

SUPPLEMENT ARTICLE

Identification of undeclared sources of animal origin in canine dry foods used in dietary elimination trials

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Summary

Failure to respond to commercial limited antigen diets can occur in dogs kept on a dietary trial for the diagnosis of adverse food reaction (AFR). The aim of this study was to assess twelve canine dry limited antigen diets (eleven novel protein diets and one hydrolysed diet) for potential contamination by ingredients of animal origin not mentioned on the label. The validity of the two methods adopted for the detection of such food antigens was also evaluated. Each dietary product was analysed by microscopy analysis using the official method described in Commission Regulation EC 152/2009 with the aim of identifying bone fragments of different zoological classes (mammalian, avian and fish) and by *polymerase chain reaction* (PCR) for the identification of DNA of animal origin. Discrepancies between the results obtained by PCR and/or microscopy analysis and the ingredients listed on pet food packages were found. Only in two pet foods did the results of both analyses match the ingredients listed on the label. In the remaining ten samples, microscopy detected bone fragments from one or two unpredicted zoological classes, revealing avian fragments in six of ten samples followed by those of fish in five of ten and mammalian fragments in four of ten. In two samples, microscopy analysis identified a contamination that would have otherwise passed unobserved if only PCR had been used. However, PCR confirmed the presence of all the zoological classes detected by microscopy and also identified the DNA of an additional unexpected zoological class in two samples. Dogs might fail to respond to commercial limited antigen diets because such diets are contaminated with potential allergens. Both PCR and microscopy analysis are required to guarantee the absence of undeclared animal sources in pet foods. Before ruling out AFR, a novel protein home-made diet should be considered if the dog is unresponsive to a commercial regimen.

Keywords adverse food reaction, allergens, contamination, pet food, microscopy analysis, PCR

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Introduction

In the last decade, pet foods claiming to be useful as limited antigen diets have gained a significant market position with the aim of diagnosing adverse food reaction (AFR) and controlling the related symptoms. The gold-standard method to diagnose an AFR consists in feeding the animal a limited antigen diet until clinical symptoms improve and then reintroducing the diet previously fed to demonstrate a relapse of symptoms (Scott et al., 2001). The objective of limited antigen diets is to avoid exposing animals to potential allergens during the 8- to 10-week elimination period. These diets can either be home-cooked or commer-

cially produced. Both typically contain a single source of protein (novel protein diets) and a single source of carbohydrate, neither of which is usually contained in maintenance diets, and therefore, there is less probability of the animal ever having come into contact with them before (Jackson, 2001). Hydrolysed protein diets are limited antigen diets, which represent a valid alternative to novel protein diets because they are made of protein fragments with molecular weights of <10 kDa that confer higher digestibility and lower allergenicity (Cave, 2006). Whenever no clinical improvement is observed during the dietary trial, AFR is ruled out and other diseases such as atopic dermatitis or non-allergic pruritic disorders may be suspected.

Choosing the correct commercial limited antigen diet is a very important step in diagnosing canine AFR and begins by collecting an accurate feeding history from the owner that reveals the ingredients the dog has already eaten. Therefore, careful reading of the ingredients on the label is of utmost importance in avoiding exposure to potential allergens. The diet must not contain any of the ingredients previously mentioned by the owner.

A dietary elimination trial remains the only effective tool to definitively diagnose AFR because skin and serologic tests, skin biopsy and gastroscopic food sensitivity testing have all been demonstrated inadequate to the purpose (Scott et al., 2001).

Although most veterinary dermatologists consider home-made diets the first choice when submitting a dog to a diagnostic food elimination trial (Roudebush and Cowell, 1992; Bloom, 2005), many owners prefer the convenience of commercial diets, and for this reason, several studies have assayed the efficacy of commercial limited antigen diets in diagnosing AFR. Some have found commercial novel protein diets appropriate for the long-term management of pruritus caused by AFR (Leistra et al., 2001); others have shown that they cannot replace home-made diets for food hypersensitivity testing and control of clinical signs (Jeffers et al., 1991; Leistra and Willemse, 2002). It is interesting to note that certain protein sources are no longer considered novel; fish protein, for example, has been extensively used as a dietary component in canine maintenance diets, and this has led some dogs to become sensitized to it (Tapp et al., 2002). The novelty of a specific protein is therefore not an absolute concept and must be evaluated whenever a dog is submitted to a dietary elimination trial. Although hydrolysed diets have been tested, their efficacy has not been uniformly recognized (Biourge et al., 2004; Loeffler et al., 2004, 2006; Olivry and Bizikova, 2010; Ricci et al., 2010).

