Development of a polymerase chain reaction-based method to identify species-specific components in dog food

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Objectives—To determine whether there is a relationship between species-specific mitochondrial DNA (mtDNA), especially canine and feline mtDNA, and detectable amounts of pentobarbital in previously analyzed dog food samples.

Sample Population—31 dog food samples previously analyzed for pentobarbital (limit of detection, 1 µg/kg).

Procedure—Polymerase chain reaction (PCR) analysis was performed on dog food samples by use of PCR primers specific for either canine, feline, equine, bovine, porcine, ovine, or poultry mtDNA.

Results—PCR amplicons specific for feline or canine mtDNA at a 0.007% (70 µg/g [wt/wt basis]) or 0.0007% (7 µg/g) level, respectively, were not found in the 31 dog food samples. Most of the 31 dog food samples had a PCR amplicon on PCR analysis when a PCR primer set capable of simultaneously detecting mtDNA of cows, pigs, sheep, goats, deer, elk, and horses was used. Results of PCR analysis by use of primers specific for bovine, swine, sheep and goat, or horse mtDNA revealed amplicons specific for bovine or swine mtDNA only in 27 of the 31 samples. Analysis of the remaining 4 samples failed to yield amplicons for any mammalian mtDNA. Pentobarbital was detected in 2 of these 4 samples. Results of PCR analysis correlated with the stated ingredient list for most, but not all samples.

Conclusions and Clinical Relevance—Because canine and feline mtDNA were not found in a set of retail dog food samples, these results indicate that the source of pentobarbital in dog food is something other than proteins from rendered pet remains. (*Am J Vet Res* 2004;65:99–103)

Pentobarbital is a drug used to euthanatize unwanted animals as well as animals in situations of severe pain and suffering.[1] In the past, some euthanatized animal carcasses were disposed of by rendering into products such as meat and bone meal, tallow, and other products.[2] In 2 separate studies, several lots of commercial dog food were found to contain confirmable amounts of pentobarbital.[3,4]

It has been presumed that pentobarbital was present in these dog food samples because euthanatized animals were included with other animal by-products used in preparing dog food. This presumption has been difficult to test because of the limitations of existing analytic methods.

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The purpose of the study reported here was to determine whether there is a relationship between species-specific **mitochondrial DNA (mtDNA)**, especially canine and feline mtDNA, and pentobarbital in previously analyzed dog food samples. Therefore, we developed a **polymerase chain reaction (PCR)**-based approach to identify species-specific products that might be present in dog food.

The current approach is a modification of the PCR-based method validated for detecting bovine-derived materials in complete animal feed.[5] The underlying principle of the method is the amplification of a specific **mtDNA** sequence by use of PCR primer pairs that permit species-specific amplification. The use of mtDNA sequences increases the number of targets available for amplification relative to genomic DNA, thereby increasing the sensitivity of the method. Accordingly, PCR primers specific for canine, equine, or feline mtDNA sequences were developed and used to test for the possible presence of rendered materials from these species in dog food. In addition, other species-specific PCR primers were used to assess the accuracy of the label claims by comparing the PCR assay results with the ingredient statements from the package label.

Materials and Methods

Dog food samples were obtained from retail outlets in the Baltimore-Washington DC area. These samples had been used previously in a study[4] to determine whether the presence of pentobarbital could be confirmed and the amounts measured. These samples were purchased in December 2000. They were stored at room temperature (approx 23°C) until ground to a powder, then stored at 4°C until analyzed. The sample identification numbers used in this study do not correspond with the previously published table of results.[6]

The PCR primers were designed by use of a software program.[a] The design of the PCR primers for canine, equine, and feline mtDNA used published sequences.[7-9]

The experimental procedure was previously described in detail.[5,10] Briefly, a 0.5-g subsample of ground dog food was mixed with 4.5 mL of extraction buffer (5M guanidine thiocyanate; 0.02M EDTA, pH 8.0; 0.05M Trizma-HCL, pH 6.4; 1.3% Triton X-100) and allowed to incubate overnight (16 to 18 hours) at 60°C. The supernatant was placed in a new tube following centrifugation (4,200 × *g*) to remove undissolved material. Five hundred microliters of the clarified supernatant was mixed with 0.5 mL of extraction buffer and 0.04 mL of silica suspension. The silica was prepared according to Boom.[11] After a 10 minute incubation at room temperature the silica was precipitated by centrifugation (13,000 × *g*), then washed 3 times with wash buffer (extraction buffer minus EDTA and Triton X-100), 2 times with 70% ethanol, and once with acetone. Each wash step was accomplished by resuspending the silica pellet followed by centrifugation

