Title

Detection of DNA from undeclared animal species in commercial canine and feline raw meat diets using qPCR.

Journal

The Canadian veterinary journal. La revue veterinaire canadienne, 61(9)

ISSN

0008-5286

Authors

Cox, Allison

Defalque, Vincent

Udenberg, Tyler

et al.

Publication Date

2020-09-01

Peer reviewed

Article

Detection of DNA from undeclared animal species in commercial canine and feline raw meat diets using qPCR

Allison Cox, Vincent E. Defalque, Tyler J. Udenberg, Samantha Barnum, Cara Wademan

Abstract — The best diagnostic test for cutaneous adverse food reactions (CAFR) in companion animals is an elimination diet and subsequent provocation trials. Many commercial diets contain novel protein ingredients used in elimination diets, and selection is based on label ingredients. Raw meat-based diets (RMBD) have become increasingly commercially available, gaining popularity despite potential health risks. Reliability of RMBD based on label ingredients has not been investigated. Using quantitative polymerase chain reaction (qPCR), 9 canine and 9 feline commercial RMBD were assessed for reliability of species-specific animal DNA. Two separate batches of each diet were assessed for content consistency. The DNA of 1 or more unlisted animal species was identified in > 60% of diets, as was discrepancy between batches. The unlisted DNA most frequently detected was lamb in canine diets and turkey in feline diets. Based on these findings, use of commercially available RMBD cannot be recommended as an elimination diet in clinical diagnosis of CAFR.

Résumé — Détection par qPCR d'ADN d'espèces animales non-déclarées dans des aliments crus pour chien et chat. Le meilleur test diagnostique pour les réactions cutanées adverses aux aliments (CAFR) chez les animaux de compagnie est une diète d'élimination et des essais subséquents de provocation. Plusieurs diètes commerciales contiennent des ingrédients protéiques nouveaux utilisées dans les diètes d'élimination, et la sélection est basée sur la liste des ingrédients sur l'étiquette. Les diètes à base de viande crue (RMBD) sont devenues de plus en plus disponibles commercialement, gagnant en popularité malgré les risques potentiels pour la santé. La fiabilité des RMBD basée sur les ingrédients listés n'a pas été examinée. En utilisant la réaction d'amplification en chaîne par la polymérase quantitative (qPCR), neuf RMBD canines et neuf RMBD félines commerciales furent évaluées pour la fiabilité de l'ADN spécifique d'espèces animales. Deux préparations séparées de chaque diète furent évaluées pour l'uniformité du contenu. L'ADN d'une ou plus d'espèces animales non-listées fut identifié dans > 60 % des diètes, ainsi que des différences entre les préparations. L'ADN non-listé le plus fréquemment détecté était de l'agneau dans les diètes canines et de dinde dans les diètes félines. Sur la base de ces trouvailles, l'utilisation de RMBD commercialement disponible ne peut être recommandée comme une diète d'élimination dans le diagnostic clinique de CAFR.

(Traduit par Dr Serge Messier)

Can Vet J 2020;61:977–984

Introduction

An adverse food reaction (AFR) is defined as any abnormal clinical response that occurs following ingestion of a food or food component (1). It is often unclear if the pathologic mechanisms of AFR represent a specific immune-mediated response (i.e., type I hypersensitivity) to food antigens, or if the mechanism is related to non-immune intolerance to a component in the food (2). When manifested as dermatological signs, an AFR is termed a cutaneous adverse food reaction (CAFR) (3). In addition to cutaneous signs, gastrointestinal signs, symmetric lupoid onychodystrophy, conjunctivitis, sneezing, and anaphylaxis have been associated with AFR in dogs; gastrointestinal and respiratory signs, conjunctivitis, and hyperactive behavior have also been reported in cats (4). Of these clinical signs, diarrhea and frequent defecation were most often diet-responsive in dogs; in the cat, diet-responsive clinical signs included vomiting and diarrhea (4). Among dogs and cats presented to their veterinarian for pruritus, the median prevalence of CAFR is estimated to be between 15% and 20% (5). In dogs in Australia, Europe, and North America, the most common food antigens causing

North West Veterinary Dermatology Services, Vancouver, British Columbia (Cox, Defalque, Udenberg); Department of Medicine and Epidemiology, University of California Davis, Davis, California, USA (Barnum, Wademan).

Address all correspondence to Dr. Allison Cox; e-mail: a.cox@vetderm.ca

Table 1a. Diet, brand, and declared composition of canine commercial raw meat diets in analysis.

