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

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A Thesis by

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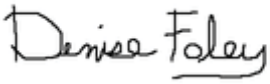

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DEDICATION

This thesis is dedicated to my family for believing in me and being understanding of the time and dedication required. It is especially dedicated to my niece and nephew Arya Hyunh and Thomas Hyunh, in hopes that it will serve as inspiration that hard work will always pay. To my sister giving me the joy of being an aunt, and my brother with his empathetic talks. I would also like to dedicate it to my grandparents who always have had food ready, so that I did not have to cook as much. They followed every advancement during this thesis very closely and reminded me to keep focus.

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Finally, I would like to dedicate to all the students changing careers to remind them that it is never too late to do what you love.

ABSTRACT

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

by Sunjung Magrit Chung

While the presence of ≥1% of an undeclared species in ground meat generally used as an indicator of intentional mislabeling as opposed to cross-contamination, the actual percent of undeclared species resulting from cross-contamination has not been experimentally determined. The objective of this study was to quantify the effect of sanitation procedures on the cross-contamination of animal species in ground meat products, using undeclared pork in ground beef. Pork (13.6 kg) was processed using a commercial grinder, then one of three sanitation treatments was completed ("no cleaning", "partial cleaning", or "complete cleaning"). Next, beef (13.6 kg) was ground using the same equipment. For "no cleaning," beef was ground immediately after pork without any cleaning step; for "partial cleaning," the hopper tray was wiped, and excess meat was taken out from the auger; for "complete cleaning," all parts of the grinder were disassembled and thoroughly cleaned with water and soap. 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. Real-time polymerase chain reaction (PCR) was used to quantify pork in ground beef. For "no cleaning," the first 100-g sample of ground beef run through the grinder contained 24.42 ± 10.41% pork, while subsequent samples contained <0.2% pork. With "partial cleaning," 4.60 ± 0.3% pork was detected in the first 100-g sample of ground beef, and subsequent samples contained <0.2% pork. Pork was not detected in ground beef following "complete cleaning." These results indicate that incomplete cleaning of the grinding equipment can lead to cross-contamination of species at initial levels of <25%; however, subsequent samples processed on

the same equipment would be expected to contain <1% of the contaminating species. Proper sanitation procedures must be followed when grinding multiple species in order to prevent cross-contamination and product mislabeling.

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LIST OF ABBREVIATIONS

BOLD - Barcode of Life Database

COI - cytochrome c oxidase subunit I

ddPCR - droplet digital Polymerase Chain Reaction

dddPCR – duplex droplet digital Polymerase Chain Reaction

ELISA - Enzyme-linked immunosorbent assay

FDA - U.S. Food and Drug Administration

FSAI - Food Safety Authority of Ireland

LOD – Limit of detection

LOQ – Limit of quantification

NCBI - National Center for Biotechnology Information

NTC – Negative Template Control

PCR - Polymerase Chain Reaction

PCR-RFLP - PCR-Restriction Fragment Length Polymorphism

UK – United Kingdom

USC - United States Code

USDA - United States Department of Agriculture

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. Review of Literature

2.1. Species misbranding of ground meat products

Visual detection and proper identification of secondary species when meat samples are mixed can be challenging. For example, when beef is mixed with pork, the mixture color and texture is visually similar to pure samples of beef (Figure 1). These similarities make it difficult for consumers to detect fraudulent species mixing or cross-contamination of ground meats.

A) 50/50 Raw

Figure 1 (A) Raw meat and pork mixture (50%/50%); (B) Raw 100% pork and raw 100% beef. (Photo by Sunjung M. Chung)

2.2. Ground meat misbranding in the global market

Ground meats are a target for misbranding because high-value species can be mixed with other lower-value species. A summary of undeclared species detected in a variety of ground meat products is shown in Table 1.

In 2012, the Food Safety Authority of Ireland (FSAI) found that out of 27 burgers tested with PCR, 10 contained equine DNA. One result enabled FSAI to confirm that

high levels of equine DNA were from adulteration and not cross-contamination from processing. The tests were performed at the beginning of the meat scandal in Ireland and the United Kingdom (UK) where horse meat was an adulterant in beef products (O'Mahony, 2017).