(13,000 × *g*). The DNA was extracted from the silica by use of 0.05 mL of Tris-EDTA buffer (10mM Tris-HCL, pH 8.0; 1mM EDTA, pH 8.0). Five microliters of the DNA-containing solution was used for the PCR assay. The PCR primers specific for canine, feline, and equine mitochondria were deduced and commercially prepared.[b] Each PCR assay tube contained, in a 50-µL volume, 5 µL extracted DNA, 12.5 pmol of each primer (forward and reverse), 50 µM of each dNTP, 1.5mM $MgCl_2$, 2 to 2.5 U Taq, and 50mM KCL. Positive and negative controls were analyzed with all PCR runs. The bovine, porcine, ovine, poultry, and universal PCR primers were as previously described (**Appendix 1**).[9,12,13] Prior to use, all PCR primers were screened against a panel of animal DNA that included blood samples derived from 2 dogs (2 different breeds) and a cat, rabbit, chicken, turkey, horse, pig, cow, deer, elk, sheep, or goat. The primers used in this study were species-specific; they did not produce PCR amplicons when used with DNA from any other species.

The PCR amplification was accomplished by 29 cycles of denaturing at 94°C for 1 minute (first denaturing step is performed for 2 minutes), annealing for 0.5 minutes, followed by extension at 72°C for 1 minute (**Appendix 2**). Sizes of PCR products were determined by use of an Hae III digest of θX-174 as the standard marker.

The positive controls were species-specific DNA isolated from peripheral blood samples, whereas the negative control was prepared by use of double-distilled water. The DNA was isolated from heparinized blood samples by use of a DNA purification kit,[c] following manufacturers' instructions. This method isolates genomic and mitochondrial DNA. The swine, bovine, and canine (Beagle) blood samples were obtained from animals housed at the Office of Research. The samples of elk[d] and deer[e] blood were provided. All other blood samples were obtained from a commercial laboratory.[f] After completion of the PCR assay, 30-µL samples (20-µL PCR product mixed with 10-µL gel loading buffer) were electrophoresed through 2% agarose gels containing ethidium bromide and viewed with ultraviolet light.

Results

A PCR primer set specific for a canine mtDNA sequence was found not to amplify mtDNA-derived blood samples of a cow, sheep, goat, pig, cat, deer, elk, chicken, turkey, rabbit, or horse (**Fig 1**). The potential for components in dog food to impact the PCR process were assessed in 2 ways. Ground dog food in which pentobarbital was not found (limit of detection, 1 µg/kg) was spiked with homogenized whole canine liver, with the DNA extracted from this mixture as detailed. The second approach used samples of DNA purified from these dog food samples that were spiked with varying amounts of purified canine DNA and subjected to PCR analysis (data not shown). The results from these 2 approaches indicated that nothing in the dog food was present that would interfere with either the DNA isolation step or the PCR process.

Some dog food samples previously analyzed for the presence of pentobarbital[4] were then subjected to the DNA extraction process and tested for the presence of canine DNA. The results indicated a complete absence of canine DNA in all 31 samples (**Fig 2**) at a level exceeding 0.007% (wt/wt basis). Repeat analysis of the samples at a level of 0.0007% (wt/wt basis) by use of 50 µL of extracted DNA instead of 5 µL confirmed the absence of mtDNA (data not shown).

Cats and horses are other species that are euthanatized with pentobarbital, and thus might be the source of this drug in dog food. The PCR primer sets specific for either feline or equine mtDNA were developed as for the canine PCR primers (data not shown). These primers were used to test the dog food samples for presence of mtDNA that might have been derived from cats or horses. The results from these analyses indicated a complete absence of PCR amplicons specific for either cat or horse mtDNA in all 31 dog food samples (**Table 1**). This analysis was performed under conditions that achieved detection at the level of 0.007% (wt/wt basis).

