

## Prevalence of *Mycobacterium avium* subsp. *paratuberculosis* Fecal Shedding in Alpacas Presented to Veterinary Hospitals in the United States

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**Background:** The prevalence of Johne's disease in alpacas in the United States is unknown. The limits of polymerase chain reaction (PCR) detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in alpaca feces have not been determined.

**Objectives:** To evaluate the use of PCR for MAP detection in alpaca feces; and to estimate the prevalence of MAP fecal shedding in alpacas presented to veterinary teaching hospitals.

**Animals:** Alpacas presenting to 4 US veterinary teaching hospitals from November 2009 to February 2011.

**Methods:** Prospective study. Ten dilutions of a wild MAP strain were added to negative alpaca feces and processed for MAP detection by means of a commercial real-time PCR (RT-PCR) assay, and cultured on Herrold's Egg Yolk Medium (HEYM) and liquid broth. The limits of detection for each method were determined. Fecal samples from alpacas admitted to the veterinary teaching hospitals during the study period were evaluated for MAP via PCR and HEYM.

**Results:** The lowest MAP dilution detectable via PCR was 243 MAP colony-forming units (CFU)/g of feces, at which concentration MAP growth was detectable on HEYM. Ten (6%; 95% confidence interval: 3–9%) of the 180 fecal samples collected were positive on PCR.

**Conclusions and Clinical Importance:** Polymerase chain reaction can provide an accurate and rapid detection of MAP fecal shedding in alpacas; and the prevalence of MAP fecal shedding in hospitalized alpacas in 4 US veterinary teaching hospitals was 6%.

**Key words:** Camelid; Detection; Infectious diseases.

Johne's disease (JD) (paratuberculosis) is a chronic intestinal infection of cattle, sheep, goats, camelids, and wild ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). In cattle, the clinical disease is characterized by diarrhea, weight loss, and edema associated with protein-losing enteropathy.<sup>1</sup> Infected animals can shed MAP organisms in their feces for months to years before they show clinical signs.<sup>1</sup> As a result, these undetected shedders are responsible for spread of the disease before they themselves are suspected as being infected. The most recent studies looking at the prevalence of MAP infection in cattle in the United States suggest that nearly 70% of all US dairy herds, and 95% of herds with more than 500 cows, are infected.<sup>2</sup>

In contrast, the prevalence of JD in alpacas in the United States is unknown. There are individual case reports<sup>3,4</sup> but no surveys of regional or nationwide

### Abbreviations:

BHI	brain heart infusion
CFU	colony-forming units
Ct	cycles to positive threshold
HEYM	Herrold's egg yolk media
HPC	hexadecylpyridinium chloride
JD	Johne's disease
MAP	<i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i>
NVSL	National Veterinary Services Laboratory
RT-PCR	real-time polymerase chain reaction
TTD	time to detection

prevalence. In 1994, llamas were banned from use as pack animals in national parks in the southwestern United States, for fear of transmission of MAP to the native bighorn sheep, yet there were no data to support this policy.<sup>5,6</sup>

To date, the diagnosis of MAP infection in alpacas has been problematic. Like cattle, alpacas infected with MAP develop a granulomatous lesion in the small intestines, leading to protein-losing enteropathy and severe weight loss.<sup>4,7</sup> However, alpacas with JD often do not develop the intractable diarrhea that accompanies the weight loss in cattle, or might develop it terminally, so owners and veterinarians might not be alerted to the possibility of a MAP infection.<sup>7</sup> In addition, the weight loss is often obscured by the alpacas' fleece, so that JD will not be suspected on the basis of casual observation alone.<sup>8</sup> Diagnosis in the past has also been complicated by the lack of diagnostic tests with appropriate sensitivity to detect MAP infection in camelids. Serum tests (ELISA) for antibodies to MAP have been adapted

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Submitted September 5, 2012; Revised April 5, 2013; Accepted May 8, 2013.

