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Complementary molecular methods detect undeclared species in sausage products at retail markets in Canada

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ABSTRACT

Accurate food labelling is of utmost importance for food safety and consumer choice in the food chain. Complete or partial substitution, whether intentional or unintentional, may introduce food pathogens or allergens to a product or affect personal or religious beliefs. Several studies around the world have reported different degrees of species substitution in meat products but no similar studies have been conducted in the Canadian market for sausage products. In this study, 100 raw meat sausage samples that were labelled as single meat species products (beef, pork, chicken or turkey) were collected from retail establishments across Canada and were surveyed for the presence of a panel of non-labeled species. The predominant meat species were determined using DNA barcoding and contaminant or unclaimed meat species were detected using digital droplet PCR using species specific primers and probes. All samples were also tested for presence of horse meat using real-time PCR. All samples contained the predominant species matching the label species except for five turkey sausage samples which contained chicken as the predominant species. Second, this analysis showed that 6% of beef sausages also contained pork, 20% of chicken sausages contained turkey while 5% contained beef, and 5% of pork sausages also contained beef. Five samples labeled as turkey sausage contained no turkey and one pork sample was found to contain horse meat. The overall mislabeling rate detected in this study was 20% and the results provide a baseline for assessing species mislabeling in processed meat products in Canada.

1. Introduction

Accurate food labelling is important to ensure food safety and quality management in the supply chain, as well as to support consumer choice. Substituted species, whether trace adventitious contaminants or a product of economically motivated adulteration (EMA), may introduce toxins, pathogens, or allergens into products (Spink & Moyer, 2011). Public safety measures and testing procedures are put into place by regulatory agencies based on the declared product contents. Consumers may also choose products labeled free from certain species for personal or religious reasons. The availability of reliable testing methods helps to identify and address issues of undeclared meat species. For example, the increased and improved testing for horse in meat products throughout Europe has brought light to, and helped mitigate, one of the largest food scandals in recent history (O'Mahony, 2013). Food authenticity tests often target DNA as it is more robust to processing associated with many food products, in contrast to protein-dependent methods (Ballin, 2010). Various DNA-based methodologies can be employed to identify the component species of meat products.

Studies of species identification of commercial meat products have been conducted using DNA-based methods in several

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countries including Thailand (Kitpipit, Sittichan, & Thanakiatkrai, 2014), Turkey (Ulca, Balta, Çağın, & Senyuva, 2013), Iran (Doosti, Dehkordi, & Rahimi, 2014; Mehdizadeh et al., 2014), the United States (Kane & Hellberg, 2016; Quinto, Tinoco, & Hellberg, 2016), South Africa (Cawthorn, Steinman, & Hoffman, 2013; D'Amato, Alechine, Cloete, Davison, & Corach, 2013), Spain (Rodríguez et al., 2003), China (Cai et al., 2014), Germany and the Netherlands (Floren, Wiedemann, Brenig, Schutz, & Beck, 2015; von Bargen, Brockmeyer, & Humpf, 2014), the United Arab Emirates (Premanandh, Sabbagh, & Maruthamuthu, 2013) and Ireland (O'Mahony, 2013). Many studies focused heavily on processed meat products like ground meat or sausage where the opportunity for mislabeling is higher. These studies have revealed levels of mislabeling of up to 70%, where products are either wholly or partially substituted with species not listed on the label including pork, horse, chicken and others. Although there are examples where little or no mislabeling was found, products with undeclared ingredients were found in almost all published studies.

DNA barcoding is one method that has particular promise for species identification in food (Galimberti et al., 2013). For animal species, this method involves sequencing of a ~650bp fragment of the COI gene (Hebert, Ratnasingham, & de Waard 2003). While used successfully to identify mislabeling of meat products (D'Amato et al., 2013; Kane & Hellberg, 2016; Quinto et al., 2016), it cannot be reliably used on its own to identify species in mixtures and can only detect whole substitution. However, the extensive availability of DNA barcode sequences for commercial and game meat species allows for development of other types of testing that require DNA sequence information for development of primers and probes. This includes droplet digital PCR (ddPCR), which can identify the presence of undeclared species from a mixture and also quantify the amount of that species, allowing separation of cases of adventitious presence from adulteration. Shehata et al., 2017 have developed a method for quantitative ddPCR detection of turkey, chicken, pork and beef which can be used to test the contents of market samples.

