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Original Article

Determination of mammalian DNA in commercial canine diets with uncommon and limited ingredients

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Abstract

Over-the-counter (OTC) limited ingredient canine diets could be reliable alternatives to veterinary therapeutic formulations for the diagnosis and management of adverse food reaction (AFR). However, the possibility of undeclared ingredients jeopardizes the efficacious use of OTC options for medical purposes. The objective was to determine the presence of undeclared ingredients in OTC canine dry diets marketed as limited or single protein source diets. Twenty-one OTC adult canine diets marketed as limited or single protein source diets were purchased. Multiplex PCR was used to screen for DNA of 10 mammalian species with species-specific primers that anneal to regions of the mitochondrial cytochrome b gene. The presence of DNA from one or more species not declared on the label was identified in all 21 diets: cow *(Bos taurus*), pig (*Sus scrofa*), sheep (*Ovis* sp.), goat (*Capra hircus*) and bison (*Bison bison*). Twenty diets were positive for the declared protein source and one diet was negative for the declared species. Cat (*Felis catus*), dog (*Canis sp.*), horse (*Equus sp.*), mouse (*Mus musculus*) and rat (*Rattus norvegicus*) DNA was not identified in any samples. The presence of undeclared mammal species in OTC canine dry diets marketed as having limited or single protein source ingredients may complicate AFR diagnosis and treatment. However, PCR can detect a miniscule amount of DNA which might not be clinically significant, because the amount needed to elicit a response is unknown. Quantification of the contamination was not determined in this study, precluding discrimination of intentional adulteration from unavoidable cross-contamination.

Keywords: adverse food reaction, limited ingredient, novel, PCR analysis, canine.

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Introduction

Adverse food reaction (AFR) is a general term that refers to various immunological (food allergy) and non-immunological (food intolerance) responses of animals to diets or dietary components. Food allergy can be defined as an aberrant adverse immune response elicited by exposure to a particular food substance (antigen), typically a glycoprotein (Gaschen & Merchant 2011). Beef, dairy products, chicken, wheat and lamb are the most commonly reported ingredients causing AFR in dogs (Verlinden et al. 2006; Mueller et al. 2016). Clinical disease typically manifests as a non-seasonal pruritic dermatosis, gastrointestinal disturbances or both. It has been reported that concurrent dermatological and gastrointestinal disease accounts for 10–15% of dogs with AFR (Merchant & Taboada 1991).

Establishing a diagnosis of AFR in dogs and cats relies on elimination diet trials and challenge testing. If gastrointestinal signs are present, a trial of 2–4 weeks may be sufficient (Roudebush et al. 2000), whereas 8–12 weeks may be necessary to rule out AFR in those with dermatological signs (Rosser 1993; Gaschen & Merchant 2011). Diagnosis is confirmed by remission of clinical signs on the elimination diet, followed by recrudescence of clinical signs upon prior diet reintroduction. However, failure to improve does not necessarily rule out AFR. Ingestion of other antigens during a food trial could result in misdiagnosis. Accurate diagnosis relies on a thorough diet history, confident selection of a diet with

only ingredients novel to that individual pet, owner compliance during the elimination trial and identification of all substances consumed. Therefore, it is critical that the elimination diet only contains the declared ingredients and that no other food sources, including flavored medications, supplements or toothpastes, are fed during the trial.