Naaum et al. (2018) tested 100 sausage samples collected in Canada for species mislabeling. All samples were tested with DNA barcoding and species-specific droplet digital Polymerase Chain Reaction (ddPCR) or real-time PCR. Twenty percent of the 100 samples were adulterated with species not labeled in the product. Among the beef sausages tested, 6% were adulterated with pork; among the chicken sausages, 20% were adulterated with turkey and 5% were adulterated with beef; and 5% of the pork sausages tested were adulterated with beef. Five out of 15 turkey sausages failed to test positive for turkey, and one of 38 pork sausages tested positive for horse. Results in Naaum et al. (2018) were similar to those found in Kane and Hellberg (2016), in which 21% of samples collected contained other species.

As a follow-up to the study by Naaum et al. (2018), Shehata et al. (2019) collected 100 sausage samples and tested for pork, beef, chicken, turkey, goat, horse, sheep, and bison using a combination of ddPCR, PCR, real-time PCR, and DNA barcoding. The lowest percent of DNA detected in the samples was 1%. Out of the 30 beef sausages collected, four contained pork, one tested positive for chicken, and five were found to contain sheep. Of the 20 chicken sausage samples, one sample contained mostly beef, one contained pork and turkey, and two tested positive for turkey. For the 20 turkey sausages, one tested positive for chicken and pork, and another for bison. In total, the overall mislabeling found in this study was 14% (Shehata et al., 2019).

In Turkey, Keyvan et al. (2017) collected 37 sucuk, 33 sausages, and 32 salamis from markets to be tested for species adulteration using PCR. A total of 14.7% of all samples collected were found to be adulterated. Poultry was detected in six samples of sucuk, seven samples of salami and two samples of sausages. In addition to poultry, horse was also detected in one of the samples of sucuk. Because Turkey is a Halal country, availability of pork is very low and expensive. Therefore, Keyvan et al. (2017) concluded that the presence of secondary species in the samples was likely due to EMA (Keyvan et al., 2017).

Flores-Mungia et al. (2000) tested 23 hamburger patties and 17 sausages purchased from local stores in Mexico for species identification. Species were identified using immunodiffusion on agarose gel assay. LOD of this method was 3% of pork in mixtures. Flores-Mungia et al. (2000) found that 9 samples out of 23 hamburger patties contained horse as an undeclared species, 2 out of 17 sausages contained undeclared horse, and 3 out of 17 sausages contained undeclared pork. The study concluded that undeclared horse was present due to intentional adulteration because horse meat was less expensive and less regulated compared to beef (Flores-Mungia et al., 2000).

In South Africa, 68% species mislabeling was found in sausages, hamburgers, minced meats and deli products (Cawthorn et al., 2013). Species were identified using species-specific PCR and DNA sequencing. The top species found not declared in the labels was pork, followed by chicken. Pork was found in 46 of the 139 samples tested, and was detected in 52% of sausages, 38% of minced meats, 30% of deli meats and 10% of dried meats. The authors concluded that although there was a possibility of cross-contamination of species during processing, the presence of pork in these products was

most likely for economic gain. In the case of sausage, use of pork casings was also suspected. Chicken was mostly found in hamburger patties, sausages and deli meat, and suspected to be present in these products for economic gain (Cawthorn et al., 2013).

2.2.1. Ground meat misbranding in the U.S. market

Similar to research conducted in other countries, meat mislabeling has been detected on the U.S. commercial market (Kane & Hellberg, 2016). Kane and Hellberg (2016) used a combination of DNA sequencing and real-time PCR to identify mislabeling in 10 out of 48 ground meat samples. Some samples were adulterated with only one species while others contained multiple species. For instance, a sample of chicken tested positive for beef, turkey, and sheep/lamb; bison tested positive for beef, pork, and horse; and black bear tested positive for pork (Kane & Hellberg, 2016).

