Survival of *Mycobacterium bovis* during forage ensiling

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OBJECTIVE
To determine whether *Mycobacterium bovis* remains viable in ensiled forages.

SAMPLE
Alfalfa, mixed mostly grass, and corn silages.

PROCEDURES
For each of 10 sampling days, six 250-g replicate samples of each feedstuff were created and placed in a film pouch that could be vacuum sealed to simulate the ensiling process. Within each set of replicate samples, 4 were inoculated with 10 mL of mycobacterial liquid culture medium containing viable *M bovis* and 2 were inoculated with 10 mL of sterile mycobacterial liquid culture medium (controls) on day 0. Pouches were vacuum sealed and stored in the dark at room temperature. On the designated sampling day, 1 control pouch was submitted for forage analysis, and the other pouches were opened, and forage samples were obtained for *M bovis* culture and analysis with a PCR assay immediately and 24 hours later.

RESULTS
None of the control samples had positive *M bovis* culture or PCR assay results. Among *M bovis*–inoculated samples, the organism was not cultured from alfalfa and corn silage for > 2 days but was cultured from mixed mostly grass silage for 28 days after inoculation and ensiling initiation. *Mycobacterium bovis* DNA was detected by PCR assay in samples of all 3 feedstuffs throughout the 112-day observation period.

CONCLUSIONS AND CLINICAL RELEVANCE
Results suggested that properly ensiled forages would be an unlikely source for *M bovis* transmission to cattle. Further research is necessary to determine whether ensiling kills *M bovis* or forces it to become dormant and, if the latter, elucidate the conditions that cause it to revert to an infectious state. (*Am J Vet Res* 2019;80:87–94)

Bovine tuberculosis is endemic in wild white-tailed deer (*Odocoileus virginianus*) in northeast Michigan, and deer are considered an important wildlife reservoir for transmission of bTB in that area. Understanding potential modes of transmission for *Mycobacterium bovis*, the causative agent of bTB, is important for mitigating the risk of infection for cattle herds. Feed contaminated by infected deer is believed to be 1 mode of transmission for *M bovis* to cattle.[1] Results of a previous study[2] indicate that *M bovis* can survive for at least 16 weeks on common feedstuffs (eg, apples, corn, carrots, sugar beets, potatoes, and hay) available to deer in northeast Michigan. Ensiled corn, alfalfa, and grasses are frequently fed to cattle in northeast Michigan, including herds identified as infected with bTB. The fields from which those feedstuffs are harvested are commonly grazed by white-tailed deer. Thus, feedstuffs could be contaminated with viable *M bovis* at harvest prior to ensiling. Some bacterial pathogens of cattle can survive the ensiling process including enterococci, streptococci,[3] and *Listeria monocytogenes*.[4] The investigators of 2 studies[5,6] used PCR assays to monitor the persistence of MAP DNA in feedstuffs that were experimentally inoculated with the bacterium before being ensiled, and in both studies, MAP DNA was detected after the ensiling process was completed. Culturing of MAP was either not attempted[6] or not successful[5]; therefore, the potential for MAP to remain viable and perhaps infectious in ensiled feedstuffs is uncertain. The objective of the study reported here was to determine whether *M bovis* could survive the ensiling process of feedstuffs commonly used to feed cattle in northeast Michigan.

ABBREVIATIONS

bTB	Bovine tuberculosis
Cq	Quantification cycle
MAP	*Mycobacterium avium* subsp *paratuberculosis*
MMG	Mixed mostly grass
qPCR	Quantitative PCR

Materials and Methods

Forage samples

Specific feedstuffs evaluated included alfalfa, MMG (primarily orchard grass with some clover and alfalfa), and chopped corn plants. All feedstuffs were raised and harvested on Michigan State University farms under conditions that mimicked standard practices for the region. The 3 feedstuffs were harvested at different times during the 2013 growing season. The alfalfa and MMG forages were cut and left in the field to dry with the intent that they would be chopped and removed from the field when the dry-matter content reached approximately 40%. Corn plants were left standing in the field until the dry-matter content of the plants reached approximately 35%, and the entire corn plant was chopped to create forage.

Immediately after harvest, 1 large sample of each feedstuff was transported to the biosafety level 3 microbiology laboratory at the Michigan State University Veterinary Diagnostic Laboratory in Lansing, Michigan. At the laboratory, each large feedstuff sample was subdivided into sixty 250-g replicate samples. Each sample was placed in a 20 X 30-cm nylon-polyethylene 4-mil-thick high-performance film pouch[a] that could be vacuum sealed to simulate the ensiling process.

