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Food fraud is the act in which food is purposefully mislabeled, adulterated, or contaminated, often to make up a percentage of a missing substance when the product is being made. It is often done purposefully to mislead consumers into thinking some ingredient is or is not present in the food, which can be hazardous to the consumer. This project aimed to validate a forensic domestic animal species reference ladder and multiplex PCR kit that can be used in animal investigations, with a focus on food fraud. A multiplex PCR protocol targeting mitochondrial DNA (mtDNA) markers and a species reference ladder can quickly identify DNA from chicken, cow, sheep, turkey, goat, horse, dog, and cat. To validate the kit, it was tested on five different brands of dog food, specifically four brands of kibble alongside a canned wet stew dog food. DNA extraction was performed using the QIAamp MinElute Column kit or the Qiagen EZ1 investigator kit, then DNA quantification, multiplex PCR, gel electrophoresis, and capillary electrophoresis to build species mtDNA profiles for each food's contents. Results from one dog food kibble yielded some speculation as DNA for sheep and goat contents not listed in the ingredients were detected using both the gel and capillary electrophoresis.

Keywords: *animal food fraud, multiplex PCR, species identification*

Introduction

Food fraud remains a global threat ranging from misleading the consumer of a product purchase to subjecting them to fatal consequences through the serious dangers of unlisted ingredients to the consumer, such as a religious dietary restriction or allergens. As stated by John Spink (Spink, 2021), a well-renowned researcher and director of Food Fraud Prevention Academy at Michigan State University, food fraud appears to be a common form of "occupational crime" (p. 69) which means most incidents occur in a place of employment either by an individual or a collaborative act within a company. The potential for this crime is based on the supply and demand of products which will, in turn, create an opportunity for criminals to take advantage and benefit via financial gain (Manning & Soon, 2016).

Unfortunately, humans are not the only ones at risk of food fraud incidents. Pet food fraud is an ongoing problem, with millions of dollars in lawsuits actively being settled within the last decade. In 2018, Wilbur-Ellis Co., a company that makes ingredients used in the pet food industry, was legally obligated to pay $4.5 million dollars in restitution alongside the arrest of company officials for adulterating pet food ingredients. Officials knowingly replaced chicken and turkey meal with feather meal and feed-grade chicken bone byproduct meal, economically efficient substituents. Alongside fraudulent food production, they misbranded food products through the omission of turkey meal in products being identified as having turkey meal (Cima, 2018).

At present, the Food and Drug Administration (FDA) has procedures in place such as mandatory inspections, DNA sequencing, and food analytical chemistry testing, to monitor food products as they are imported and exported, yet these methods have not been fully efficient. To combat this threat, there has been an increase in development of various forensic DNA-based techniques to help determine if food has been adulterated or contaminated. For example, a Taq-Man Polymerase Chain Reaction (PCR) method for detection of poultry was developed. The specificity of this probe makes it possible to test poultry-based targets such as chicken, turkey, duck, and geese only, without the detection of other bird species (Scholtens, 2017). Another study that used PCR-based methods was conducted in Europe to verify any species substitution or mislabeling of meat from Italian markets (Pinto et al, 2015). They used the cytochrome b mitochondrial gene from bovine, porcine, and equine to identify any adulterants or contamination that could have been present. From this study, they noticed a "high probability of incorrect species declaration in meat products and insufficient labeling information for sausages, pate, and meat patties" (Pinto et al, 2015, p. 3). These techniques consist of extracting and amplifying mitochondrial DNA from known reference sources for analysis and comparison with the labeling on the food product. Mitochondrial DNA is used because it is extremely abundant in each cell and often the degradation rate is much slower compared to nuclear DNA.

The objective of this research was to validate a multiplex PCR detection kit and method for determining fraudulent dog food products. This method targets a universal mitochondrial DNA (mtDNA) marker present in domestic animal species and uses a reference ladder to help identify the unknown sample (Ostenson, 2023). Unlike species-specific DNA kits, this allows for food content analysis of multiple species at one time. The reference ladder is a mixture of the known species DNA generated by using the same multiplex to test

the unknown sample. It allows for accurate species determination and alignment of unknown samples to the known ones in the "molecular ladder". The species in this reference ladder are chicken, pig, turkey, cow, sheep, goat, horse, dog, and cat. These animals are commonly found around the world, making this reference ladder a versatile tool that can be used globally. To validate this kit, this study chose pet food fraud.

