What's In Your Pet's Food? A Study of Protein Contamination in Raw Pet Food Products.

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Abstract

Contamination of raw pet food products with unspecified animal DNA can jeopardize pet safety and waver the customer's trust in its quality. While raw pet food diets are becoming more popular with pet owners, research and control over protein contamination is below par. Our team took 5 raw meat products (beef, chicken, kangaroo, pork, and sheep) from the local pet food manufacturer, hypothesized to be contaminated. The sample DNA was amplified and quantified using DNA isolation, PCR and gel electrophoresis, and gels were analyzed to determine contamination or lack thereof. We found that beef and chicken products were not contaminated with any of the tested primers' DNA (beef, chicken, goat, horse, pork, sheep), but kangaroo, pork, and sheep samples showed contamination with bands that had a size predictive of beef DNA (274bp). The kangaroo meat contamination is suspected to be caused by a region of similarity between beef and kangaroo mitochondrial D-loop cytochrome B sequences, which could have led to a false positive. Further research should be done into kangaroo, sheep, and pork raw meat products to get a better understanding of the extent of protein contamination.

Introduction

Raw meat-based diets (RMBD) are a subset of commercially available diets for dogs and cats, consisting primarily of raw meat, muscles, bones, and organs[1]. Over the past decade, RMBDs have grown in popularity, despite the lack of evidence for associated health benefits and the established concerns for pet and human health[1]. However, RMBDs have well-documented use in elimination diets to diagnose pets' adverse food reaction (AFR), which manifest as dermatological and gastrointestinal disturbances to certain dietary components[2]. Upon suspecting food allergies, 33% of dog owners and 29% of veterinary specialists have been found to switch their dogs' food to a raw diet, respectively[3]. To manage their pets' AFR, proponents of elimination diets rely on accurate ingredient labeling to ensure that the pet food contains ingredients permitted in an elimination diet.

The transfer of DNA and large particles, also known as cross-contamination between two products, can occur in RMDBs due to poor handling of meat products, insufficient sanitation, or due to meat adulteration (the replacement of one meat type with another of lower economic value)[4]. Raw animal-containing products used in RMBDs are particularly susceptible to adulteration, with studies showing high numbers of tested pet foods containing undeclared species[5]. Commonly consumed beef and chicken species are more economically preferable to less popular species such as horse, goat and sheep as they are more commonly consumed and leave more animal by-products[5]. Studies on RMBDs point to chicken, beef, and pork as the most common undeclared species[6]. Beef and chicken have been found to be some of the most commonly reported ingredients causing AFR in both dogs and cats[12,14]. Elimination diets would therefore be unsuitable and potentially harmful if conducted with contaminated RMDBs and/or improper labeling, which aligns with current literature[15].

In this article, any mentions of protein contamination will refer to the presence of DNA from animal species not listed on the pet food label; the word 'protein' is used from a food source perspective to describe varying animal species in our samples. A similar definition has been outlined in studies that revealed discrepancies between protein analyses and food labeling in over 75% of tested diets[7,8]. Furthermore, multiple studies have detected chicken DNA and other undeclared DNA of animal origin within canine pet diets using polymerase chain reaction (PCR)[9,14,16,17]. In line with aforementioned methodologies, this study will use PCR technology to test commercially available raw pet food diets (beef, chicken, kangaroo, pork, sheep) for the presence of animal DNA other than what is listed on the label. This will be accomplished by using primers that can distinguish different animal meats from one another by targeting the

mitochondrial D-loop cytochrome B sequences in their DNA as each animal species has a specific number of base pairs (bp) for this sequence[18].

Additional risks for ingredient mislabeling in RMDBs include a lack of national pet food regulations[10] present particularly among small producers that sell domestically[10]. RMDBs can be purchased in most pet stores and are not regulated in ingredient labeling[11]. Canada's labeling guidelines recommend all ingredients, major or minor, to be listed on the pet food label[11]. Therefore, we are predicting contaminants to be present in our raw pet food samples, across all animal meat varieties.