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10.1111/jvim.12125

for use in camelids.<sup>9</sup> However, the sensitivity of these tests to detect MAP infection is between 50 and 70%, which is most likely explained by the fact that as an intracellular pathogen, MAP might not induce a strong humoral immune response until late in the infection.<sup>9,10</sup> Similar to cattle, tests relying on the detection of antibodies could be more useful at the herd level, and not for individual animal diagnosis. Bacteriologic culture of MAP from feces, often considered the “gold standard” for diagnosis, can require 8 to 16 weeks before colonies can be identified. Recent advances in diagnostic technology have led to the application of the real-time polymerase chain reaction (RT-PCR) to quickly and accurately identify MAP in fecal specimens.<sup>11</sup> In bovine fecal specimens, the sensitivity and specificity of the test were estimated to be around 60 and 97%, respectively.<sup>11</sup> However, to the authors’ knowledge, the limits of MAP detection in alpaca feces by use of PCR have not been determined.

The authors hypothesized that the combination of inapparent or unrecognized clinical signs, as well as difficult laboratory confirmation, might have led to an underreporting of MAP infection in alpacas in the United States. The objectives of this study were to (1) validate the PCR test for use in alpaca feces, and define the limits of MAP detection in these samples; and (2) estimate the prevalence of MAP shedding in the feces of alpacas presented to 4 veterinary teaching hospitals in the United States.

## Materials and Methods

### Prevalence Samples Collection

The study was approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Alpacas presenting to 4 US veterinary teaching hospitals (Cornell University, Oregon State University, Tufts University, and University of Pennsylvania) from November 2009 to February 2011 were enrolled in the study. After obtaining a written consent from their owner or agent, approximately 2 g of feces was collected from the enrolled alpacas and placed in individual containers. The attending clinician also completed a standardized questionnaire at the time of sampling. The information obtained from the questionnaire included the date of collection; the animal’s name or identification number; signalment; presenting complaint; tentative diagnosis; body condition score (out of 10),<sup>8</sup> if the animal appeared thin (Y/N); if the animal had diarrhea (Y/N); and if the animal was pregnant at the time of sampling. Fecal samples were kept frozen at  $-70^{\circ}\text{C}$  until processing. All fecal samples were processed for RT-PCR and mycobacterial culture in the same laboratory (UPenn) and by the same laboratory personnel. Fecal samples collected at other participating veterinary hospitals were shipped in batches on dry ice and stored at  $-70^{\circ}\text{C}$  until processing.

### Test Strain of MAP

MAP was isolated on Herrold’s Egg Yolk Medium (HEYM) from a fecal sample taken from an alpaca, which had been presented to the George D. Widener Hospital for Large Animals in December of 2001 with chronic weight loss and diarrhea, and was later confirmed to have JD.<sup>4</sup> The isolate was confirmed as

MAP with PCR targeting both the IS900 insertion sequence and the *hspX* gene; and identified as a bovine strain by IS1311 restriction endonuclease analysis.<sup>12</sup> A 0.5 McFarland stock suspension was created from this isolate by choosing colonies from the HEYM and suspending in sterile phosphate-buffered saline. The concentration of MAP in the working stock was determined by serial 10-fold dilutions on HEYM to be  $3.8 \times 10^7$  colony-forming units (CFU)/mL. Prepared stocks were then preserved in 1 mL aliquots at  $-70^{\circ}\text{C}$  and subsequently thawed for the PCR validation experiment.

The MAP parent stock ( $3.8 \times 10^7$  CFU/mL) was thawed at room temperature. Using 7H9 broth without additives, 9 5-fold dilutions were created. The tubes were then identified by their dilution: undiluted; 1 : 5; 1 : 25; 1 : 125; 1 : 625; 1 : 3,125; 1 : 15,625; 1 : 78,125; 1 : 390,625; and 1 : 1,953,125. Normal PCR and culture protocol calls for suspension of 2 g of feces in 35 mL of water. Therefore, for this experiment, 2 g of RT-PCR-negative feces from a repeatedly test negative alpaca from the UPenn teaching herd, as well as 35 mL of sterile water was weighed and added into each 50-mL conical tubes. Using the MAP dilution series described above, 1 mL of each MAP dilution was added to the corresponding fecal water tube. All tubes were rocked for 30 minutes and allowed to stand for 30 minutes. Twenty-five milliliters of supernatant were then removed from each tube: 5 mL was transferred into a 50-mL conical tube containing 25 mL of brain heart infusion (BHI)/hexadecylpyridinium chloride (HPC) for culture and 20 mL was transferred into another 50 mL conical tube to be processed for PCR. For prevalence samples, 2 g of feces was suspended in 35 mL of water, and processed as above. Although the culture and PCR methods utilize an initial 5 mL and 20 mL volume of fecal water, respectively, the difference is accounted for when back calculating the CFU/gram of feces in the original 2-g sample.