Diet number	Brand	Composition as declared
1	A	Lamb (muscle meat, hearts, livers, bone), fruits/vegetables (kale, carrot, squash, broccoli, apples, cranberries, blueberries), seeds (pumpkin, sunflower), montmorillonite clay, parsley, apple cider vinegar, oils (salmon, coconut), quinoa powder, kelp, alfalfa, Vitamin E
2	A	Turkey (muscle meat, necks, hearts, livers), whole sardines, fruits/vegetables (collard greens, squash, cranberry, blueberry, celery), seeds (pumpkin, sunflower), montmorillonite clay, apple cider vinegar, cilantro, ginger, coconut oil, quinoa powder, alfalfa, kelp, Vitamin E
3	A	Duck (muscle, necks, wings, hearts, gizzards, livers), fruits/vegetables (kale, carrots, squash, broccoli, apples, blueberries, cranberries), seeds (pumpkin, sunflower), montmorillonite clay, parsley, apple cider vinegar, oils (salmon, coconut), quinoa powder, kelp, alfalfa, Vitamin E
4	B	Muscle meat from pork chops/ribs, beef (heart, marrow, bone), pumpkin, vitamins/minerals
5	A	Rabbit (muscle meat, bone, liver, heart), fruits/vegetables (collard greens, squash, celery, cranberry, blueberry), seeds (pumpkin, sunflower), montmorillonite clay, apple cider vinegar, cilantro, ginger, oils (sardine, coconut), quinoa powder, alfalfa, kelp, Vitamin E
6	C	Duck (muscle meat, ground bone), turkey (heart, liver, bone), yeast culture, pumpkin seeds, montmorillonite clay, fruits/vegetables (apples, broccoli, butternut squash, carrots, spinach, blueberries) salt, cod liver oil, kelp, dried, chicory root
7	D	Beef (muscle meat, heart, liver, bone), ground salmon, turkey (muscle meat, liver), fruits/vegetables (cranberries, spinach, broccoli, beets, carrots, squash, apples, blueberries), vitamins/minerals, dried fermentation products as probiotics *(Pediococcus acidilactici, Lactobacillus acidophilus, Bifidobacterium longum, Enterococcus faecium)*
8	D	Beef (muscle meat, liver, kidney, heart, tripe, bone), pumpkin seed, fruits/vegetables (cranberries, spinach, broccoli, beets, carrots, squash, apples, blueberries), vitamins/minerals, dried fermentation products as probiotic *(Pediococcus acidilactici, Lactobacillus acidophilus, Bifidobacterium longum, Enterococcus faecium)*
9	E	Chicken meat, eggs, brown kelp, flaxseed, fruits/vegetables (cabbage, broccoli, chard, carrot, cauliflower, kelp, apple, pear), green mussel, vitamins/minerals, selenium yeast, sunflower oil
10	Royal Canin Ultamino	Corn starch, hydrolyzed poultry by-products aggregate, coconut oil, soybean oil, natural flavors, vitamins/minerals, chicory, L-tyrosine, fructooligosaccharides, fish oil, marigold extract, histidine, rosemary extract, mixed tocopherols, citric acid

CAFR are beef, dairy products, chicken, wheat, and lamb (6). In cats, the most common food antigens that are incriminated are beef, fish, and chicken (6).

The best diagnostic procedure for identifying CAFR in companion animals is an elimination diet with subsequent provocation trials (7). To reliably diagnose CAFR in more than 90% of dogs and cats, elimination diet trials should last at least 8 wk (8) and can be either homemade or commercially produced (9). Although veterinary dermatologists often consider homecooked diets as their first choice, many pet owners prefer the convenience of commercial "novel" or "hydrolyzed" protein diets, and rely on package labels to select diets that do not contain previously fed ingredients (9). There are concerns that these diets may contain unlisted food sources, and that unidentified ingredients might cause clinical reactions in hypersensitive patients (10). Unidentified dietary allergens could preclude a resolution of clinical signs in CAFR-affected patients, yielding misleading results for the elimination diet trial. Inadvertent cross-contamination of pet foods appears common, even in those with "limited ingredients" proposed for elimination diets (10). Rigorous quality control to screen for accidental contamination using real-time or quantitative polymerase chain reaction (qPCR) has been used in both human and animal food manufacturing as a rapid and sensitive point-of-care application to screen for food-borne bacteria, viruses, or allergens, which can be identified at low concentrations (11). This screening method has also been used in the production of veterinary prescription diets to validate their contents in order to avoid inadvertent allergen exposures for sensitized pets (12). Of the quality control analysis methods available, DNA-based protocols are considered most reliable for detecting animal species in processed pet food manufacturing (13).

A subset of commercially available diets includes raw meat-based diets (RMBD). Historically, these diets were fed to racing greyhounds and sled dogs; this feeding practice began to be extended to pets and became increasingly popular in the 1990's (14). Despite evidence of nutritional deficiencies or excesses and potential health risks of feeding raw or undercooked animal-source proteins, advocates of RMBD claim anecdotal health benefits, such as improvement in coat and skin, and a reduction in medical conditions such as allergies (15). The increasing popularity of RMBD has been demonstrated in multiple studies. A 2008 telephone survey revealed approximately 30% of dogs and 15% of cats in the United States and Australia consumed a combination of "unconventional" diets (i.e., homemade diets, table scraps, and/or RMBD) and commercial pet foods (16). A more recent anonymous Internet-based American survey indicated that 46% of dog owners and 38% of cat owners had fed RMBDs to their pets (17). Additionally, sales of RMBD have increased annually by as much as 15% in recent years (18). Over the past decade, this feeding practice has continued to increase, and market locations have expanded to include grocery stores, mass merchandisers, pet specialty stores, and veterinary clinics (19). A 2019 Italian-based survey shed light on dog owners' motivations for adopting this feeding method (20). About 80% of respondents reported that they abandoned feeding commercial diets due to distrust in the clarity of ingredients

Table 1b. Diet, brand, and declared composition of feline commercial raw meat diets in analysis.