Despite the fact that ingredient species must be listed in Canadian food, a national market survey looking at incidence of mislabeling in sausage meat products has not been conducted. In this study, we provide a baseline for occurrence of species mislabeling in sausage products purchased on the Canadian market using a combination of DNA-based methods, including DNA barcoding, digital PCR and real-time PCR. This tiered approach allows the identification of whole substitution using DNA barcoding, as well as identification of undeclared species from a panel of ddPCR assays targeting pork, beef, chicken or turkey (Shehata et al., 2017) and one horse-specific real-time PCR assay. The ddPCR approach also provides a measure of the level of adulteration to differentiate cases that are likely purposeful, from adventitious cases.

2. Materials and methods

2.1. Sample collection

Samplers under contract to the Canadian Food Inspection Agency (CFIA) were utilised to collect a total of 100 sausage samples in three major Canadian cities (Montreal n = 40, Toronto n = 25 and Calgary n = 35) at various retail locations (Table 1). All sampling took place between January 17 and February 25, 2016. Products were purchased from national grocery store chains, regional and local grocery stores, and speciality stores. Sausages were labeled as containing only pork, beef, chicken or turkey (pork n = 38, beef n = 27, chicken n = 20, and turkey n = 15) and did not include more than a single source animal species as identified on the ingredients list. Wieners ("hot dog"), Vienna sausages and sausages containing cheese were excluded from the survey. Sausages were raw, fresh or previously frozen, ready-to-cook, in casings that were prepackaged, or partitioned in-store. No bulk sausage meat samples (without casings) were selected for this study. Digital images of labels and packaging were taken at the time of submission to the laboratory along with details of purchase, and submitted electronically to the CFIA office. Samples were shipped directly to the laboratory in coolers with cold packs. Ninety samples originated from Canada while five samples were imported from the USA and five samples were from unknown origin.

2.2. Sample preparation and controls

The sausage samples were removed from casing, cut into ~1.0–1.5 cm pieces, and then ~25–30 g of representative sample pieces combined from every sausage in the package were homogenized in a Cuisinart® grinder for 3–5 min. The grinder was cleaned and treated with 20% bleach to remove residual DNA between samples. The homogenized samples were then used for DNA extraction. An artificial DNA fragment cloned into a plasmid was used as an internal control to monitor ddPCR procedures (Shehata et al., 2017). For ddPCR, pure bovine, pork, chicken and turkey muscle meat tissues were used as reference materials. For each of the four meat species, tissue of equal weight from five representative single meat species sausage samples, as verified by DNA barcoding and species-specific ddPCR assays, were pooled to establish calibration curves between ddPCR output (copies) and DNA amount (ng) in sausages. The standard curves were established at three-fold dilutions between concentrations ranging from 0.0039 to 0.32 ng for beef, pork and chicken (five points in total), and from 0.0013 to 0.32 ng for turkey (six points in total). Fortified samples, prepared by adding 0.1% (DNA by mass) of the target animal species to a non-target animal species, were used as positive controls. A fish tissue sample, reagent blank and sterile water were used as negative controls.

2.3. DNA extraction

A representative portion (~500 mg) of each homogenized sausage sample was used for DNA extraction in the lysis stage, to increase the amount of representation from the full sausage package. The volume of lysis buffer was increased to accommodate this. After lysis, the usual process was followed for extracting genomic DNA using DNeasy Blood and Tissue® Kit (Qiagen, Mississauga, ON) according to the manufacturer's protocol for "Purification of Total DNA from Plant/Animal Tissue". DNA concentrations and quality (A_{260nm} and A_{280nm}) were determined using both NanoDrop ND-2000 UV–Vis Spectrophotometer (Thermo Fisher Scientific, Ottawa, ON) and Qubit® Fluorometer with the Qubit® dsDNA BR Assay Kit (Thermo-fisher Scientific). The DNA samples were diluted to 10–20 ng/μL in AE buffer, and then were either tested directly or were frozen at −20 °C for testing at a later date.

