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Authors: Anderson, Jennifer L., Meece, Jennifer K., Koziczkowski, Jeff J., Clark, Dorn L., Radcliff, Roy P., et al.

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Mycobacterium avium subsp. *paratuberculosis* in Scavenging Mammals in Wisconsin

Jennifer L. Anderson,[1] Jennifer K. Meece,[2] Jeff J. Koziczkowski,[1] Dorn L. Clark, Jr.,[1] Roy P. Radcliff,[1] Cherrie A. Nolden,[3] Michael D. Samuel,[4] and Jay L. E. Ellingson[1,5] [1] Marshfield Clinic Laboratories, Food Safety Services, Marshfield Clinic, Marshfield, Wisconsin 54449, USA; [2] Clinical Research Center, Marshfield Clinic Research Foundation, Marshfield Clinic, Marshfield, Wisconsin 54449, USA; [3] Department of Wildlife Ecology, University of Wisconsin–Madison, Madison, Wisconsin 53706, USA; [4] Wisconsin Cooperative Wildlife Research Unit, University of Wisconsin–Madison, Madison, Wisconsin 53706, USA; [5] Corresponding author (email: jellingson@kwiktrip.com)

ABSTRACT: The presence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in nonruminant wildlife has raised questions regarding the role of these species in Johne's disease transmission. In this study we tested 472 tissues from 212 animals of six different species of scavenging mammals. All animals were taken from within a 210-square-mile area in Dane and Iowa counties of south central Wisconsin from September to May in 2003–04 and tested for the presence of MAP. We detected MAP-specific DNA in 81 of 212 (38%) scavenging mammals, in 98 of the 472 (21%) tissues; viable MAP was cultured from one coyote's ileum and lymph node tissue. Despite the low numbers of viable MAP isolated in this study, our data adds to the increasing evidence demonstrating the potential for transmission and infection of MAP in nonruminant species and provides possible evidence of interspecies transmission. The apparently high exposure of nonruminant wildlife provides potential evidence of a spillover of MAP to wildlife species and raises the question of spillback to domestic and wild ruminants. These results demonstrate the importance of understanding the role of wildlife species in developing management strategies for Johne's disease in domestic livestock.

Key words: Johne's disease, *Mycobacterium avium* subsp. *paratuberculosis*, scavenging mammals, Wisconsin.

Johne's disease is a chronic granulomatous infection of wild and domestic ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The digestive tract is the primary site of infection and is the typical site of lesions in animals infected with MAP (Williams, 2001). The disease results in significant economic losses for individual dairy farms and the dairy industry as a whole due to reduced milk production and death of ruminants. In the United States, annual dairy industry losses are estimated as high as $250 million (Ott et al., 1999). There also is growing concern of a possible link between MAP and Crohn's disease in humans (Nasser et al., 2004).

MAP infection is rarely reported in free-ranging wildlife populations, but has been documented in a number of wild ruminants including: white-tailed deer (*Odocoileus virginianus*; Chiodini and Van Kruningen, 1983); red deer (*Cervus elaphus*), fallow deer (*Dama dama*), roe deer (*Capreolus capreolus*), and moufflon (*Ovis musimon*; Pavlik et al., 2000); bighorn sheep (*Ovis canadensis*) and mountain goats (*Oreamnos americanus*; Williams et al., 1979); tule elk (*Cervus nannodes*; Jessup et al., 1981); bison (*Bison bison*; Buergelt et al., 2000); ibex (*Capra ibex*; Ferroglio et al., 2000); and saiga antelope (*Saiga tatarica*; Dukes et al., 1992). Recent studies have also reported the presence of MAP in nonruminant wildlife such as rabbits (*Oryctolagus cuniculus*; Greig et al., 1999); red fox (*Vulpes vulpes*), stoats (*Mustela erminea*), weasel (*Mustela nivalis*), vole (*Microtus agrestis* and *Clethrionomys glareolus*), crow (*Corvus corone*; Beard et al., 2001); and feral cats (*Felis familiaris*; Palmer et al., 2005). One study of wild animals collected from farms with endemic Johne's disease in Georgia (USA) and Wisconsin (USA) reported MAP prevalence of 2.7% to 8.3% per premises in Wisconsin and documented the shedding of viable MAP in seven different animals (Corn et al., 2005). The goal of our study

was to test common scavenging mammals from a geographically defined area in southern Wisconsin for the presence of MAP infection. The extent of MAP infection in wild animal populations and the role of these animals in maintenance and spread of the disease must be understood to facilitate appropriate and effective disease management programs.