Diet number	Brand	Composition as declared
1	D	Duck (muscle meat, bone, gizzard), turkey (muscle meat, liver), goose, pumpkin seed, vitamins/minerals, dried fermentation products as probiotics *(Pediococcus acidilactici, Lactobacillus acidophilus, Bifidobacterium longum)*
2	C	Rabbit (muscle meat, bone, liver, kidney, lung), pork (liver, heart, bone, fat), yeast culture, pumpkin seeds, montmorillonite clay, fruits/vegetables (apples, broccoli, butternut squash, carrots, kelp, spinach, blueberries), salt, cod liver oil, taurine, dried chicory root
3	A	Beef heart, beef liver, ground beef bone
4	A	Turkey (muscle meat, necks, hearts, livers), fruits/vegetables (collard green, squash, celery, cranberries, blueberries, cilantro), seeds (pumpkin, sunflower), almonds, apple cider vinegar, oils (sardine, cod liver, coconut), vitamins/minerals, quinoa powder, kelp, ginger
5	A	Chicken (muscle meat, necks, gizzards, livers), salmon, fruits/vegetables (kale, yams, carrots, apples, broccoli, cranberries, blueberries), seeds (pumpkin, sunflower), vitamins/ minerals, apple cider vinegar, oils (salmon, coconut, cod liver), quinoa powder, kelp
6	A	Rabbit (muscle meat, bone, livers, hearts), fruits/vegetables (collard greens, squash, celery, cranberries, blueberries), seeds (pumpkin, sunflower), montmorillonite clay, apple cider vinegar, oils (sardine, coconut, cod liver), vitamins/minerals, quinoa powder, kelp, ginger
7	F	Chicken (muscle meat, heart, liver, gelatin), water, dried egg yolk, dulse powder, egg shell powder, organic psyllium husk powder, Vitamin E, manganese gluconate
8	F	Beef (muscle meat, heart, liver, gelatin), water, organic dried egg yolk, dulse powder, egg shell powder, psyllium husk powder, Vitamin E, manganese gluconate
9	F	Lamb (muscle meat, heart, liver, gelatin), water, dried egg yolk, dulse powder, egg shell powder, psyllium husk powder, Vitamin E, manganese gluconate
10	Royal Canin Ultamino	Corn starch, hydrolyzed poultry by-products aggregate oils (coconut, vegetable, fish) powdered cellulose, natural flavors, dried chicory root, vitamins/minerals, fructo-oligosaccharides, marigold extract, rosemary extract, glycine, mixed tocopherols, citric acid

used, and 94% reported that they believed in the absolute safety of this feeding method (20). Of the meats selected for canine RMBD, the most frequently purchased were beef, chicken, and turkey (20).

Although feeding RMBD to dogs and cats is discouraged by veterinary organizations and governmental public health organizations (21,22) up to 1/3 of pet owners may choose to use RMBD for the purpose of an elimination diet (23). When a pet owner categorically declines to feed a homecooked or commercial prescription veterinary diet, veterinarians may acquiesce to such requests. However, the reliability of RMBD for this purpose has not been evaluated.

The primary aim of this study was to use PCR to test commercially available RMBD for the presence of DNA of animal origin (beef, chicken, duck, turkey, salmon, lamb, rabbit, kangaroo, pork) other than that declared on the labels. A secondary objective was to determine the consistency of DNA presence between different batches of the same diets. The hypothesis was that the diets would contain unlisted protein ingredients, and that these unlisted proteins would vary between batches. To the authors' knowledge, no previous studies have examined these issues.

Materials and methods

Diets

Nine commercial canine and feline RMBD (Tables 1a, 1b) were selected for analysis. All diets (with the exclusion of feline diet 3) were marketed as balanced for complete feeding. The selected diets included a variety of commonly available North American RMBD, some formulated with novel (less commonly fed) animal source proteins (e.g., rabbit), or limited ingredients (e.g., single protein diets), or grain-free diets which may be potentially selected for use as an elimination diet. The diets evaluated were not specifically marketed for feeding as elimination diets, but contained ingredients which may be considered by pet owners for this feeding purpose. Two lot numbers of RMBD representing separately prepared batches of each diet were selected for analysis in order to assess for consistency of any ingredient contamination between the 2 batches. One canine and one feline veterinary prescription extensively hydrolyzed poultry feather-based diet (Royal Canin Ultamino; labelled as Anallergenic in Canada) were used as negative controls.