Methods

A single sample of each type of meat (beef, chicken, kangaroo, pork, sheep) was obtained from a local raw pet food store in British Columbia (Figure 1). From each sample, 3 replicates were extracted, giving us a sample size of n=3 for each raw meat type. A control of sterile distilled H_2O was also used, resulting in a total sample count of 16. DNA forward and reverse primers from Integrated DNA Technologies were used in PCR. Band sizes observed were 157bp, 227bp, 274bp, 331bp, 398 bp, 439bp for goat, chicken, beef, sheep, pig and horse respectively[18].

Figure 1. Raw pet food purchased from the local raw pet food supplier
(Left to right) packaged raw meat containing kangaroo, pork, sheep, chicken, and beef respectively.

DNA Isolation

Small samples from each replicate, the size of ¼ of a pinky nail were added to 1.5mL Eppendorf tubes and carefully mashed with a toothpick. 300µL of Cell Lysis Solution were added with Proteinase K to the tube and incubated at 65°C for 15 minutes, vortexing every 5 minutes. Once cloudy, samples were put on ice for 5 minutes. We then added 150µL of Protein Precipitate Reagent to the tubes and vortexed for 10 seconds, and then we centrifuged all samples together for 10 minutes at full speed (see Appendix, Figure 3). The clear layers of suspended DNA supernatant (300µL) were pipetted to new 1.5mL Eppendorf tubes and the old tubes of fat and protein precipitate were discarded. 500µL of cold isopropanol was added to the new tubes, and carefully inverted around 30 times. The tubes were centrifuged again at maximum speed for 10 minutes. Once complete, the isopropanol was carefully poured off, being cautious of the DNA pellet, and 2 rinses with 500µL of ethanol were done. After carefully pouring off the last of the ethanol, the Eppendorf tubes were left open and on their side on a new piece of paper towel overnight at room temperature to dry overnight. They were rehydrated by laboratory technicians the next day with 30µL of TE buffer solution.

Polymerase Chain Reaction

The master mix for PCR was made using 42.5µL 10X PCR buffer, 8.5µL 10µM dNTPs, 34µL 25µM $MgCl_2$, 17µL of each of the 10µM 5' Primer (Meat Forward SIM) and 3' Primers (sheep, beef, chicken, pig, horse, goat), 8.5µL Taq polymerase, 85µL 50% glycerol, and 195.5µL dH_2O (see Appendix, Table 2). Animal primers were encoded for mitochondrial D-loop cytochrome B regions according to Integrated DNA Technologies. The order of added solvents by largest volume first, and the Taq polymerase was added last. These volumes make 17 tubes, in our case this was 16 running samples and an extra tube's volume for contingency. 29µL of the Master Mix were put into each tube, along with 1µL of the DNA sample, and 1µL sterile distilled water to the control, while the tubes were on ice. Tubes were placed into the PCR machine with a cycle of 5 minutes at 95°C, 35 repeats of (95°C for 40 seconds, 50°C for 80 seconds, 72°C for 80 seconds), and then 72°C for 7 minutes. Once complete the samples were removed and placed in a freezer overnight.

Gel Electrophoresis

Samples were rested on ice as 1µL of 6X loading dye was added to each sample and gently mixed. 10µL of the dyed PCR sample was pulled out of each tube and arranged on a 3% agarose gel by meat type and separated by ladders (see Appendix, Figure 4). The gel was run for 10 minutes at 80V, followed by 45 minutes at 120V. Gel was analyzed under UV light.