To ensure that DNA from species other than dogs and cats could be amplified from these dog food sam-

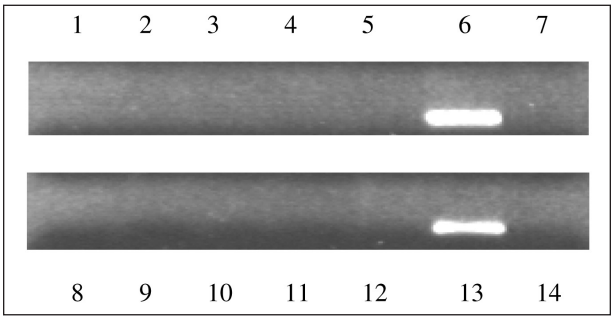

Figure 1—Detection of canine mitochondrial DNA (mtDNA) by use of polymerase chain reaction (PCR) primers specific for *Canis familiaris*. The PCR primers specific for *Canis familiaris* were used to amplify mtDNA obtained from numerous species. The PCR product was separated in a 2% agarose gel containing ethidium bromide. Lanes 1 to 7 (top panel) contained DNA obtained from the blood samples of a cow, elk, horse, goat, sheep, dog (Beagle), and pig, respectively. Lanes 8 to 14 (bottom panel) contained DNA obtained from blood samples of a chicken, goose, cat, rabbit, turkey, dog (mixed breed), and deer, respectively. Only mtDNA from the 2 dogs produced a PCR amplicon. Similar results were obtained when either the feline-specific primers or the equine-specific primers were used. That is, these latter primers only amplified mtDNA from their respective species.

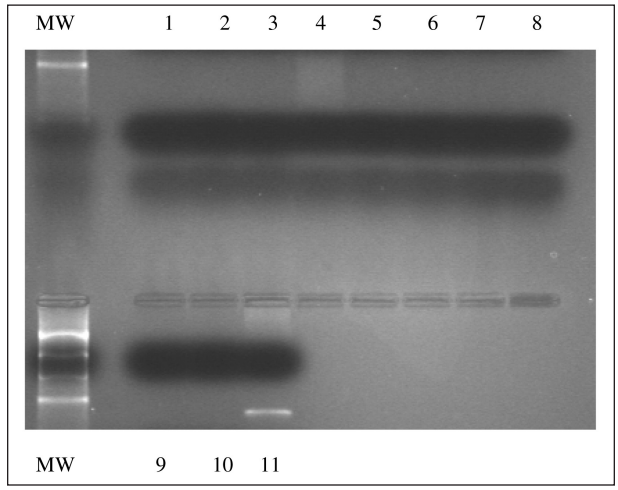

Figure 2—Results of PCR assay for canine mtDNA in dog food. Results are representative of those of the 31 dog food samples analyzed. All these particular samples had previously been found to contain pentobarbital. The DNA from the dog food samples was extracted and subjected to PCR amplification by use of the canine specific PCR primers. The PCR product was separated in a 2% agarose gel containing ethidium bromide. Lanes 1 to 9 contained dog food samples. Lane 10 contained the negative control. Lane 11 contained purified canine DNA (positive control). Only results of the positive control sample (Lane 11) indicated the presence of a PCR amplicon. MW = Molecular weight standards.

Table 1—Polymerase chain reaction (PCR) assay results for species-specific PCR primers used to analyze 31 dog food samples*

	Specificity of the PCR primer pair								
Sample No.	PtB†	Universal	Canine	Feline	Equine	Bovine	Porcine	Ovine	Avian
1	Pos	Pos	Neg	Neg	Neg	Pos	Neg	Neg	Pos
2	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos
3	Neg	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Pos
4	Pos	Pos	Neg	Neg	Neg	Pos	Neg	Neg	Pos
5	Neg	Pos	Neg	Neg	Neg	Pos	Pos	Neg	Neg
6	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos
7	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos
8	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos
9	Pos	Pos	Neg	Neg	Neg	Pos	Pos	Neg	Pos
10	Neg	Pos	Neg	Neg	Neg	Pos	Pos	Neg	Neg
11	Pos	Pos	Neg	Neg	Neg	Pos	Pos	Neg	Pos
12	Neg	Pos	Neg	Neg	Neg	Pos	Pos	Neg	Pos
13	Pos	Pos	Neg	Neg	Neg	Pos	Neg	Neg	Pos
14	Neg	Pos	Neg	Neg	Neg	Pos	Neg	Neg	Pos
15	Neg	Pos	Neg	Neg	Neg	Pos	Neg	Pos	Pos
16	Pos	Pos	Neg	Neg	Neg	Pos	Neg	Neg	Pos
17	Pos	Pos	Neg	Neg	Neg	Pos	Neg	Neg	Neg
18	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos
19	Pos	Pos	Neg	Neg	Neg	Pos	Pos	Neg	Pos
20	Pos	Pos	Neg	Neg	Neg	Pos	Neg	Neg	Pos
21	Neg	Pos	Neg	Neg	Neg	Pos	Neg	Neg	Pos
22	Neg	Pos	Neg	Neg	Neg	Pos	Neg	Neg	Pos
23	Pos	Pos	Neg	Neg	Neg	Pos	Neg	Neg	Neg
24	Pos	Pos	Neg	Neg	Neg	Pos	Neg	Neg	Pos
25	Neg	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Pos
26	Neg	Pos	Neg	Neg	Neg	Pos	Neg	Neg	Pos
27	Pos	Pos	Neg	Neg	Neg	Pos	Pos	Neg	Pos
28	Pos	Pos	Neg	Neg	Neg	Pos	Neg	Neg	Pos
29	Neg	Pos	Neg	Neg	Neg	Pos	Neg	Neg	Pos
30	Neg	Pos	Neg	Neg	Neg	Pos	Neg	Neg	Pos
31	Pos	Pos	Neg	Neg	Neg	Pos	Pos	Neg	Pos