Table 1 Species substitution of meat, poultry and game meats

Product label	**Other species found**	**Methods used**	**Source**
Beef	Pork	ddPCR	(Naaum et al., 2018)
	Pork	Species-specific PCR	(Cawthorn et al., 2013)
	Horse	PCR	(O'Mahony, 2017)
	Horse	Immunodiffusion gel	(Flores-Mungia et al., 2000)
	Chicken	Species-specific PCR	(Cawthorn et al., 2013)
	Buffalo	Species-specific PCR	(Cawthorn et al., 2013)
Chicken	Beef, Turkey,	DNA sequencing	(Kane & Hellberg, 2016)
	Sheep/Lamb	DNA sequencing	(Kane & Hellberg, 2016)

	Beef, Turkey,	Real-time PCR	(Kane & Hellberg, 2016)
	Sheep/Lamb	Real-time PCR	(Kane & Hellberg, 2016)
	Beef, Turkey	ddPCR	(Naaum et al., 2018)
	Pork	Immunodiffusion gel	(Flores-Mungia et al., 2000)
Turkey	Beef, Sheep/Lamb,	DNA sequencing,	(Kane & Hellberg, 2016)
	Chicken	real-time PCR	(Kane & Hellberg, 2016)
	Chicken	ddPCR	(Naaum et al., 2018)
Pork	Beef	ddPCR	(Naaum et al., 2018)
	Horse	Real-time PCR	(Naaum et al., 2018)
	Horse	Immunodiffusion gel	(Flores-Mungia et al., 2000)
	Horse, chicken,	PCR	(Keyvan et al., 2017)
	turkey, unidentified	PCR	(Keyvan et al., 2017)
Sheep	Pork, Sheep, Horse	DNA sequencing,	(Kane & Hellberg, 2016)
	Pork, Sheep, Horse	real-time PCR	(Kane & Hellberg, 2016)
	Beef	Species-specific PCR	(Cawthorn et al., 2013)
Black	Pork	DNA sequencing,	(Kane & Hellberg, 2016)
Bear	Pork	real-time PCR	(Kane & Hellberg, 2016)
Bison	Beef, Pork, Horse	DNA sequencing, real-time PCR	(Kane & Hellberg, 2016)
Yak	Beef	DNA sequencing, real-time PCR	(Kane & Hellberg, 2016)

Kangaroo	Beef	DNA sequencing, real-time PCR	(Kane & Hellberg, 2016)

2.3. U.S. regulations on meat species mislabeling

In the U.S, the USDA monitors and inspects most meats and poultry with the Food Safety and Inspection Service (FSIS) (USDA, 2017). The Food and Drug Administration (FDA) has jurisdiction when raw meat is 3% or less in a product, less than 2% of cooked meat, or a close faced sandwich (2017). Game meats in the U.S. are under the jurisdiction of FDA and inspection for game meats by the USDA is voluntary for a fee (USDA, 2011).

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 misbranded when the label of the product is misleading, intended to be sold under a different name, or is an imitation but is not labeled as such on the packaging. 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).

In 9 CFR 320 the required items that should be documented while grinding beef include the date and time of grinding, name of supplier, lot number with pack date, number of establishments supplying the material, time and date when grinder and equipment was last cleaned, and a comment section (USDA 2015) (Figure 2). However,

these requirements are only for beef. Currently, there are no sanitation procedures established for grinding pork or other species.

Store Name: _____
Store Address: _____
_____

FRESH GROUND BEEF PRODUCTION LOG/TRACKING LIST

Employee Name: _____ Today's Date: _____

Time of Grind	Lot/ Batch Number (lot = same source material)	Exact Name/ Type of Product Produced	Package Size of Product Produced	Amount (in pounds) of Product Produced	Production Code of Product Produced	Manufacturer, Name of Source Material Used for Product Produced	Supplier Lot Numbers, Product Code and/or Pack Date of Source Material Used	Establishment Information from label of Source Product Used (Est. #, ph #, contact info)	Grinder Cleaned and Sanitized Between Source Materials? If Y, Date and Time	Comments

_____ _____
Signature of Store Management Reviewer Date

Figure 2 Log template of meat grinding sanitation (USDA 2015)

2.4.Consequences of species mislabeling

Mislabeling of species can lead to possible health consequences and interfere with religious practices. These concerns are addressed in the following subsections.