In their study, Tapp et al. (2002) further emphasized that the use of commercial limited antigen diets provides better compliance than using home-made diets, which is an important element to take into consideration to make a correct diagnosis, but although failure to respond to commercial limited antigen diets may occur, it does not definitively rule out an AFR (Bloom, 2005). For this reason, even when a dog shows no evident improvement of clinical signs during the elimination trial, dietary-related factors cannot be excluded from playing a role in preventing the recovery of skin inflammation and pruritus. This failure to recover may occur because the veterinarian has not correctly selected the diet based on the ingredients

list, residual allergenic fragments remain in the hydrolysed diet, or contamination has occurred during the production process of the limited antigen diet. In all cases, a correct diagnosis of AFR would be compromised.

In this study, the authors collected commercial dog foods used in dietary elimination trials with the objective of detecting the presence of sources of animal origin not declared on the label that amount to contamination. An additional aim was to assess the validity of the methods adopted for food antigen detection.

Materials and methods

Samples

Twelve dry dog foods (eleven novel protein diets and one hydrolysed diet) from five different manufacturers, both international and Italian, were collected from the market. Each sample's label was read carefully to identify ingredients containing proteins of animal origin. Samples were then submitted to the official method for the detection of constituents of animal origin in feedstuffs (Commission Regulation EC 152/2009), which is based on microscopic bone fragment identification. The presence of bone fragments of three zoological classes (M: mammalian, A: avian and F: fish) was assessed in this study. Given, however, that not all the animal sources used in pet foods contain bones but may contain proteins (e.g. blood, viscera, feathers), even if only in traces, such as fats, *polymerase chain reaction* (PCR) was also adopted to detect contamination in the samples by the identification of mammalian, avian and fish DNA. The procedures employed are described in detail below.

Microscopy analysis

The microscopic examination was carried out by a well-trained pathologist (MV) using the technique described in Commission Regulation EC 152/2009 dated 27 January 2009. This is recognized as the official method for the detection of constituents of animal origin in feedstuff, and the procedure is here briefly reported. Fifty grams of each sample were ground, and 5 g were successively treated in a separating funnel with a concentration agent (tetrachloroethylene, density 1.62). The mixture was shaken twice for 1 min and left to stand for 3 min. The sediment obtained was separated and dried in a fume cupboard. At least three slides for each sample, prepared with a small amount of glycerol, were examined under a microscope at different magnification. Results are

expressed as either negative or positive for the following zoological classes: mammalian, avian and fish. The pathologist distinguished mammalian from avian and fish bone fragments by looking at the characteristics of the *lacunae* (Fig. 1).

DNA extraction

Total DNA was extracted from 200 mg of pet food samples and prepared in duplicate using the Wizard Magnetic DNA Purification System for Food (Promega, Madison, WI) according to manufacturer's