A set of 6 replicate samples was created for each of 10 sampling days (days 0, 1, 2, 6, 8, 10 [corn] or 13 [alfalfa and MMG], 15, 28, 56 [alfalfa and corn] or 66 [MMG], and 112). For each sampling day, 4 of the 6 replicates were inoculated with *M bovis* in 10 mL of mycobacterial liquid culture medium, and the remaining 2 replicates were inoculated with 10 mL of sterile mycobacterial liquid culture medium to serve as control samples. The contents of each pouch were mixed thoroughly after the assigned inoculum was applied. A temperature data logger[b] was placed in each pouch containing a control sample so that temperature could be monitored while the forage fermented. For all pouches except those created for day 0, approximately 90% of the air was evacuated immediately following inoculation and mixing, and the pouches were sealed with a chamber vacuum packager.[c] The sealed pouches were placed in black plastic trash can liners to eliminate exposure to light and stored at room temperature (18° to 22°C) in a class 3 biological safety cabinet until analyzed.

M bovis inoculum

The *M bovis* strain (MDCH No. 358258) used in this study was originally isolated from a white-tailed deer harvested in northeast Michigan and was closely related to the *M bovis* strain isolated from infected cattle in that region. *Mycobacterium bovis* was propagated to mid-log growth phase in Middlebrook 7H9 broth with albumin, dextrose, and catalase supplement. To determine the number of CFUs of *M bovis* in each broth culture, serial 10-fold dilutions of the broth culture were inoculated onto Middlebrook 7H11 selective agar plates. Serial 10-fold dilutions of each broth culture were also used to determine the limit of detection for *M bovis* DNA by the qPCR assay. The broth cultures served as the source of inoculum for all feedstuffs. Because forage availability for ensiling varied throughout the season, broth culture inoculums were prepared separately for each feedstuff. Thus, the *M bovis* concentration within the broth cultures varied. The *M bovis* concentration was 1.0×10^8 CFUs/mL in broth cultures created for alfalfa and MMG silage samples and 1.3×10^6 CFUs/mL in broth cultures created for corn silage samples.

Sampling protocol

The sampling protocol was the same for all 3 feedstuffs. From each set of 6 replicate samples, 1 control pouch was surface decontaminated and submitted for forage analysis on the designated sampling day. Samples were collected from the remaining pouches for *M bovis* culture and qPCR analysis. From the set of replicate samples created for day 0, 1 silage sample was obtained from each of the 5 pouches (4 pouches inoculated with *M bovis* and 1 control pouch) not submitted for forage analysis. From each set of replicate samples created for each of the other sampling days, 2 samples were collected from each of the 4 pouches inoculated with *M bovis* and 1 sample was collected from the control pouch that was not used for forage analysis on the designated day and 24 hours later to determine the effect of oxygen exposure.

M bovis culture

From each sample designated for *M bovis* culture and PCR analysis, 10 g of silage was placed in a sterile blender jar to which approximately 50 mL of tryptose broth with phenol red indicator was added (ie, enough to cover the silage sample). The contents were finely chopped with a blender, and 7.5 mL of the resulting slurry was poured into a graduated 50-mL conical centrifuge tube. Five milliliters of 0.5N sodium hydroxide was added to the slurry in the conical tube. The contents of the tube were mixed, and the mixture was allowed to sit and digest for 45 minutes at room temperature. Following digestion, 6N hydrochloric acid was added to the mixture in a dropwise manner until a pH of approximately 5.0 was obtained (determined on the basis of a color change on pH paper). Then, the pH was brought back to 7.0 by the addition of 1N sodium hydroxide in a dropwise manner. The tubes were centrifuged at 3,000 X *g* for 20 minutes to concentrate any *M bovis* organisms at the bottom of the tubes. The resulting supernatant was decanted until approximately 2 mL of the sample remained. A 1-mL aliquot of the sample was placed in a sterile cryovial, frozen, and stored at –70°C until qPCR analysis. The 1-mL sample remaining in the centrifuge tube was treated with 1 mL of a combination of vancomycin (10 mg/mL), nalidixic acid (10 mg/mL), and amphotericin B (10 mg/mL) and incubated at 36°C for 1 hour. The resulting mixture was used to inoculate solid culture medium plates (Middlebrook

7H11 and Middlebrook 7H11 selective agar plates[d]) and mycobacterial liquid culture medium.[e] All inoculated culture media were incubated at 36°C for 8 weeks. Solid culture medium plates were visually inspected on a weekly basis for bacterial colonies with morphological characteristics consistent with mycobacteria. Bottles containing liquid culture medium were read with an automated reader[e] on a weekly basis. For any presumptive *M bovis*–positive culture, a portion of the liquid culture medium or bacterial colonies obtained from the solid culture medium plates was smeared onto a glass slide and stained to detect acid-fast bacteria. Only cultures that contained acid-fast bacteria were considered positive for *M bovis*.