Nucleic acid preparation

A 100-mg sample of pet food was added to a 96-well deep well grinding block (Greiner Bio-One; Monroe, North Carolina, USA) with 600 µL of ATL Buffer, 60 µL of Proteinase K (Qiagen, Valencia, California, USA), and 2 stainless-steel beads (Fisher Scientific, Waltham, Massachusetts, USA). The grinding block was sealed and the pet food pulverized in a 2010 Geno/Grinder homogenizer (SPEX SamplePrep, Metuchen, New Jersey, USA) at 1750 rpm for 2.5 min. Lysate was incubated for 15 min at 56°C before 200 µL of lysate were removed and used for total nucleic acid (TNA) extraction. Total nucleic acid extraction was performed on a semi-automated extraction system (QIAamp 96 DNA, QIAcube HT Kit; QIAcube, Qiagen) according to manufacturer's instructions and eluted in 100 µL of diethylpyrocarbonate (DEPC)-treated water.

qPCR assay design and validation

Nine species-specific qPCR assays were designed to detect beef, chicken, duck, turkey, salmon, sheep, rabbit, kangaroo, and pig DNA (Table 2). Sequences for each species were found in the National Institute of Biotechnology Information (NCBI) database. Two primers and an internal hydrolysis probe (5′ end, reporter dye FAM, 6-carboxyfluorescein), 3′ end, quencher dye

Table 2. qPCR assay and sequence information for DNA of each of 9 targeted species.

Species	Forward primer name/ Sequence	Reverse primer name/ Sequence	Probe name/ Sequence with labeling	Gene location	GenBank accession number	Amplicon size (bp)
Beef	B.taurusHBB-1084f GTTGTGCTGGCT CGCAAT	B.taurusHBB-1147r CCTTCTGAAAGT CAGCCTGCA	B.taurusHBB-1103p FAM-TGGCAAGGAATTCA-MGB	HBB	X00376	64
Chicken	GallusBactin-3323f CAGCTGGCCTG CCGG	GallusBactin-3419r AAAGGACCAGGA CCAGTATTGC	TGGCAGAG	TGF-BETA3	X60091	97
Duck	CairinaBactin-1588f CTTTGATTTGTT CAGCGAGCG	CairinaBactin-1710r TCAGTGTACAGG TAGCCCCTCTCT	CairinaBactin-1656p FAM-CCTGCCTAGGAGAGGT-MGB	beta actin	NM_001310421	123
Turkey	Meleagris-303f CGGCCTATATTA TGGTTCGTACCT	Meleagris-398r ACATAGCCTACA AAGGCTGTTGC	Meleagris-354p FAM-AGTCTTACTTCTCA CCCTC-MGB	cytochrome b	L08381	96
Salmon	Oncorhynchus-1453f CCCTGTTGCCTG ATGAACG	Oncorhynchus-1534r ATTGGGCTCACG ATGGAGTC	Oncorhynchus-1479p FAM-CTGAACAAGATATTCCTG-MGB	growth hormone 1	X61938	82
Sheep	O.ariescytoB-513f CAAAGCTACCCT CACCCGATT	O.ariescytoB-642r GTCCGATGGAAT TCCTGTGG	O.ariescytoB-573p FAM-CCTCGCCATAGTTCA CCT-MGB	cytochrome b	KY110724	130
Rabbit	LeporidaeBactin-74f CCATGGATGACG ATATCGCC	LeporidaeBactin-150r ATCGTCGCCCG CGAA	LeporidaeBactin-99p FAM-CGTGGTCGACAATGG-MGB	beta actin	NM_001101683	77
Kangaroo	MacropusNADH-118f GTAGGCCCCTAC GGACTCCT	MacropusNADH-237r GAGGGCTAGGAT TGGGGC	MacropusNADH-177p FAM-ACCCCTACGACCTTT-MGB	NADH	JN003396.1	120
Pig	SusBactin-297f GACGAGGCTGCC GTAAAGG	SusBactin-406r TGCAAGGAACAC GGCTAAGTG	SusBactin-352p FAM-TCTGACGTGACTCCCCGA-MGB	Beta actin	DQ452569	110

NFQMGB (Non-Fluorescent Quencher Minor Grove Binding) were designed using Primer Express Software (Thermo Fisher Scientific, Carlsbad, California, USA) for all species, with the exception of chicken which used a locked nucleic acid probe (Roche Molecular Systems, Pleasanton, California, USA). A Basic Local Alignment Search Tool (BLAST, NCBI) of the amplicons confirmed unique species detection. To ensure these assays did not cross-react with DNA of other species, a cross-reactivity evaluation was performed by running all assays with control DNA from each species (Table 3). A housekeeping gene, eukaryotic 18S assay (Hs99999901_s1, Applied Biosystems, Thermo Fisher Scientific), was run with each sample to confirm successful DNA extraction. All assays were validated for efficiency and sensitivity by running 10-fold standard curves in triplicate from serial dilutions of control DNA. Each assay was 90% to 100% efficient and sensitive enough to detect as few as 10 copies of the target gene.

qPCR reaction

Each qPCR reaction contained 20× primers and probe with a final concentration of 400 nM for each primer and 80 nM for the probe, commercial PCR master mix (TaqMan Universal PCR Master Mix; Thermo Fisher Scientific) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl2, 2.5 mM deoxynucleotide triphosphates, 0.625 U AmpliTaq Gold DNA polymerase per reaction, 0.25 U AmpErase UNG per reaction, and 5 μL of diluted extracted TNA. qPCR was performed using an automated fluorometer (ABI PRISM 7900 HT FAST; Thermo Fisher Scientific). The following amplification conditions were used: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, and 60 s at 60°C. Fluorescent signals were collected during the annealing phase and cycle quantification values (Cq) extracted with a threshold of 0.1 and baseline values of 3 to 10. A no template control (DEPC-treated water) was run with all assays to ensure absence of non-specific binding of the primers and probes. Positive controls (extracted DNA from animal tissue or blood) were run with their respective assay to ensure the assay was working properly.