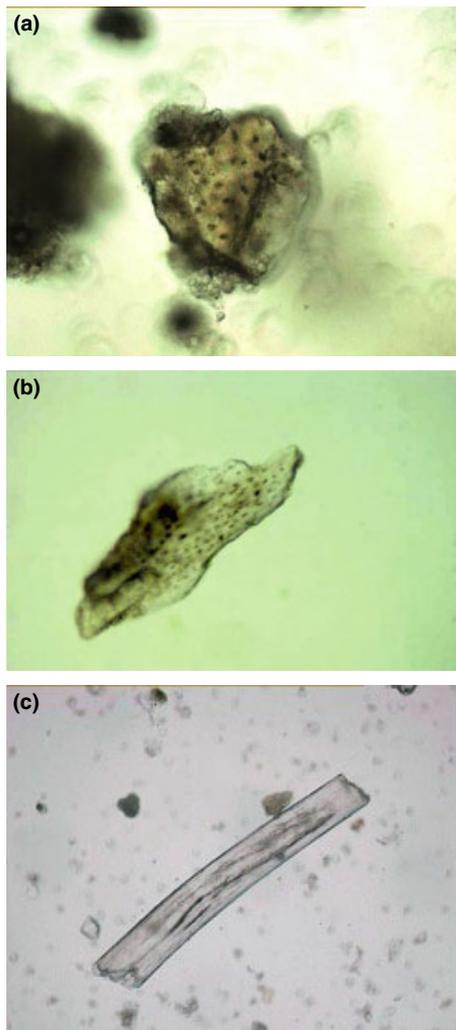


Fig. 1 Microscopic images of (a) mammalian, (b) avian and (c) fish bone fragments. The characteristics of the *lacunae* differ from one zoological class to another one, and this allows pathologists to distinguish them through microscopy. In mammalian fragments, *lacunae* range from round to elliptical; avian fragments show typically jagged edges with elongate *lacunae*, whereas fish bone fragments have a peculiar stick-shape with stringy *lacunae*.

instructions. Negative controls were included in each extraction. DNA concentration was determined by spectrophotometry (Nanodrop ND1000; NanoDrop Technologies Inc., Wilmington, DE, USA).

Polymerase chain reaction (PCR) protocol

Three pairs of previously published PCR species-specific primers (Table 1), designed on different regions of mitochondrial DNA (12S ribosomal RNA mitochondrial gene or 16S ribosomal RNA mitochondrial gene), were used.

In a preliminary step of this study, primer specificity was tested with DNA extracted from different animal tissue (cattle, pig, sheep, goat, horse, chicken, turkey, fish). Positive and negative controls were included in all PCR assays.

DNA amplification was performed in a final volume of 50 μ l containing 5 μ l of DNA (100–250 ng), 1X PCR buffer, 0.2 mM of each dNTP and the final concentrations of MgCl₂, primers, AmpliTaq Gold DNA polymerase and bovine serum albumin listed in Table 2. Amplification was performed in a Perkin Elmer GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) or in a Veriti Thermal Cycler (Applied Biosystem, Foster City, CA) with the following amplification profile: i) denaturation at 95 °C for 10 min; ii) 35 cycles at 94 °C for 30 s, annealing temperature according to species (Table 1), extension at 72 °C for 30 s; iii) final extension at 72 °C for 3 min.

PCR products were analysed by electrophoresis on 7% acrylamide gel and visualized by silver staining.

Results

Table 3 shows the protein and fat sources of animal origin listed on the label of the twelve pet food samples collected. The results expected and those obtained from microscopy and PCR analyses are also reported and expressed as zoological classes (A, F or M). The expected zoological classes were gathered from the ingredients list, and whenever the label reported the term 'animal fats', both mammalian and avian classes were considered.

Discrepancies between the results expected and those obtained from PCR and microscopy analyses were observed. Only in two pet foods (samples 11 and 12) did the results of both analyses match the ingredients listed on the label. Food 11, the only hydrolysed diet considered in this study, contained hydrolysed soy as its main protein source, but animal fats, hydrolysed chicken liver and fish oil were also declared on the label. PCR clearly detected the DNA of all zoological

Table 1 DNA sequence of species-specific primers, annealing temperature, fragment size, and references

Primer	Sequence (5' – 3')	Annealing Temperature/time	PCR product (bp)	Reference
Mammal for	GGT TGG GGT GAC CTC GGA GA	61°C/30 s	117	Chiappini et al., 2005;
Mammal rev	GGG TAA CTT GTT CCG TTG ATC AA			
Poultry for	TGA GAA CTA CGA GCA CAA AC	60°C/1 min	183	Dalmaso et al., 2004;
Poultry rev	GGG CTA TTG AGC TCA CTG TT			
Fish for	TAA GAG GGC CGG TAA AAC TC	60°C/1 min	224	Dalmaso et al., 2004
Fish rev	GTG GGG TAT CTA ATC CCA G			