### DNA Extraction and RT-PCR

The DNA from each sample was extracted from 20 mL of the fecal water suspension with a commercial DNA extraction kit.<sup>a</sup> For each extraction performed, a positive control (a bovine fecal sample known to contain MAP) and negative control (a bovine fecal sample known not to contain MAP) were extracted alongside the experimental (prevalence or PCR validation) samples to serve as quality control samples.

Each DNA sample was analyzed with a commercially available RT-PCR assay<sup>a</sup> by means of a fluorogenic probe,<sup>b</sup> which targets the MAP-specific sequence of the *hspX* gene. Briefly, 6  $\mu\text{L}$  of extracted DNA is added to 54  $\mu\text{L}$  of the RT-PCR master mix, containing *Taq* polymerase, according to manufacturer’s instructions and each sample run in duplicate wells containing 25  $\mu\text{L}$  each of the DNA/mastermix combination with a real-time PCR thermal cycler.<sup>c</sup> Included on each PCR plate of unknown samples a standard curve consisting of 10-fold dilutions from 25,000 genomic copies of MAP was run in triplicate. Amplification conditions included an initial melting step at  $95^{\circ}\text{C}$  for 10 seconds, followed by 50 cycles of a 2-step PCR consisting of  $95^{\circ}\text{C}$  for 15 seconds and  $62^{\circ}\text{C}$  for 60 seconds. Samples that reached the end of 42 cycles without crossing the threshold were considered negative for MAP DNA. For prevalence samples, a sample was considered positive on PCR if the number of cycles to positive threshold (Ct) of at least 1 well was  $<42$  cycles.

### Mycobacterial Cultures

The laboratory utilizes a HPC decontamination-double incubation method for the culture of MAP on HEYM as recommended by the National Veterinary Services Laboratory

(NVSL) and maintains an annual NVSL certification for the detection of MAP by this method.<sup>13</sup> Fecal samples from the PCR validation experiment were cultured by both HEYM and a commercial liquid broth method.<sup>d</sup> Prevalence fecal samples were cultured by the HEYM method only. Five milliliters of fecal water suspension was transferred to a 50-mL tube containing 30 mL of BHI, incubated at 37°C overnight, and on the second day spun for 30 minutes at 900 × g. The tube was then decanted and the pellet was resuspended in 1 mL of antibiotic solution (amphotericin B, nalidixic acid, and vancomycin) and incubated at 37°C overnight. On the 3rd day, the tube was vortexed and 100 µL was inoculated onto each of 4 tubes (for the PCR validation experiment) or 2 flasks (for the prevalence samples) containing HEYM. Cultures were read every 2 weeks for a period of 16 weeks, at which time, the total number of colony-forming units (CFU)/tube was recorded. Colonies were verified as MAP by subculture on HEYM without mycobactin and by acid-fast staining.

For the liquid broth method (PCR validation experiment only), the commercial tubes<sup>d</sup> were first supplemented with egg yolk<sup>e</sup> and growth supplement.<sup>f</sup> Tubes were then inoculated with 100 µL of each MAP dilution (1 tube/sample), and incubated at 37°C in the commercial instrument,<sup>d</sup> which automatically detects mycobacterial growth by the measure of bacterial oxygen consumption. The time to detection (TTD) for each tube was recorded in days. Any tube that had not signaled by 56 days was considered negative for mycobacterial growth.

### Statistical Analysis

For each dilution included in the PCR validation experiment, the PCR Ct value, the number of CFU/tube on HEYM, and the TTD in the commercial detection system<sup>d</sup> were recorded. The lowest limit of detection for each method was determined as the lowest concentration of MAP that resulted in a positive test for each method. In addition, the relationship among Ct versus CFU, Ct versus TTD, and CFU versus TTD was evaluated by the Spearman's rank correlation coefficient.