As part of a larger study to evaluate potential chronic wasting disease infection, we collected raccoons (*Procyon lotor*), opossums (*Didelphis virginiana*), skunks (*Mephetis mephetis*), coyotes (*Canis latrans*), red foxes (*Vulpes vulpes*), and feral cats (*Felis familiaris*) from a 210 square mile area in Dane and Iowa counties (43°05′N, 89°50′W) of south central Wisconsin from September to May 2003–04. The landscape in this area is characterized by rolling hills and small stream valleys with a mixture of dairy farms and oak-hickory woodlots, almost exclusively in private ownership. We obtained animal carcasses opportunistically from road kills, collaborating trappers and hunters, and with box traps. Sampling was neither random nor uniform across the study area, but primarily based on the distribution of trapper effort. Data recorded for each animal included a unique ID, geographic location, date collected, date processed, and prosector. Carcasses were either refrigerated at 4 C or frozen at −20 C until dissected for tissue sampling. Tissues were removed at necropsy, placed in individually labeled bags, and stored frozen at −20 C at University of Wisconsin–Madison. Batches of frozen tissues were periodically shipped to Marshfield in insulated coolers. Upon receipt, the ileum and jejunum were dissected from partially thawed intestinal tracts and placed into individual, sterile 1.5 ml microcentrifuge tubes for testing. Lymph node tissues were transferred to individual, sterile 1.5 ml microcentrifuge tubes. All tissues were stored at −80 C until tested.

Each tissue sample (approximately 2 g each for ileum and jejunum samples) was thawed on ice and minced with a sterile scalpel. Phosphate buffered saline (200 µl: pH 7.2) was added to each sample in a 1.5 ml microcentrifuge tube. Samples were then vortexed using a disruptor genie (Scientific Industries, Inc., Bohemia, New York, USA) on the highest setting for 3 min and centrifuged at 2000 × G for 10 min; supernatant (100 µl) was removed for DNA extraction (described below). The remaining tissue and liquid were added to 1 ml of Dubos medium containing PACT (Polymyxin B sulfate, Amphotericin B, Carbenicillin, and Trimethoprim) (International Dairy Federation, 2001) and incubated 24 hr at 35–37 C. Supernatant was then placed into 1 ml 0.9% Hexadecylpyridinium chloride in one-half strength Brain Heart Infusion broth and incubated at 35–37 C for another 24 hr. Samples were centrifuged at 1500 × G for 15 min. Supernatant was discarded and the pellet was resuspended in 500 µl of para-jem antibiotic supplement prepared according to the manufacturer's instructions (TREK Diagnostic Systems, Sun Prairie, Wisconsin, USA) and incubated at 35–37 C overnight. Each sample was inoculated (300 µl) onto a Herrold's Egg Yolk Agar (HEYA) Slant with Mycobactin J and Amphotericin B, Nalidixic Acid, and Vancomycin, (HEYA; Becton Dickenson, Sparks, MD). The slants were incubated horizontally with the caps loosened at 35–37 C until the surface of the slant was dry (approximately 1 wk). The caps were then tightened and slants were oriented vertically. The inoculated slants were evaluated for typical colonies at 8 wk and 16 wk. At 16 wk, any typical colonies were obtained with a loop for acid-fast staining and DNA extraction.

DNA was extracted from the sample supernatants set aside during processing and any typical colonies obtained from HEYA using the MagNA Pure LC Instrument and the MagNA Pure LC DNA Isolation Kit III (bacterial, fungi) according to the manufacturer's instruction (Roche Diagnostics, Indianapolis, Indiana,

USA). Extracted DNA was stored at −20 C until polymerase chain reaction (PCR) amplification could be performed.