Table 2 Final concentration of MgCl₂, primers, AmpliTaq Gold DNA polymerase and bovine serum albumin in species-specific PCR protocol

PCR protocol	MgCl ₂ final concentration (mM)	Primers final concentration (μM)	AmpliTaQ Gold DNA polymerase(U)	BSA (0.1 mg/ml)
Mammal	2.5	0.6	2.5	No
Avian	2	0.5	1	No
Fish	3	0.5	2	Yes

classes (mammalian, avian and fish), whereas no bone fragments were identified by light microscopy, as expected. In pet food 12, fish meal was claimed as the only ingredient of animal origin, and this was confirmed by both PCR and microscopy analysis.

Among the remaining ten samples, microscopy analysis detected bone fragments belonging to one unexpected zoological class in five pet foods, whereas bone fragments from two unexpected zoological classes were identified in the other five products. Avian bone fragments were the most representative and were detected in six of ten samples; mammalian fragments were present in four samples, whereas those from fish were found in five samples.

Table 3 List of the protein and fat sources of animal origin declared on the labels and the results expected and obtained from microscopy analysis and PCR expressed as zoological class (A = avian; F = fish; M = mammalian)

Food N	Declared animal protein source	Declared animal fat source	Expected results for microscopy analysis	Microscopy analysis results	Expected results for PCR analysis	PCR analysis results
1	Duck	–	A	A F M	A	A F M
2	Rabbit	–	M	A F M	M	A F M
3	Fish	Fish oil	F	F M	F	A F M
4	Deer	Fish oil	M	A F M	F M	A F M
5	Rabbit	Fish oil	M	A F M	F M	A F M
6	Horse	–	M	A M	M	A M
7	Duck	Fish oil	A	A F	A F	A F M
8	Lamb	–	M	A M	M	A M
9	Fish	Animal fats	F	A F M	A F M	A F M
10	Fish, hydrolysed chicken liver	Animal fats, fish oil	F	F M	A F M	A F M
11	Hydrolysed chicken liver	Animal fats, fish oil	None	nd	A F M	A F M
12	Fish	–	F	F	F	F

nd = not detected bone fragments.

PCR analysis results from samples 9 and 10 revealed DNA of all zoological classes: fish, avian and mammalian. This result was predictable because fish and animal fats were listed on the label of sample 9, and fish, fish oil, hydrolysed chicken liver and animal fats were named on the label of sample 10. In sample 9, however, not only were fish bone fragments identified by microscopy, as expected, but also mammalian and avian fragments were found. Similarly, unpredicted mammalian bone fragments were identified in sample 10.

In the remaining eight samples (1–8), PCR confirmed the presence of all the zoological classes detected by microscopy. Moreover, poultry and mammalian DNA were also identified in samples 3 and 7 respectively.

Discussion

Two elements play a pivotal role in making a dietary elimination trial effective: the owner's compliance, as he/she is recommended not to feed the dog with anything but the prescribed diet for the entire trial (up to 8–10 weeks), and the adequacy of the diet adopted.

When no amelioration of clinical signs is observed during the diagnostic dietary trial, other pruritic allergic or non-allergic diseases may be the cause. The slightest lack of pet or owner compliance should be promptly assessed, while also considering that the diet itself may have impaired the improvement of clinical signs (Fig. 2).

While it has been demonstrated that the client's understanding and cooperation can be significantly enhanced by an approach that includes emphasizing the importance of severity during the dietary trial (Chesney, 2002), the adequacy of the limited antigen diet depends on the pet food manufacturers. This signifies that whenever a veterinarian chooses a limited antigen diet for a dog based on a careful feeding history and reading of the ingredients list, perfect correspondence between the ingredients in the list and those actually included in the formula must be guaranteed by the pet food producer.

Biourge et al. (2004) suggested that the use of several limited antigen diets may be required when a dog is unresponsive to the dietary trial. We believe this to be overly time-consuming, however, and likely to lessen the owner's collaboration. On the contrary, the reliability of the dietary trial should be guaranteed to avoid waste of time.