2.4.1. Health consequences of species mislabeling

Misbranding of meats can cause serious health consequences, especially for patients with red meat allergies. Pork, beef, and other mammalian meats are considered red meats. Anaphylaxis to red meat is a condition that starts after a lone star tick bites the victim. Upon consuming red meat, the onset of an allergic reaction presents itself a few hours later as hives (Wolver et al., 2013). The lone star tick bite triggers sensibility of mast cells to the alpha-gal present in mammalian meat, triggering the antibody IgE to

binds the antigen and release mediating products such as histamine (Wolver et al., 2013). It is recommended that patients with this condition avoid mammal meats like beef, pork, lamb, and venison because exposure could be life threatening in severe reactions (Wolver et al., 2013). However, undeclared red meats have been found in turkey purchased from local butchers and an online specialty store (Kane & Hellberg, 2016).

Kemp, Lockey, Wolf, and Lieberman (1995) found that out of 266 subjects with a history of allergic reactions in the U.S, six allergic reactions were due to beef and four to pork. Also, the degree of seriousness of allergic reactions depends on the concentration of meat and the type of tissue used. Hilger et al. (2016) found that pork kidney caused the fastest allergic reaction compared to the muscle tissues of pork and beef. Skin prick test results showed 100% sensitivity to pork kidney, but 42-63% for raw pork muscle and 33-38% for raw beef muscle (Hilger et al., 2016).

Another problem to consider is the presence of toxins or drug residues that the adulterating species might contain. For example, European Union Law (EUR-Lex, 2013) requires testing for phenylbutazone when there is horse meat present. Phenylbutazone can interfere with blood clotting, cause ulceration, and damage kidneys and liver if more than 4.4 mg/kg body weight is consumed (Lees, UK, Toutain, & France, 2017). A burger containing 100% horse meat made from a horse where a therapeutic dose of 4.4 mg/kg body weight of phenylbutazone was given and slaughtered 12 hours later would be expected to contain around 20 µg of phenylbutazone. Children weighing 40 kg would experience a concentration of 0.5 µg/kg bodyweight of phenylbutazone. However, more studies should be performed to determine the actual amount of phenylbutazone consumers may be exposed to from adulterated horse meat (Lees et al., 2017).

Another safety issue is that the potential pathogens in meat can vary depending on the species. Pork is associated with the parasite *Trichinella spiralis*, which can be contracted when pork contaminated with the organism is consumed. Additionally, the bacterium *Yersinia enterocolitica* is largely associated with undercooked and contaminated pork intestines (2013b). Therefore, the USDA recommends an internal cooking temperature for pork to be 145 °F (USDA, 2013b). The presence of undeclared pork in an undercooked beef hamburger patty could give rise to additional health concerns other than those associated with beef.

2.4.2. Religious consequences

In Jewish and Muslim communities, consumption of pork is prohibited. However, pork has a lower cost compared to beef and it has been detected in commercial beef products (Erwanto et al., 2014). Addition and mislabeling of pork in beef products infringes on religious practices (Ali et al., 2012). In Erwanto et al. (2014), twenty meatballs from nine shops in Indonesia were tested using PCR-Restriction Fragment Length Polymorphism (RFLP) and nine tested positive for pork. Eight meatball shops claimed that their meatballs only contained beef meat while only one shop declared pork as one of their meatball ingredients. About 90% (3 million) of the population around the restaurants serving the experiments' sample meatballs were Muslims. It was concluded that adulteration occurred as unintentional cross contamination or as EMA because pork is cheaper than beef (Erwanto et al., 2014).

Products marketed as Halal should not contain pork. However, Amaral et al. (2017) found that 6 out of 15 canned meat products collected in Canada and labeled as Halal tested positive for pork (pork proportion mean ranged from 0.26 to 6.61 (%w/w).

Additionally, 16 out of 26 processed ground meats and sausages labeled as Halal contained pork (Amaral et al., 2017).

2.5. DNA-based methods of identification of meat

DNA-based methods, such as DNA barcoding (Hellberg, Hernandez, & Hernandez, 2017; Kane & Hellberg, 2016; Quinto et al., 2016) and PCR (Keyvan et al., 2017; Quinto et al., 2016) have been used for species identification in meat products. However, qPCR or ddPCR are preferred in instances where quantification of targeted species is necessary (Amaral et al., 2017; Soares, Amaral, Oliveira, & Mafra, 2013).