Real-time qPCR assay for detection of *M bovis* DNA

All 5 silage samples collected on sampling day 0 underwent analysis with a qPCR assay. For each sampling day other than day 0, all samples obtained from control pouches underwent analysis. However, only 1 of the 2 samples collected from each *M bovis*–inoculated pouch underwent analysis to detect *M bovis* DNA initially, and if that sample yielded negative results, then the second sample also underwent analysis. Thus, the number of silage samples that underwent analysis at each sample acquisition time ranged from 5 to 8.

Each 1-mL aliquot designated for qPCR assay analysis was thawed, and 200 µL of the sample was transferred to a sterile 1.5-mL microcentrifuge tube that contained 0.1-mm glass beads for extraction of bacterial DNA. The tube was placed in a water bath, which was degassed. Then, the tube was sonicated at high power for 5 minutes, boiled for 10 minutes, flash frozen in an ethanol–dry ice bath, boiled for another 5 minutes, and centrifuged for 5 minutes at 10,000 × *g*. The supernatant was aspirated from the pellet and transferred to a sterile 1.5-mL microcentrifuge tube. To purify the bacterial DNA, a volume of 100% ethanol equal to that of the supernatant was added to the tube, and the contents of the tube were mixed. The mixture was then transferred to a silica membrane spin column[f] to capture the DNA. The column was centrifuged at 12,000 × *g* for 1 minute and washed once with 500 µL of buffer (2mM Tris-Cl, 150mM sodium chloride, and 80% ethanol). Bound DNA was eluted from the column in 50 µL of molecular biology–grade water. The eluted DNA was frozen and stored at –20°C until qPCR analysis.

A real-time qPCR assay was performed to quantify the amount of *M bovis* DNA present in silage samples. For each feedstuff, a standard curve against which silage sample results could be compared was created by quantification of *M bovis* DNA in serial 10-fold dilutions of freshly prepared *M bovis* broth cultures. The primer and probe set used for the PCR assay was described previously[7] and was specific for IS6110, an insertion sequence present in the genome of organisms in the *Mycobacterium tuberculosis* complex. For each sample, the PCR assay reaction contained 2 µL of the sample, 0.4µM of each primer, 0.1µM of the probe, and sufficient PCR master mix[g] to bring the reaction volume to 20 µL. The reaction conditions included 1 cycle of 95°C for 10 minutes and 40 cycles of 95°C for 30 seconds and 60°C for 1 minute. All reactions were performed with a commercially available high-performance, multicolor, real-time PCR assay system[h] that included software for data analysis.

All qPCR assays were performed in 96-well plates, and the standard dilution curve was applied to each plate in triplicate. The Cq for each test sample was compared with the standard dilution curve, which served as a proxy measure for the number of CFUs of *M bovis* in the broth culture used to inoculate the silage samples. To monitor for laboratory-acquired or potential environmental DNA contaminants, a DNA sample obtained from a control pouch was included for every 8 wells that contained DNA samples from *M bovis*–inoculated pouches. For each sampling time, the range of Cq values for PCR-positive silage samples was reported in conjunction with the corresponding range for the estimated concentration of *M bovis* in those samples (reported in CFUs/µL), which was extrapolated from the standard dilution curves.

Forage analyses

On each sampling day, 1 control pouch of silage was submitted to a forage analysis laboratory[i] for a standard fermentation profile. That analysis included determination of the percentages of dry matter, lactic acid, acetic acid, butyric acid, propionic acid, isobutyric acid, and total acids; lactic acid-to-acetic acid ratio; pH; absolute and percentage of crude protein; crude protein equivalent from ammonia; and ammonia nitrogen as a percentage of total nitrogen.

Data analysis

The primary outcome variables of interest included detection of *M bovis* in silage samples by culture on solid and liquid mycobacterial media and qPCR assay. Descriptive statistics were generated to summarize *M bovis* detection by those methods in each feedstuff before and during ensiling.