Results

The data from the 15 samples of raw pet food (3 beef, 3 chicken, 3 kangaroo, 3 pork, and 3 sheep) were collected from the gel electrophoresis (Figure 2). Each sample band size (bp) was measured and compared against known literature values for the proteins found in the primers (beef, chicken, goat, horse, pork, sheep) and are displayed in Table 2. All three beef (~274bp) and three chicken (~227bp) samples presented singular bands on the agarose gel that were consistent with the band sizes expected from their respective proteins. The three kangaroo samples (~280bp) presented singular bands, indicating contamination as there was no primer added specific to kangaroo meat. The band sizes observed in the kangaroo samples were most comparable to the band size expected from beef protein (Table 1). For the three pork (~398bp & 300bp) and three sheep (~331bp & 280bp) samples there were two distinct bands detected indicating that two different animal protein types were found in each of these sample types. The two distinct bands observed in the pork samples matched the band sizes expected from pork protein and beef protein; the bands observed in the sheep samples aligned best with band sizes expected from sheep protein and beef protein (Table 1).

Figure 2. Image of gel electrophoresis on a 3% agarose gel for each PCR product.
PCR products consist of each of our raw pet food samples treated with primers targeting specific proteins from beef, chicken, goat, horse, pig and sheep with expected base pair sizes of 274, 227, 157, 439, 398, and 331 respectively. Samples consisted of three replicates (1,2,3) of beef (B), chicken (C), kangaroo (K), pork (P) and sheep (S). Distilled water (H2O) was used as a control. Ladders (L) were inserted between approx. 3 samples. The gel was exposed to UV light and a photo of the gel taken with an iPhone 11.

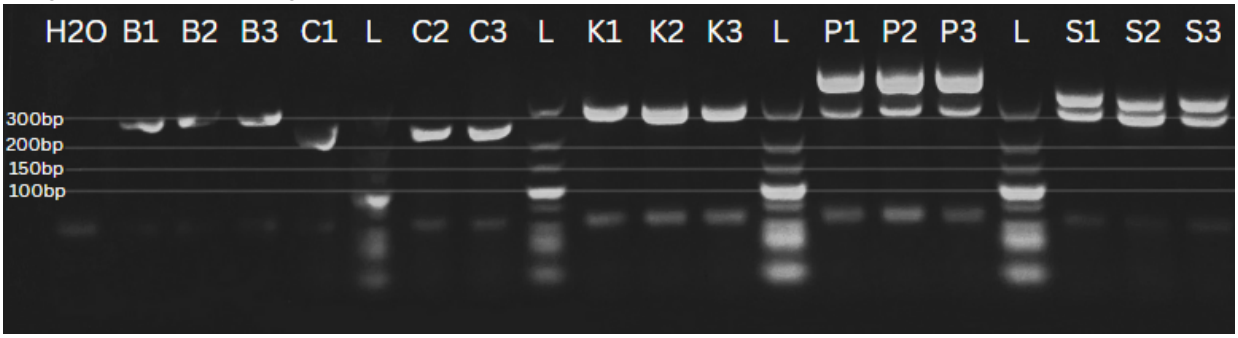


Table 1. Observed data from analyzing bands from gel electrophoresis.
Expected band size and approximated band sizes observed on 3% agarose gel for each sample. Approximated band sizes for samples with protein contaminants were compared to expected band sizes for each protein type to determine the type of contaminant present.

Sample ID	Replicate	Raw Meat Types	Expected Band Size (bp)	Approximate Observed Band Size(s) (bp)	Contaminants Present: lamb, beef, chicken, pig, horse, goat
B1	1	Beef	274	274	-
B2	2	Beef	274	274	-
B3	3	Beef	274	274	-
C1	1	Chicken	227	227	-
C2	2	Chicken	227	227	-
C3	3	Chicken	227	227	-
K1	1	Kangaroo	No band	280	Beef
K2	2	Kangaroo	No band	280	Beef
K3	3	Kangaroo	No band	280	Beef
P1	1	Pork	398	300 & 398	Beef
P2	2	Pork	398	300 & 398	Beef
P3	3	Pork	398	300 & 398	Beef
S1	1	Sheep	331	280 & 331	Beef
S2	2	Sheep	331	280 & 331	Beef
S3	3	Sheep	331	280 & 331	Beef
Control		Distilled H_2O	No band	No band	-

Discussion

Overall, the gel electrophoresis results (Figure 2) from the 15 samples indicate that out of the 5 different raw pet food types, only 2 (beef and chicken) did not contain any protein contaminants as per the primers they were tested against, whereas the remaining 3 varieties (kangaroo, pork and sheep) tested positive for protein contamination (Table 1). The contaminated samples contained band sizes that were identified as beef DNA (~274bp). We initially predicted that we would find contamination in all meat types but our results did not completely support our hypothesis as we only found contaminants in the sheep, pork and kangaroo samples, whereas the chicken and beef samples had no contaminants.