*The 31 dog food samples were all analyzed for the potential presence or absence of rendered meat and bone meal derived from various species. †Results for the presence or absence of pentobarbital.[6] PtB = Pentobarbital. Pos = Positive result. Neg = Negative result.

ples, they were subjected to PCR amplification by use of a set of PCR primers (termed universal primers) shown to amplify only mtDNA from cows, deer, elk, sheep, goats, horses, and pigs.[13] These species were considered as potential candidates for presence in these samples on the basis of the ingredient statements of the dog food samples. The results indicated that most, but not all samples had a PCR amplicon, indicating that rendered materials from 1 or more of these species were present in these dog food samples (Table 1). Interestingly, 2 of the 31 samples were positive for pentobarbital, but did not produce a PCR amplicon when the universal primers were used.

Further characterization of the dog food samples was performed by use of PCR primer sets specific for bovine, swine, or sheep mtDNA (Table 1). These results indicated the presence of rendered material derived from 1 or more of these species. As expected, samples that did not produce a PCR amplicon by use of the universal primers failed to produce amplicons when the species-specific primers were used. For the most part, the PCR assay results confirmed the ingredients as listed on the package label (**Table 2**). The exceptions to this were found in 4 of the 31 samples (samples 15, 22, 25, and 29). Of these 4 samples, only 2 had poultry and lamb-derived proteins listed (15 and 22), yet an amplicon specific for bovine DNA was observed in both samples, with no amplicon specific for sheep (lamb) observed in the results of 1 of the 2 samples (22). The remaining 2 of the 4 samples did not have a source of mammalian protein listed, only animal fat. The DNA from 1 sample produced PCR amplicons when the universal primers and swine-specific primers were used. The DNA from the other sample produced amplicons by use of universal and bovine-specific primers.

For the sake of completeness, the dog food samples were analyzed for the presence of poultry-derived products, although poultry are not euthanatized with pentobarbital. Poultry-derived products were listed as an ingredient for 21 samples. Of the 21 samples, 20 had a PCR amplicon when the poultry primer was used. Seven samples positive for poultry by PCR analysis did not have poultry products listed on the label, whereas 3 samples had negative results for poultry by PCR analysis and did not have poultry-derived products listed on the label.

Discussion

It is widely presumed that the rendered remains of animals euthanatized at animal shelters is the principal source of pentobarbital in pet food. However, the absence of detectable feline or canine mtDNA in the samples indicates that, within the context of our limited survey, rendered proteins from euthanatized dogs and cats were not present in these dog food samples. The detection limit of the method as used in our study is, at a minimum, at a level of 0.0007% (wt/wt basis).