2.5.1. DNA sequencing

DNA sequencing has been used to identify species in meat samples. For example, Kane and Hellberg (2016) tested 48 samples using a DNA sequencing method called DNA barcoding to confirm the identity of the species, and then proceeded with qPCR testing for the samples that failed sequencing. DNA barcoding is highly effective in identifying species in meat, but not in mixed-species samples. However, qPCR can target a species for identification in samples with mixed species (Kane & Hellberg, 2016). Similarly, Naaum et al. (2018) tested 100 samples with DNA barcoding for species identification and ddPCR or real-time PCR for targeted detection and quantification of some species. DNA barcoding identified five adulterated sausage meats of turkey with chicken. Following DNA barcoding, ddPCR confirmed that other samples were also adulterated with multiple species, and real-time PCR identified one sample with horse meat.

Hellberg et al. (2017) used full and mini DNA barcoding to identify red meat and poultry species in a variety of products. The study targeted cytochrome *c* oxidase subunit

I (COI) and sequences were identified to the species level with the Barcode of Life Database (BOLD) and GenBank. Overall, DNA barcoding was successful in identifying most beef, pork, bison, buffalo, lamb, and poultry products, except for ground chicken. DNA barcoding may have been unsuccessful in ground chicken because the sample could have been mixed with other species that were not declared in the label. In total, full barcoding identified species in 68% of samples tested, while mini barcoding identified species in 38% of products. However, mini barcoding identified species in 24% of canned products compared to 19% with full barcoding (Hellberg et al., 2017).

2.5.2. Real-time PCR (qPCR)

Real-time PCR is a DNA-based method that uses a fluorescent dye to signal DNA amplification by cycle (Ali et al., 2012). The amount of DNA amplified can also be quantified based on the level of fluorescence released (Ali et al., 2012). qPCR is advantageous over PCR because it is more accurate, rapid, and does not require the use of gel electrophoresis (Amaral et al., 2017).

For pork quantification, Evagreen® with qPCR has been used in raw and autoclaved pork and beef (Amaral et al., 2017). The target gene for pork was cytochrome b. A binary mixture of these two species ranging from 0.0001% to 10% pork was tested and a standardized curve was made to be used for quantification of pork. Amaral et al. (2017) found that qPCR could detect as low as 0.01 pg of pork DNA. After building standardized curve to validate the results, known mixtures of pork and beef were tested against the curve. Results showed that binary mixtures of pork and beef could be quantified using the procedure described in the experiment (Amaral et al., 2017). Kang and Tanaka (2018) used the same method as described in Amaral et al. (2017) but

adjusted the number of cycles to 30 because signals were shown at cycle 40 for negative controls. Similarly, Safdar and Abasiyanik (2013) quantified pork in a mixed sample consisting of beef, poultry, mutton, vitamin A, wheat, and rice to create a sample reference to later test for mislabeled species on pet foods, using Evagreen® fluorescence dye with qPCR targeting 12S rRNA and mitochondrial DNA. Results showed that pork DNA was detected at 50 ng and at 0.003% of pork (Safdar & Abasiyanik, 2013).

In Ren et al. (2017), qPCR was used to identify and quantify poultry and mammal species using the gene *myostatin*. Samples (n=12) consisted of different mixtures of minced chicken, pork, beef, sheep, duck, and rat. Fluorescent signals showed that the assay was able to detect very low levels of DNA (minimum 0.001% DNA) at Ct 23.13 to 24.51 and LOQ was 0.01% DNA. Test method used a standard curve where linearity was proven by calculating regression correlation and was proved to be specific, reproducible and repeatable (Ren et al., 2017).

Real-time PCR with TaqMan probe targeting mitochondrial (mt) genes is highly sensitive and reliable (Ali et al., 2012). The limit of detection was 0.01% when pork was mixed with beef burger patties, with an accuracy of at least 90% (Ali et al., 2012). Similarly, the limit of detection was also 0.01% when targeting mitochondria D-loop section on mixed samples (Kim, Yoo, Lee, Hong, & Kim, 2016). However, TaqMan™ probes are more expensive than EvaGreen, which can also detect and quantify 0.01% pork (Amaral et al., 2017). EvaGreen Master Mix for qPCR is $0.35 per reaction (Lamda Biotech 2017), compared to TaqMan™ Fast Reagent Starter Kit of US $0.43 per reaction, in addition to expensive TaqMan™ probes (ThermoFisher Scientific 2017).