DNA samples were tested by PCR for the presence of MAP genetic material using the P90/P91, IS*900*, and *hsp*X primer sets. Conventional PCR using the IS*900* and *hsp*X primers was conducted as previously described (Miller et al., 1999). Each set of PCR reactions included a master mix negative control (in which sterile PCR-grade water was substituted for extracted DNA) and a previously extracted MAP DNA (ATCC# 19698) positive control. Reactions were amplified on a GeneAmp® PCR System 9700 instrument (Applied Biosystems, Warrington, UK). Conventional PCR products were analyzed by agarose gel electrophoresis (1.5% agarose). The electrophoresis buffer contained 5 mg/l of ethidium bromide and gels were imaged for bands using a Gel Doc 2000 (Bio-Rad Laboratories, Hercules, California, USA). A sample was considered positive if amplified product was observed at 229 base pairs (bp) for the IS*900* primer set and 211 bp with the *hsp*X primer set. The P90/P91 primer set was used to amplify products on the Roche LightCycler® in the presence of SYBR green as described (O'Mahony and Hill, 2002). The annealing temperature in this procedure was adjusted to 75 C for 5 sec during in-house optimization. Each set of PCR reactions included negative and positive controls as was described for conventional reactions. A sample was considered positive if it showed an increase in fluorescence during amplification and a corresponding melting curve was observed at 90–92 C. A sample was considered PCR positive if it was positive by at least one of the following methods: IS*900* conventional PCR, *hsp*X conventional PCR, or P90/P91 LightCycler® real-time PCR.

Culture positive samples were sequenced using the PCR confirmation product from the IS*900* primers. The PCR product was purified using the QIAquick® PCR Purification Kit (QIAGEN, Valencia, California, USA) according to the manufacturer's instructions. One µl of the purified PCR product was amplified in a sequencing reaction using the BigDye® Terminator v3.1 cycle Sequencing Kit (Applied Biosystems) and the manufacturer's recommended protocol. Each reaction contained 1.6 µM of either the forward or reverse primer. After amplification, the dye terminators were removed from each reaction using the DyeEx 2.0 Spin Kit (QIAGEN) according to the manufacturer's instructions. The reactions were then loaded onto a 96-well PCR plate and dried down on the GeneAmp® PCR System 9700 (Applied Biosystems) at 70 C for about 30 min. Reactions were resuspended in 10 µl Hi-Di formamide and denatured at 95 C for 2 min. Sequencing was performed on the ABI model 3100 automated DNA sequencer (Applied Biosystems). DNA sequences were aligned using Lasergene99 software (DNASTAR Inc., Madison, Wisconsin, USA). Sequence analysis was performed using the Basic Local Alignment Search Tool (BLAST) program for database searches at the National Center for Biotechnology Information website (Altschul et al., 1990).

The presence of MAP-specific DNA was detected in 81 of 212 (38%) scavenging mammals and 98 of the 472 (21%) tissues tested with at least one of the following methods: IS*900* conventional PCR, *hsp*X conventional PCR, or P90/P91 LightCycler® real-time PCR (Table 1). Several tissue samples were positive for more than one primer set (Table 2). Most PCR positives were found using the P90/P91 or the IS*900* primer sets because these primers amplify a portion of the IS*900* insertion sequence; 15 to 20 copies of this sequence exist in the MAP genome (Green et al., 1989), whereas the *hsp*X primers amplify a single copy heat shock-like protein. All PCR master mix reagent and extraction reagent negative controls were negative and all PCR

TABLE 1. Summary of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) PCR and culture results from 472 scavenging mammal tissues collected from 212 animals in southern Wisconsin September to May 2003–04. Tissue types tested for each animal depended on the condition of the individual carcass. More than one sample of a tissue type was tested for some animals.

	Animals			Tissues			
Species	No. tested	PCR positive[a]	% positive	No. tested	PCR positive[a]	% positive	Culture positive[b]
Coyote	59	28	47	159	34	21	1
Feral cat	5	1	20	11	1	9	0
Skunk	5	3	60	8	3	38	0
Opossum	63	15	24	141	16	11	0
Raccoon	73	29	40	133	37	28	0
Red fox	7	5	71	20	7	35	0
Totals	**212**	**81**	**38**	**472**	**98**	**21**	**1**

[a] PCR positive by any of the following methods: IS*900* conventional PCR, *hsp*X conventional PCR, or P90/P91 LightCycler® real-time PCR. Tissue samples that were PCR positive with more than one primer set were only tallied once as positive.

[b] Culture positive on Herrold's egg yolk agar with mycobactin J and ANV, acid-fast positive by staining, and PCR confirmed.

MAP DNA positive controls were positive during sample testing.