Materials and Methods

Sample Preparation

Four dog kibble brands referred to as "Dog Kibble #1", "Dog Kibble #2", "Dog Kibble #3", "Dog Kibble #4" were provided alongside a canned wet dog food referred to as "Dog Wet Food #1". One piece of kibble per brand was crushed and soaked in 1X Phosphate buffered saline (PBS) buffer and Buffer G2 from the Qiagen QIAamp DNA Micro Kit or the Qiagen EZ1 investigator kit, with varying amounts of each buffer according to how viscous the kibble mixture was. Then, 20 µL of Proteinase K was added to all samples prior to each mixture being vortexed extensively, until a slurry was formed. Samples were incubated at 56 degrees Celsius for 20 minutes and then weighed out at ~0.1 grams prior to the addition of another 200 µL of Buffer G2.

DNA Extraction. To perform the DNA extraction, the Qiagen QIAamp DNA Micro Kit or the Qiagen EZ1 investigator kit was used following the manufacturer's instructions. To perform the EZ1 DNA extraction, previously prepared samples were loaded into the EZ1 instrument and run using the manufacturer's "Trace Protocol".

DNA Quantification. For quantification of the extracted DNA, the Qubit 2.0 fluorometer (ThermoFisher Scientific, Waltham, Massachusetts) was used. Using Qubit protocols, 198 µL of working solution was added to each tube. Then 2 µl of sample were added into the tube, which was then inserted into the Qubit instrument. Samples were read three times with the DNA yield averaged.

The final stock solutions following the DNA extraction varied from yields as low as 0.05 ng/µL to some as high as 14 ng/µL, which meant certain samples needed to be diluted prior to the sensitive Multiplex PCR protocol, which works best with samples of 1.0 ng/µL concentrations. The calculations were done using M1V1=M2V2, with the goal of obtaining the volume of water to add to dilute our aqueous extracts. Dilution of products prior to performing the Multiplex PCR was not done for the EZ1 products due to low DNA quantitation yields.

Multiplex PCR. The following step was to perform the multiplex PCR protocol targeting mitochondrial DNA (mtDNA) markers and amplify it on the Proflex PCR System (ThermoFisher Scientific, Waltham, Massachusetts).

Master mixes consisting of 2X Qiagen Multiplex Mix, multi-primer mix (5uM), and Hyclone water were made for samples depending on their final DNA concentration. The final reaction mix was: 6.25 µL 2X Qiagen Multiplex Mix, 1.25 µL Primer mix, and 4.0 µL Hyclone water with the 1.0 ng/µL DNA sample, for a final volume of 12.5 µL. It is crucial to include positive and negative controls while running a PCR, in this case

the negative control consisted of the master mix with an additional one microliter of Hyclone water with no DNA added. The positive control was a known sample of chicken DNA or pig DNA.

Capillary Electrophoresis. A capillary electrophoresis was performed to analyze the fragment lengths for precise identification of species-specific PCR products. The SeqStudio Genetic Analyzer (ThermoFisher Scientific, Waltham, Massachusetts) was programmed using the DS-30 program. The master mix for the loading consisted of HiDi® Formamide and size standard GS ROX 500. The results are interpreted through the ThermoFisher Fragment Analysis app on the Applied Biosystems portal.

Results

In Dog Kibble #1, the main listed ingredients in this brand's dog food were all chicken products which are consistent with the results obtained (**Figure 1**). In Dog Kibble #2, the ingredients in this brand of pet food are consistent with the company claims with animal products listed as: lamb and poultry-by-product meal. With an analytical threshold of 150 Relative fluorescent units (RFUs), the porcine amplicon is called but due to the sensitivity of the instrument and the intense signal from the other peaks, it is most likely an artifact of the highly sensitive capillary electrophoresis (**Figure 2**). In Dog Kibble #3, the main listed ingredients in this brand's dog food are all chicken products which are consistent with the results (**Figure 3**).