Results

Forage quality and temperature during ensiling

For each of the 3 feedstuffs, select characteristics from the forage analysis profiles over time during ensiling were summarized **(Table 1)**. The dry-matter content was approximately 36%, 65%, and 32% for alfalfa, MMG, and corn silage samples, respectively, on day 0 (ie, immediately after harvest prior to ensiling) and remained fairly consistent throughout the duration of the observation period. As expected, the pH decreased and percentages of lactic acid and total acid increased in silage samples as the ensiling process progressed,

Table 1—Characteristics of alfalfa, MMG, and corn silage samples at various times before (day 0) and during ensiling.

Forage type	Characteristic	Duration of ensiling (d)			
		0	6	10 or 13*	112
Alfalfa	Dry matter (%)	35.76	35.61	35.90	37.16
	pH	5.10	4.80	4.80	4.40
	Lactic acid (%)	2.72	3.41	4.01	5.06
	Butyric acid (%)	0.00	0.00	0.00	0.00
	Total acid (%)	4.39	5.43	5.76	7.17
MMG	Dry matter (%)	65.41	65.17	60.98	59.72
	pH	6.20	6.40	5.80	5.00
	Lactic acid (%)	0.20	0.09	0.96	2.41
	Butyric acid (%)	0.00	0.01	0.01	0.00
	Total acid (%)	0.42	0.26	1.43	3.4
Corn	Dry matter (%)	32.47	31.46	33.71	33.33
	pH	4.50	4.7	3.9	4.2
	Lactic acid (%)	2.46	3.91	4.25	3.83
	Butyric acid (%)	0.00	0.00	0.00	0.00
	Total acid (%)	3.73	5.10	5.57	5.81

*Alfalfa and MMG silage samples were analyzed 13 days after initiation of ensiling, and corn silage samples were analyzed 10 days after initiation of ensiling.

Table 2—*Mycobacterium bovis* culture and qPCR assay results at various times before (day 0) and during ensiling for alfalfa silage samples.

Ensiling duration (d)	*M bovis* culture		*M bovis* qPCR assay			
	No. of samples cultured	No. of samples with positive results	No. of samples assayed*	No. of samples with positive results	Cq for PCR-positive samples	Estimated *M bovis* concentration in PCR-positive samples (CFUs/μL)
0	4	2	5	3	32.07–34.52	22–95
1	8	7	5	3	31.60–34.75	19–125
1†	8	1	4	4	31.55–38.19	2–129
2	8	8	4	4	28.21–30.67	219–950
2†	8	1	5	4	26.81–35.73	11–1,023
6	8	0	6	2	31.20–32.76	63–159
6†	8	0	5	3	34.83–38.78	2–18
8	8	0	5	3	27.19–30.53	237–1,758
8†	8	0	6	2	34.12–36.72	6–28
13	8	0	4	4	26.39–36.72	6–2,835
13†	8	0	7	1	34.68	18
15	8	0	5	3	31.28–37.83	3–152
15†	8	0	4	4	33.89–38.74	2–32
28	8	0	5	4	30.52–36.99	5–239
28†	8	0	4	4	32.14–35.68	11–91
56	8	0	7	1	34.11	14
56†	8	0	8	0	ND	ND
112	8	0	5	3	28.20–29.39	218–434
112†	8	0	7	1	30.87	80

Values represent the range unless otherwise specified. For each of 10 sampling days (days 0, 1, 2, 6, 8, 13, 15, 28, 56, and 112), six 250-g replicate samples of the forage were created and placed in a film pouch that could be vacuum sealed to simulate the ensiling process. Within each set of 6 replicate forage samples, 4 were inoculated with 10 mL of mycobacterial liquid culture medium containing *M bovis* (concentration, 1.0×10^8 CFUs/mL), whereas the other 2 (controls) were inoculated with 10 mL of sterile mycobacterial liquid culture medium on day 0. From the set of replicate samples created for day 0, 1 silage sample was obtained from each of the 4 pouches inoculated with *M bovis* and 1 control pouch. From each set of replicate samples created for each of the other sampling days, 2 samples were collected from each of the 4 pouches inoculated with *M bovis* and 1 sample was collected from 1 control pouch on the designated day and 24 hours later to determine the effect of oxygen exposure. All silage samples collected underwent *M bovis* culture. All silage samples collected on day 0 underwent analysis with a qPCR assay. However, for each of the other sample acquisition times, only 1 of the 2 samples collected from each *M bovis*–inoculated pouch underwent analysis initially, and if that sample yielded negative results, the second sample then underwent analysis.