2.4. Identification of the predominant meat species using DNA barcoding

Primers (Table 2) targeting the mitochondrial cytochrome oxidase subunit I (COI) gene were designed using the PrimerQuest Tool www.idtdna.com/Primerquest/Home/Index) based on NCBI sequences for the following species: *Bos taurus* (AF493542), *Sus scrofa* (KP301137), *Gallus gallus* (KM096846), and *Meleagris gallopavo* (JF275060). The accession numbers represent the reference sequences used, however multiple sequences from each species were considered during primer design. Each PCR reaction mix (25 μL) contained 1x HotStarTaq Master Mix (Qiagen), 0.5 μM of each of the

Table 1
Raw meat sausage sample declared species, origin, and sampling regions.

Sausage species (As labelled)	Sample origin		Sampling region	
	Origin	No of samples	Region	No of samples
Beef (n = 27)	Canada	26	Toronto	1
	Unknown	1	Montreal	15
			Calgary	11
Chicken (n = 20)	Canada	17	Toronto	6
	Unknown	3	Montreal	7
			Calgary	7
Pork (n = 38)	Canada	32	Toronto	11
	USA	5	Montreal	17
	Unknown	1	Calgary	10
Turkey (n = 15)	Canada	15	Toronto	7
			Montreal	1
			Calgary	7

Table 2
Primers used in this study for DNA barcoding and ddPCR.

	Primer name	Sequence (5′-3′)	Amplicon Length	Reference
DNA barcoding	COI-animal-F4	TCRTHAAYCGHTGAYTATWYTC	712	This study
	COI-animal-R716	CCRAARAATCARAAYARRTGTTG		
ddPCR	Bovine-F8108	CCATATACTCTCCTTGGTGAC	270	(Krcmar & Rencova, 2003)
	Bovine-R8357	GTAGGCTTGGGAATAGTACGA		
	[a]Bovine-probe	TAGACACGTCAACATGACTGACAATGATC		(Shehata et al., 2017)
	Chicken-Cytob-F	TCTGGGCTTAACTCTCATACTCACC	106	(Tanabe et al., 2007)
	Chicken-Cytob-R	GGTTACTAGTGGGTTTGCTGGG		
	[a]Chicken-probe	CATTCCTAACACTAGCCCTA		
	Swine-F7773	CTCAATGGTATGCCACAACTAG	313	(Krcmar & Rencova, 2003)
	Swine-R8064	CATTGTTGGATCGAGATTGTGC		
	[a]Swine-probe	ATCTCAAACTACTCATACCCAGCAAGCCCA		(Shehata et al., 2017)
	Turkey-12SFW	CCACCTAGAGGAGCCTGTTCTGTAAT	122	(Abuzinadah, Yacoub, Ashmaoui, & Ramadan, 2015)
	Turkey-12SRV2	TTGAGCTCACTATTGATCTTTCATTTT		(Shehata et al., 2017)
	[a]Turkey-probe	TCCACCCAACCACCTCTTGCCAACAC		
Internal control	IC-Forward	AAGACATTGTGGATGCAGATGAGTA	134	(Shehata et al., 2017)
	IC-Reverse	TAGGCAAGTGCATCCTCCTC		
	[a]IC-probe	CTTGTCCCTCCTGTTGGTACTAGAGA		

[a] Probes were labeled with FAM or Cal Fluor Orange (for IC) at 5′ and BHQ-1 at 3'.

primers (COI-animal-F4 and COI-animal-R716) (Table 2), 0.15 μg of Bovine Serum Albumin (BSA) and 20–40 ng of template DNA. PCR thermal cycling was conducted using a GeneAmp™ PCR System 9700 (Applied Biosystems, Foster City, CA). The PCR cycling conditions were 95 °C for 15 min, 40 cycles of 94 °C for 20 s, 52 °C for 20 s and 72 °C for 1 min, followed by 72 °C for 7 min. To confirm successful amplification of target gene, PCR products were visualized on 2% agarose gels. PCR products were then purified using NucleoFast® 96 PCR clean-up kit according to the manufacturer's protocol (Macherey-Nagel, Duren, Germany). The purified PCR fragments were sequenced bidirectionally with the same primers as for PCR using an ABI 3730 Genetic Analyzer (Applied Biosystems). The retrieved sequences were analyzed using ABI Prism™ Sequencing Analysis software (Applied Biosystems) to obtain a single high quality consensus sequence for each sample (Q > 20 and length > 650 bp). The consensus sequences were queried against the Barcode of Life Data (BOLD) species ID engine and were queried against NCBI GenBank using BLASTN to get the taxonomic identification. Positive (tissue samples with confirmed target animal species) and negative (reagent blank) controls were included and analyzed with each batch of test samples.