In Dog Kibble #4, the main listed ingredients in this brand of pet food are chicken and rice with beef fat being an additional animal product. After three trials, chicken is the only listed ingredient that appeared in the sample. There were no beef products amplified even though beef fat was listed as an ingredient. However, sheep/goat consistently appeared and was amplified at levels high enough for valid allele calls (intensity of signal heights over 150 RFUs). In this multiplex PCR protocol, sheep and goat will cross amplify due to certain primer interactions. Even with those primer interactions in consideration, goat and sheep amplified consistently as a reproducible result (**Figure 4**).

In Dog Wet Food #1, the main listed ingredients in this brand's wet food are all chicken products which are consistent with company claims (**Figure 5**). A known source of DNA from pig tissue was run alongside the trials as a positive control which ensures the PCR was valid and as shown, amplifies and calls as such (**Figure 6**).

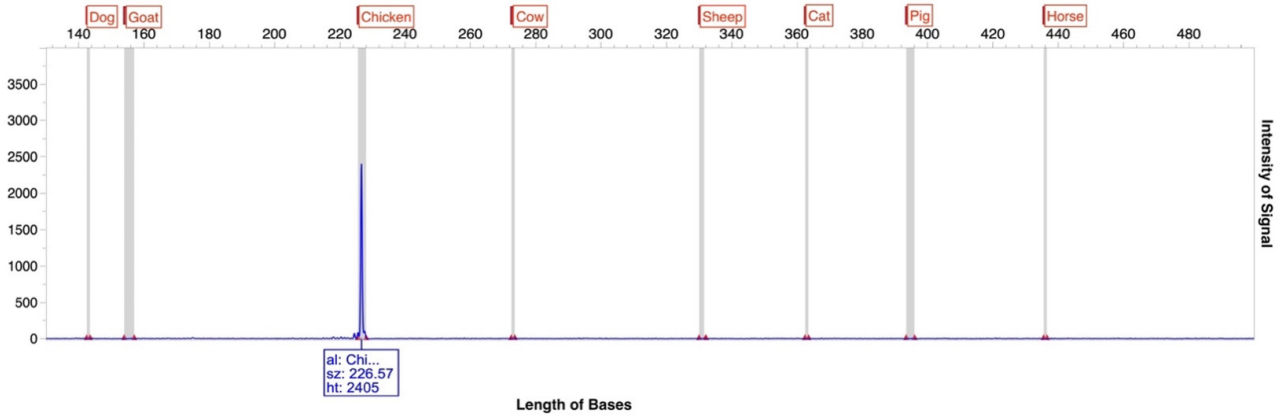
Table 1

Summarized Comparison of Capillary Electrophoresis Plots to Ingredient Lists

Dog Food Brand	Amplicons Found (Ht: RFUs)	Major to Minor Ingredients listed on Food label
Dog Kibble #1	Chicken (ht: 2405)	Chicken, Chicken Liver Flavor, Chicken Fat, Pork Liver Flavor
Dog Kibble #2	Goat (ht: 516) Chicken (ht: 29189) Sheep (ht: 3660) *Pig (ht: 168); above the analytical threshold*	Lamb, (rice, corn), Poultry By-product Meal, Beef Fat Preserved with Mixed-tocopherols
Dog Kibble #3	Chicken (ht: 6328)	Chicken Meal, Chicken Fat, Chicken Liver Flavor, Pork Liver Flavor
Dog Kibble #4	Goat (ht: 176) Chicken (ht: 2574) Sheep (ht: 1347)	Chicken, Poultry By-product Meal, Beef Fat Preserved with Mixed-tocopherols
Dog Wet Food #1	Chicken (ht: 1771)	Chicken Broth, Chicken, Pork Liver flavor, Chicken Liver Flavor