*Includes only silage samples obtained from *M bovis*–inoculated pouches (does not include forage samples collected from control pouches, all of which yielded negative results on both culture and qPCR assay). †Samples obtained 24 hours after the pouches were opened.

ND = Not detected.

although the rate and extent of those changes varied among the feedstuffs. The percentage of butyric acid, which is an indicator of poor fermentation and associated with rotten or putrefied forage, remained at or only slightly greater than 0 in all silage samples over time. For both alfalfa and MMG silage samples, the temperature in control pouches increased to 26.5°C within 24 hours after initiation of ensiling (ie, vacuum sealing of the pouches) and then gradually decreased and fluctuated between

Table 3—*Mycobacterium bovis* culture and qPCR assay results at various times before (day 0) and during ensiling for MMG silage samples.

	M bovis culture		*M bovis* qPCR assay			
Ensiling duration (d)	No. of samples cultured	No. of samples with positive results	No. of samples assayed*	No. of samples with positive results	Cq for PCR-positive samples	Estimated *M bovis* concentration in PCR-positive samples (CFUs/μL)
0	4	4	6	2	28.46–35.58	6–374
1	8	8	8	1	35.87	3
1†	8	8	8	0	ND	ND
2	8	8	7	2	25.27–30.88	119–2,868
2†	8	8	6	3	26.81–35.73	11–1,023
6	8	8	5	4	29.48–39.03	1–262
6†	8	7	6	3	25.88–36.32	5–1,945
8	8	5	4	4	22.20–37.32	3–16,404
8†	8	5	5	4	25.64–36.23	6–2,323
13	8	2	5	4	25.67–34.76	13–2,280
13†	8	0	7	2	30.65–38.10	2–135
15	8	3	5	3	25.73–38.36	2–2,208
15†	8	0	5	3	27.51–27.98	617–805
28	8	2	7	3	26.86–34.30	9–1,041
28†	8	0	7	1	31.78	71
66	8	0	4	4	26.06–27.77	695–1,829
66†	8	0	4	4	26.42–37.89	2–1,494
112	8	0	5	3	29.76–36.42	5–225
112†	8	0	4	4	24.58–31.64	77–4,245

For each of 10 sampling days (days 0, 1, 2, 6, 8, 13, 15, 28, 66, and 112), six 250-g replicate samples of the forage were created and placed in a film pouch that could be vacuum sealed to simulate the ensiling process. Within each set of 6 replicate forage samples, 4 were inoculated with 10 mL of mycobacterial liquid culture medium containing *M bovis* (concentration, 1.0×10^8 CFUs/mL), whereas the other 2 were inoculated with 10 mL of sterile mycobacterial liquid culture medium (controls) on day 0.

See Table 2 for remainder of key.

18° and 22°C (essentially reflected room temperature) for the remainder of the observation period. For the corn silage samples, the temperature in control pouches peaked at 24°C within 24 hours after initiation of ensiling and then decreased and fluctuated between 19° and 23°C for the remainder of the observation period.

M bovis culture results

Mycobacterium bovis was not cultured from any of the control samples. *Mycobacterium bovis* culture results for *M bovis*–inoculated samples of alfalfa **(Table 2)**, MMG **(Table 3)**, and corn **(Table 4)** silage were summarized. Among samples obtained immediately after pouches were opened, *M bovis* was cultured from at least 1 alfalfa or corn silage sample by use of both solid and liquid culture medium systems on days 0, 1, and 2. *Mycobacterium bovis* was cultured from at least 1 MMG silage sample on days 0, 1, 2, 6, 8, and 13 by use of a solid culture medium system and on days 0, 1, 2, 6, 8, 13, 15, and 28 by use of a liquid culture medium system. Among silage samples obtained from pouches 24 hours after they were opened, *M bovis* was cultured from at least 1 alfalfa silage sample on days 1 and 2, from at least 1 MMG silage sample on days 1, 2, 6, and 8, and from at least 1 corn silage sample on day 1 only.

