

# Culture- and Quantitative IS900 Real-Time PCR-Based Analysis of the Persistence of *Mycobacterium avium* subsp. *paratuberculosis* in a Controlled Dairy Cow Farm Environment

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The aim of this study was to monitor the persistence of *Mycobacterium avium* subsp. *paratuberculosis* in environmental samples taken from a Holstein farm with a long history of clinical paratuberculosis. A herd of 606 head was eradicated, and mechanical cleaning and disinfection with chloramine B with ammonium (4%) was carried out on the farm; in the surrounding areas (on the field and field midden) lime was applied. Environmental samples were collected before and over a period of 24 months after destocking. Only one sample out of 48 (2%) examined on the farm (originating from a waste pit and collected before destocking) was positive for *M. avium* subsp. *paratuberculosis* by cultivation on solid medium (Herrold's egg yolk medium). The results using real-time quantitative PCR (qPCR) showed that a total of 81% of environmental samples with an average mean *M. avium* subsp. *paratuberculosis* cell number of  $3.09 \times 10^3$  were positive for *M. avium* subsp. *paratuberculosis* before destocking compared to 43% with an average mean *M. avium* subsp. *paratuberculosis* cell number of  $5.86 \times 10^2$  after 24 months. *M. avium* subsp. *paratuberculosis*-positive samples were detected in the cattle barn as well as in the calf barn and surrounding areas. *M. avium* subsp. *paratuberculosis* was detected from different matrices: floor and instrument scrapings, sediment, or scraping from watering troughs, waste pits, and cobwebs. *M. avium* subsp. *paratuberculosis* DNA was also detected in soil and plants collected on the field midden and the field 24 months after destocking. Although the proportion of positive samples decreased from 64% to 23% over time, the numbers of *M. avium* subsp. *paratuberculosis* cells were comparable.

*Mycobacterium avium* subsp. *paratuberculosis* is the causal agent of a chronic inflammatory intestinal infection known as paratuberculosis or Johne's disease. Its worldwide spread in ruminants means that it bears a high financial impact, especially in dairy cattle. Losses attributed to clinical infection include decreases in milk production, increasing calving intervals, reductions in slaughter weight, infertility, and increased incidence of mastitis (12, 24). Additionally, the potential role of *M. avium* subsp. *paratuberculosis* in triggering Crohn's disease has been discussed for many years (22, 38, 42). Furthermore, new studies describing a significant association of *M. avium* subsp. *paratuberculosis* with diabetes mellitus type I and multiple sclerosis patients have appeared (4, 5).

Due to the long incubation period that is necessary for the development of clinical signs as well as variable immunological responses and irregular shedding of *M. avium* subsp. *paratuberculosis* in feces, it is difficult to diagnose paratuberculosis and eliminate this pathogen from the farm and pastures before it spreads to other susceptible animals. It has been documented that cows with clinical signs can shed pathogens in average amounts of  $10^6$  *M. avium* subsp. *paratuberculosis* cells per gram of feces (6). Therefore, the natural excretion of *M. avium* subsp. *paratuberculosis* in the feces of infected animals as well as the land application of their fresh manure onto fields and pastures contributes to the spread of the pathogen in the environment and its persistence. Interspecies transmission between cattle and wild animals sharing the same habitats was documented by some authors (9, 17).

Although *M. avium* subsp. *paratuberculosis* is generally unable to replicate outside the host, it is able to survive for long periods in different environments (44, 46). This is attributed to the pathogen's thick waxy cell wall that possesses large amounts of lipids responsible for properties such as acid fastness, hydrophobicity,

biofilm formation, increased resistance to chemicals, and physical processes (47). Furthermore, a dormant state as well as spore-like forms are thought to occur in this pathogen (20, 46).

Livestock manure provides nutrients for vegetables and helps build and maintain soil fertility; hence, it is routinely applied on agricultural lands. However, before application on land, proper maturing processes need to be followed in order to eliminate potential pathogens excreted in feces. For that reason, it is generally recommended that manure be stored (fermentation) for 6 months before being spread on land. Unfortunately, *M. avium* subsp. *paratuberculosis* is more resistant than other fecal bacteria, and the survival of the pathogen depends on the treatment methods used, which include anaerobic and aerobic lagoons, composting systems as static piles, aerated static piles, and turned and aerated turned windrows. Grewal et al. (10) reported that with anaerobic liquid lagoon storage treatment, *M. avium* subsp. *paratuberculosis* can survive for 56 days, and other bacteria such as *Escherichia coli*, *Salmonella* species, and *Listeria* species can survive for 28 days. This was in comparison to thermophilic composting at 55°C, when numbers of all bacteria were reduced over 3 days. Nevertheless, *M. avium* subsp. *paratuberculosis* DNA was detectable for up to 175 days in an anaerobic liquid lagoon and for up to 56 days in response to other treatments.