Results

Of the 9 species of animal DNA tested, 8 species, including pork, chicken, duck, rabbit, lamb, beef, salmon, and turkey, were detected in at least 1 sample of the canine and feline RMBD tested. Only kangaroo DNA was not detected in any of the RMBD.

The 2 extensively hydrolyzed poultry feather-protein based diets (negative controls) contained either trace amounts of chicken DNA (canine) or no detectable DNA (feline).

In the canine RMBD, DNA of 1 or more animal species not indicated on the label was identified in 9 out of 9 diets, in either 1 or both of the tested batches (Table 4a). Of the 18 batches tested, 89% tested positive for unlisted animal-source DNA. An average of 4.4 unlisted proteins was detected in each diet. A total of 2 batches (test diets 1a and 4a) were found to contain

Table 3. Results of cross-validation qPCR assay.

	qPCR assay (Cq)								
	Beef	Chicken	Duck	Turkey	Salmon	Sheep	Rabbit	Kangaroo	Pig
Controls (DNA obtained from tissue or other biological material)									
Beef	20.45	—	—	—	—	—	—	—	—
Chicken	—	21.40	—	—	—	—	—	—	—
Duck	—	—	28.66	—	—	—	—	—	—
Turkey	—	—	—	25.55	—	—	—	—	—
Salmon	—	—	—	—	27.08	—	—	—	—
Sheep	—	—	—	—	—	22.90	—	—	—
Rabbit	—	—	—	—	—	—	25.58	—	—
Kangaroo	—	—	—	—	—	—	—	19.44	—
Pig	—	—	—	—	—	—	—	—	24.41

Quantification cycle (Cq) values out of 40 cycles reported in positive circumstances, '—' indicates no amplification detection (qPCR negative).

DNA consistent with the stated label ingredients. The diet with the greatest number of unlisted proteins was a single batch (2a), labeled as containing turkey and sardine. It contained a total of 6 unlisted proteins (pork, chicken, duck, lamb, beef, and salmon). The unlisted DNA most frequently detected was lamb (n = 12). Discrepancy in the unlisted DNA between batches was noted in 78% of batches.

In the feline RMBD, DNA of 1 or more animal species not indicated on the label was identified in 7 of 9 diets, in either 1 or both of the tested batches (Table 4b). Of the 18 batches tested, 61% were positive for unlisted animal-source DNA. An average of 2.6 unlisted proteins was detected in each diet. A total of 7 batches (test diets 3a, 6a and b, 8a and b, 9a and b) were found to contain DNA consistent with the stated label ingredients. The diet with the highest number of unlisted proteins was a single batch (7a), labeled as containing chicken and salmon. It contained 5 unlisted proteins (pork, rabbit, lamb, beef, and turkey). The unlisted DNA most frequently detected was turkey (n = 7). Discrepancy between batches was noted in 56% of batches. All of the canine and feline RMBD included in the analysis were found to contain the proteins listed on their labels.

Discussion

Contamination of one or both batches in all canine RMBD and most of the feline RMBD tested was detected in this study, which supports the hypothesis that cross-contamination would be found in many RMBD. Numerous independent studies have also demonstrated significant discrepancies between label claims and actual contents of dry or canned over-the-counter (OTC) commercial diets, including those marketed for the management of CAFR (24–28). While this finding may have been expected in RMBD due to the prevalence of unlisted DNA detected in other such studies, diet purity could theoretically have been improved in RMBD as they are purported to undergo less processing before distribution, allowing less opportunity for protein contamination. An additional finding of this study was that unlisted animal source proteins varied among batches in most batches tested, including both canine and feline RMBD that were analyzed. Discrepancy among batches has not been previously studied for comparison, but these results showed that differences in unlisted ingredients were common in RMBD. Due to cost limitations restricting the analysis to only 2 batches of each RMBD, a statistically significant batch contamination rate could not be determined. However, the finding of discrepancy among batches represents yet another variable which could impact interpretation of an ED trial in a patient fed a commercially prepared RMBD.

While no particular manufacturer's diets were found to be more likely to contain unlisted proteins, brand F, the producer of feline diets 7 to 9, had the least number of contaminants, as well as the most consistent agreement between each batch. It is possible that this finding is due to the nature of the processing practices of this particular manufacturer, the production of smaller batch sizes to minimize opportunity for contamination, or more limited sourcing of ingredients to restrict the potential for supplier cross-contamination. Overall, the number of animal protein ingredients included in each diet was not a predictor of the number of unlisted proteins isolated in the analysis. Even diets restricted to single proteins were as likely to contain 1 or more sources of unlisted animal DNA as those with multiple animal proteins and batch contamination was unpredictable.

Previous studies have shown that in rare cases, ingredients listed on product packaging were found to be missing from the analysis (10). Our study showed that no animal DNA was missing from that declared on the packaging of any RMBD included in the analysis. Due to the target DNA of the qPCR assay, the study was unable to validate the presence of sardine (listed in canine test diet 2) or goose (listed in feline test diet 1) to confirm the inclusion of these ingredients. This was again due to cost limitations precluding the addition of these proteins in the analysis.