We analyzed 15 samples of raw pet food from a local raw pet food store (undisclosed) from 5 different varieties of meat that they offer, to investigate the presence of protein contaminants in the meats. Each variety of meat (beef, chicken, kangaroo, pork, sheep) was advertised as only containing the labeled protein, yet every single kangaroo, pork and sheep sample contained a band of DNA with a similar base pair size to what would be expected from beef proteins. The detection of two separate bands on the agarose gel for both pork and sheep are also further evidence that there was protein contamination as there should only be one band if the products were pure as advertised.

These results are particularly concerning as there are no requirements in Canada for pet food to have an ingredient statement nor a guaranteed analysis of the food product - the only three requirements that must be on a pet food label are the amount of pet food, the type of pet food (dog or cat) and the location and name of the company producing the food[19]. This is problematic for pet owners that have their pet on an elimination diet as there may be ingredients present that are not listed on the label that could potentially trigger a pet's food allergy or medical condition.

According to the raw pet food manufacturer, the chicken and beef that they supply are obtained locally (British Columbia) but all other meats such as the kangaroo, pork and sheep are imported. This is particularly interesting as the only samples that contained no protein contaminants were the beef and chicken raw meats. Protein contamination of pet foods can occur at any point throughout production (stockyard, manufacturing, packaging) and it's clear from these results that regulations for imported meats may not be as strict as those for meats obtained and processed locally[20].

A finding from our study that we did not expect was the appearance of a DNA band from the kangaroo samples. As we did not have a kangaroo primer added to the PCR master mix, the

presence of no bands would have indicated a pure raw meat sample with no protein contaminants. The band sizes observed in the kangaroo samples closely match the expected size of a beef protein, so it is possible that these samples were contaminated with beef (Figure 2). Alternatively, the primer that was used to identify beef protein could have had enough similarities to the same matching DNA sequence in the kangaroo genome and thus produced a band on the gel if the beef primer aligned with this section of kangaroo DNA. Using of the National Library of Medicine site, it was determined that the mitochondrial D-loop DNA (which codes for mitochondrial cytochrome B) found in beef has a sequence that has a significant similarity such that it can align with the mitochondrial D-loop DNA found in kangaroo meat[21] (Figure 3 & 4). A primer using a different sequence of DNA would need to be utilized to distinguish and verify beef versus kangaroo meat. As a result, it cannot be determined with confidence whether the samples of kangaroo meat contained true protein contamination due to the interactions of the beef primer with kangaroo meat. For future studies, the sequence of beef mitochondrial cytB primer could be compared to the kangaroo mitochondrial cytB sequence for further analysis.

Figure 3: NCBI BLAST Search Parameters

BLAST results were obtained by searching for a nucleotide matchup between GenBank ID MN746797.1 (Giant Kangaroo cytochrome B complete genome) and Bos taurus.