Recently, the number of biogas plants which produce biogas

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but also fermented mass that is used as land fertilizer has increased. During such treatment *M. avium* subsp. *paratuberculosis* was isolated from the fermentors for up to 2 months, and *M. avium* subsp. *paratuberculosis* DNA was detected 16 months after manure introduction (40).

Due to the economic impact of paratuberculosis, control programs for this disease in cattle, sheep, and goats have been introduced in many countries. Programs are based on early detection and elimination of infected animals. The most important steps are considered to be the culling of heavy shedders and separation of young animals from adults (potentially infected animals). Regardless, complete information about *M. avium* subsp. *paratuberculosis* transmission, survival, and pathogenesis is also still missing, and therefore these control programs are not as successful as expected.

The first aim of our study was to monitor the distribution and persistence of viable *M. avium* subsp. *paratuberculosis* cells and *M. avium* subsp. *paratuberculosis* DNA on one dairy cattle farm before and after destocking of all animals and following the application of recommended measures (disinfectant and lime application). The second aim of this study was to determine the extent of environmental contamination with *M. avium* subsp. *paratuberculosis* outside the farm and to study the persistence of *M. avium* subsp. *paratuberculosis* cells or DNA over time.

## MATERIALS AND METHODS

**Farm history.** Clinical and subclinical infection of *M. avium* subsp. *paratuberculosis* was determined in one dairy cattle herd containing 606 head. The prevalence of *M. avium* subsp. *paratuberculosis* (based on quantitative PCR [qPCR] examination of fecal samples from animals older than 15 months) reached 75.6% with a maximum shedding of around  $10^8$  *M. avium* subsp. *paratuberculosis* cells per gram. An outbreak of *M. avium* subsp. *paratuberculosis* infection was declared, and due to an unsuccessful control program, it was necessary that the herd be eradicated. Therefore, measures were taken in accordance with the declaration of sources of infection. A hot pressure cleaner with steam was used for mechanical cleaning, and activated chloramine B with ammonium (4%) was applied for subsequent disinfection in stables and operational areas around the farm. Chloramine B was applied outside the stables to concrete and asphalt, while lime was applied to soil and green areas. Lime was also used for decontamination of the field and field midden in doses of 1,000 kg/ha.

**Sample collection.** To study the effect of herd elimination on *M. avium* subsp. *paratuberculosis* persistence on and outside the dairy farm, a total of 84 environmental samples were analyzed (Tables 1 and 2). Environmental samples were collected into sterile plastic bags or vials. Sampling sites were located inside and outside the stables and the building for storing animal feed (scrapings from floor, spider webs, silage, plants, and liquid lagoon storage). These samples were collected before and 12, 18, and 24 months after the removal of all animals (Table 1). The samples from outside the farm (the field midden with the remains of the manure from an infected cow, a field with a history of intensive fertilization with the manure of infected cows, and a pond that caught the water from the fertilized field) were collected at 12, 18, and 24 months after the removal of all animals from the farm (Table 2). After transportation to the laboratory, samples were stored at 4°C in the refrigerator until the next day or at -70°C in the freezer for up to 4 weeks prior to laboratory examination.

**Sample preparation and identification of isolates.** All environmental samples were submitted for *M. avium* subsp. *paratuberculosis* culture and qPCR. Methods for processing, decontamination, and inoculation of environmental samples on solid medium were published previously (16, 30) and were carried out as follows. Fifty milliliters of liquid sample was centrifuged at  $4,500 \times g$  for 45 min, and the sediment was mixed with 25 ml of sterile distilled water and used for the following procedure. Three grams of each sample was mixed with 25 ml of sterile distilled water and

shaken for 30 min, followed by sedimentation for 30 min at room temperature. After that, 5 ml of the supernatant was added to 20 ml of 0.9% hexadecylpyridinium chloride (HPC; Merck, Darmstadt, Germany) to reach a final concentration of 0.72% HPC; samples were then put on a shaker for 30 min. After 72 h in a dark room at room temperature, 100  $\mu$ l of sediment was inoculated on each of three vials containing Herrold's egg yolk medium (HEYM) with mycobactin J (Allied Monitor, Fayette, MO). The incubation was at 37°C for 3 to 4 months. The presence of acid-fast bacilli was determined by Ziehl-Neelsen (ZN) staining, and ZN-positive cultures were examined for *M. avium* subsp. *paratuberculosis* by conventional multiplex PCR detecting IS900 as described previously (25).