Table 2—Results of PCR assay for mammalian DNA in 31 dog food samples and feed ingredients from animals listed on the sample labels

		Listed ingredients			
Sample No.	Mammalian DNA*	Meals	Animal fat	Digest	Beef tallow
1	Pos	MBM, BBM	AF	AD	None
2	Neg	CK, PBPM	None	None	BT
3	Pos	MBM, CBPM	AF	AD	None
4	Pos	CBPM, BBM	None	None	None
5	Pos	BBM, MBM, PBPM, DCBP	AF	None	None
6	Neg	CBPM, DEP	AF	None	None
7	Neg	CBPM, PD	AF	None	None
8	Neg	CM	AF	None	None
9	Pos	MBM, FM	AF	AD	None
10	Pos	MBM	AF	AD	None
11	Pos	MBM, CBPM	AF	AD	None
12	Pos	MM, PBPM	None	None	None
13	Pos	MBM, CBPM	AF	AD	None
14	Pos	MBM	AF	AD	None
15	Pos	LM, DCBP	AF	None	None
16	Pos	MBM, CBPM	AF	AD	None
17	Pos	BBM	AF	None	None
18	Neg	PM, PD	None	None	BT
19	Pos	MBM	AF	AD	None
20	Pos	MBM, CBPM	AF	AD	None
21	Pos	BF, BBM	None	AD	BT
22	Pos	PM, LM, PD	None	None	BT
23	Pos	BBM	AF	AD	None
24	Pos	MBM, PBPM	AF	AD	None
25	Pos	PM, DEP	AF	None	None
26	Pos	PBPM, MBM, CK	PF	None	None
27	Pos	MBM	AF	BD	None
28	Pos	MBM	AF	AD	None
29	Pos	CBPM	AF	None	None
30	Pos	BBM, CBPM	None	None	BT
31	Pos	MBM, CBPM	AF	AD	None

*PCR assay results for mammalian DNA.
AD = Animal digest. AF= Animal fat. BBM = Beef and bone meal. BD = Beef digest. BF = Beef. BT = Beef tallow. CBPM = Chicken by-product meal. CK = Chicken. CM = Chicken meal. DCBP = Digest of chicken by-product. DEP = Dried egg product. FM = Fish meal. LM = Lamb meal. MBM = Meat and bone meal. MM = Meat meal. PBPM = Poultry by-product meal. PD = Poultry digest. PF = Poultry fat. PM = Poultry meal.
See Table 1 for remainder of key.

This translates to 7 kg of rendered protein in 1,000 metric tons of dog food. Although it can be argued that there may be the rendered remains of dogs or cats below the detection limit, this amount of rendered meat and bone meal is insufficient to produce the amounts of pentobarbital detected in some of these dog food samples.

Horses are the other species euthanatized with pentobarbital in large numbers. Because of their large size, and the amount of drug needed for euthanasia, 1 horse would represent a substantial portion of a large batch of meat and bone meal. However, none of the 31 dog food samples examined in our study tested positive for equine-derived proteins. Similarly, no evidence of the presence of PCR amplicons specific for feline mtDNA was found in the dog food samples. Thus, the presence of pentobarbital in the dog food samples analyzed in our study do not appear to be the result of contamination of meat and bone meal containing the remains of euthanatized dogs, cats, or horses.

Further support for the hypothesis that meat meals derived from euthanatized pets are not the source of the pentobarbital contamination in dog food comes from the analysis results of 2 of the 31 samples. These 2 samples were negative for mammalian mtDNA (cow, deer, elk, sheep, goat, horse, pig, cat, dog) but positive for poultry by-products. These results are in agreement with the product labels, which list only poultry-derived protein products. Although this method cannot conclusively state that there are no pet-derived proteins in these 2 samples (below the level of 0.007% [wt/wt basis]), the low amount of pentobarbital in these samples would preclude them from being the source of drug residue.

Results of analysis of the dog food samples by use of the universal primer and the various species-specific PCR primers indicate that rendered materials from cattle, swine, or sheep were present. Cattle are only occasionally euthanatized with pentobarbital, and thus are not considered a likely source of pentobarbital in dog food. Comparison of the PCR assay results with the product labels reveals a lack of correlation between the various protein sources and the presence of pentobarbital. The only report[2] to follow the fate of pentobarbital through the rendering process found it was equally distributed in the meat and bone meal and tallow fractions. In our study, PCR assay results on the species of origin in the various dog food samples does not support a single point source of protein for the origin of the pentobarbital. The only common feature of all samples containing pentobarbital was the presence of animal fat. This suggests that animal fat might be the source for pentobarbital.

This hypothesis is supported by observations from the initial survey[6] for pentobarbital in dog food. A relationship was observed between the ingredients listed on the package label and the likelihood a sample contained pentobarbital. Specifically, the higher the ranking of animal fat (tallow) on the ingredient list, the greater the likelihood that a given sample would be positive for pentobarbital. Although the results of our study narrow the search for the source of pentobarbital, it does not define the source (ie, species) responsible for the contamination.

