mixture at levels down to 10.0% w/w (Table 1; Sample 5). Although pork was  
207 detected at levels as low as 5.00% w/w (Sample 4), this result was only found with one of the  
208 duplicate samples. The beef-specific ELISA test showed greater sensitivity compared to the  
209 pork-specific test, with the lowest detection at 0.50% w/w (Sample 12) and the lowest consistent  
210 detection level at 1.00% w/w (Sample 11) for beef within a binary mixture. In comparison, the  
211 USDA lists adulteration detection limits of 4% w/w for pork and 1% w/w for beef in binary

212 mixtures of sample extracts when using the ELISA-TEK™ Cooked Meat Species Test Kits, with  
213 the caveat that the sensitivity and specificity of each assay may vary depending on the lot that is  
214 being tested (USDA 2005). It is possible that the assay was less sensitive for pork in the current  
215 study due to differences in preparation methods for the binary mixtures. However, the USDA  
216 protocol does not provide details on this point, making it difficult to elaborate further.  
217 Interestingly, the USDA protocol is based on a sandwich ELISA with polyclonal antibodies  
218 (pAbs); however, previous studies using a sandwich ELISA assay with monoclonal antibodies  
219 (mAbs) have reported lower detection limits for both pork and beef. For example, studies using a  
220 sandwich ELISA with porcine-specific mAbs have reported the ability to detect pork at levels of  
221 0.05-0.5% w/w in various meat mixtures (Chen & Hsieh, 2000; Liu et al., 2006). Similarly,  
222 Yamamoto et al. (2015) were able to detect beef at levels of 0.1% w/w in a beef and pork meal  
223 mixture using a sandwich ELISA with two bovine-specific mAbs. Although the goal of the  
224 current study was to compare the USDA protocol for ELISA to a real-time PCR assay, future  
225 research along these lines should include consideration of ELISA with mAbs.

226 As shown in Table 1, the real-time PCR assay showed greater sensitivity as compared to  
227 the commercial ELISA kit for both the beef and pork assays. The lowest consistent level of  
228 detection for pork within the binary mixture was 0.10% w/w using real-time PCR (Sample 1), as  
229 compared to 10.0% with ELISA. This was the lowest percentage of pork contained in a reference  
230 sample, meaning that the assay may be capable of even greater sensitivity. Similarly, Laube et al.  
231 (2003) were able to detect pork species at 0.1% w/w in a beef/pork mixture by applying real-time  
232 PCR with TaqMan™ probes. Rodriguez et al. (2005) also showed detection of pork at the lowest  
233 level tested (0.5% w/w) in cooked pork/beef mixtures using real-time PCR with TaqMan™  
234 MGB probes. In the case of beef, the lowest level consistently detected with real-time PCR in the

235 current study was 0.50% w/w within the binary mixture (Sample 12), as compared to 1.00% for  
236 ELISA. This detection level was also found in a previous study utilizing a real-time PCR assay  
237 with TaqMan<sup>TM</sup> probes for the detection of meat species in mixtures Dooley et al. (2004). On the  
238 other hand, Laube et al. (2003) showed slightly greater sensitivity for beef detection at 0.1% w/w  
239 in a beef/pork mixture tested with real-time PCR.

### 240 3.2. Commercial samples

241 The ability of ELISA and real-time PCR to detect pork and beef in commercial samples  
242 was tested with a variety of processed meat products, including ground meat, sausage, deli meat,  
243 pet treats, and canned meat (Table 2). Overall, the two methods showed agreement as to the  
244 species detected in the products for 26 out of the 30 samples. Among the 26 samples showing  
245 agreement between the two methods, 23 of these were found to be correctly labeled and 3 were  
246 potentially mislabeled (Samples 34, 38, and 40). Among the four samples with inconsistent  
247 results between the two methods, two were found to be correctly labeled by at least one method  
248 (Samples 37 and 39) and two were found to be potentially mislabeled (Samples 44-45). These  
249 samples are discussed in detail in the following paragraphs.