Some diets also contained animal fat sources such as salmon oil, cod oil, or sardine oil. Through purification processes, fish oils undergo refinement to remove proteins from the oil to render them free of proteins (29). While cod and sardine were not included in the analysis, our study did evaluate for salmon DNA. Both canine diets 1 and 3 contained salmon oil and tested negative for salmon DNA. Feline diet 5 contained salmon oil, but also contained salmon meat, and tested positive for salmon DNA as would be expected. If extrapolating from the finding that salmon DNA was not found in the diets containing salmon oil, it may be expected that the other diets containing fish

Table 4a. Results of analyses of canine diets.

Diet number	Animal proteins in ingredients	Animal fats in ingredients list	Batch	Animal species DNA detected in samples "+" = present, "−" = not detected in 40 cycles of amplification								
				Pork	Chicken	Duck	Rabbit	Lamb	Beef	Salmon	Kangaroo	Turkey
1	Lamb	—	a	−	−	−	−	+	−	−	−	−
			b	−	−	−	−	+	−	−	−	+
2	Turkey, sardine	—	a	+	+	+	−	+	+	+	−	+
			b	−	+	−	−	+	−	−	−	+
3	Duck	—	a	−	+	+	−	+	−	−	−	+
			b	−	+	+	−	+	+	−	−	+
4	Pork, beef	—	a	+	−	−	−	−	+	−	−	−
			b	+	−	−	−	+	+	−	−	−
5	Rabbit	—	a	−	+	−	+	−	−	−	−	+
			b	−	+	−	+	+	−	−	−	+
6	Duck, turkey	—	a	−	+	+	−	−	+	−	−	+
			b	+	+	+	−	−	−	+	−	+
7	Beef, salmon, turkey	—	a	−	+	−	+	+	+	+	−	+
			b	−	+	−	+	+	+	+	−	+
8	Beef	—	a	−	−	−	−	+	+	−	−	−
			b	−	−	−	−	+	+	−	−	+
9	Chicken	—	a	−	+	−	−	+	−	−	−	−
			b	−	+	−	−	+	−	−	−	−
10	Hydrolyzed poultry feather protein	—	a	−	+	−	−	−	−	−	−	−

sources of oil did not contain cod or sardine DNA as potential allergens. In general, plant- and animal-based oils are not considered allergenic when highly purified (30).

Of additional note was the finding that no diet contained the DNA of kangaroo. The commercial RMBD tested were obtained in California due to the proximity to the testing facility (Real-time PCR Research and Diagnostics Core Facility, University of California, Davis, California, USA). Since kangaroo meat is not used by any of the manufacturers in their RMBD, this finding serves as an additional negative control for this study to validate that no DNA of kangaroo origin was detected in the analysis, as would be expected.

Analysis of the 2 extensively hydrolyzed poultry feather-based diets (Royal Canin Ultamino) revealed no detection of unlisted animal DNA. This is consistent with previous reports that contaminants are detected less commonly and in lower numbers in hydrolyzed diets (10,24). The extensive hydrolysis of poultry feather proteins into component amino acids or very short oligopeptides is intended to avoid inducing IgE-mediated mast cell activation that can occur with proteins 10 kDa in size or greater (12,31). Extensive hydrolysis to reduce poultry allergenicity has been validated in both serum IgE and feeding trials to show the clinical benefits for CAFR (32). These negative control diets were selected because of the rigorous quality control methods undertaken by the manufacturer to ensure cross-contamination does not occur before market release (12). While the canine diet did test positive for chicken DNA, the manufacturer does list the feathers of chicken, turkey, and duck as their sourced raw materials (12). The target gene of the analysis for chicken DNA, transforming growth factor beta 3, is a protein expressed in chicken feathers (33). Additionally, the manufacturer is aware that cross-contamination needs to be avoided in a therapeutic diet and has developed and clinically validated calibration curves to prevent contamination (12). These calibration curves correspond to a known DNA level that was clinically tolerated based on Global Skin Scores (GSS) in feeding trials in order to set a tolerance level for ancillary proteins known as the NPPI (no protein pollution index %), which is strictly monitored in each diet prior to allowing market release (12). Based on the manufacturer's quality control data, 72.3% of these extensively hydrolyzed diets contain DNA below the limit of detection (LOD = 0.003 μg/g), and 25.7% may have DNA above the LOD but below a safety threshold of 1.2 μg/g (12). However, no diet released to market will exceed the established cut-offs of the NPPI based on the pre-established calibration curves (12). Therefore, trace copies of chicken DNA may be expected on PCR in some of the diets released to market, as was found in our study. The DNA in the feline extensively hydrolyzed diet in this study was below the LOD, and no animal DNA was detected in the assay.

Based on the sensitivity of qPCR, it can be argued that these assays, being sensitive enough to detect as few as 10 copies of the target gene, are of greater sensitivity than that required to detect clinically meaningful contamination that would trigger CAFR. There is no established maximum tolerable level of a contaminating protein that may elicit a pruritic reaction in a food sensitized pet. In humans, soy protein concentration as low as 10 ppm (0.01 μg/mg) may evoke a reaction in a soy-sensitized individual (34). Additionally, dose distribution has been demonstrated to vary between different food allergens in sensitized humans, showing that a tolerance range may exist for different food antigens

Table 4b. Results of analyses of feline diets.