The methods of our study may also be used in assuring the validity of label claims. Of the 31 samples, 27 had agreement between the PCR assay results and the package label for mammalian derived components. Only 4 samples had PCR assay results that did not agree with the label claims. In all 4 instances, bovine materials were detected by the PCR assay; however, no bovine protein sources were listed on the labels for these samples. However, 3 of these samples had either animal fat (2 samples) or beef tallow (1 sample) listed on the product label, suggesting that this component might be the source of the bovine material. Residual amounts of animal derived proteins contaminating the animal fat might explain these findings; whether this is the case cannot be determined at present. The results from the remaining 1 of the 4 samples are more difficult to explain. The only animal-derived products on the label for this sample were poultry meal and lamb meal. However, PCR analysis failed to detect lamb (sheep) specific mtDNA, but detected bovine mtDNA.

This finding could be the result of experimental error or sample misbranding. On the basis of previous results, the rates of false negatives and false positives are 1.25% and 0.83%, respectively.[5] Finding bovine mtDNA but not finding lamb (sheep) mtDNA could represent a false positive and false negative, respectively. However, 2 different investigators analyzed this sample on 2 different occasions, with both analysts obtaining the same result, suggesting that the product is incorrectly labeled.

Similarly, 27 of the 31 samples were positive by PCR analysis for poultry, even though only 21 samples had poultry products listed on the label. Only 20 of these 21 samples yielded a PCR amplicon when the poultry primer was used. Seven samples were positive for poultry by PCR analysis but did not list poultry by-products on the ingredient list. These 7 samples had animal digest or animal fat listed as ingredients, however.

One sample with 2 different poultry products on the ingredient list was negative by PCR analysis for poultry. The absence of a PCR amplicon in this sample could be because of experimental error or sample misbranding. However, repeat analysis by different investigators yielded the same result, suggesting that this sample is also incorrectly labeled.

The results of our study indicate that a lack of correlation exists between species identity and the presence of pentobarbital in dog food. They also provide evidence against the presumption that euthanatized pets are routinely rendered and used in pet food. In addition, our study establishes a method for identification of the types (ie, species) of meat and bone meal present in dog food. This method should prove useful for analysis of dog (and cat) food for the accuracy of the label claims.

[a]Primer Premier V 5.00 software program, Premier BioSoft Intl, Palo Alto, Calif.
[b]InVitrogen, Gaithersburg, Md.
[c]Wizard Genomic DNA Purification Kit, Promega, Madison, Wis.
[d]Gift of Dr. Beth Williams, University of Wyoming, Laramie, Wyo.
[e]Gift of Vickie Solberg, Washington, DC.
[f]Rockland Laboratories, Gilbert, Pa.

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Appendix 1

Polymerase chain reaction (PCR) primer sequences

Species	PCR primer sequences	
Bovine	S	GCCATATACTCTCCTTGGTGACA
	AS	GTAGGCTTGGGAATAGTACGA
Porcine	S	GCCTAAATCTCCCCTCAATGGTA
	AS	ATGAAAGAGGCAAATAGATTTTCG
Ovine	S	TTAAAGACTGAGAGCATGATA
	AS	ATGAAAGAGGCAAATAGATTTTCG
Poultry	S	GGGACACCCTCCCCCTTAATGACA
	AS	GGAGGGCTGGAAGAAGGAGTG
Universal	S	ACTTTGAAAAATGATCTGCATCAA
	AS	TCGTTCATTTTGTTTCTCAAGGGGT
Canine	S	ACTGATCGTCATATTCCCTTCCAT
	AS	TCCCTGCTCATAGGGGAATTGCTA
Feline	S	AGCATTAACCTTTTAAGTTAAAGAC
	AS	CCTATTATTGTTGGGGTAG
Equine	S	TGGCGGTGCTTTACATCCCT
	AS	TTTGAGTGAAATCTTCTAGGTGTAA

S = Sense. AS = Antisense.

Appendix 2

Expected reverse transcriptase (RT)-PCR fragment size, annealing temperature, and number of cycles used for amplification

Gene specificity	Annealing temperature (°C)	RT-PCR product (base pairs)	Final extension time (min)
Bovine	58	271	5
Canine	54	271	7
Feline	54	510	7
Equine	56	500	5
Swine	58	212	5
Poultry	58	280	5
Universal	48	271	5
Ovine	58	255	5