2.5. Detection and quantification of contaminant or undeclared meat species using droplet digital polymerase chain reaction (ddPCR)

Species-specific primers and probes (Table 2) were used to amplify mitochondrial DNA sequences of the bovine, porcine, chicken and turkey genomes based on the 5′-nuclease assay chemistry. All samples were tested for presence of all four target species regardless of results from primary species testing. The probes were labeled with 6-carboxyfluorescein (6-FAM) as the reporter for the animal species targets or with CAL Fluor Orange for the internal control, and BHQ-1 as the quencher. Each PCR reaction mix (25 μL/reaction) contained 1x ddPCR Supermix for Probe (Bio-Rad, Mississauga, ON), 96 nM each of the primers and 64 nM probe for the animal targets, 40 nM each of the primers and 32 nM probe for the internal control, and template DNA according to the upper limits of the standard curve for that species (details in Shehata et al., 2017). QX200 Droplet Generator (Bio-Rad) was used to generate PCR droplets where 20 μL from each PCR reaction mixture were mixed with 70 μL of droplet generation oil (Bio-Rad) in a DG8 Cartridge; and 40 μL from each droplet mix were then transferred to a 96-well PCR plate (Bio-Rad). The plate was sealed with a foil heat seal using PX1 ™ PCR plate Sealer (Bio-Rad). The GeneAmp™ PCR System 9700 (Applied Biosystems) was used for thermal cycling. The PCR reaction conditions used were initial denaturation at 95 °C for 10 min, followed by 48 cycles of 20 s at 95 °C and 40 s at 59–60 °C, followed by final extension at 98 °C for 10 min, and then a holding step at 10 °C until reading. Each PCR reaction was run in duplicate. When the PCR was complete, the QX200™ Droplet Reader (Bio-Rad) was used to read the amplification signals, and QuantaSoft software (Bio-Rad) was used to analyze the data. The data were then recorded as copies/μL and then converted into % by

DNA mass based on copy numbers obtained from standard curves for each of the sausage meat species (Fig. 1). Positive and negative controls were included for testing with each batch of samples. Results were accepted only if all QC had passed.

2.6. Real-time PCR for detection of horse

For all samples, extracted DNA was tested for the presence of horse meat using the qualitative InstantLabs Horse Real-Time PCR kit (InstantLabs, Baltimore, USA), which included both a probe for detecting horse meat and an internal control to monitor reaction success. For initial tests on all samples, manufacturer instructions were followed for real-time PCR, with the exclusion of the DNA extraction steps. Samples that initially tested positive were re-tested in triplicate starting from the tissue sampling stage. Samples were run on the Cepheid Smart Cycler II system according to cycling parameters suggested by the kit manufacturer.

3. Results and discussion

A total of 100 raw meat sausages were tested for the predominant meat species using DNA barcoding based on COI gene sequences and also for potential contamination with other meat species mixed with the claimed species on the labels using ddPCR assays for bovine, chicken, porcine and turkey meats (Supplementary Table 1). High quality sequences of >650bp were obtained from all samples. For undeclared species, a cut-off value of 1% (by DNA mass) was used to distinguish the samples where undeclared species detection may be due to adventitious contamination rather than purposeful substitution. Where the quantity of undeclared species was more than 1%, two ranges (1%–5% and more than 5%) were used to identify the proportion of the sample containing these species. Providing ranges, rather than exact percentages, helped to mitigate some of the potential issues related to copy number in different tissue types that may arise when using a mitochondrial marker for quantification (Floren et al., 2015). Generally, a proportion of more than 1% undeclared species may indicate some breakdown in proper production or purposeful adulteration, rather than trace contamination (Premanandh et al., 2013).