The overall prevalence in the sample population was determined as the fraction of PCR-positive samples. The estimated standard error of the proportion and the 95% confidence limit were then calculated. Descriptive statistics were used to report the epidemiologic results obtained from the questionnaire.

### Results

The lowest MAP dilution detectable via PCR was 243 MAP CFU/g of feces (Table 1). At that concen-

tration, MAP growth was also detectable on 3 of the 4 HEYM tubes with 1 MAP CFU/tube. The lowest dilution of MAP to trigger a TTD in the commercial detection system<sup>d</sup> culture medium was 1,216 CFU/g of feces. The Spearman rank correlation coefficient between the PCR Ct value and the number of CFU/g of feces on HEYM was  $-1.0$  ( $P = .0004$ ). The Spearman rank correlation coefficient between the number of CFU/g and the TTD in MGIT was  $-0.972$  ( $P = .003$ ). Finally, the Spearman rank correlation coefficient between Ct and TTD was  $0.991$  ( $P < .0001$ ).

A total of 180 fecal samples were obtained from the 4 participating referral hospitals (Cornell, 31; Oregon, 61; Tufts, 50; and UPenn, 38). Of the 180 fecal samples, 10 (6%) were positive on PCR with Ct values ranging from 33.0 to 39.6 cycles. The 95% confidence interval was determined to be between 3 and 9% of the population. None of the samples was positive on HEYM culture. The prevalence of PCR-positive animals was variable between referral hospitals, with 0, 1, 2, and 7 test-positive animals for Cornell, UPenn, Oregon, and Tufts, respectively; or 0, 3, 3, and 14% of the number of tested animals for Cornell, UPenn, Oregon, and Tufts, respectively. Three of the 7 PCR-positive alpacas from Tufts were from the same farm. The 2 PCR-positive alpacas from Oregon came from 2 different farms. The 10 PCR-positive alpacas (8 females, 2 males) ranged from 1 to 10 years of age (median, 6 years). Their body condition scores ranged from 2/10 to 9/10 (median, 5/10). The presenting complaints varied between animals and were not limited to the gastrointestinal system. Those included weight loss/diarrhea (2), mandibular swelling (1), dystocia (1), corneal ulcer (1), colic (2), and recumbency (1). The 2 remaining animals were healthy on presentation and included a dam admitted with a sick cria, and a companion admitted with the alpaca with the corneal ulcer. Of the 10 PCR-positive animals, 4 had diarrhea on presentation. Final diagnoses were available for 6 of the 8 sick alpacas, and included copper deficiency (1), tooth root abscess (1), uterine tear (1), keratopathy (1), abdominal abscess (1), and rickets (1). The 2 remaining alpacas with "unknown" diagnoses included

**Table 1.** Real-time polymerase chain reaction (RT-PCR) and bacterial culture results for 10 dilutions of a test strain of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) added to negative alpaca feces.

MAP Concentration (CFU/g feces)	RT-PCR Triplicates (Ct)	HEYM Culture on 4 Tubes (MAP CFU/tube)	Liquid Culture TTD (days)
$1.9 \times 10^7$	20,21,26	>300,>300,>300,>300	7.7
$3.8 \times 10^6$	21,22,22	250–300,100–150,250–300,150–200	10.4
$7.6 \times 10^5$	25,25,25	250–300,>300,250–300,>300	14.6
$1.52 \times 10^5$	28,28,29	150–200,150–200,100–150,150–200	16.9
$3.04 \times 10^4$	30,30,31	12,12,6,19	22.3
6,080	31,31,31	3,12,9,7	26.6
1,216	36,37,39	2,3,2,2	45.7
243	36,38,41	1,1,0,1	Negative
49	Negative	0,0,0,0	Negative
10	Negative	0,0,0,0	Negative

CFU, colony-forming units; Ct, cycles to positive threshold; HEYM, Herrold's Egg Yolk Medium; TTD, time to detection.

1 alpaca admitted for recumbency, and for which a final diagnosis could not be determined; and 1 alpaca admitted for colic and diarrhea, which resolved shortly after its admission to the hospital.