Diets appropriate for elimination trials contain a limited number of uncommon ingredients, ideally novel to the individual or contain hydrolyzed ingredients. There are a variety of veterinary therapeutic diets formulated with these strategies. They are manufactured in separate or clean facilities to minimize cross-contamination with other ingredients. Over-the-counter (OTC) limited ingredient canine diets, which are sold without veterinary authorization and oversight, provide a less expensive substitute to veterinary therapeutic formulations, and in some cases, a convenient alternative to a home-cooked diet. Over-the-counter diets are increasingly formulated with limited and uncommon ingredients. In some cases, this may simply be a flavor or nutritional choice. In other cases, these diets may be marketed with implied, perceived or explicit indication for diagnosis or management of AFR. The misperception surrounding associated health benefits may be that of the owner or veterinarian and not necessarily the intended goal for the pet food companies point of sale. Controversy persists over the use of OTC limited ingredient diets for this purpose due to concerns regarding contamination with ingredients not declared on the product label. Studies conducted in Europe, using PCR technology, have identified animal DNA from one or more species other than those declared on the label in the majority of diets tested (Ricci *et al.* 2013; Horvath-Ungerboeck *et al.* 2017). Such findings raise concern regarding the adequacy of quality control measures to prevent cross-contamination with ingredients other than those declared on the label, as well as the potential for intentional adulteration. Additionally, these results highlight a diagnostic and therapeutic challenge for veterinary practitioners utilizing these diets in dogs with AFR.

The objective of this study was to evaluate the presence of declared and undeclared mammalian DNA using PCR methodology in commercially available, OTC and veterinary therapeutic, limited ingredient, canine dry diets, in order to determine if OTC options are a reliable alternative to veterinary therapeutic diets for the diagnosis and long-term management of AFR. Our hypothesis is that undeclared mammalian DNA is present in the majority of OTC diets, but that this is uncommon in veterinary therapeutic diets.

Materials and methods

Samples

The inclusion criteria for the diets selected for this study were that they had to be commercially available, OTC and veterinary therapeutic, canine, dry diets obtained from local pet stores and an online source at one time point between March and April 2017. Only diets with a marketing term or product name using limited ingredient, single protein or similar verbiage on the label's principal display panel (PDP) were included. To be included, the selected diets were required to have declared mammalian ingredients on the label that could be detected by the 12 species PCR panel available at the laboratory (Veterinary Genetics Laboratory, University of California, Davis, CA): bison, cat, cow, dog, goat, horse, deer (*Odocoileus* sp.), mouse, pig, rat, rabbit (*Oryctolagus cuniculus*) and sheep. Diets containing fish, poultry or other species on the ingredient list were excluded due to limited DNA primer availability. Diets not available for purchase via online or local sources were excluded. A total of 29 diets were identified and were only from one batch. Ideally, samples would have been run in duplicate and from several different lots; but funding limited our ability to do so. After submission of the samples to the laboratory, we were subsequently informed that the primers used for venison and rabbit would not detect the specific species of venison and rabbit typically used in pet foods. Given the inability to determine exactly which species were in the impacted diets and the potential for both false-positive and -negative results, we elected to remove those eight diets from the study (six deer and two rabbit-based diets; these included all three of the veterinary therapeutic

diets). Therefore, only 21 OTC diets were analyzed in this study.

Procedures

The primary marketing term or product name on the PDP was recorded for each diet and categorized as either limited ingredient or single animal protein (or similar verbiage) diet. Species specification, nutritional adequacy statement, ingredient list, name and address of manufacturer or distributer, lot number and expiration date were also recorded. Marketing terms and related claims were obtained from the PDP and manufacturer websites.

To ensure even distribution of the contents, all products were mixed prior to opening. Only one diet was opened at a time for sample collection, the work space was cleaned in between sampling and new sterile gloves were used to collect each sample. Approximately 5 g of dry food was weighed and then placed into individual unused resealable plastic bags. Each bag was labelled with a number code correlating to the specific product to ensure that the laboratory was masked to sample identities. The samples were stored in a controlled environment at room temperature until they were submitted on the same day to the laboratory for processing and analysis.