Figure 1

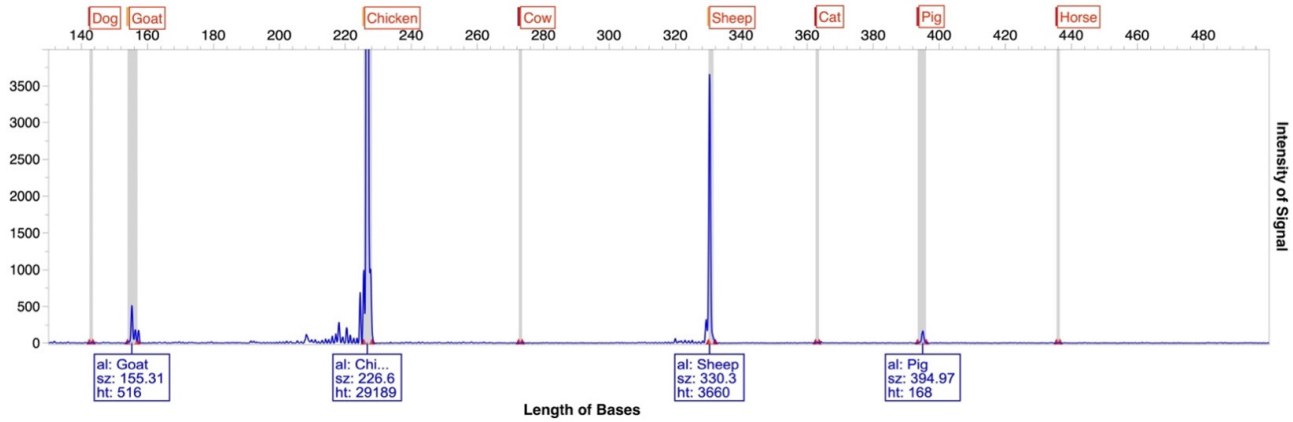
An electropherogram of the results from Dog Kibble #1



Note: The only ingredient, chicken, was consistent with the labeling on the package. The X axis is the length of the amplicon in base pairs, the Y axis is the intensity of the signal in in Relative fluorescent units (RFUs). The box under the peak defines the allele (al) species peak, sz defines the amplicon length, and ht defines the intensity of the peak. The red boxes at the top of the electropherogram are the species marker designations, the vertical grey lines are the binning ranges based on the reference ladder of known species (reference ladder not shown).

Figure 2

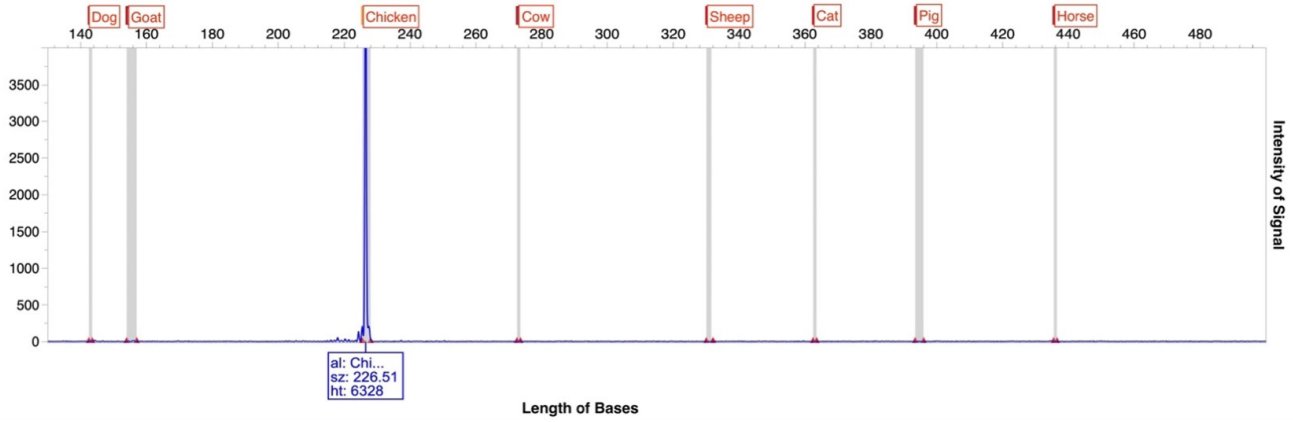
An electropherogram of the results from Dog Kibble #2



Note: Multiple amplicons, representing several species, were shown. The results were reproducible with replication. While it was expected to find sheep/goat as it was sold as Lamb & Rice kibble, it was unexpected that the most intense peak would be chicken.