2.5.3 Droplet digital PCR (ddPCR)

Another quantification method using PCR is droplet digital PCR (ddPCR) (Shehata et al., 2017). The ddPCR uses an artificially made recombinant plasmid with DNA sequences derived from mitochondrial DNA. Each mixture is separated into ten thousand or more droplets where PCR reactions takes place. This method is specific, with a limit of quantification of 0.01-1% for heat treated pork and chicken and 0.005-10% for beef and turkey (Shehata et al., 2017). Repeatability and reproducibility of this method was tested with three different samples and the results showed a relative standard deviation of below 20%. Shehata et al. (2017) concluded that ddPCR was a reliable method to detect species substitution. This method was used by Naaum et al. (2018) and Shehata et al. (2019) in their market survey of sausages sold in Canada (discussed in section 2.2).

Cai et al. (2017) tested mixed samples of pork, beef, chicken, and mutton with duplex ddPCR for identification and quantification. Samples were tested individually and as mixtures and quantification of DNA was calculated by comparing the linear relationship of quantity of meat in micrograms (mg) and nucleic acid in nanograms (ng). Cai et al. (2017) determined that the LOD for pork and beef was 0.1 ng/µl and the LOQ was 0.5 ng/µl. However, when unknown samples were tested with the derived linear curve, deviation for pork from observed to actual amount of pork was from -21.88% to 23.33%.

2.6. Rationale and Significance

Adulteration of meats, poultry, and game meat is a global problem that needs to be addressed. It not only is misleading and fraudulent, but it can cause serious harm such as allergic reactions to proteins, and religious interference (Ali et al., 2012; Wolver et al., 2013). If meat products are substituted with secondary meats not suitable for

consumption, such as horse that has been treated with phenylbutazone, there is a chance of developing kidney, liver, and digestive system problems (Lees et al., 2017).

Real-time PCR can be used to identify target species in meat products. Many researchers have used this method to identify undeclared species in ground meat products, but it has been difficult to determine whether undeclared species were present as a result of EMA or cross-contamination (Kane & Hellberg, 2016; Keyvan et al., 2017; Naaum et al., 2018). Therefore, the objective of this study is to quantify the effect of poor sanitation procedures on the cross-contamination of animal species in ground meat products

The expected outcome of this study is that it will serve as the basis to differentiate intentional adulteration as opposed to cross-contaminated product and deter fraudulent meat adulteration by providing more information to enforcers regarding appropriate thresholds. Additionally, the results may incentivize meat processing facilities to better sanitize their equipment to avoid cross-contamination in their ground meat products. Misbranding of meat species is misleading and may be dangerous, therefore there is a need for better control and sanitation practices.

3. Materials and methods

3.1. 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 3.4. 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.

3.2. Reference binary species mixture samples

The ability of the qPCR assay to accurately quantify pork in beef was assessed using reference binary species mixtures prior to performing the meat grinding experiments described in section 3.3. 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). The DNA was extracted as described in section 3.4. 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. Following homogenization, the reference sample mixtures underwent DNA extraction in triplicate as described below.

3.3. 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 independent retail establishments (Gould et al., 2011). Upon arrival at the laboratory, the meat samples were cut into 1-inch cubes with autoclaved knives to facilitate grinding. Meat from each species was prepared separately using separate cutting boards and knives to avoid cross-contamination. Prior to grinding, a subsample (50 g) of each species was collected for to verify the presence or absence of pork DNA with real-time PCR (described below). Meat samples were ground using a Kitchener #8 Commercial Grade Electric Stainless-Steel Meat Grinder .5 HP 370W (Shanghai, China). Each grinding session began with 13.6 kg of pork, followed by one of the cleaning treatments described below, then 13.6 kg of beef. A 100-g sample of ground beef was collected at the beginning of each 0.91 kg (2 lb) of meat exiting the grinder for a total of 15 samples. Each 100-g subsample was homogenized with 100 mL of sterile deionized water using a 12 speed Oster® blender (Neosho, MO, USA) for 1 min at speed 6, as described in Perestam et al. (2017). The blender parts were cleaned and autoclaved after each use. Following homogenization, the samples underwent DNA extraction as described in section 3.4.