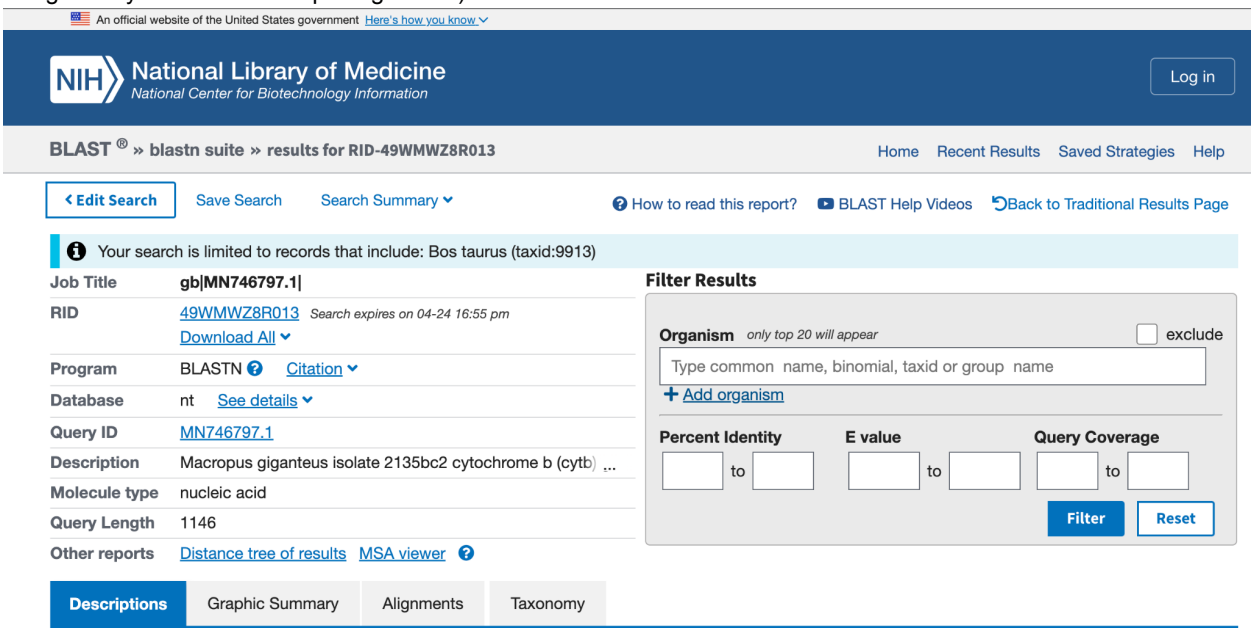
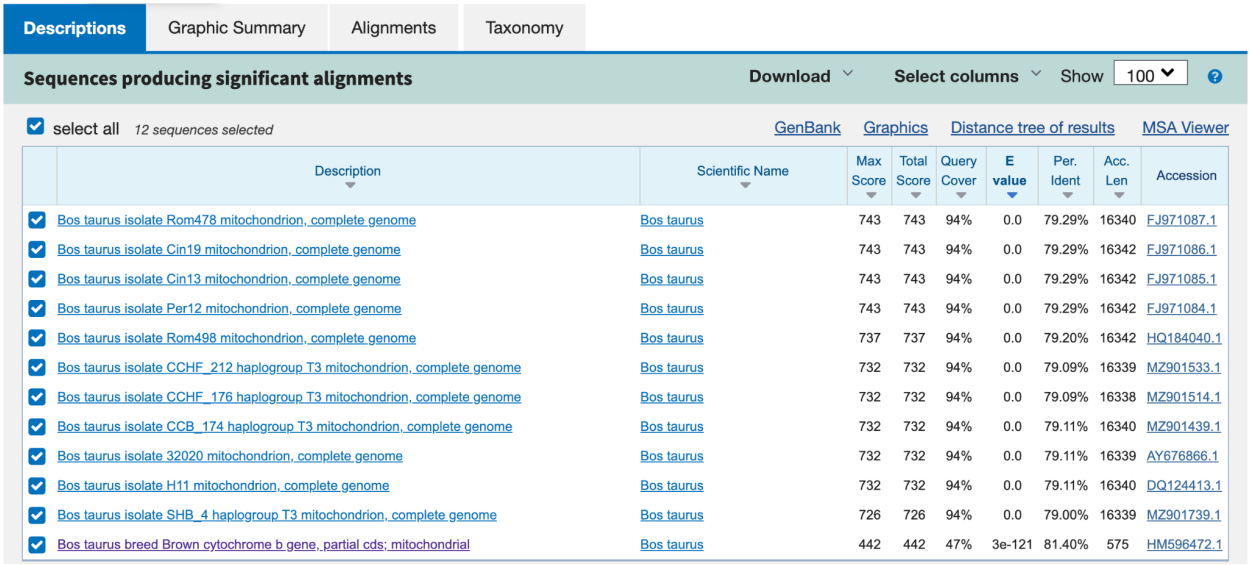