**DNA isolation and qPCR.** Fifty milliliters of water or 0.25 g of another type of sample (e.g., soil, scrapings, cobwebs, and plants) was employed for DNA isolation. Before the isolation, water samples were concentrated by centrifugation at  $4,500 \times g$  for 45 min. For DNA isolation the resulting sediment was used. The roots of plants were washed in phosphate-buffered saline (PBS) buffer at the beginning of the processing. DNA from the upper part of the plants was isolated using a PowerFood Microbial DNA isolation kit (MoBio, Carlsbad, CA) according to the manufacturer's instructions. DNA from roots and other environmental samples was isolated using a MoBio PowerSoil DNA isolation kit (MoBio) with slight modifications (14).

The conditions for qPCR using the primers for IS900 and an internal amplification control as well as the calculation of the absolute number of *M. avium* subsp. *paratuberculosis* cells per g or per ml of environmental sample (depending on the type of sample) was described previously (39). qPCR was performed on a LightCycler 480 instrument (Roche Molecular Diagnostic, Mannheim, Germany), and subsequent analysis was carried out using the fit point analysis option of the LightCycler 480 software (Roche Molecular Diagnostic). Each sample was analyzed in duplicate using qPCR assays.

**Statistical analysis.** Data analysis was performed using the statistical software Statistica, version 9 (StatSoft, Inc., Tulsa, OK) and GraphPad Prism, version 5 (GraphPad Software, Inc., San Diego, CA). Proportions of *M. avium* subsp. *paratuberculosis* DNA-positive samples were evaluated by Fisher's exact test and a chi-square test for trend. Logarithmically transformed values of *M. avium* subsp. *paratuberculosis* cells were compared by an unpaired *t* test and Kruskal-Wallis test.

## RESULTS

**Distribution of *M. avium* subsp. *paratuberculosis* on the farm studied by culture and qPCR.** A total of 48 environmental samples were collected inside and outside the building on the studied dairy farm with only 1 sample (2%) culturing positive for *M. avium* subsp. *paratuberculosis*. This sample originated from a waste pit (water lagoon) and was collected at time zero (when the infected herd was present). Applying the qPCR method, 28 environmental samples (58%) were positive for *M. avium* subsp. *paratuberculosis* DNA during the studied periods. Significant differences in *M. avium* subsp. *paratuberculosis* positivity were shown before (0 months) and in the period after the removal of the infected herd (months 12, 18, and 24), with 81% and 47% positivity, respectively ( $P < 0.05$ , Fisher's exact test). The proportion of *M. avium* subsp. *paratuberculosis* DNA-positive samples decreased over time from 81% at time zero to 43% at 24 months after the removal of the infected herd (Table 1). This trend was statistically significant ( $P < 0.05$ , chi-square test for trend).

A total of 82% of *M. avium* subsp. *paratuberculosis* DNA-positive samples were detected in the cattle barn environment with heavily shedding animals, while other locations with low expected levels of *M. avium* subsp. *paratuberculosis* DNA detection (58% sample positivity for the calf barn and mow and feed preparation rooms), but the difference was not statistically significant ( $P > 0.05$ , Fisher's exact test) (Table 1).

TABLE 1 Environmental samples from the farm collected before and after removal of the infected herd studied by qPCR and cultivation

<i>M. avium</i> subsp. <i>paratuberculosis</i> prevalence by time and method of analysis <sup>a</sup>														
Location or source	Matrix <sup>b</sup>	Spring (0 mo)			Spring (12 mo)			Autumn (18 mo)			Spring (24 mo)			Total no. of samples examined/no. of positive samples (% positive)
		No. of samples examined/no. of positive samples	qPCR	C	No. of samples examined/no. of positive samples	qPCR	C	No. of samples examined/no. of positive samples	qPCR	C	No. of samples examined/no. of positive samples	qPCR	C	
Shed from cattle	Cobweb	3/3	10 <sup>2</sup> –10 <sup>5</sup>	0	1/0	0	1/0	0	1/0	0	1/1	10 <sup>2</sup>	0	11/9 (82)
	Organic substrate	1/1	10 <sup>2</sup>	0	3/3	10 <sup>2</sup>	0	1/1	0	1/1	0	10 <sup>2</sup>	0	
Shed from calves	Cobweb	4/4	10 <sup>3</sup>	0	4/3	0	2/1	1/0	0	0	1/1	0	0	11/6 (55)
	Organic substrate	3/2	10 <sup>2</sup> –10 <sup>3</sup>	0	1/0	0	0	1/0	0	2/2	2/2	10 <sup>2</sup> –10 <sup>3</sup>	0	
Mow and feed preparation room	Cobweb	7/4	10 <sup>0</sup>	0	1/0	10 <sup>0</sup>	0	2/1	1/0	0	1/0	10 <sup>0</sup>	0	5.86 × 10 <sup>2</sup>
	Organic substrate	0	0	0	3/2	10 <sup>2</sup>	0	2/1	0	2/1	0	10 <sup>0</sup>	0	
Subtotal	Organic substrate	0	0	0	0	0	2/2	2/2	0	0	0	10 <sup>1</sup> –10 <sup>2</sup>	0	8/5 (63)
	Waste pit	5/5	10 <sup>3</sup> –10 <sup>4</sup>	1	1/1	10 <sup>2</sup>	0	1/1	0	1/0	0	10 <sup>4</sup>	0	
Outside environment	Silage	0	0	0	2/0	0	2/1	0	0	0	2/0	0	0	0
	Fecal	0	0	0	1/0	0	1/0	0	0	0	0	0	0	
	Others	0	0	0	0	0	2/0	0	0	0	1/0	0	0	
	Subtotal	5/5	0	0	4/1	0	6/2	0	3/0	0	3/0	0	0	
Total (% positive)	16/13 (81)	3.09 × 10 <sup>3</sup>	1	12/6 (50)	2.98 × 10 <sup>2</sup>	0	13/6 (46)	2.20 × 10 <sup>2</sup>	0	7/3 (43)	5.86 × 10 <sup>2</sup>	0	48/28 (58)	
Geometric mean <sup>c</sup>														