Extraction of DNA was accomplished by digesting 0.3 g of material in 1.0 mL of 200 mmol/L NaOH at 95°C for 10 min followed by neutralization with an equal volume of 200 mmol/L Tris-HCl, pH 8.5. PCR amplification was performed in 25 μL reactions on a thermal cycler (Applied Biosystems 2720 Thermal Cycler, Thermo Fisher Scientific, NY, USA) using 1 μL DNA extract, 0.2 μL Taq polymerase (Clontech Laboratories, CA, USA), 2.5 μL 109 Taq polymerase PCR Buffer (Clontech Laboratories, CA, USA), 2.5 μL 2.0 mmol/L dNTPs (Bioline USA, Tauton, MA, USA), 7 μL primer mix and molecular grade water (Thermo Fisher Scientific, MA, USA) to final volume. The PCR began with a 1-min activation step at 95°C followed by 31 cycles of 30 s at 95°C, 30 s at 60°C, 1 min at 72°C and a final extension at 72°C for 30 min. A quantity of 1 μL of PCR product was then serially diluted out to 1:100 into deionized water, and 1 μL of each dilution was further diluted into 10 μL HiDi formamide (Thermo Fisher Scientific, NY, USA) and 0.0625 μL size standard (Thermo Fisher Scientific, Waltham, MA, USA). Fragment separation was carried out on a DNA analyser (Applied Biosystems 3730 DNA Analyzer, Thermo Fisher Scientific, Grand Island, NY, USA) using the GeneMapper36_POP7 run module and a 10 s injection. Fragment analysis was performed using allele calling STRand Analysis Software (Toonen & Hughes 2001).

The primer mix incorporated three fluorescently labelled universal primers that anneal to highly conserved regions of the mitochondrial cytochrome b gene (Tobe & Linacre 2008). Reverse species- or genus-specific primers, two for each target species, were included in the primer mix with the dye-labelled primers to create a multiplex capable of detecting bison, cat, cow, deer, dog, goat, horse, mouse, rat, pig, rabbit and sheep DNA. The primers produce fragments of 247 and 257 bp for bison, 98 and 183 bp for cat, 92 and 286 bp for cow, 90 and 272 bp for deer, 172 and 305 bp for dog, 275 and 316 bp for goat, 212 and 334 bp for horse, 188 and 364 bp for mouse, 193 and 312 bp for rat, 202 and 220 bp for pig, 186 and 196 bp for rabbit, and 101 and 338 bp for sheep.

The negative control was run on the same plate as the samples but separated by at least six lanes. It consisted of water and all the reagents that were present with the samples. The negative passed if there were no peaks present. The positive control was made up of known DNA from all of the 12 species listed above. This was run similarly to the negative control; it was on the same plate as the samples, separated by at least six lanes. The positive control passed if both signals were detected for each species in the test.

Statistics

Descriptive statistics (median and range) were calculated using computer-based software (Microsoft Office Excel 2016, Microsoft Corp., Redmond, WA USA).

Results

Twenty-one OTC canine dry diets were purchased from March to April 2017; 20 diets were purchased from an online source and one diet from a local pet store. Eight diets were intended for adult canine maintenance while 13 diets were adequate to feed all life stages according to the nutritional adequacy statements (Association of American Feed Control Officials, 2017). The term limited ingredient appeared as part of the primary marketing term or product name on the PDP on 17 diets (81%) and single animal protein or similar verbiage appeared on the PDP of four diets (19%, Table 1). Grain-free or another negative claim (highlighting lack of certain ingredients) was often part of the product name or included as a term on the PDP (19/21). The number of marketing terms and claims found reported on company websites were often greater than on the PDP for the same diet. For example, three of the four diets with the term single animal protein on the PDP also used the term limited ingredient on their respective website. Other claims that were found on the PDP and website that implied use for AFR included sensitivities, allergies, hypoallergenic, digestive health or skin and coat benefits. One diet contained two animal-origin protein sources (bison and egg), yet was still marketed as a limited ingredient diet, and thus met the inclusion criteria. The remaining diets (Table 2) declared just one mammalian species as the main protein source on the ingredient list. The most commonly declared species was ovine (n = 14), with fewer diets containing bison (n = 4), bovine (n = 2) and porcine (n = 1).

Of the 21 samples, 20 were positive for the declared mammalian component. Only one sample had a PCR assay result negative for the declared species. All 21 diets were found to have the presence of mammalian DNA from at least one animal species not declared on the ingredient list (Table 2). Bovine DNA was the most frequently undeclared species (n = 18), with porcine the second most common (n = 16). One diet that declared only bison was positive for DNA from five species (bison, cow, goat, pig and sheep). The median number of undeclared animal protein sources per sample was 3 (range 1–4).