Out of 100 sausage samples, 95% contained the predominant species matching the label (Table 3). All sausage samples labelled as beef, chicken or pork (total n = 85) contained the predominant species matching the label on the sample packages. For turkey sausage (n = 15), ten samples contained turkey as the predominant meat species while five samples contained chicken as the predominant species. All 100 raw meat sausages were also tested for potential contamination with other meat species mixed with the claimed species on the labels using ddPCR assays for bovine, chicken, porcine and turkey meats. Table 4 summarizes the presence of unlabeled species found in the sausage samples tested while individual results from each sample can be found in Supplementary Table 1. From 27 beef sausages, seven samples also contained pork. Two of these contained more than 5% pork. From 20 chicken sausages, four contained turkey, two at more than 5%, and one sample contained beef at 1–5%. Two out of the 38 pork sausages were mixed with beef at 1–5%. These concentrations were determined based on calibration curves created from

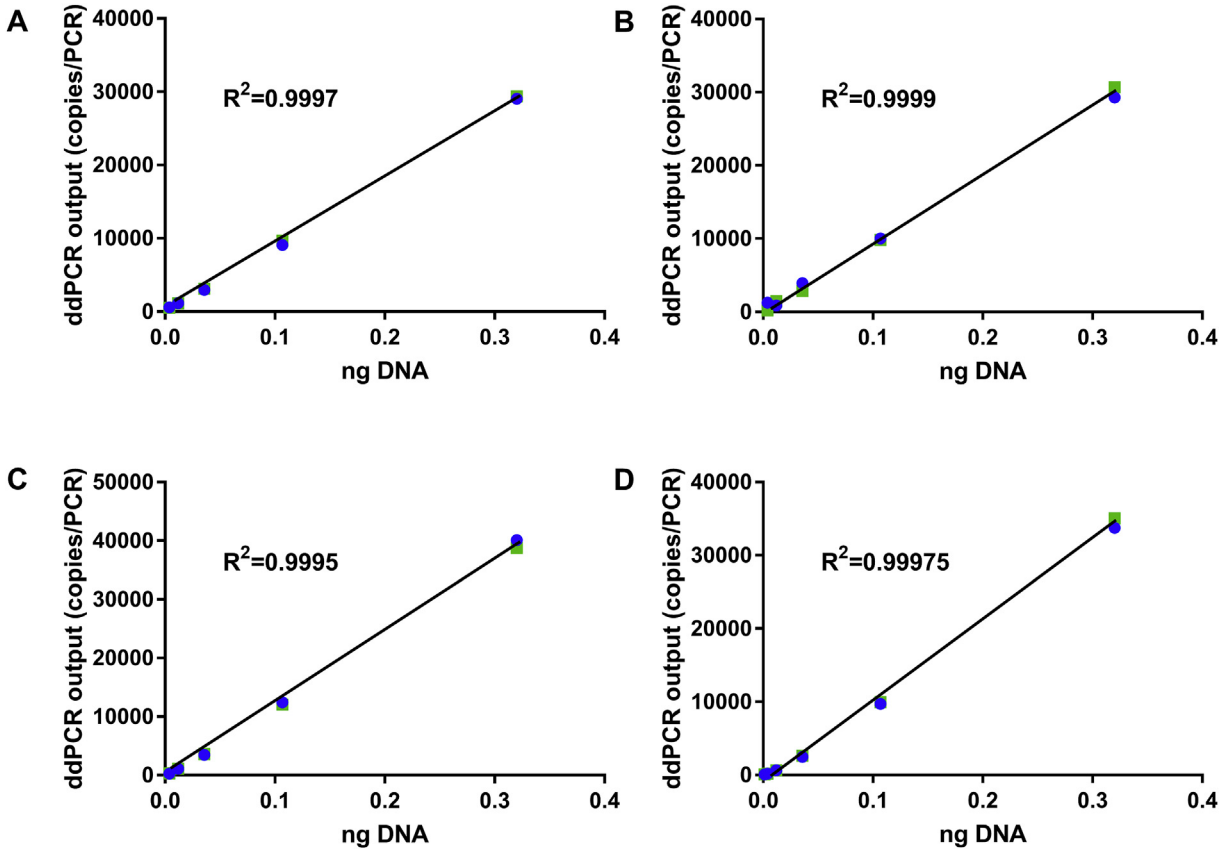

Fig. 1. Relationship between ddPCR output (copies) and DNA amount (ng) from single species sausages of beef (A), pork (B), chicken (C) and turkey (D). Five representative single species sausage samples (types) were pooled for each of the four meat species; and DNA was extracted from the pooled sausage samples for each of the species to establish the relationships.