M bovis qPCR assay results

Mycobacterium bovis DNA was not detected in any of the control samples. *Mycobacterium bovis* DNA was detected in at least 1 alfalfa (Table 2), MMG (Table 3), or corn (Table 4) silage sample throughout the duration of the observation period regardless of whether those samples were obtained immediately or 24 hours after the pouch was opened, with the exception of alfalfa silage samples obtained 24 hours after the day 56 pouches were opened, at which time none of the samples had a PCR-positive result. The proportion of PCR-positive MMG silage samples on day 1 (1/8) was substantially lower than the proportion of PCR-positive alfalfa (3/5) and corn (4/4) silage samples on day 1.

The estimated concentration of *M bovis* in silage samples (range, 10 to 1,000 CFUs/μL) remained fairly constant throughout the observation period for all 3 feedstuffs, although there was some day-to-day and sample-to-sample variation observed **(Figure 1)**. When the dilutions made to the starting concentration of *M bovis* in the 10-mL broth culture inoculum were factored in, the anticipated concentration of *M bovis* in the inoculated samples ranged from 100 to 1,000 CFUs/μL. Thus, it appeared that there was little degradation of *M bovis* DNA during or after ensiling.

Discussion

Results of the present study indicated that *M bovis* was not cultured from alfalfa and corn silages samples that were experimentally inoculated with the bacterium > 2 days after inoculation and initiation of ensiling. However, *M bovis* was intermittently cultured from MMG silage samples that were experimentally inoculated with the bacterium for up to 28 days after inoculation and initiation of ensiling. The

Table 4—*Mycobacterium bovis* culture and qPCR assay results at various times before (day 0) and during ensiling for corn silage samples.

Ensiling duration (d)	*M bovis* culture		*M bovis* qPCR assay			
	No. of samples cultured	No. of samples with positive results	No. of samples assayed*	No. of samples with positive results	Cq for PCR-positive samples	Estimated *M bovis* concentration in PCR-positive samples (CFUs/μL)
0	4	4	4	4	26.33–31.37	214–1,853
1	8	8	4	4	32.34–37.17	18–141
1†	8	8	6	4	33.99–36.11	28–70
2	8	8	5	3	35.37–37.99	13–38
2†	8	0	5	3	35.25–36.82	22–41
6	8	0	5	3	29.57–34.81	49–462
6†	8	0	7	1	35.48	37
8	8	0	4	4	30.52–36.59	23–307
8†	8	0	4	4	33.58–36.95	20–83
10	8	0	4	4	29.27–33.78	76–525
10†	8	0	5	3	33.62–36.15	28–82
15	8	0	4	4	29.42–32.60	126–492
15†	8	0	4	4	33.66–37.29	17–80
28	8	0	4	4	28.27–32.88	112–808
28†	8	0	7	3	31.55–34.46	57–198
56	8	0	4	4	26.65–36.82	21–1,612
56†	8	0	4	4	32.93–35.80	3–21
112	8	0	4	4	29.07–33.22	18–252
112†	8	0	5	3	29.48–35.66	4–194

For each of 10 sampling days (days 0, 1, 2, 6, 8, 10, 15, 28, 56, and 112), six 250-g replicate samples of the forage were created and placed in a film pouch that could be vacuum sealed to simulate the ensiling process. Within each set of 6 replicate forage samples, 4 were inoculated with 10 mL of mycobacterial liquid culture medium containing *M bovis* (concentration, 1.3 X 10^6 CFUs/mL), whereas the other 2 (controls) were inoculated with 10 mL of sterile mycobacterial liquid culture medium on day 0.

See Table 2 for remainder of key.