A sample of ileum from one coyote (of 59 tested; 2%) was culture positive, showing typical growth of >50 colonies. Colonies looped from the positive slant were PCR positive for all three primer sets, and showed presence of acid-fast bacilli when stained. Six additional tissues (spleen, liver, kidney, lung, heart, and an additional lymph node) from this positive coyote were subsequently tested according to the methods described earlier. The kidney sample was PCR positive and the additional lymph node sample was culture positive. Colonies from the second lymph node were also PCR positive for all three primer sets and showed presence of acid-fast bacilli when stained. Figure 1 shows conventional PCR results from the two culture positive coyote tissues and several other samples tested. Sequenced IS*900* PCR products from the culture positive lymph node and ileum were identical to *Mycobacterium avium* subsp. *paratuberculosis* sequence.

Recent studies have reported the presence of MAP in both ruminant and

TABLE 2. Frequency of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) PCR positives in culture negative scavenging mammal tissues with P90/P91 LightCycler®, IS*900*, and *hsp*X primer sets. Every tissue was tested with each of the three primer sets.

	Ileum				Jejunum				Lymph Node			
Species	P90/91	IS*900*	*hsp*X	Totals	P90/91	IS*900*	*hsp*X	Totals	P90/91	IS*900*	*hsp*X	Totals
Coyote	5	8	1	14/59	7	5	0	12/56	2	3	2	7/44
Feral cat	0	0	0	0/4	1	0	0	1/4	0	0	0	0/3
Skunk	0	0	0	0/1	1	2	0	3/5	0	0	0	0/2
Opossum[a]	3	2	1	6/62	4	6	0	10/65	0	1	1	2/14
Raccoon[b]	3	4	1	8/25	19	9	0	28/78	2	5	1	8/30
Red fox	2	1	0	2/7	2	0	0	3/6	2	1	0	3/7
Totals	**13**	**15**	**3**	**30/158**	**34**	**22**	**0**	**57/214**	**6**	**10**	**4**	**20/100**
% pos	**8**	**9**	**2**	**19**	**16**	**10**	**0**	**27**	**6**	**10**	**4**	**20**

[a] Two separate opossum jejunum tissues were positive for both the IS*900* and P90/91 primer sets.

[b] Five separate raccoon jejunum tissues and one lymph node tissue were positive for both the IS*900* and P90/91 primer sets. One raccoon lymph node tissue was positive for both the P90/91 and *hsp*X primer sets.

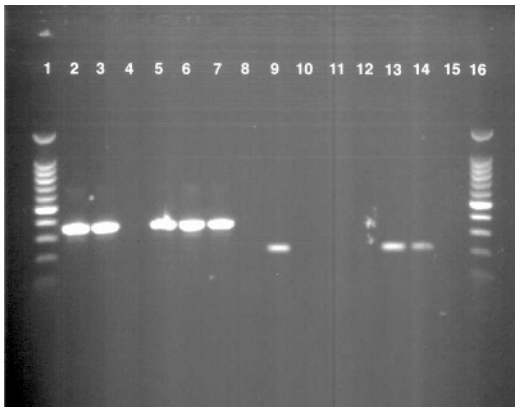

FIGURE 1. PCR detection of *Mycobacterium avium* subsp. *paratuberculosis* in scavenging mammal tissue samples with IS*900* and *hsp*X primer sets. Lanes 1 and 16 contain 100 bp molecular weight markers. Lanes 2 and 9 contain amplification products from scavenging mammal sample 117, a coyote ileum tissue that was culture positive for *Mycobacterium avium* subsp. *paratuberculosis* (MAP), with the IS*900* and *hsp*X primers, respectively. Lanes 3 and 10 contain amplification products from scavenging mammal sample 123 with the IS*900* and *hsp*X primers, respectively. Lanes 4 and 11 contain amplification products from scavenging mammal sample 125 with the IS*900* and *hsp*X primers, respectively. Lanes 5 and 12 contain amplification products from scavenging mammal sample 128 with the IS*900* and *hsp*X primers, respectively. Lanes 6 and 13 contain amplification products from scavenging mammal sample 117, a coyote lymph node tissue that was culture positive for MAP, with the IS*900* and *hsp*X primers, respectively. Lanes 7 and 14 contain amplification products from a positive control with the IS*900* and *hsp*X primers, respectively. Lanes 8 and 15 contain amplification products from a negative control with the IS*900* and *hsp*X primers, respectively.

