An Investigation into the Protein Mislabelling in Commercially Available Dog Food

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Abstract
According to The Canadian Animal Health Institute, 7.9 million domestic dogs were owned in 2022 across the country, with an estimated $1,200 spent on food alone for each pet annually. Thus, mislabeling in commercially sold dog food poses a vast and concerning issue, and many studies have shown this to be a continuing problem. In this study, different types of dog food were investigated with the intent of finding a correlation between commercial quality and degrees of mislabeling. It was hypothesized that higher-quality foods should have a lower degree of mislabeling compared to their counterparts. 4 samples of dried dog food and 1 sample of freeze-dried dog food underwent standard PCR analysis protocol, including DNA analysis, PCR amplification, and gel electrophoresis with an additional sample preparation process. Results were unfortunately inconclusive, with most samples failing to develop analyzable molecular bands on the agar gel. Hence, no conclusion could be made regarding the relationship between commercial quality and mislabeling, but valuable insight was provided into the procedure of dried dog food PCR analysis.

Introduction

The Canadian Animal Health Institute estimates that as of 2022 there are 7.9 million domestic dogs owned across Canada - with this number steadily increasing year by year (Bedford). With this many domestic dogs across Canada, the pet food market share is equally as large with Canadians estimated to be spending $1,200 on dog food alone for their pets in 2022 (Bedford). Furthermore, on a national scale, the pet food industry is estimated to generate 4.93 billion dollars in Canada as of 2022 (Bedford, 2023). With the vast number of modern Canadian pet owners comes a powerful market influence, and the shifts they cause based on market trends have been well-documented in the past. For example, the prominent rise in popularity of grain-free and raw dog food diets (Conway and Saker, 2018) was promoted by pet owner decisions, showing the influence consumers have on the pet food industry.

Pet food is highly regulated from processing to labelling. In Canada, there are a number of policies that must be followed before pet food is sold to consumers such as the Canadian Government's *Guide for Labelling and Advertising of Pet Foods* (2001). Canadian labelling standards entail that all components must be present on the label in order of largest percent composition to smallest. Canada is among many nations with such regulations, with the most established regulating body in this sector being the Association of American Feed Control Officials (AAFCO) (Zicker *et al.*, 2008). This is apparent, as within Canada's regulations they refer to AAFCO guidelines repeatedly as to where to look for important data, such as the definition of ingredient categories (Government of Canada, 2001)

Even with labelling guidelines, mislabelled dog food has been detected in multiple studies testing dog food ingredients from multiple source countries. For example, Burdett et al. analyzed 27 Canadian extruded dog and cat foods against their nutrition statements and ingredient lists, and only 9 were found to match what was on the label (Burdett et. al., 2018). Of those foods whose amino acids were analyzed, 25 did meet the general nutrition requirements as modelled by AAFCO, even if the package contents did not align with the outer label. In Italy, a similarly concerning study on dog food sold in Korea determined that mislabelling was present in 4 of 10 samples through the use of DNA barcoding (Lee et. al. 2023), suggesting the global severity of pet food mislabeling.

This experiment aims to extract DNA from 5 locally available pet food products to ascertain if the labelled protein ingredients are all that are present and to determine if higher-quality dog foods were more or less prone to mislabeling than lower-quality dog foods. It was hypothesized that higher-quality dog foods should have fewer instances of mislabelling than those of lower-quality. However, due to the methods applied during dry dog food, or 'kibble' manufacturing, inconclusive results were also expected. Kibble is processed through extrusion in which the kibble is exposed to high heat and pressure environments

(Trang et. al., 2008). During this process, many cell components are damaged and this can affect DNA as well. It was our prediction that if any DNA was extractable, it would be from the higher-quality, more minimally processed samples.

Methods

This study was conducted over four days, using an additional day for preparation. Four types of dried dog food and one freeze-dried dog food were analyzed. The given code names utilized throughout the study are detailed below in Table 1.

Type of Dog Food	Code Name
Performatrin Ultra - Freeze-dried raw bites	FDB
Orijen Regional Red	ORR
Performatrin Ultra - Prairie	PUP
Pedigree Vitality	PV
Blue Buffalo Life Protection Chicken	BBL

Table 1. Sample code names for each type of dog food.

Firstly, all pet food samples were prepared for DNA isolation, with three samples made for each type of dog food. For each sample, material about the size of a pinky fingernail was placed into a 1.5 mL Eppendorf tube. After breaking down the material, 300µL of cell lysis solution lacking proteinase K was pipetted into each sample to be left overnight with the intent of softening samples for DNA isolation.