Diet number	Animal proteins in ingredients	Animal fats in ingredients list	Batch	Animal species DNA detected in samples "+" = present, "−" = not detected in 40 cycles of amplification								
				Pork	Chicken	Duck	Rabbit	Lamb	Beef	Salmon	Kangaroo	Turkey
1	Duck, turkey, goose	—	a	−	+	+	−	+	−	−	−	+
			b	−	−	+	−	+	−	−	−	+
2	Rabbit, pork	Pork fat	a	+	−	−	+	−	−	−	−	+
			b	+	−	−	+	+	−	−	−	+
3	Beef	—	a	−	−	−	−	−	+	−	−	−
			b	−	−	−	−	+	+	−	−	+
4	Turkey	Cod-liver oil	a	+	+	−	−	−	+	−	−	+
			b	−	+	−	−	+	+	−	−	+
5	Chicken, salmon	—	a	+	+	−	+	+	+	+	−	+
			b	−	+	−	−	+	−	+	−	+
6	Rabbit	—	a	−	−	−	+	−	−	−	−	−
			b	−	−	−	+	−	−	−	−	−
7	Chicken	—	a	−	+	−	−	−	−	−	−	+
			b	−	+	−	−	−	−	−	−	+
8	Beef	—	a	−	−	−	−	−	+	−	−	−
			b	−	−	−	−	−	+	−	−	−
9	Lamb	—	a	−	−	−	−	+	−	−	−	−
			b	−	−	−	−	+	−	−	−	−
10	Hydrolyzed poultry feather protein	—	a	−	−	−	−	−	−	−	−	−

themselves (35). The additional concern for CAFR pets is that, as opposed to most humans, pets are often fed a specific commercial diet with daily regularity, increasing their risk of chronic re-exposure to a food antigen contained therein. As a result, even small amounts of unknown allergens may lead to a cumulative reaction in a CAFR-affected pet and skew the clinical impression of their response to a particular ED. These reactions may even be sporadic if there is significant variation of the protein constituents of the diet between batches. The need to validate food allergic threshold distributions in canine and feline CAFR is an important area for future research. Until such time, rigorous quality control using protein analysis methods such as qPCR or enzyme-linked immunosorbent assay (ELISA) remains a sensitive method to confirm such contaminants are not detected in therapeutic diets fed for the purpose of the clinical diagnosis of CAFR. This remains the industry standard for quality control of commercially produced diets, including extensively hydrolyzed diets.

In conclusion, this study confirms that commercial RMBD should not be considered appropriate for selection as ED in the diagnosis of CAFR as a result of their tendency to include unlisted protein ingredients, which can differ from batch to batch. A clinician should use caution when interpreting the results of an owner-directed ED trial using RMBD to exclude CAFR as a cause of their pet's pruritic dermatopathy, and veterinarian-guided elimination diet oversight is still recommended. Until further evidence is presented, an elimination diet and provocation trial with a patient-appropriate prescription-based diet subjected to applicable quality control or a home-prepared novel protein diet remain the current diagnostic standard for CAFR. CVJ

References

1. Cave N. Canine and Feline Gastroenterology. Philadelphia, Pennsylvania: Saunders, 2013:398–408.
2. Gaschen FP, Merchant SR. Adverse food reactions in dogs and cats. Vet Clin North Am Small Anim Pract 2011;41:361–379.
3. Mauldin E, Peters-Kennedy J. Integumentary system. In: Maxie MG, ed. Jubb, Kennedy, and Palmer's Pathology of Domestic Animals. Vol 1. 6th ed. Philadelphia, Pennsylvania: Saunders, 2016:509–736.
4. Mueller RS, Olivry T. Critically appraised topic on adverse food reactions of companion animals (6): Prevalence of noncutaneous manifestations of adverse food reactions in dogs and cats. BMC Vet Res 2018;14:341.
5. Olivry T, Mueller RS. Critically appraised topic on adverse food reactions of companion animals (3): Prevalence of cutaneous adverse food reactions in dogs and cats. BMC Vet Res 2017;13:51.
6. Mueller RS, Olivry T, Prélaud P. Critically appraised topic on adverse food reactions of companion animals (2): Common food allergen sources in dogs and cats. BMC Vet Res 2016;12:9.
7. Mueller RS, Olivry T. Critically appraised topic on adverse food reactions of companion animals (4): Can we diagnose adverse food reactions in dogs and cats with in vivo or in vitro tests? BMC VetRes 2017; 13:275.
8. Olivry T, Mueller RS, Prélaud P. Critically appraised topic on adverse food reactions of companion animals (1): Duration of elimination diets. BMC Vet Res 2015;11:225.
9. Jackson HA. Diagnostic techniques in dermatology: The investigation and diagnosis of adverse food reactions in dogs and cats. Clin Tech Small Anim Pract 2001;16:233–235.
10. Olivry T, Mueller RS. Critically appraised topic on adverse food reactions of companion animals (5): Discrepancies between ingredients and labeling in commercial pet foods. BMC Vet Res 2018;14:24.
11. Salihah NT, Hossain MM, Lubis H, Ahmed MU. Trends and advances in food analysis by real-time polymerase chain reaction. J Food Sci Technol 2016;53:2196–2209.
12. Lesponne I, Naar J, Planchon S, Serchi T, Montano M. DNA and protein analyses to confirm the absence of cross-contamination and support the clinical reliability of extensively hydrolyzed diets for adverse food reaction-pets. Vet Sci 2018;5:63.
13. Ballin NZ, Vogensen FK, Karlsson AH. Species determination — Can we detect and quantify meat adulteration? Meat Sci 2009;83:165–174.