Figure 4: BLAST Results

BLAST results from searching for a nucleotide matchup between GenBank ID MN746797.1 (Giant Kangaroo cytochrome B complete genome) and Bos taurus. Best match highlighted at bottom of page; GenBank ID HM596472.1.



Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments — Download — Select columns — Show 100

select all 12 sequences selected — GenBank | Graphics | Distance tree of results | MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bos taurus isolate Rom478 mitochondrion, complete genome	Bos taurus	743	743	94%	0.0	79.29%	16340	FJ971087.1
Bos taurus isolate Cin19 mitochondrion, complete genome	Bos taurus	743	743	94%	0.0	79.29%	16342	FJ971086.1
Bos taurus isolate Cin13 mitochondrion, complete genome	Bos taurus	743	743	94%	0.0	79.29%	16342	FJ971085.1
Bos taurus isolate Per12 mitochondrion, complete genome	Bos taurus	743	743	94%	0.0	79.29%	16342	FJ971084.1
Bos taurus isolate Rom498 mitochondrion, complete genome	Bos taurus	737	737	94%	0.0	79.20%	16342	HQ184040.1
Bos taurus isolate CCHF_212 haplogroup T3 mitochondrion, complete genome	Bos taurus	732	732	94%	0.0	79.09%	16339	MZ901533.1
Bos taurus isolate CCHF_176 haplogroup T3 mitochondrion, complete genome	Bos taurus	732	732	94%	0.0	79.09%	16338	MZ901514.1
Bos taurus isolate CCB_174 haplogroup T3 mitochondrion, complete genome	Bos taurus	732	732	94%	0.0	79.11%	16340	MZ901439.1
Bos taurus isolate 32020 mitochondrion, complete genome	Bos taurus	732	732	94%	0.0	79.11%	16339	AY676866.1
Bos taurus isolate H11 mitochondrion, complete genome	Bos taurus	732	732	94%	0.0	79.11%	16340	DQ124413.1
Bos taurus isolate SHB_4 haplogroup T3 mitochondrion, complete genome	Bos taurus	726	726	94%	0.0	79.00%	16339	MZ901739.1
Bos taurus breed Brown cytochrome b gene, partial cds; mitochondrial	Bos taurus	442	442	47%	3e-121	81.40%	575	HM596472.1

Some limitations to our experiment that affected the accuracy of the results include potential imperfections in the formation of the 3% agarose gel used during gel electrophoresis. As seen in Figure 2. the bands produced on the left side of the gel appear to be distorted and fainter than the other bands. Due to this discrepancy, it is possible that the approximation of the band sizes for beef and chicken might not be accurate, and were misidentified as being the correctly labeled proteins.

Conclusion

This study looked at five raw meat pet food varieties (beef, chicken, kangaroo, pork, sheep) sourced from a local raw pet food store, to test whether there was protein contamination present. It was predicted that all animal meat varieties would show evidence of protein contamination but it was found that only the beef and chicken samples were not contaminated. The kangaroo, pork, and sheep samples showed evidence of contamination matching that of the beef PCR primer. Due to similarities between the mitochondrial D-loop (cytochrome B region) of cow and kangaroo DNA, it cannot be said with certainty whether the kangaroo sample was contaminated with beef, or the beef primer annealed to kangaroo DNA. As our study only determines the presence of contaminants, future studies could be conducted to investigate the amount of contaminants present within the samples to find out the percent composition of contaminant protein.