## Discussion

The 1st objective of this study was to validate the PCR test for use in alpaca feces, and define the limits of MAP detection in these samples. The main advantage of RT-PCR over traditional MAP culture is that it provides a faster result regarding MAP fecal shedding of a given animal. In a previous study, RT-PCR results were highly and inversely correlated with HEYM culture results, where the higher the number of MAP CFUs per sample, the lower the Ct value for that sample,<sup>14</sup> with the advantage that results are available in days instead of months. This study confirms the utility of RT-PCR for estimating the level of MAP shedding in alpaca feces. It is recognized that MAP organisms can clump in culture suspension or in feces, so that CFU counts might not correspond 1 : 1 with the number of organisms in the sample. However, our results showed that, as the number of organisms or clumps of organisms (CFU) are reduced in the sample through dilution, results of HEYM culture (CFU counts), liquid culture (TTD), and RT-PCR (Ct values) all changed in a proportional and highly correlated fashion. Thus, the CFU values shown in this study should not be construed to indicate the specific number of organisms in the samples because of the possibility of clumping, but it is reasonable to assume that the 2 values are related, such that higher CFU values represent a greater number of organisms in the sample. Finally, many clinicians have experience interpreting HEYM CFU values from fecal samples, so that these values represent a convenient and familiar reference to which RT-PCR Ct values can be compared.

The 2nd objective of this study was to estimate the prevalence of MAP shedding in feces of alpacas presented to 4 veterinary teaching hospitals in the United States. In the study reported here, the overall prevalence for the population tested was 6%, with a 95% confidence interval between 3 and 9%. The population tested was comprised of alpacas of all ages admitted to 4 veterinary teaching hospitals including 3 teaching hospitals located in the Northeast and 1 from the northwestern part of the United States. It is also acknowledged that the prevalence reported here was obtained from a population comprised of hospitalized alpacas, and might not be a reflection of the prevalence of MAP fecal shedding in the general alpaca population in the United States. Regardless, a MAP fecal shedding prevalence of 6%, even in hospitalized patients, represents a higher prevalence than would be suggested by cases of JD in alpacas seen at those hospitals (no confirmed cases) during the study period. Possible explanations for the discrepancy between the number of clinical cases of JD in alpacas admitted to the participating veterinary hospitals and the preva-

lence of MAP fecal shedding in the population tested might include 1) some animals with clinical JD that are misdiagnosed; 2) the minority of infected camelids that go on to develop clinical JD; 3) some animals that are only transiently infected with MAP and self-cure the infection; or 4) some animals that are passive shedders (pass-through) and are not really infected with MAP.

Of the 7 PCR-positive alpacas from 1 veterinary teaching hospital, 3 were from the same farm. This fits with previous reports of both farm-related outbreaks and sporadic individual cases.<sup>3,4</sup> Genotyping of the MAP isolates could not be performed because MAP was not cultured. Therefore, it is not possible to know if the farm with multiple positive animals harbors a unique MAP genotype or several different genotypes. In a case report describing high MAP fecal shedding in an alpaca and its implications for the rest of the herd, several MAP genotypes were found in the described herd.<sup>4</sup> In the same report, 1 positive alpaca was found to be infected with 2 different MAP genotypes.<sup>4</sup> In the study reported here, it was also not possible to know if the MAP strain(s) were of bovine, ovine, or other origins. It is believed that most strains can infect across ruminant species lines.<sup>7,15</sup> The practice of feeding bovine colostrum to neonatal crias could represent a possible source of MAP introduction within a herd.<sup>16</sup> Cohousing with cattle or grazing of pastures fertilized with cattle manure could represent additional routes of transmission. Interestingly, in the study presented here, the farm with the 3 positive alpacas also housed cattle. Further epidemiologic studies are needed to determine risk factors associated with the presence of MAP on alpaca farms.

Of the 10 PCR-positive animals, 4 displayed potential signs of JD (weight loss and/or diarrhea) on admission. However, 3 of those 4 alpacas were diagnosed with other diseases, copper deficiency (1), rickets (1), and abdominal abscess (1), which could also explain their clinical signs. The remaining alpaca with diarrhea was in excellent body condition and was presented for colic that quickly resolved, although the cause of her colic episode was never determined. Because these animals were discharged alive from the hospitals, postmortem examinations were not performed. Follow-up information regarding response to treatment after discharge was not available. It is therefore impossible to know for certain if JD played a role in the clinical presentation of those animals or if the MAP fecal shedding was purely an incidental finding. This emphasizes the importance of ruling out other known causes of diarrhea or weight loss or both when presented with an alpaca displaying chronic weight loss, change in fecal character, /or hypoproteinemia.