250 Both ELISA and real-time PCR were able to positively identify pork in 16 of the 20  
251 products (80%) that specifically listed pork or a pork-derived ingredient on the label. One of the  
252 pet treats (Sample 38) and two canned meats (Samples 44-45) were negative for pork with both  
253 ELISA and real-time PCR. The pet treat listed bacon fat as the twelfth ingredient in the product  
254 and the two canned meats each listed pork as the fifth ingredient in the product. The inability of  
255 ELISA to detect pork in bacon fat is not surprising, given that ELISA is a protein-based assay;  
256 however, real-time PCR would have been expected to detect DNA in this product, based on  
257 previous studies involving pet foods (Okuma & Hellberg, 2015). Species detection in the canned

258 meat samples may have been limited due to the extensive processing that these products undergo  
259 as well as the presence of inhibitory ingredients present in these samples, or these could  
260 represent instances of mislabeling. Because neither method was able to detect the target species  
261 in these three products, they were determined to be potentially mislabeled.

262         There were two pet treats (Samples 37 and 39) for which only one of the two methods  
263 was able to detect pork. Sample 37 tested positive for pork with ELISA but negative with real-  
264 time PCR, even though it listed pork as the first ingredient on the label. This result may have  
265 been due to the presence of numerous plant-based ingredients in the sample, which are known to  
266 have compounds that are inhibitory to PCR (Schrader, Schielke, Ellerbroek, & Johne, 2012). On  
267 the other hand, Sample 39 tested positive for pork with real-time PCR and negative with ELISA.  
268 This sample listed bacon seventh in the ingredient list and may have contained pork below the  
269 level of detection for ELISA. Interestingly, one of the pet treats (Sample 34) tested positive for  
270 pork with both ELISA and real-time PCR even though pork was not specifically stated on the  
271 label. This product listed animal liver flavor as an ingredient, which was likely the source of the  
272 detected pork.

273         Of the 19 commercial samples that declared beef or a beef-derived ingredient on the  
274 label, ELISA identified beef in 14 products (74%) and real-time PCR identified beef in 16 of the  
275 products (84%). Two pet treats (Samples 34 and 38) and one canned meat product (Sample 40)  
276 tested negative for beef with both methods. Similar to the results discussed above involving  
277 bacon fat, one of the pet treats (Sample 34) listed beef tallow, a form of beef fat, as the only beef  
278 ingredient. The other pet treat that tested negative for beef (Sample 38) listed beef as the eighth  
279 ingredient on the package and the canned meat product (Sample 40) that tested negative for beef  
280 was a corned beef hash that listed beef as the first ingredient. These three products were

281 determined to be potentially mislabeled due to the negative test result with both real-time PCR  
282 and ELISA. Two additional canned meats (Samples 44-45) tested positive for beef with real-time  
283 PCR, but negative with ELISA. These samples listed beef as the second and fourth ingredient,  
284 respectively.

285         When comparing the five categories of commercial products tested, the ground meat  
286 samples were the most identifiable by ELISA testing. The sausage and deli meat samples only  
287 showed one inconsistent ELISA result each between the two products. For real-time PCR, the  
288 ground meat, sausage, and deli meat samples were all identifiable and showed consistent results  
289 among duplicate samples. The pet treat and canned meat products also showed a high level of  
290 consistency among duplicate samples for both ELISA and real-time PCR, with only one  
291 inconsistent result found for one of the samples tested with real-time PCR. However, there were  
292 several instances in which a declared species in these product types was not detected by one or  
293 both methods. This was likely due to a number of factors, including high heat treatment, the  
294 presence of inhibitory ingredients, an inability to identify species in animal-derived fats, and  
295 mislabeling of the products. The findings indicate that in some cases, it may be beneficial to use  
296 a combination of real-time PCR and ELISA in order to more accurately authenticate the product  
297 label. For example, when detecting species within pet food products, real-time PCR would be  
298 advantageous for the detection of meat species at low levels, while ELISA may help to overcome  
299 false negatives due to PCR inhibition.