nonruminant wildlife. We tested 472 tissues from 212 animals across different species of scavenging mammals for the presence of MAP infection, and to provide complete information, data are reported, by animal and by tissue. We detected MAP-specific DNA in 21%, 9%, 38%, 11%, 28%, and 35% of tissues and 47%, 20%, 60%, 24%, 40%, and 71% of coyotes, feral cats, skunks, opossum, raccoon, and red fox tested, respectively (Table 1). Results indicate that viable MAP was once present in the digestive tracts of these animals, and viable MAP was cultured from one coyote's ileum and lymph tissue.

In a study of free-ranging birds and mammals from MAP infected farms in Wisconsin and Georgia (Corn et al., 2005), viable MAP was isolated from a higher percentage of animals than reported in this study. The difference between rates of viable MAP isolation in this study and others might be due to a number of factors, including the decontamination procedures used in this study, freezing and thawing of samples during processing, the type of culture media used, and the prevalence of Johne's infected farms in our study area. Decontamination methods similar to those used in our study have been shown to reduce the recovery of viable MAP (Dundee et al., 2001). Another factor that could have affected the viability of MAP in this study was the numerous freeze-thaw cycles that the samples underwent before being processed for culture. The results of this study also might have been affected by the type of culture media used as other studies used either a liquid culture media, such as BACTEC (Corn et al., 2005), or a 7H11 solid media (Beard et al., 2001; Greig et al., 1999). The prevalence of Johne's infected farms in our study may have differed from those reported by Corn et al. (2005), a reflection of geographically distinct study areas. Iowa and Dane counties contain a large number of infected herds (320 and 406 operating dairy herds respectively; United States Department of Agriculture, 2005), but because Johne's disease testing programs are voluntary and results are confidential, it is unknown how many infected herds were located in our study area. Such differences in techniques and study sites make comparisons of Johne's disease prevalence with presence of MAP in wildlife difficult.

No recent data are available on the rates of Johne's disease in cattle herds within our study area. A 1994 study reported MAP prevalence of 7.3% in 4,990 dairy

cows tested from 158 Wisconsin herds. More infected herds were found in the western and southern (including our study area) districts (Collins et al., 1994). According to the 2004 Animal Health Report (United States Department of Agriculture, 2004), the number of herds participating in the Johne's control program is increasing, and the percentage of Johne's test negative herds is decreasing. Therefore, we suspect that prevalence in Wisconsin (and our study area) has increased since 1994, but further research is needed to understand the relationship between MAP-positive cattle herds and wildlife populations.

Despite the low numbers of viable MAP isolated in this study, these data add to the increasing evidence demonstrating the potential ability of MAP to infect many different species. Our results are the first to document infection in free-ranging coyotes and indicate that MAP is, at a minimum, commonly ingested by and potentially infecting the scavenging species of mammals found in our study area. If individual animals within this area are being exposed to MAP (and possibly infected), they might have the potential to transmit the bacteria to other wild animals and cattle as they travel throughout their normal range. This potential must be investigated because it could severely impact the domesticated ruminant populations in this and other areas. We recommend further research to determine the extent of MAP infection in wildlife populations and the role of infected animals in the maintenance and spread of the disease to both free-ranging and domestic species. In particular, information is needed on the rates of infection and prevalence of MAP in free-ranging species, whether these species are contracting MAP at infected farms, whether a reservoir has been established in wildlife populations, and whether infected wildlife can transmit disease to domestic species. In addition, the potential impact of MAP on the health of wildlife populations of both ruminant and nonruminant species is completely unknown.

Future research should determine which species of wildlife are most susceptible to MAP infection and whether infected wildlife are associated with Johne's-infected farms. We recommend integrated research and surveillance studies that consider the role of farm animals, wild animals, and environmental sources of MAP within a geographically defined area, using sequencing and pulsed field gel electrophoresis (PFGE) of all MAP isolates discovered. A study of this type would help determine how MAP transmission occurs between species, the potential for spread of Johne's disease, and potential barriers to prevent the spread of this bacterium. Ideally, this study should be conducted in several areas with varying levels of Johne's infected cattle to facilitate comparison of MAP prevalence in wild animals and domesticated ruminants.

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