Following the overnight soak, DNA isolation of samples occurred following typical protocol. A positive control for ground beef was prepared at this point. 1µL of proteinase K was pipetted into all 16 current samples which were incubated at 65^{o}C for a total of 15 minutes, being vortexed every 5 minutes until the solution appeared murky. After icing the samples for 15 minutes following incubation, 15μL of protein precipitate reagent was added to each tube. Each tube was further vortexed and then centrifuged at maximum speed for the first time. The supernatant of all samples was extracted and transferred into a new set of labelled 1.5mL Eppendorf tubes. 500μL of isopropanol was pipetted into each sample and inverted 30-40 times. The samples were centrifuged again for 10 minutes, after which isopropanol was poured off and two sets of ethanol washing occurred. All tubes were left overnight to dry with their cap open.

For PCR analysis, a bulk Master Mix (MM) was created to prepare all samples for PCR. 30μL of a Tris-EDTA (TE) buffer solution was first added to all DNA-isolated samples for proper suspension. Our MM used meat primers in various proportions which amplified different regions of the DNA of goat, chicken, cattle, sheep, pig, and horse meat. 24mL of the MM was pipetted into each of the 17 PCR tubes, along with 1μL of the respective DNA. A negative control was introduced, using 1μL of dH_2O in place of DNA. Each tube was then placed in the PCR machine and subjected to the following cycle: 95^{o}C for 2 minutes, 95^{o}C for 30 seconds, 95^{o}C for 30 seconds, 95^{o}C for 30 seconds, repeat ii-iv 35 times, and 72^{o}C for 5 minutes. Samples were stored in the freezer.

Lastly, our PCR samples were analyzed using gel electrophoresis. 5μL of the 6X buffer was pipetted into each PCR sample and mixed by resuspension. 12μL-15μL of each sample mixture was loaded into the agar gel. The gel was run at 50V until all samples left the wells, which was followed by 120V for a little over an hour until completion. Pictures were taken of the results of the gel for later analysis. A spectrophotometry analysis of BBL and FDB was conducted by Tessa Blanchard after results were recorded to diagnose possible errors in the procedure.

Results

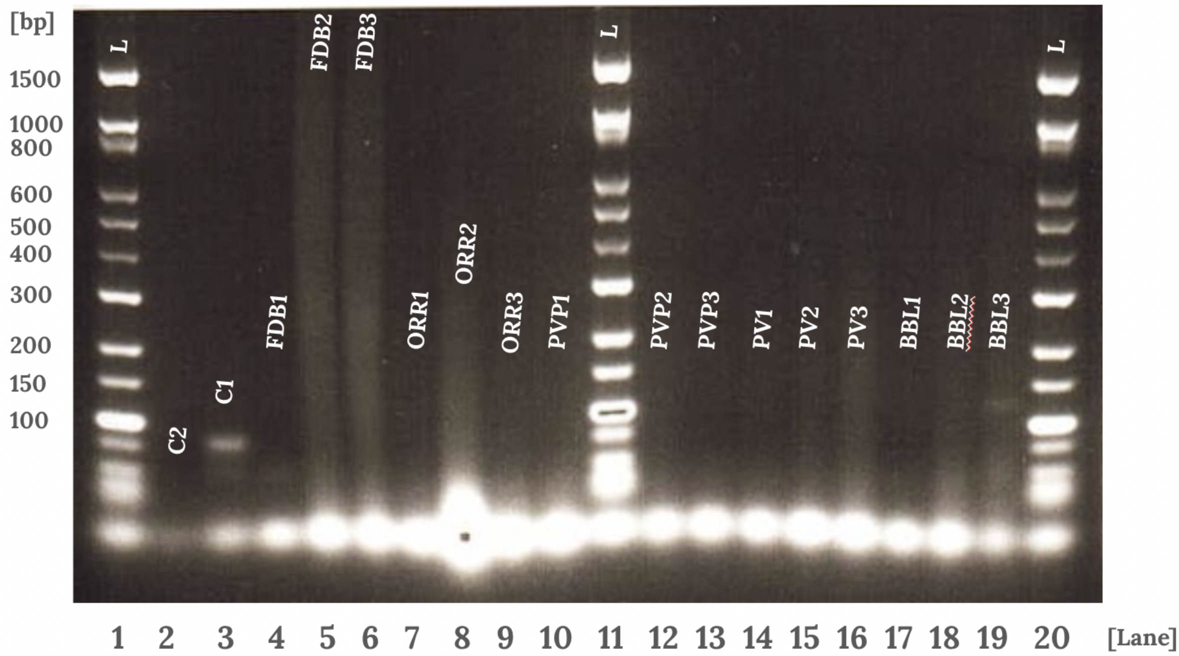

Figure 1: Agarose gel-electrophoresis image of sample DNA and molecular ladder. Sample DNA was extracted and then amplified using PCR. PCR products (amplified DNA) were prepared with 6X buffer solution and loaded into agarose gel wells. Lanes 1, 11, and 20 were loaded with a DNA ladder for reference in determining band sizes of sample DNAs. Gel was run at 50V until all samples left the wells then additionally at 120V for 2-3 hours.