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Appendix

Table 2. PCR Master Mix Calculations

MM for 15 raw pet food samples,one control, and a 'contingency' amount (17 total)

Component	Amount	MM (x17) for samples
10X PCR buffer	2.5 μL	(42.5) μL
10 μM dNTPs	0.5 μL	(8.5) μL
25 μM $MgCl_2$	2.0 μL	(34) μL
5' Primer 10µM (meat forward (*SIM*))	1.0 μL	(17) μL
3' Primer 10μM (sheep "S")	1.0 μL	(17) μL
3' Primer 10μM (beef "B")	1.0 μL	(17) μL
3' Primer 10μM (chicken "C")	1.0 μL	(17) μL
3' Primer 10μM (pig "P")	1.0 μL	(17) μL
3' Primer 10μM (horse "H")	1.0 μL	(17) μL
3' Primer 10μM (goat "G")	1.0 μL	(17) μL
Taq polymerase	0.5 μL	(8.5) μL
50% Glycerol	5.0 μL	(85)μL
dH_2O	11.5 μL	(195.5) μL
Total	29 μL	(493) μL
Sample DNA or control	1 μL	N/A

Figure 5. DNA extraction of samples after first centrifuge
Each sample had a lipid layer at the top and a protein precipitate at the bottom.

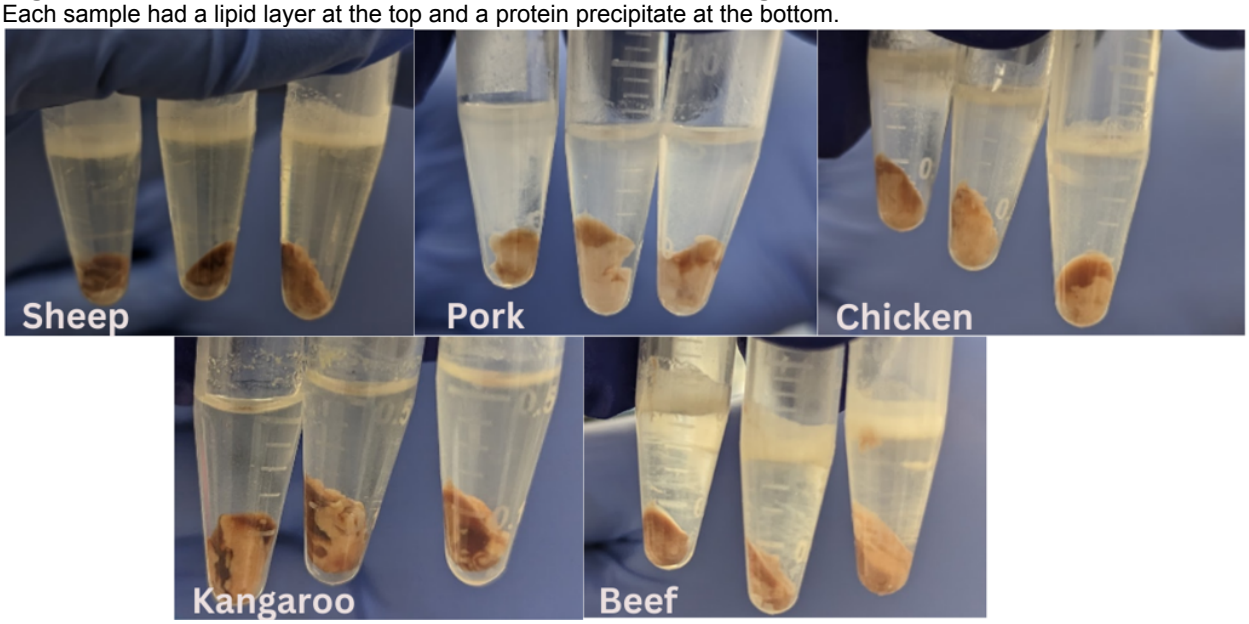

Figure 6. 3% agarose gel loaded with each PCR sample mixed with dye
There is a green ladder separating approx. each three separate samples