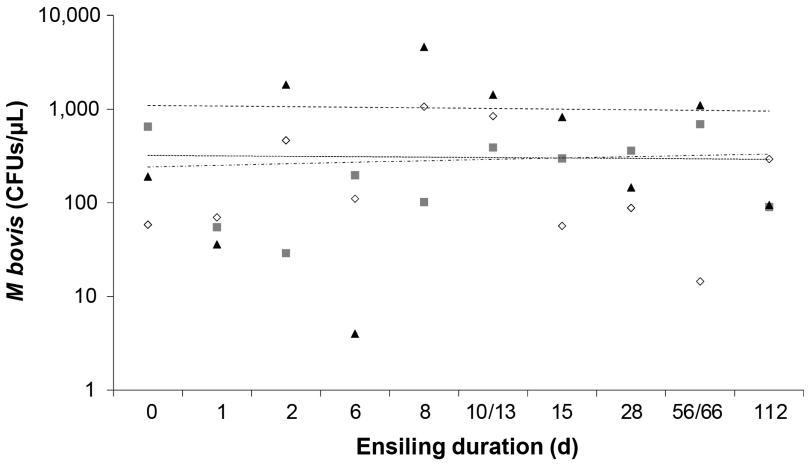

Figure 1—Mean estimated *Mycobacterium bovis* concentration in alfalfa (diamonds), MMG (triangles), and corn (squares) silage samples with positive qPCR assay results at various times before (day 0) and after ensiling as determined by extrapolation from the standard dilution curves created for the qPCR assay. For each of 10 sampling days (days 0, 1, 2, 6, 8, 10 [corn] or 13 [alfalfa and MMG], 15, 28, 56 [alfalfa and corn] or 66 [MMG], and 112), four 250-g replicate samples were inoculated with 10 mL of mycobacterial liquid culture medium spiked with *M bovis* (concentration, 1.0 X 10^8 CFUs/mL for alfalfa and MMG silage samples and 1.3 X 10^6 CFUs/mL for corn silage samples). On the designated day, 1 silage sample was obtained from each of the 4 pouches prepared for day 0, and 2 silage samples were obtained from each of the 4 pouches prepared for the other sampling days. All forage samples collected on day 0 underwent analysis with a qPCR assay. However, for each of the other sampling days, only 1 of the 2 samples collected from each pouch underwent analysis initially, and if that sample yielded negative results, the second sample then underwent analysis. Therefore, each symbol represents the mean *M bovis* concentration for 1 to 8 PCR-positive samples. The dotted, dashed, and dashed-and-dotted lines represent the line of best fit for the data for alfalfa, MMG, and corn silage samples, respectively. Notice that all 3 lines of best fit are close to horizontal, which indicated that the estimated *M bovis* concentration remained fairly stable for the duration of the observation period.

fermentation profiles of MMG, alfalfa, and corn silages can vary markedly.[8] Typically, MMG forages produce less acid and thereby become less acidic during the ensiling process than do alfalfa or corn silages,[8] a finding that was supported by the results of this study. In fact, for the MMG silage samples evaluated in the present study, organic acid production in the form of lactic acid was approximately 50% and 60% that detected for alfalfa and corn silages, respectively, following completion of the fermentation process (day 112). This translated to a higher pH for the MMG silage (5.0), compared with the pH for the alfalfa (4.4) and corn (4.2) silages. That higher pH might have created a less hostile environment for *M bovis*, which allowed the organism to remain viable and cultivatable for a longer period in MMG silage relative to alfalfa and corn silages.

The dry-matter content of the MMG evaluated in the present study was 65% at the time of harvest. For optimal ensiling, the target dry-matter content of forages at harvest ranges from 35% to 40%.[9] The fairly high dry-matter content for the MMG at harvest might have extended respiration and decreased the forage sugar content resulting in suboptimal fermentation relative to the other 2 feedstuffs

evaluated. Thus, it is possible that the risk for extended survival of *M bovis* is greater for forages that are ensiled under suboptimal conditions.

Mycobacterium spp generally do not grow or survive well in acidic environments, although the extent of growth inhibition in acidic conditions varies among species. For example, *Mycobacterium avium* grows well, whereas growth of *M tuberculosis* is inhibited at a pH of 6.0.[10] Additionally, chemotherapeutics that are weak acids appear to be more effective than those with a neutral or basic pH for the treatment of *M tuberculosis* in human patients.[11]

The infectious dose of *M bovis* required to induce bTB in calves following oral ingestion is estimated to be 5×10^3 CFUs.[12] Given that the primary objective of the present study was to determine whether *M bovis* survived the ensiling process of forages commonly used to feed cattle, the concentration of *M bovis* (range, 10^6 to 10^8 CFUs/mL) included in the experimental inoculums was purposely selected to be substantially greater than that expected under natural conditions, and the results should be interpreted accordingly. In fact, under natural conditions, it is highly unlikely that an *M bovis*–infected white-tailed deer would shed the organism in an amount similar to that used for the experimental inoculums of this study.[12,13] That does not, however, negate the value of the findings of the present study because it is important to understand all potential transmission risks for a disease such as bTB, for which a federally regulated eradication program is currently underway in the United States.

In the present study, *M bovis* DNA was detected by use of a real-time qPCR assay in samples of all 3 feedstuffs for the duration of the observation period. Moreover, the amount of DNA quantified in PCR-positive samples remained fairly stable, which suggested that degradation of *M bovis* DNA was minimal during ensiling. That finding was consistent with results of other studies,[5,6,14] which indicate that DNA of MAP, another mycobacterial pathogen of cattle, is detected at fairly consistent levels over extended periods in ensiled forages and fermented dairy products.