### 300 *3.3. Agreement among duplicate samples*

301         Of the 15 reference samples, the pork-specific ELISA showed agreement among  
302 duplicates for all but one sample, which contained 5.00% pork and 95.0% beef (Sample 4; Table  
303 1). The beef-specific ELISA assay also had one reference sample that did not show agreement



304 among duplicates, which contained 99.5% pork and 0.50% beef (Sample 12). Both samples were  
305 just below the lowest consistent level of detection for the assay. Of the 30 commercial products,  
306 the pork-specific ELISA showed 100% agreement among duplicates and the beef-specific  
307 ELISA showed agreement for all but two samples (Table 2; Samples 26 and 32). In total, ELISA  
308 showed agreement among duplicate samples for 86 out of 90 tests performed, with the pork-  
309 specific assay showing greater agreement (97.8%) compared to the beef-specific assay (93.3%)  
310 (Table 3).

311 As compared to ELISA, real-time PCR showed slightly greater overall agreement among  
312 duplicate samples, with 100% agreement for all reference samples and 95% agreement for  
313 commercial samples (Tables 1-2). Of the 30 commercial products, two canned meats (Samples  
314 44-45) showed inconsistent results when attempting to detect beef using real-time PCR and a pet  
315 treat product (Sample 36) showed inconsistent results for pork detection (Table 2). Overall, real-  
316 time PCR analysis showed agreement among duplicate samples in 87 out of 90 tests performed,  
317 with the pork-specific assay demonstrating higher agreement (97.8%) than the beef-specific  
318 assay (95.6%) (Table 3).

#### 319 *3.4. Time requirements and ease of use*

320 The commercial ELISA kit was found to be easier to carry out and have shorter time  
321 requirements as compared to the real-time PCR assay (Table 3). The hands-on technician time  
322 required to complete the ELISA test was approximately 2 min faster per sample and 1.9 h faster  
323 per 24 samples as compared to real-time PCR (Table 3). Both assays involved the use of  
324 multichannel and/or electronic pipets, which improved ease of use and reduced the hands-on and  
325 total time required.

326 Overall, ELISA was found to be easy to moderate to carry out. The main technical  
327 challenge of ELISA was found to be timing the addition of the Stop Solution in order to achieve  
328 the absorbance values for the positive controls called for in the USDA protocol (USDA 2005).  
329 The real-time PCR assay was considered to be moderately difficult to perform. The main  
330 technical challenge of this assay was the need to determine the DNA concentration for each  
331 sample and perform dilutions prior to performing PCR. This is especially tedious when large  
332 numbers of samples are being analyzed.

333 The total assay time required for ELISA was about 3.3 h for one sample and about 3.8 h  
334 for 24 samples (Table 3). These values include the time required for processing the four positive  
335 controls, four negative controls and two blanks, which adds approximately 9 min to the total  
336 assay time. The total assay time does not include sample preparation and collection of the  
337 supernatant, which is dependent on the nature of the sample and can range from 20 min per  
338 sample for cooked products to 30 min per sample for uncooked products. The times observed in  
339 the current study are similar to those reported by the manufacturer of the ELISA-TEK kits: about  
340 30 min of hands-on time for sample preparation and collection of the supernatant, followed by 3  
341 h to conduct a complete ELISA test (ELISA Technologies Inc. 2016).

342 The total assay time required for real-time PCR was about 3.4 h for one sample and about  
343 5.6 h for 24 samples (Table 3). The total assay time includes the use of the three positive  
344 controls, a reagent blank, and a no-template control, which adds approximately 10 min. Total  
345 assay time for sample preparation and collection of tissue is not included. Unlike ELISA,  
346 uncooked samples do not require heat treatment prior to analysis with PCR. Therefore, the time  
347 required for sample preparation and collection of tissue is estimated to take an additional 20 min  
348 per sample.

### 349 3.5. Cost of assay

350 The sample costs associated with real-time PCR were found to be less expensive than the  
351 sample costs associated with ELISA. The 2016 list price for a 96-well, ELISA-TEK™ Cooked  
352 Meat Specification Kit was US\$550, resulting in a cost per well of US\$5.73. However, this price  
353 does not factor in the positive controls, negative controls, or blanks, which all together occupy an  
354 additional eight wells and add a cost of US\$46 to each ELISA run. Since each run must include  
355 controls and blanks, the maximum value will be obtained by running a full plate of samples  
356 rather than testing a few samples at a time. For example, when including the costs of the  
357 controls, the price would be US\$52 to test one sample at a time, but would be reduced to  
358 US\$7.64 per sample if 24 samples were run simultaneously (Table 3).