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

3.4. 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.

3.5. 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.

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.

4. Results and Discussion

4.1. 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. 3). 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).

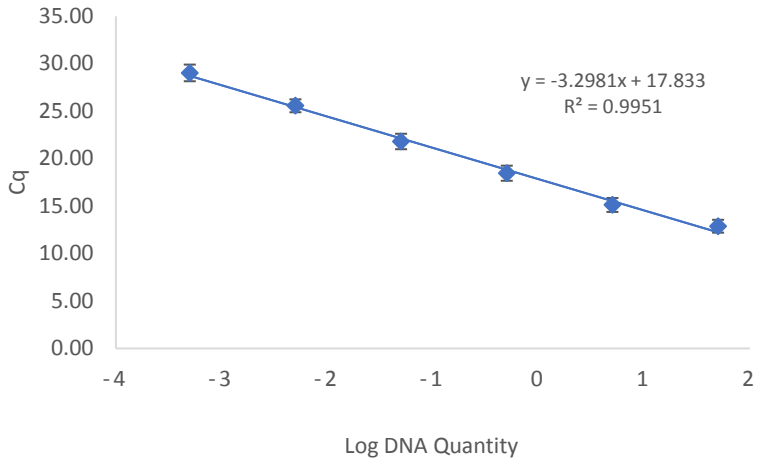

Figure 3 Standard curve obtained for serially diluted pure pork DNA using real-time PCR with a pork-specific primer. Starting DNA quantity ranged from 0.001 ng to 100 ng. Error bars are based on the standard deviation.

4.2. Reference binary species mixtures

Pork was detected down to a level of 0.01% in the pork/beef reference binary species mixtures (Fig. 4). 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 2). The coefficient of variation (CV) for reference binary species mixtures ranged from 6.65% to 53.0% and bias ranged from -45.4% to 97.8%. These values have a wider range than previous studies using the same quantification method, possibly due to the use of a different real-time instrument. Specifically, Amaral et al. (2017) reported CVs ranging from 5.7 to 19.7% and bias of 5.6-10.1% for pork in raw binary mixtures of pork and beef, while Kang and Tanaka (2018) reported bias values of -19.10% to 2.34% using the same quantification method as in the current study for binary mixtures of pork and beef containing 0.25-50% pork (CVs were not reported).

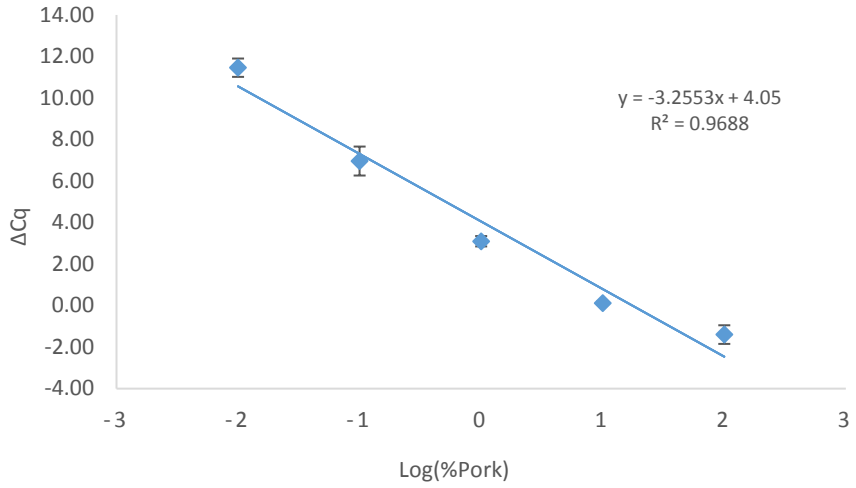

Figure 4 Standard curve for reference binary species mixtures (0.01%, 0.1%, 1.0%, 10%, and 100% pork in beef) analyzed with real-time PCR. The Cq obtained with the universal eukaryotic primers was subtracted from the Cq for the pork-specific primers to obtain ΔCq. The average ΔCq is reported based on three independent assays, and error bars represent standard deviation.

Table 2 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

4.3.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 3).

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).

Table 3. **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

5. 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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