The interpretation of a positive PCR result in an asymptomatic alpaca can represent a challenge for a clinician. The RT-PCR for MAP is highly specific (around 97%) in other species.<sup>11</sup> In this study, most PCR-positive prevalence samples had high Ct values,



were positive in only 1 well, and all were negative on HEYM culture. All this suggests low levels of fecal shedding. Although in cattle, it is not uncommon that fecal samples with Ct values near the positive cut-off (38–42) are negative on culture<sup>17</sup>; it is acknowledged that, based on the results obtained in the validation part of the study presented here, PCR-positive animals with a Ct value between 35 and 38 were expected to have at least 1 MAP CFU per HEYM tube. The difference in culture sensitivity between the 2 experiments presented here could be attributed to several factors: 1) fecal samples from the prevalence animals were shipped and frozen for up to 8 months before being cultured, which might have affected MAP viability; 2) the PCR-positive animals from the prevalence study might have been harboring a different strain of MAP that can be more difficult to culture than the test strain used in the validation part of the study; 3) the test strain used in the validation part of the study had already gone through one passage and therefore might have been more adapted to laboratory conditions; and 4) in animals that are naturally infected, MAP might be more intracellular and can be more difficult to grow than in feces that have been spiked with a known amount of MAP.

In cattle and other ruminant species including camelids, animals that are shedding high numbers of MAP in their feces can contribute to substantial environmental contamination,<sup>1,4</sup> and the presence of 1 or several high shedders can contribute to “passive shedding” or “pass-through shedding” of MAP by herd mates. As MAP is deposited on pasture by infected animals, it can be ingested by other animals and detected in their feces by RT-PCR.<sup>1</sup> Those “passive shedders” typically have a high Ct number and are found negative on follow-up fecal RT-PCR testing, especially after removal of a high shedder from a group.<sup>5</sup> In the study reported here, alpacas were tested only once. Whether these low-level shedders were transiently infected, colonized but not likely to develop JD, or simply identified before the development of JD remains unknown. Furthermore, as documented in other species, an animal can shed significantly different levels of MAP over relatively short (days) and long (weeks to months) periods of time. Clinicians and owners must keep in mind that intermittent shedding could potentially lead to the misclassification (false negative) of animals, especially when tested only once. Additional studies aimed at following the progression of PCR-positive alpacas are necessary to evaluate the clinical significance of a positive result.

In conclusion, the authors acknowledge that this is a limited study, which included a small number of animals from 4 teaching hospitals, and involving a disease process that is still poorly characterized in camelids. However, from this experiment, we were able to show that RT-PCR could be used to identify MAP fecal shedding in alpacas; and that MAP fecal shedding was found in 6% of the alpacas presenting to the 4 veterinary teaching hospitals included in this study.

## Footnotes

- <sup>a</sup> Tetracore Vet Alert; Tetracore Inc, Rockville, MD
- <sup>b</sup> Stratagene Mx3000P qPCR System; Agilent Technologies, Santa Clara, CA
- <sup>c</sup> TaqMan; Tetracore Inc
- <sup>d</sup> BACTEC MGIT, BD Diagnostic Systems, Sparks, MD
- <sup>e</sup> BD Difco Egg Yold Enrichment 50%, BD Diagnostic Systems
- <sup>f</sup> BD BACTEC MGIT Para TB Supplement, BD Diagnostic Systems

## Acknowledgments

This study was conducted at the University of Pennsylvania, School of Veterinary Medicine. Financial support for the study was graciously provided by the Alpaca Research Foundation. The results of this study were presented at the 54th Annual Conference of the American Association of Veterinary Laboratory Diagnosticians, September 2011, Buffalo, NY; and the 11th International Colloquium on Paratuberculosis, February 2012, Sydney, Australia. The authors thank Ms. Gretta Riffin of the New Bolton Center-Johne's disease Laboratory for her technical support.

*Conflict of Interest:* Authors disclose no conflict of interest.

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