Table 3
Summary of predominant meat species identified in sausage samples.

Sausage species (as labelled)	No of samples tested	No of samples with predominate species as labeled	No of samples predominate species not as labeled
Beef	27	27	0
Chicken	20	20	0
Pork	38	38	0
Turkey	15	10	5[a]
Total	100	95	5

[a] Chicken was the predominant species in these samples.

Table 4
Summary of meat species identified in sausage samples at levels greater than or equal to 1%, with undeclared species noted in bold text.

Sausage Species (as labelled)	Detection Target				
	ddPCR Assay				Real-time PCR[a]
	Bovine	Chicken	Porcine	Turkey	Horse
Beef (n = 27)	27	0	**7**	0	0
Chicken (n = 20)	**1**	20	0	**4**	0
Pork (n = 38)	**2**	0	38	0	**1**
Turkey (n = 15)	0	**5**	0	10	0

[a] Qualitative test only.

representative sausage samples with up to 20% technical variations. None of the samples contained more than one other species in addition to the predominate species.

Overall, the rate of mislabeling of 20% was similar to another study of ground meat products in North America (Kane & Hellberg, 2016) and showed that undeclared meat species are present in a significant percentage of products in the Canadian market. Our results suggest that the vast majority of products contain mostly the declared species. This is encouraging, but even small amounts of undeclared species can have potential human health implications. For example, the presence of beef in one chicken and two pork products was unexpected as beef is a more expensive meat. This could mean that "waste" beef products, rework, or other non-conforming materials that may not otherwise be consumed are being introduced as a cheap addition to these sausage products. Alternatively, it could mean that insufficient cleaning between grinding of different meats is occurring. Either way this could represent a means for pathogens to enter the food supply as beef products, particularly by-product, would be subject to different screening, specifically screening for *E. coli* O157:H7. In the event of a recall, products where beef is present, but undeclared, would not be removed from sale, presenting another potential health risk. In addition, a third of turkey products were found to be wholly substituted with chicken. The price of ground turkey in Canada for 2016 was more than that for ground chicken (Agriculture and Agri-Food Canada, 2016), suggesting that these instances of substitution may be economically motivated or that a gross mislabeling event occurred during production or packaging. All these samples were produced in the same establishment but purchased in different cities. Additional regulatory actions were undertaken to address the issue.

One of the major drivers for testing for the presence of unlabeled pork is due to religious concerns. For example, a number of studies have focused on identifying the presence of pork in Halal products, which is a concern to some religious communities *(e.g.* Nakyinsige, Man, & Sazili, 2012). In this study, 6% of the beef samples collected contained pork, confirming that this is an ongoing issue that requires monitoring. Though this study focused on the meat contents of the products, sausage casings may represent another potential source of unlabeled pork ingredients. Interestingly, although chicken was found to be a common undeclared ingredient in beef and pork products tested in other studies (Cawthorn et al., 2013; Mehdizadeh et al., 2014; Ulca et al., 2013), we found no examples of undeclared chicken or turkey in non-poultry products. Though rare, allergies to poultry meat have been described (Zacharisen, 2006), so the absence of evidence of poultry in beef or pork products is encouraging.

Finally, horse was also detected in one sample of pork sausage among the 100 sausage samples analyzed in this study (Table 4). This result was confirmed after multiple real-time PCR tests of original DNA extract and of new DNA extracts taken from homogenized new tissue samples from the original sausage sample. According to Canadian regulations, horse can be sold for human consumption but must be labeled on packages as with other ingredients (Department of Justice, 2017).

4. Conclusions

Meat mislabeling continues to be an issue within the food chain and was studied here for the first time in sausage products sold in Canada. This is also the first application of ddPCR to not only identify species present in commercial meat products, but also to differentiate turkey and chicken in commercial samples. Though the primary species in most products was as labeled, the presence of undeclared species was detected in some samples at levels above what would be considered adventitious. This work, completed in partnership with the CFIA, provided an opportunity for a baseline assessment of authenticity in sausage meat and highlighted the need for ongoing monitoring of these products. Overall, digital PCR provides a powerful tool to determine the component species of processed meat products. Additional availability of assays for more common meat species and potential adulterants will further strengthen the utility of this tool for regulatory testing.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.foodcont.2017.07.040.

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