As shown in Figure 1, one band below 100bp appeared in lane 3, for C1, and a faint band among streaks appeared in lanes 5 and 6, representing samples FDB2 and FDB3, between the range of 200-300bp. There is a last band in lane 19, for sample BBL3, between the range of 100-150bp. No bands are seen in the remaining lanes.

	FDB	**BBL**
DNA content	1817µg/mL	732µg/mL

Table 2: Spectrophotometer results on FDB and BBL. Spectrophotometer tests run on an FDB sample and a BBL sample to observe DNA content.

Spectrophotometer tests showed a reading of 1817µg/mL for the FDB sample and 732µg/mL for the BBL sample.

Discussion

Throughout this study, we aimed to find a correlation between the degree of mislabeling and the perceived quality of dog foods by PCR analysis. We tested 12 samples of dried dog food, 3 samples of freeze-dried dog food, and a positive and negative control. After DNA isolation, PCR amplification, and gel electrophoresis of the samples, our results were unfortunately inconclusive due to the failure of most samples to develop molecular bands as seen in Figure 1. Despite the observations of two bands and two more possible bands, all other lanes in the gel failed to produce analyzable results. Therefore, our hypothesis stating higher grade dog foods would have less mislabeling can neither be refuted nor supported as no conclusion can be made.

Although the majority of our samples were unable to generate molecular bands on the gel, our positive control, C1, was able to generate a distinct band while our negative control, C2, did not. It is worth noting that the protein band in C1 is less than 100 base pairs (b.p) and does not correspond to the previously researched molecular band of beef in a study by Matsunaga et al., which would be expected to be around 274 b.p (1999). This result is particularly intriguing, as C1 should be a sample of pure, unaltered beef, so a mystery band may suggest contamination as pork was shown to have a second band below 100 b.p in the study. However, given the general unreliability of results, no cause for this phenomenon can be reasonably justified.

In Figure 1, potential molecular bands can be observed in lanes 5 and 6, representing samples FDB2 and FDB3, between the range of 200-300 b.p. Molecular bands of chicken do exist at 227 b.p. and beef at 274 b.p. (Matsunaga et al. 1999), although the observed bands are not very discrete amongst the streaks left on the gel, therefore, it cannot be clearly determined what meat is being recognized. Given that FDB had only beef content listed in its ingredients, we can infer that these bands correlate to bovine DNA and align with the ingredient labelling. The last molecular band can be observed in lane 19, corresponding to BBL3, between the range of 100-150 b.p. Similar to the result observed in lane 2, this band does not correlate to any meat previously researched by Matsunaga et al. and introduces yet another mystery to the data (1999). BBL samples were labelled with high amounts of chicken ingredients, so the lack of a band at 227 b.p. may suggest mislabeling, but given the general failure of results, no conclusion can be made regarding this observation.

After gel electrophoresis, spectrophotometry was used to analyze samples of FDB and BBL in an attempt to diagnose any potential errors in DNA isolation. With FDB at 1817μg/mL and BBL at 732μg/mL, DNA content should have been high enough for valid PCR results from gel electrophoresis in both samples. However, as previously discussed we only observed possible bands in lanes 5 and 6 and lane 19 yielded a clear, but unidentified, molecular band. Thus, an error likely occurred during the PCR process to yield these results which would not be unexpected given the volatility of PCR.