Figure 3

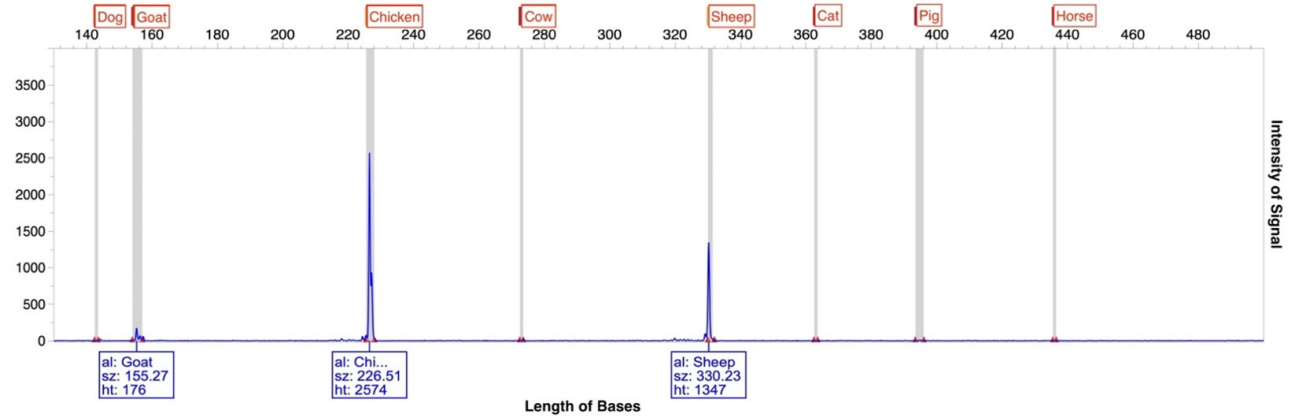
An electropherogram of the results from Dog Kibble #3



Note: This shows chicken as the only animal protein present. This is consistent with the labeling of the product.

Figure 4

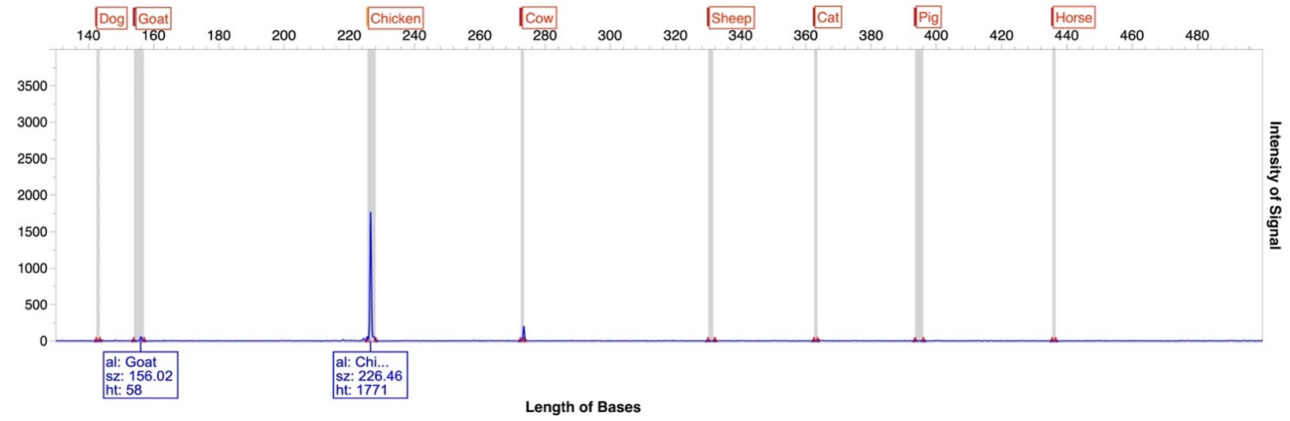
An electropherogram of the results from Dog Kibble #4



Note: This indicates the presence of both chicken and sheep/goat meat products which is not consistent with the label. The label does not indicate any sheep/goat protein should be in the product.