Bearing this in mind, twelve commercial limited antigen diets (eleven novel protein diets and one hydrolysed diet) were assessed in this study for potential contamination with ingredients of animal origin not listed on the label. The authors hypothesize that such presence may be responsible for a lack of improve-

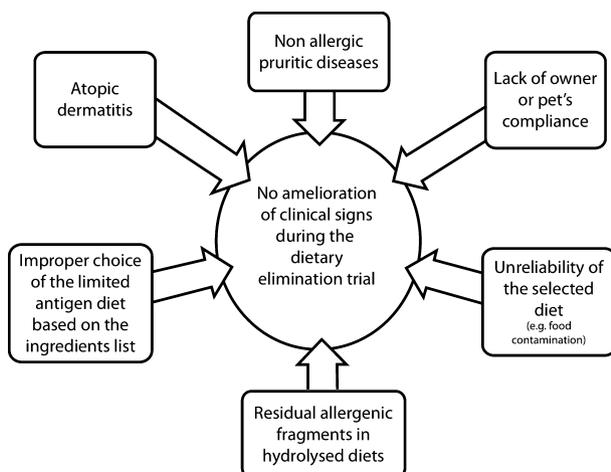


Fig. 2 Causes of lack of clinical improvement in AFR-suspected dogs after an 8- to 10-week dietary elimination trial. In this figure, we assume that parasitic infestation and bacterial and/or fungal infections had already been investigated and ruled out at the time of dietary test set-up.

ment of clinical signs during diagnostic dietary trials and failures in diagnosing canine AFR.

The achieved results showed that ten of the twelve selected commercial dry limited antigen diets, all novel protein diets, were unsuitable for use in diagnostic food elimination trials because they contained ingredients belonging to one or two zoological classes (mammalian, avian or fish) not listed on the label.

The sources of animal origin in the samples were detected through both microscopy analysis to identify bone fragments from animal meal and PCR analysis to detect the DNA of bone-free animal ingredients, such as animal fats, fish oil and hydrolysed chicken liver, which would otherwise not be detected by microscopy.

In our study, all ten samples were found positive by microscopy examination, and this indicates that the unpredicted animal sources are most likely animal meals, which confirms the authors' hypothesis of pet food contamination.

Pet food ingredients are generally by-products of the meat, poultry and fishing industries. When animals are slaughtered, muscle tissue and few organs are used for human consumption, while the remaining parts of the carcass (by-products) are used in pet food. By-products are submitted to a special thermal process known as rendering that separates fat from bone and lean parts to obtain meat meals and meat-and-bone meals that are the principal sources of animal proteins in dry pet foods.

It is worth noting that the authors considered not only protein but also fat sources of animal origin as potential allergens because cases of allergy in humans have been observed after the ingestion of vegetable oils or animal fats due to the presence of residual proteins from the extraction process (Olszewski et al., 1998; Crevel et al., 2000; Zitouni et al., 2000). Therefore, it cannot be excluded that this may happen also during the rendering of animal by-products.

Our results also demonstrated that both analytical methods, microscopy and PCR analysis, should be performed on each sample to rule out any contamination because they provide different information equally required for a complete safety assessment of the product. For example, PCR-detected avian DNA in sample 3 and mammalian DNA in sample 7 whose bone fragments were not identified by microscopy analysis. This can be explained by the fact that the *lacunae* in a sample can be limited in number, entirely missing or only partially visible depending on the quality of the extraction procedure. The non-identification of *lacunae* can also be due to heavy milling of raw materials that can damage bone histological traits. Furthermore,

the DNA positivity for a zoological class detected by PCR indicates the presence of any organic tissue, such as fat, blood, offal and feathers, which like animal meals may have the potential for being allergenic. Precisely which animal proteins cause AFR, in fact, is still a matter of debate, and therefore, any part of the 'banned' animal species should be considered potentially allergenic, and its administration should be prohibited during the dietary trial.