359 The real-time PCR beef and pork assay used in this study was determined to cost  
360 US\$4.49 per sample tube. These costs include the 2016 list prices of a DNeasy Extraction Kit  
361 (50 reactions), beef and pork TaqMan MGB probes, beef and pork forward and reverse primers,  
362 and 2X TaqMan Fast Universal PCR Master Mix (250 reactions). As with the ELISA cost  
363 calculations, these costs do not factor in the use of positive and negative controls, which must be  
364 included with each run. The controls included in this study for each assay were the reagent blank  
365 from DNA extraction, three positive DNA controls for PCR, and a non-template PCR control,  
366 which would add a total cost of US\$9.57 to each run. When the controls are included in the  
367 calculation, the total cost becomes US\$14 to test one sample at a time and US\$4.89 per sample  
368 when testing 24 samples simultaneously (Table 3).

### 369 4. Conclusions

370 Overall, the results show that the real-time PCR assay used in this study was a more  
371 sensitive method for pork and beef species detection within ground meat products as compared

372 to the ELISA protocol described by the USDA. However, due to the potentially inhibitory  
373 ingredients that are found within some processed meat products, it may be beneficial to use both  
374 real-time PCR and ELISA for species detection when testing products with additional  
375 ingredients, such as pet foods. The results of this study also suggest that the real-time PCR assay  
376 was a more reliable and less expensive method to perform when compared to the ELISA  
377 protocol. On the other hand, ELISA was found to be less time consuming and easier to perform  
378 than real-time PCR. It is important to note that the findings of this study are based on specific  
379 protocols, and other real-time PCR and ELISA protocols may show different results. To further  
380 compare real-time PCR and ELISA methodologies, it is suggested that sandwich ELISA assays  
381 with sensitivity limits closer to that of real-time PCR methods be used.

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**Table 1.** Results of meat species identification testing in cooked porcine and bovine reference sample mixtures. The results of real-time PCR and ELISA are reported as positive (+) or negative (-) for each duplicate sample

Sample no.	% Pork <sup>a</sup>	% Beef	Real-time PCR results		ELISA results	
			Pork	Beef	Pork	Beef
1	0.10	99.9	+ / +	+ / +	- / -	+ / +
2	0.50	99.5	+ / +	+ / +	- / -	+ / +
3	1.00	99.0	+ / +	+ / +	- / -	+ / +
4	5.00	95.0	+ / +	+ / +	- / +	+ / +
5	10.0	90.0	+ / +	+ / +	+ / +	+ / +
6	25.0	75.0	+ / +	+ / +	+ / +	+ / +
7	50.0	50.0	+ / +	+ / +	+ / +	+ / +
8	75.0	25.0	+ / +	+ / +	+ / +	+ / +
9	90.0	10.0	+ / +	+ / +	+ / +	+ / +
10	95.0	5.00	+ / +	+ / +	+ / +	+ / +
11	99.0	1.00	+ / +	+ / +	+ / +	+ / +
12	99.5	0.50	+ / +	+ / +	+ / +	- / +
13	99.9	0.10	+ / +	- / -	+ / +	- / -
14	100	0	+ / +	- / -	+ / +	- / -
15	0	100	- / -	+ / +	- / -	+ / +

<sup>a</sup>Total weight of each sample was 50 g

**Table 2.** Results of meat species identification in porcine and bovine commercial samples using real-time PCR and ELISA. The results of real-time PCR and ELISA are reported as positive (+) or negative (-) for each duplicate sample