Failure to culture *M bovis* from silage samples that tested positive for *M bovis* DNA by qPCR assay might be attributed to multiple factors including the decontamination method (ie, mixing the silage slurry with a combination of vancomycin, nalidixic acid, and amphotericin B and duration of sodium hydroxide digestion) used for silage samples prior to culture, actual culture methods and conditions, physical nature of *M bovis*, and potential for the bacterium to transition to a dormant state. Organisms of the *M tuberculosis* complex tend to have fastidious culture requirements and grow slowly. Consequently, mycobacterial cultures can become overgrown with more rapidly growing bacterial contaminants. Preculture decontamination of samples and the type of culture medium used can affect the rate of growth and detection of *Mycobacterium* spp.[15–17] In the present study, we used standard diagnostic solid and liquid mycobacterial culture media for *M bovis* cultures. The addition of mycobacterial growth factors to the culture media and an extended incubation period might have facilitated detection of viable *M bovis* organisms. Because it was anticipated that silage samples would contain substantial bacterial contamination, we extended the sodium hydroxide digestion phase from the usual 10 minutes to 45 minutes during preculture sample decontamination, which effectively minimized bacterial overgrowth of cultures but might have adversely affected the viability of *M bovis*.

Culture detects viable *M bovis*, whereas the qPCR assay detects *M bovis* DNA regardless of the viability of the organism. In another study,[6] nonviable mycobacteria were experimentally inoculated into forage samples immediately before ensiling, and mycobacterial DNA was detected in those samples for an extended period after ensiling. As part of a pilot project in preparation for this study, we inoculated grass samples with nonviable *M bovis* immediately prior to ensiling, and the amount of *M bovis* DNA subsequently detected in those samples remained fairly stable for 122 days. Although the ensiling process may inactivate (ie, kill) *M bovis*, the organism's thick waxy cell wall likely protected and slowed the degradation of its DNA for an extended period.

In human patients infected with *M tuberculosis*, the organism can persist for prolonged periods by transitioning to a nonreplicating dormant state that is associated with noncultivability.[18] A review[19] of in vitro conditions indicates that *M tuberculosis* can enter a dormant-like state in response to oxygen depletion, exposure to nitric oxide, or nutritional deficiency. While in a dormant state, *M tuberculosis* is characterized as an ovoid cell with a thickened cell wall, low metabolic activity, and abnormally increased resistance to antimicrobials and heating.[20] *Mycobacterium avium* subsp *paratuberculosis* has similar dormancy characteristics.[21,22] Results of an in vitro study[23] indicate that *M bovis* Bacille Calmette-Guérin, an attenuated vaccine strain of *M bovis*, also enters a dormant nonreplicating state during periods of oxygen depletion. To our knowledge, the ability of wild strains of *M bovis* to enter a dormant state has not been investigated, but it seems reasonable to assume that, like other mycobacteria, *M bovis* becomes dormant when conditions for growth and reproduction are suboptimal.

Failure to culture *M bovis* from feedstuffs that were experimentally inoculated with the organism shortly after initiation of ensiling suggested that properly ensiled forages would be an unlikely source for transmission of *M bovis* to cattle. Further studies are necessary to determine the optimum preculture decontamination method and mycobacterial culture medium necessary to facilitate recovery of viable *M bovis* from silage samples. In the present study, *M bovis* DNA was detected in silage samples throughout the observation period, even those from which

M bovis was not cultured; however, we were unable to determine whether that was because the ensiling process inactivated the organism or caused it to enter a dormant state. Additional research is necessary to determine the effect of ensiling on *M bovis* and, if ensiling causes the organism to become dormant, elucidate the conditions that cause it to revert to a nondormant and infectious state.

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Footnotes

a. Bunzl Koch Supplies, St Louis, Mo.
b. EasyLog, Lascar Electronics Inc, Erie, Pa.
c. MV 31, Minipack America, Orange, Calif.
d. Hardy Diagnostics, Santa Monica, Calif.
e. BACTEC, Becton Dickinson and Co, Franklin Lakes, NJ.
f. EconoSpin, Epoch Life Science, Missouri City, Tex.
g. TaqMan Universal PCR Master Mix, Applied Biosystems, Waltham, Mass.
h. 7500 Real-Time PCR System, Applied Biosystems, Waltham, Mass.
i. Dairy One, Ithaca, NY.

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