Thanks to their high specificity, sensitivity and rapidity, PCR-based methods have been increasingly used for the detection of allergenic food constituents in humans (Hird *et al.*, 2003; Stephan and Vieths, 2004; Mane *et al.*, 2009). It must be taken into account, however, that food products are typically complex matrixes and that a variety of their constituents (e.g. salts, proteins, lipids) are potent inhibitors of DNA amplification during PCR (Poms *et al.*, 2004). Moreover, DNA is only a marker of potentially allergenic food because it does not guarantee the presence of the allergenic protein (Poms *et al.*, 2004). For these reasons, the authors find it more helpful not to use PCR as the sole method to detect food allergens in the samples collected, while microscopy analysis should always be adopted as well.

The discrepancies observed between the results expected and those obtained in both PCR and microscopy analysis raise doubts regarding the actual efficacy of commercial limited antigen diets, and novel protein diets in particular, in diagnosing AFR.

Although several studies have focused on the clinical efficacy of different commercial diets used in elimination trials (Jeffers *et al.*, 1991; Leistra *et al.*, 2001; Leistra and Willemse, 2002; Biourge *et al.*, 2004; Loeffler *et al.*, 2004, 2006), few have considered the dietary characteristics that make these products valid diagnostic tools (Ricci *et al.*, 2009; Raditic *et al.*, 2010). The results here presented agree with those provided recently by Raditic *et al.* (2010), who found that three of the selected four venison canine dry foods used in dietary elimination trials were ELISA positive for soy and one for beef, with neither soy nor beef being named in the ingredient list. The study does not say whether or not the samples were positive also for poultry because the ELISA test for poultry protein was found unreliable. In our study, the avian zoological class was the most representative source of contamination in the ten pet foods tested positive for unexpected animal sources.

The discovery that commercial limited antigen diets contained ingredients not declared on the label is discouraging because feeding an actually food-hypersensitive dog a product unpredictably contaminated

with a potentially allergenic protein may preclude significant remission of symptoms and mislead the clinician in diagnosing AFR. The observation that more than 80% of the selected diets were contaminated signifies that the risk of a dog failing to recover during the dietary elimination trial is high, and this raises questions regarding the diagnostic validity of the products used. A previous study (Ricci *et al.*, 2009) showed commercial limited antigen diets to contain significantly higher amounts of omega-3 fatty acids than regular maintenance diets. Considering the omega-3 anti-inflammatory potential described by Scott and Miller (1993), Ricci *et al.* (2009) hypothesized this as the reason behind partial amelioration of clinical symptoms (i.e. pruritus) following dietary elimination trials and another reason for incorrect AFR diagnosis.

The results of our study suggest that feeding dogs commercial limited antigen diets may not prevent them from ingesting potential allergens. Finding that the only hydrolysed diet tested resulted free of unexpected ingredients does not permit generalized conclusions on the adequacy of hydrolysed in relation to novel protein diets, first, because the few other hydrolysed diets available in the market were not tested and, second, because this was not the aim of our study. Various hypotheses may explain the contamination observed in the majority of the selected pet foods, such as inadequate cleaning of the production line and the improper storage and/or transportation of the raw materials.

We, therefore, advise manufacturers to pay more attention to the entire processing line in order to avoid risk of contamination of the dietary products used in diagnostic elimination trials. Furthermore, mandatory official controls using both PCR and microscopy analysis should be performed on each batch to guarantee the absence of undeclared animal sources.

Species-specific PCR analysis should also be adopted in the future to detect the animal species and not only the affiliated zoological class involved in contamination. A quantitative analysis of bone fragments should also be considered to assess the extent of the contamination. The amount of allergenic protein capable of triggering symptoms in dogs and cats is unknown, while bearing in mind, however, that threshold levels for specific allergic reactions vary for humans and range from less than 1 mg to more than 1 g allergenic protein, depending on both the food and the individual (Poms *et al.*, 2004). The detection of even traces of unpredicted animal proteins in pet foods formulated as limited antigen diets is unacceptable due to the risk of invalidating the diagnostic trial.

In conclusion, the use of ten of twelve pet foods tested herein as limited antigen diets may not reliably rule out a diagnosis of AFR, and the use of home-cooked diets should be considered whenever the dog fails to respond to dietary restriction.

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Conflicts of interest

None of the authors have any conflicts of interest to declare.