Sample No.	Product type	Meat ingredients on label	Real-time PCR results		ELISA results	
			Pork	Beef	Pork	Beef
16	Ground meat	Beef	- / -	+ / +	- / -	+ / +
17	Ground meat	Beef	- / -	+ / +	- / -	+ / +
18	Ground meat	Beef	- / -	+ / +	- / -	+ / +
19	Ground meat	Pork	+ / +	- / -	+ / +	- / -
20	Ground meat	Pork	+ / +	- / -	+ / +	- / -
21	Ground meat	Pork	+ / +	- / -	+ / +	- / -
22	Sausage	Beef	- / -	+ / +	- / -	+ / +
23	Sausage	Beef	- / -	+ / +	- / -	+ / +
24	Sausage	Pork	+ / +	- / -	+ / +	- / -
25	Sausage	Pork	+ / +	- / -	+ / +	- / -
26	Sausage	Pork	+ / +	+ / +	+ / +	- / +
		Beef				
		Turkey				
27	Sausage	Pork	+ / +	+ / +	+ / +	+ / +
		Beef				
28	Deli meat	Beef	- / -	+ / +	- / -	+ / +
29	Deli meat	Beef	- / -	+ / +	- / -	+ / +
30	Deli meat	Pork	+ / +	- / -	+ / +	- / -
31	Deli meat	Chicken	+ / +	- / -	+ / +	- / -
		Pork				
32	Deli meat	Pork	+ / +	+ / +	+ / +	+ / -
		Beef				
33	Deli meat	Chicken	+ / +	+ / +	+ / +	+ / +
		Beef Hearts				
		Pork				
34	Pet treats	Chicken meat	+ / +	- / - <sup>a</sup>	+ / +	- / - <sup>a</sup>
		Beef tallow				
		Animal liver flavor				
		Salmon				

35	Pet treats	Fish				
		Beef lung	- / -	+ / +	- / -	+ / +
		Beef liver				
		Beef				
36	Pet treats	Pork liver	- / +	- / -	+ / +	- / -
		Bacon				
		Chicken fat				
37	Pet treats	Pork	- / - <sup>a</sup>	- / -	+ / +	- / -
38	Pet treats	Beef	- / - <sup>a</sup>	- / - <sup>a</sup>	- / - <sup>a</sup>	- / - <sup>a</sup>
		Chicken by-product meal				
		Liver				
		Bacon fat				
39	Pet treats	Beef	+ / +	+ / +	- / - <sup>a</sup>	+ / +
		Bacon				
40	Canned meat (corned beef hash)	Beef	- / -	- / - <sup>a</sup>	- / -	- / - <sup>a</sup>
41	Canned meat (roast beef)	Beef	- / -	+ / +	- / -	+ / +
42	Canned meat (pork)	Pork with ham	+ / +	- / -	+ / +	- / -
		Chicken				
43	Canned meat (shredded pork meat)	Pork	+ / +	- / -	+ / +	- / -
44	Canned meat (chili)	Beef	- / - <sup>a</sup>	- / +	- / - <sup>a</sup>	- / - <sup>a</sup>
		Pork				
45	Canned meat (Vienna sausage)	Chicken	- / - <sup>a</sup>	- / +	- / - <sup>a</sup>	- / - <sup>a</sup>
		Beef				
		Pork				

<sup>a</sup>Meat species listed on the product label could not be detected

**Table 3.** Comparison of real-time PCR and ELISA testing for the detection of pork and beef in mixed samples, based on observations from the current study. Time and cost calculations include the use of positive and negative controls

<b>Characteristics</b>	<b>Real-time PCR<sup>a</sup></b>	<b>ELISA</b>
Sensitivity <sup>b</sup>	0.10% Pork; 0.50% Beef	10.0% Pork; 1.00% Beef
Specificity with reference samples	100%	100%
Agreement among duplicate samples <sup>c</sup>	97.8% Pork; 95.6% Beef	97.8% Pork; 93.3% Beef
Hands on technician time (per sample; per 24 samples)	0.4 h; 2.6 h	0.3 h; 0.7 h
Total time required (per sample; per 24 samples)	3.4 h; 5.6 h	3.3 h; 3.8 h
Cost (per sample; per 24 samples)	US\$14; \$117	US\$52; \$183
Ease of use	Moderate	Easy-moderate

<sup>a</sup>Including DNA extraction

<sup>b</sup>Lowest consistent detection level (w/w) in a binary mixture of pork and beef.

<sup>c</sup>Percentages are based on a total of 45 samples tested in duplicate with each assay

**Highlights**

- Real-time PCR detected beef consistently at 0.50%, compared to 1.00% for ELISA.
- Real-time PCR detected pork consistently at 0.10%, compared to 10.0% for ELISA.
- Compared to ELISA, real-time PCR showed greater agreement among duplicate samples.
- ELISA was found to be less time consuming and easier to perform than real-time PCR.
- ELISA and real-time PCR showed 100% specificity during reference sample testing.