Table 1. Marketing terms and claims identified on the principal display panel and manufacturer websites

Primary marketing term or product name on the principal display panel	Number of diets
Limited ingredient	17
Verbiage with single (animal, protein or meat)	4
Additional marketing terms and claims on the principal display panel	
Rotational diet	3
Grain-free or lack certain ingredients (grain, corn, wheat, potato, dairy, egg, chicken, beef, fish, soy, by-products or gluten)	19
Sensitivities, allergies or hypoallergenic	3
Skin and coat or digestive health	10
Additional marketing terms or claims on manufacturer websites	
Limited ingredient	20
Single animal, protein or meat	8
Grain-free or lack certain ingredients (grain, corn, wheat, potato, dairy, egg, chicken, beef, fish, soy, by-products, gluten, etc.)	19
Sensitivities, allergies or hypoallergenic	13
Skin and coat or digestive health	14
Veterinary alternative	1

Samples with sheep as the animal-derived source reported on the label (n = 14) had the highest average of undeclared ingredients (median of 3). Bovine was the species most often detected and not declared (median of 2.5) and pork was the least common contaminant (median of 1). In all diets positive for goat DNA, sheep was the labelled protein source and they were also positive for sheep DNA. All samples positive for bison were also positive for undeclared cow DNA. Bison DNA was not detected in either of the two diets that declared bovine as the mammalian protein source. Bison DNA was never present when undeclared, but one diet that declared bison was negative for this ingredient. No samples were positive for the DNA of cat, dog, horse, rat and mouse species.

Discussion

While the exact incidence of AFR is unknown, food hypersensitivity has been reported to affect approximately 1% of the canine population (Day 2005). Implicated foods are often based on owners'

Table 2. List of diets with declared animal protein source(s) and PCR analysis results*†‡§

	Declared animal protein	Bison	Bovine	Caprine	Porcine	Ovine
1	Lamb	–	+	+	+	+/d
2	Lamb	–	–	–	+	+/d
3	Lamb	–	+	–	–	+/d
4	Lamb	–	+	+	+	+/d
5	Lamb	–	+	–	+	+/d
6	Lamb	–	+	+	+	+/d
7	Lamb	–	+	–	+	+/d
8	Bison, egg	+/d	+	–	+	+
9	Lamb	–	+	+	+	+/d
10	Lamb	–	+	+	+	+/d
11	Lamb	–	+	+	+	+/d
12	Beef	–	+/d	–	+	+
13	Bison	+/d	+	–	–	–
14	Lamb	–	+	+	+	+/d
15	Beef	–	+/d	–	+	–
16	Lamb	–	+	+	–	+/d
17	Bison	+/d	+	+	+	+
18	Lamb	–	+	+	+	+/d
19	Bison‡	–/d	+	–	–	–
20	Pork	–	+	–	+/d	–
21	Lamb	–	+	+	+	+/d