Figure 5

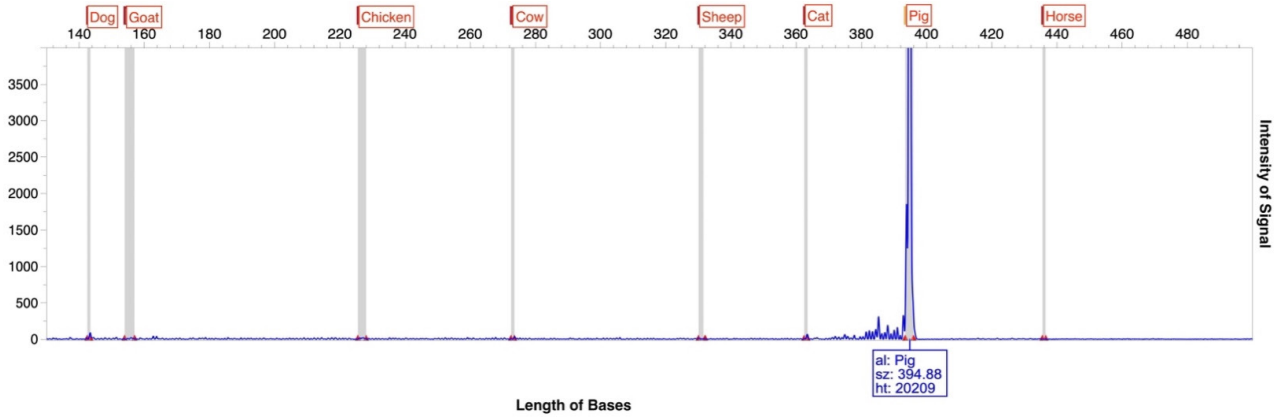
The electropherogram of the results from Dog Wet Food #1



Note: This indicates chicken as the main ingredient. The presence of an amplicon representing goat is below the analytical threshold set for calling a true peak and is therefore, an artifact.

Figure 6

An electropherogram of Positive Control (Pig Tissue)



Note: Used as a positive control and quality assurance marker.

Discussion

Domestic pets are also at risk of being victims of food fraud. Within this study, different dog food brands were analyzed using a non-human species-specific animal reference ladder and PCR multiplex kit with results being compared to the ingredients listed on the packaging for validation purposes. The main forensic intention of using this kit is as a preliminary screening method for food fraud cases where adulteration occurs. Pet food companies can include undefined products in commercially packaged food, and it may pass inspection based on the main protein despite not listing the undefined ingredient. Common examples include: "poultry by-product" and "meat and bone meal" which are likely scraps of unidentified meat which, in some cases, could be from diseased or dying animals. This is illegal (Janisse, 2021, para. 8), but difficult to pinpoint. However, not listing all the protein products could be the cause of many pet allergies because of the undefined diet ingredients.

Racheal Ray "Nutrish Lamb Meal and Brown Rice Recipe" dog food had a lawsuit filed against them in April of 2020 with traces of dog and horse being found after NGS (Next-Gen Sequencing) DNA sequencing (Thixton, 2022). The demand for jury trial documentation's original nature was to file against the claim that the food does not contain wheat, soy, or gluten. However, further analysis showed that the food contained both wheat and soy, as well as dog and horse products (Thixton, 2022). While this motion has not moved forward, there are serious contamination and mislabeling concerns in even organic brands (Thixton, 2022). This form of fraud has become common due to its economic implications, that companies such as Synergy Food Ingredients (Pet food, n.d.) have partnered with private food forensic laboratories for the public to seek services on what ingredients they are feeding their pets.

In this study, "Dog Kibble #1", "Dog Kibble #3", and "Dog Wet Food #1" samples are consistent with the company's listed ingredients. "Dog Kibble #2" samples are consistent with company ingredient claims, but as shown, pig was also amplified. Due to the overloaded samples and the extremely sensitive capillary electrophoresis instrument, this can create "noise" which causes pull up of certain amplicons. The intensity of

the other samples compared to the pig fragment can be interpreted as the pig amplicon being an instrument artifact. Further research should include 1:100 dilutions of samples to avoid the pullup. "Dog Kibble #4" samples had ingredients which are not listed but amplified during this study, specifically sheep/goat. In a forensic setting, the next steps after using this multiplex protocol as a preliminary screening tool would be to run several species-specific kits as confirmatory tests. Findings such as the ones found in this research study would garner further legal investigation in a real-world setting in whether these samples were contaminated (ex: potential factory production line contamination of different meat products without thorough cleaning) or purposefully adulterated (ex: mislabeled for economic gain). Even though the results from this study indicated additional meat products in some of the kibble, it cannot detect where that cross contamination or possible adulteration occurred.