14. Lee E. Raw dog food: Dietary concerns, benefits, and risks. Available from: https://pets.webmd.com/dogs/guide/raw-dog-food-dietary-concerns-benefits-and-risks#1 Last accessed June 30, 2020.
15. Freeman LM, Chandler ML, Hamper BA, Weeth LP. Current knowledge about the risks and benefits of raw meat-based diets for dogs and cats. J Am Vet Med Assoc 2013;243:1549–1558.
16. Laflamme DP, Abood SK, Fascetti AJ, et al. Pet feeding practices of dog and cat owners in the United States and Australia. J Am Vet Med Assoc 2008;232:687–694.
17. Morgan SK, Willis S, Shepherd ML. Survey of owner motivations and veterinary input of owners feeding diets containing raw animal products. Peer J 2017;5:e3031.
18. Wall T. Raw pet food sales growing despite health warnings. Available from: https://www.petfoodindustry.com/articles/6928-raw-pet-food-sales-growing-despite-health-warnings Last accessed June 30, 2020.
19. Parr JM, Remillard RL. Handling alternative dietary requests from pet owners. Vet Clin North Am Small Anim Pract 2014;44:667–688.
20. Morelli G, Bastianllo S, Catellani P, Ricci R. Raw meat-based diets for dogs: Survey of owners' motivations, attitudes and practices. BMC Vet Res 2019;15:74.
21. Raw meat based diets for pets — position statement. Canadian Veterinary Medical Association c2018. Available from: https://www.canadianveterinarians.net/documents/raw-meat-based-diets-for-pets Last accessed June 30, 2020.
22. Raw or undercooked animal-source protein in cat and dog diets. American Veterinary Medical Association c2012. Available from: https://www.avma.org/policies/raw-or-undercooked-animal-source-protein-cat-and-dog-diets Last accessed June 30, 2020.
23. Tiffany S, Parr JM, Templeman J, et al. Assessment of dog owners' knowledge relating to the diagnosis and treatment of canine food allergies. Can Vet J 2019;60:268–274.
24. Ricci R, Conficoni D, Morelli G, et al. Undeclared animal species in dry and wet novel and hydrolyzed protein diets for dogs and cats detected by microarray analysis. BMC Vet Res 2018;14:209.
25. Raditic DM, Remillard RL, Tater KC. ELISA testing for common food antigens in four dry dog foods used in dietary elimination trials. J Anim Physiol Anim Nutr (Berl) 2011;95:90–97.
26. Ricci R, Granato A, Vascellari M, et al. Identification of undeclared sources of animal origin in canine dry foods used in dietary elimination trials. J Anim Physiol Anim Nutr (Berl) 2013;97:32–38.
27. Okuma TA, Hellberg RS. Identification of meat species in pet foods using a real-time polymerase chain reaction (PCR) assay. Food Control 2015;50:9–17.
28. Horvath-Ungerboeck C, Widmann K, Handl S. Detection of DNA from undeclared animal species in commercial elimination diets for dogs using PCR. Vet Dermatol 2017;28:1–6.
29. Yin, H. Purification of fish oils and production of protein powders with EPA and DHA enriched fish oils. LSU Doctoral Dissertations 2011. Available from: https://digitalcommons.lsu.edu/gradschool_dissertations/2084 Last accessed June 30, 2020.
30. Health Canada's position on highly refined oils derived from food allergen sources. Health Canada c2013. Available from: https://www.canada.ca/en/health-canada/services/food-nutrition/food-labelling/allergen-labelling/position-highly-refined-oils-derived-food-allergen-sources.html Last accessed June 30, 2020.
31. Verlinden A, Hesta M, Millet S, Janssens GPJ. Food allergy in dogs and cats: A review. Crit Rev Food Sci Nutr 2006;46:259–273.
32. Olivry T, Bexley J, Mougeot I. Extensive protein hydrolyzation is indispensable to prevent IgE-mediated poultry allergen recognition in dogs and cats. BMC Vet Res 2017;13:251.
33. Jakowlew SB, Ciment G, Tuan RS, Sporn MB, Roberts AB. Expression of transforming growth factor-beta 2 and beta 3 mRNAs and proteins in the developing chicken embryo. Differentiation 1994;55:105–118.
34. Koppelman SJ, Lakemond CM, Wlooswijk R, Hefle SL. Detection of soy protein in processed foods: Literature overview and new experimental work. J AOAC Int 2004;87:1398–1403.
35. Ballmer-Weber BK, Rivas-Fernandez M, Beyer K, et al. How much is too much? Threshold dose distributions for 5 food allergens. J Allergy Clin Immunol 2015;135:964–971.