*Cat, dog, horse, mouse and rat DNA were not detected in any samples. †'Buffalo' was declared in the ingredient list and front display panel; manufacturer confirmed this is bison. ‡(+) indicates that DNA from that species was detected yet was undeclared on the label (positive, undeclared); (+/d) indicates that detected DNA was a declared species (positive, declared); (–/d) indicates that declared species was not detected (negative, declared); (–) indicates that undeclared DNA was not detected (negative, undeclared). §(to maintain the anonymity, the list below is in random sequence and does not correspond to the order of the diets in Table 2): Natural Balance® L.I.D. Limited Ingredient Diets® Legume and Wagyu Beef Formula. Natural Balance Pet Food® Inc, San Francisco, CA.; CANIDAE PURE® Petite® Grain-Free Small Breed Bison Formula. CANIDAE® Corporation©, San Luis Obispo, CA.; Great Life Dr. E's Grain-Free Limited Ingredient Buffalo Dog Food. Great Life Performance Pet Products, Simi Valley, CA.; Merrick® Limited Ingredient Grain Free Real Lamb and Sweet Potato Recipe. Merrick Pet Care Inc, Amarillo, TX.; FirstMate Grain Free Formula Australian Lamb Meal Formula. FirstMate Pet Foods©, North Vancouver, B.C. Canada.; Acana® Lamb and Apple Singles Formula. Champion Petfoods©, Auburn, KY.; Wild Calling!™ Wild Calling Western Plains Stampede® Beef Recipe. Wild Calling! Pet Foods©, St. Greely, CO.; California Natural™ Grain Free Lamb Meal Formula. ©Mars®, Fremont NE.; Acana® Pork and Squash Singles Formula. Champion Petfoods©, Auburn, KY.; Natural Balance® L.I.D. Limited Ingredient Diets® Lamb Meal and Brown Rice Formula. Natural Balance Pet Food® Inc, San Francisco, CA.; Nature's Recipe® Pure Essentials® Limited Ingredient Recipe. Nature's Recipe®, LCC, San Francisco, CA.; Zignature® Lamb Limited Ingredient Formula Dry Dog Food. Pets Global©, Inc, Gardena, CA.; Rachael Ray™ Nutrish® Just 6® Lamb and Rice. Ainsworth Pet Nutrition©, LCC, Meadville, PA.; Wild Calling!™ Xotic Essentials Bison Meal Recipe. Wild Calling! Pet Foods©, St. Greely, CO.; Chicken Soup for the Soul® Limited Ingredient Diet Lamb, Pea & Green Lentil Grain-Free dry dog food. Chicken Soup for the Soul Pet Food©, LCC, Cos Cob, CT.; Wellness® Simple Limited Ingredient Diet Lamb and Oatmeal Formula. Wellpet© LCC, Tewksbury, MA.; Natural Balance® L.I.D. Limited Ingredient Diets® Sweet Potato and Bison Formula. Natural Balance Pet Food® Inc, San Francisco, CA.; Instinct® by Nature's Variety® Limited Ingredient Diet Lamb Meal and Pea Formula. Nature's Variety®, St Louis, MO.; Blue Basics® Limited Ingredient Grain Free Formula Lamb and Potato Recipe. Blue Buffalo Co©, Joplin, MO.; Canine Caviar Open Meadow Holistic Entrée for All Life Stages. Canine Caviar Pet Foods© Inc, Norco, CA.; Nutro™ Lamb and Rice Adult Recipe. ©Mars®, Franklin, TN.

perceptions through uncontrolled dietary modifications. Pets may improve during elimination diet trials, but in the absence of rechallenge, it is not possible to identify the exact food evoking the response. Some pet food manufacturers have highlighted the exclusion of certain foods such as corn or soy concurrent with a focus on more exotic ingredients in certain products to imply or explicitly claim benefits for preventing or managing AFR. These claims strengthen owners' misconceptions surrounding the management of their pet's perceived food allergy and create confusion for veterinary practitioners when making decisions about diet choices.

Over-the-counter canine diets are intended to support healthy dogs in various life stages. Unlike veterinary therapeutic diets, they are not intended to diagnose, treat or cure a disease process, so the presence of undeclared mammalian DNA was not unexpected. However, some of the OTC diets evaluated in this study used terms such as sensitivities, allergies, and healthy skin and coat on labels or websites, which may imply or make explicit their suggested use in the diagnosis or treatment of AFR. Depending on the exact wording, these could be considered drug claims, which identify the diet as intended for diagnosis, cure, mitigation or prevention of disease, and are not allowed on OTC diet labels or on manufacturer communications (including websites) that are available directly to the consumer. More ambiguous claims may make these diets exempt from Federal Food, Drug and Cosmetic Act regulations and thus can be used without the direction of a licensed veterinarian; however, this is dependent on the wording and is always at the enforcement discretion of the US FDA (Federal Food, Drug, and Cosmetic Act**,** 1999). Regardless, pet owners and veterinarians may perceive such marketing as indications or endorsements for use in treatment of AFR, and this category of diets is often considered a reliable alternative to veterinary therapeutic options. This underscores the need to confirm the role of these diets in the diagnosis and management of AFR.