As mentioned, the multiplex kit tested would serve as a screening tool prior to using a species-specific kit. Currently most kits on the market are species-specific, for example, the "RapidFinder Poultry ID kit" by ThermoFisher Scientific. This species-specific kit is used for the detection of poultry meats using real-time PCR. This kit includes a primer/probe set targeting mitochondrial DNA. Because this kit will only identify poultry-based products if there was another contaminant of a different species present, the results would not show this leading to a "false positive" in regard to food fraud. Real-time PCR assays are the common in most species-specific kits used today. The multiplex kit validated in this study would make it possible to first narrow down a potential source, allowing for confirmatory analysis using a species-specific kit to identify the unknown source(s).

Another kit with wider implications currently going through the validation process is the "Low Cost and Density (LCD) Array", which uses DNA biochip technology to detect 24 animal species simultaneously (Beltramo C et al., 2017). The biochip detects the mitochondrial cytochrome b gene in both wild and domestic species that are used for human consumption. The kit is also highly sensitive, as it detects DNA in meat with a sensitivity at 0.5% and milk matrices at 0.1%, specificity, with reproducible results (Beltramo C et al., 2017). The study showed that the kit was able to draw analysis from samples of raw/pasteurized meat, heated meat, and milk matrices. Therefore, it proves promising as it can handle processed food. However, unlike the method used in Beltramo et al., the multiplex PCR kit observed in our study can obtain results from using standard forensic analysis equipment. Labs would not need to worry about additional costs of new instrumentation. In addition, the array protocol would take more time as it takes 90 minutes for the PCR to run and then an additional 45 minutes for the array protocol (Pacific Image Electronics, 2021). The multiplex kit used in this current study can produce results in 90 minutes.

In this validation study, two methods of DNA extraction were used: manual extraction using the Qiagen QIAamp DNA Micro Kit and an automated "modern" approach with the EZ1 instrument. While any method of DNA extraction requires reagents and buffers for lysis, separation, precipitation, and purification; it can be performed manually, which is time-consuming, or with access to instrumentation such as the EZ1. Both capillary electrophoresis and gel electrophoresis (data not shown) methods were performed to view results. While both methods have advantages and limitations when it comes to observing results, the capillary electrophoresis produces an electropherogram, a graph which used the non-human species reference ladder

with a size standard to make very accurate allele calls.

In comparison, the gel electrophoresis, while effective for screening, can still leave room for ambiguity if some amplicons from different species are close in base pair range. In a research study done by T. Matsunaga and team, a forward primer using a sequence from the mitochondrial cytochrome b sequence was made. This primer amplifies sequences from goat, chicken, cattle, sheep, pig, and horse meats (Matsunaga et al., 1999). One of the difficulties faced in this study was being able to tell if the bands on the gel electrophoresis was indicating pig or horse because of how close they were in base pair length. In this study, the capillary electrophoresis allowed for better visualization and reproducibility of results. Ultimately, the validation of this non-human species reference ladder and multiplex PCR represents its potential on a multitude of products including physically and chemically manipulated products like dog food.

Conclusion

This reference ladder and multiplex PCR protocol is a beneficial addition to the forensic field as a screening method prior to using confirmatory species-specific kits when identifying unknown animal products. The multiplex kit consists of nine domestic animals that can be tested in one run as opposed to testing for each species individually. Based on the trials performed, the results were shown to be reproducible making this a reliable tool to use. Future applications of this research could be used to continue animal food testing to combat pet food fraud, a form of fraud with a lack of awareness despite having serious consequences to domestic animals, alongside generalized forensic methods of distinguishing non-human species products or evidence.

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