Despite our failure in results, previous research has shown similar procedures and analyses to bear success. In the review conducted by Olivry and Mueller, many of the studies reviewed used dried dog food as samples for PCR analyses and mislabeling discussions, proving that PCR analysis is possible with these types of samples (2018). Specifically, in the study conducted by Okuma and Hellberg, 9 samples of dry dog food were successful in PCR analysis and were used as valid evidence of pet food mislabeling (2015). The preparation of samples in their study differed from our own, as dry samples were prepared by incubating for 1 hour at room temperature with sterilized water and homogenized with a lab blender. Our procedure instead involved soaking our samples in cell lysis solution without proteinase K overnight instead of incubating. Given the success of this previous study's results, it is possible the success of our experiment was impacted by this procedural choice, as many samples were not adequately softened in time for DNA isolation. Consequently, supernatants were difficult to remove from centrifuged samples due to limited space and layer separation as imaged in Figure 2, likely causing contamination and lower amounts of DNA being extracted, thus causing the lack of molecular bands for ORR, PUP, and PV samples. It also can be considered that our previous research on the extrusion process involved in dry dog food production contributed to the lack of molecular bands in these samples (Trang et al. 2008). From our research process, it can be taken away that PCR analysis of dried dog food does not follow typical processes, and alternative methods of preparation must be attempted to achieve successful results. Future studies analyzing dried pet food should consider using incubation to soften samples or alternative strategies aside from overnight soaking.

Figure 2: DNA isolation samples following initial centrifugation. Following the initial centrifugation, different levels of softening and sample volume impacted supernatant extraction.

Conclusion

The health of household pets is always of utmost importance for pet owners, and the correct labelling of pet food brands is a critical factor in a dog's health. Unfortunately due to the lack of observable data in our study, we are not able to refute or accept our hypothesis that higher-grade pet food brands would have a lower degree of mislabeling. However, this should not discourage future research investigating undeclared meat species in pet foods that could compromise the health of our pets. Research into the best methodology of sample preparation for PCR analysis of dried dog foods would be very beneficial to this area of research, as it would allow for more standardized testing and valuable mislabeling conclusions to be made on a concerning global issue.

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Appendix

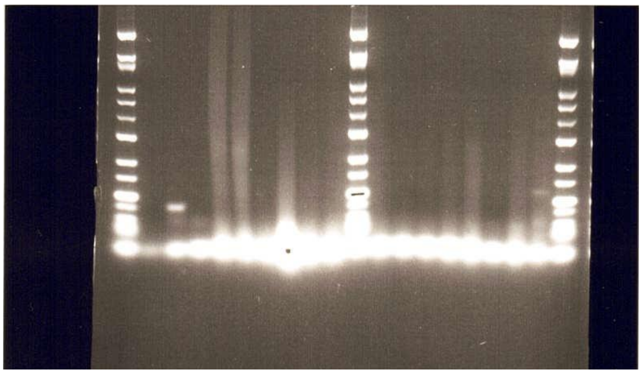

Figure 3. Enhanced contrast image of gel results

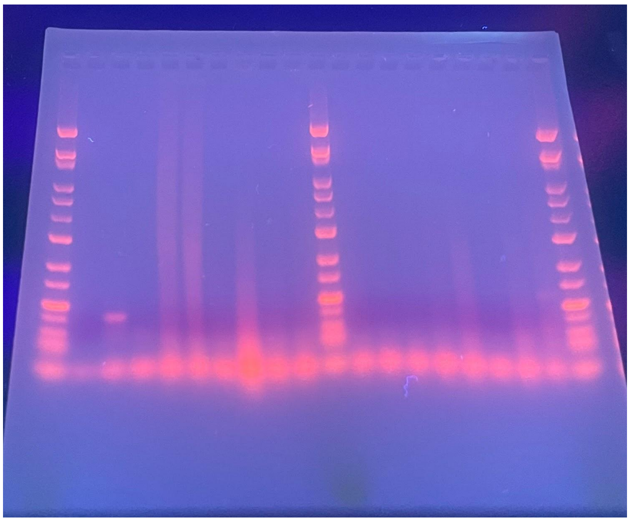

Figure 4. Raw image of gel results.

Meat PCR Master Mix		
COMPONENT	**VOLUME (per tube)**	**VOLUME (Bulk)**
dH_2O	3.6µL	90.0µL
50% glycerol	5.0µL	125.0µL
10X PCR buffer	2.5µL	62.5µL
10mM dNTPs	0.5µL	12.5µL
25mM $MgCl_2$	1.5µL	37.5µL
5’ Primer Meat Forward SIM (10µM)	1.0µL	25.0µL
3’ Primer Goat “G” (10µM)	0.2µL	5.0µL
3’ Primer Chicken “C” (10µM)	3.0µL	75.0µL
3’ Primer Cattle “B” (10µM)	0.6µL	15.0µL
3’ Primer Sheep “S” (10µM)	3.0µL	75.0µL
3’ Primer Pig “P” (10µM)	0.6µL	15.0µL
3’ Primer Horse “H” (10µM)	2.0µL	50.0µL
Taq Polymerase (1000U/200µL)	0.5µL	12.5µL
TOTAL	24µL	600µL

Table 3. Master Mix ingredients and volumes used for the bulk mixture.