This study was able to identify discrepancies between the PCR testing results and the ingredient declarations, with all OTC diets sampled containing one or more undeclared mammalian DNA fragments. These findings are consistent with previously reported studies (Ricci et al. 2013; Okuma & Hellberg 2015; Horvath-Ungerboeck et al. 2017; Kanakubo et al. 2017), and supports our hypothesis that undeclared mammalian DNA is found in OTC, limited ingredient diets. Although our study was able to qualify the presence of undeclared ingredients, quantification was not possible, nor the possible reason for their presence. Cross-contamination can occur at numerous points along the production chain, including at ingredient procurement, processing and production, especially if equipment is not thoroughly cleaned between production lines (Premananda 2013). Contamination in this sense is referred to as unavoidable when using good manufacturing practices (Association of American Feed Control Officials, 2017), and is the responsibility of the manufacturer to implement stringent quality control measures to minimize the risk and degree of occurrence. Another reason for mislabelling is intentional substitution with alternative ingredients for economic gain. Regardless, either reason reduces the predictably of contained ingredients and therefore potentially jeopardizes the reliability of OTC limited ingredient diets as diagnostic tools for canine AFR.

Currently, DNA is the most appropriate biomarker to identify the source of animal-derived materials (Ali et al. 2012). The most widely used approach for the amplification of specific DNA sequence in food and feed is by the means of PCR (Frezza et al. 2008). PCR is an efficient methodology for species authentication in processed animal feed and human foods (Okuma & Hellberg 2015; Hsieh et al. 2016; Kanakubo et al. 2017). Molecular-based methods are ideal in the identification of meat species in highly processed foods, as DNA is stable at high temperatures and pressure. In addition, DNA is present in the majority of cells and the higher copy number of the mitochondrial genome the higher the sensitivity (Ballin et al. 2009). This method has been reported (Yancy et al. 2009) to detect DNA at levels as low as 0.1%; however, the clinical significance of such high sensitivities to patients with AFR is not known. Detailed, controlled clinical studies are necessary to determine the quantity required for an antigen to have biological effect in a patient with confirmed AFR. Moreover, *in vivo* studies have not yet been performed to evaluate the significance of feeding diets positive for DNA of any species to known canine AFR patients.

Another potential reason for positive PCR results of undeclared ingredients in pet foods is cross-reactivity between closely related species. The closer the taxonomic relationship between species, the higher the risk of cross-reactivity. However, the PCR panel used in the current study has been validated to ensure accuracy, as mitochondrial DNA has a

relatively high mutation rate (10 times greater than nuclear DNA), allowing for the differentiation between closely related species (La Neve et al. 2008). In addition, the laboratory confirmed that the signals observed between individual species-specific primers for goat and sheep as well as between cow and bison were not consistent for cross-reactivity. Therefore, goat and cow DNA that were inconsistent with declared species on the product label were likely true contaminants in diets with declared sheep and bison, respectively (Table 2). Experimental error also represents a potential cause for cross-contamination between sampled diets. Contamination may have occurred during the collection, transportation and storage of the sealed samples, as well as at the reference laboratory where PCR analysis was conducted. Experimental error was considered an unlikely cause for the contamination reported in this study as adequate control measures were implemented throughout the study and performed in controlled environments.

Only one product in the current study was negative for a declared species (bison). Negative results can either suggest the species was not present or is present at concentrations less than the detection limit. The PCR analysis utilized in the present study distinguishes among water buffalo species (*Bubalus bubalis*), American bison (*Bison bison*) and beef cattle (*Bos taurus*), so there is no potential for cross-reactivity. Other negative results can occur if primers are not sufficiently selective, the temperature is not adequate, or too many amplification cycles are used in the PCR (Pelt-Verkuil et al. 2008). The likelihood of false positive and or false negative due to insufficient primer selectivity was the main consideration for excluding diets containing deer and rabbit. The word venison originally described meat of any game animal killed by hunting (*Merriam-Webster Dictionary*, 2017), and in various contexts may be applied to a range of species such as boar and goats as well as any deer species including elk. In the current study, the deer primers that were available at the laboratory specifically targeted North American deer species such as whitetail and mule deer. Thus, the inclusion of other types of deer species more likely to be used in pet foods such as English red deer and elk (imported sources from managed herds free of transmissible spongiform encephalopathy), would result in a false-negative result. Likewise, given the common use of rabbit hybrids in meat production, it was unclear whether the rabbit specie(s) used in pet foods would be consistent among sources. Furthermore, the available primer was specific for European rabbit and not previously validated for use in various hybrids or other species with the exception of not being positive for hare and jackrabbit species. There have been minimal studies published on the identification of common rabbit species in processed animal meals (Bottero et al. 2003; Martin et al. 2009), and published studies on the detection of hare species are even scarcer. Moreover, the PCR platform used in the past has been correlated with a poor sensitivity and has been restricted to raw tissues, therefore limiting its application to processed feeds (Santos et al. 2012). The use of real-time PCR in one study demonstrated suitability to detect rabbit and hare material in processed feeds, which could be considered as a useful method for the quantification of low amounts of rabbit-based ingredient (Pegels et al. 2013). Future studies that expand the target species and validate those specifically used in pet foods are still required to improve the specificity of the current methodology.

This study is the first to utilize PCR technology in this category of pet food in the US market, as well as the largest sample size compared with previous studies. However, the set number of target species of the primers offered by the laboratory was a significant limitation that impacted the sample size and scope. Diets were excluded if the product declared fish, poultry or other species in the ingredient list, due to the lack of corresponding primers. Availability also may have limited the sample size to include diets only available for purchase at local pet stores and one online source. Another important limitation was available funding, such that the study could only evaluate one sample from one batch of each diet. Analysis of different lot batches at two time points may help differentiate incidental from repeatable contamination. A longitudinal study design using samples from different batches would have increased the predictive value of our analyses and provided a

greater scope for the relationships among repeatable presence or absence of disclosed and undisclosed ingredients. Another consideration is that utilization of complementary methodologies together may reduce the number of false negatives as described in a previous study (Ricci et al. 2013), and some techniques may estimate or determine actual concentrations of DNA in the diets. DNA-based methods in combination with immunological techniques such as enzyme linked immunosorbent assay may provide sufficient information to make conclusive remarks on the accuracy of pet food labels. It should be acknowledged that PCR methodology is often used as a tool for confirming exclusion of specific food products into selected countries. For example, PCR analysis is the preferred methodology to manage the risk of transmissible bovine spongiform encephalopathy introduction into Australia (AGDA, 2015), and is used to determine the presence of restricted animal material in imported products such as meat meal, meat and bone meal, fish meal and feather meal.

Conclusion

Despite the limitations reported, these results highlight a significant percentage of OTC limited ingredient diets contained undeclared mammalian DNA. As a consequence, these diets cannot be used as reliable diagnostic or treatment tools in AFR patients. Because many consumers purchase a product based on the presence of a specific ingredient, efforts to reduce cross-contamination during diet production are necessary to ensure accurate ingredient declarations. One way to achieve this is to test for contamination as part of good quality control practices, most likely using a quantification method to better assess the scope of any identified issues. Implementing these strategies helps ensure practicing veterinarians and pet owners can make informed decisions about diet choices.

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Conflict of interest

The authors declare no conflict of interest related to this report.

Ethics statement

The authors confirm that the manuscript complies with the ethical standards presented in the journal's author guidelines. No ethical approval was required as this study did not use animals.

Contributions

Lara A. Fossati acquired the samples and materials, designed the protocol, performed the experiment, data collection, data analysis and interpretation, wrote the paper, prepared the tables and reviewed the drafts. Jennifer A. Larsen designed the protocol, data analysis and interpretation, and reviewed the drafts. Cecilia Villaverde designed the protocol, performed the experiment, data analysis and interpretation, statistical analysis and reviewed the drafts. Andrea J. Fascetti designed the protocol, data analysis and interpretation, and reviewed the drafts.

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