<sup>a</sup> Examinations were done before removal of animals from the farm (0 months) or 12, 18 and 24 months after removal of animals from the farm. qPCR values are the numbers of *M. avium* subsp. *paratuberculosis* cells per g or per ml of sample. C, cultivation.

<sup>b</sup> Organic substrate, floor scraping, instruments, sediment, or scraping from watering troughs; fecal, fecal samples from wild animals (roe deer; *Capreolus capreolus*); others, vegetables and flushing.

<sup>c</sup> Geometric mean calculated according to absolute values.

TABLE 2 Environmental samples originating from close to the farm housing the infected cattle studied by qPCR

		<i>M. avium</i> subsp. <i>paratuberculosis</i> prevalence <sup>a</sup>						Total no. of samples examined/no. of positive samples (% positive)
		Spring (12 mo)		Autumn (18 mo)		Spring (24 mo)		
Locality	Matrix	No. of samples examined/no. of positive samples	qPCR	No. of samples examined/no. of positive samples	qPCR	No. of samples examined/no. of positive samples	qPCR	
Field midden	Soil	2/2	10 <sup>2</sup> –10 <sup>3</sup>	1/1	10 <sup>2</sup>	1/0	0	12/7 (58)
	Vegetable	1/1	10 <sup>2b</sup>	4/2	10 <sup>2b,c</sup>	2/0	0	
	Biofilm	1/1	10 <sup>0</sup>	NA	NA	NA	NA	
Subtotal		4/4		5/3		3/0		
Ditch at field midden	Soil	ND	ND	1/1	10 <sup>3</sup>	1/1	10 <sup>3</sup>	6/5 (83)
	Water sediment	1/1	10 <sup>3</sup>	1/1	10 <sup>3</sup>	NA	NA	
	Vegetable	ND	ND	ND	ND	2/1	10 <sup>3c</sup>	
Subtotal		1/1		2/2		3/2		
Field	Soil	2/0	0	1/0	0	1/0	0	9/1 (11)
	Vegetable	1/1	10 <sup>2c</sup>	2/0	0	2/0	0	
Subtotal		3/1		3/0		3/0		
Pond	Water sediment	2/1	10 <sup>2</sup>	1/0	0	1/1	10 <sup>1</sup>	9/3 (33)
	Biofilm	1/0	0	1/1	10 <sup>2</sup>	ND	ND	
	Vegetable	ND	ND	ND	ND	3/0	0	
Subtotal		3/1		2/1		4/1		
Total (% positive)		11/7 (64)		12/6 (50)		13/3 (23)		36/16 (44)
Geometric mean			5.03 × 10 <sup>2</sup>		8.76 × 10 <sup>2</sup>		5.43 × 10 <sup>2</sup>	

<sup>a</sup> Values were determined at the indicated times after removal of the infected cattle. qPCR values are the numbers of *M. avium* subsp. *paratuberculosis* cells per g or per ml of sample. NA, not available; ND, not done.

<sup>b</sup> Upper part of the vegetable.

<sup>c</sup> Roots of the vegetable.

<sup>d</sup> Geometric mean calculated according to absolute values.

*M. avium* subsp. *paratuberculosis* DNA was isolated from organic substrate (85% sample positivity for scrapings of the floor, walls, instruments, and sediment from a watering trough) as well as from cobwebs (53% positivity) found usually above the animal level (Table 1). Regardless, statistically significant differences were not found ( $P > 0.05$ , Fisher's exact test).

All examined samples from the water lagoon (waste pit) were *M. avium* subsp. *paratuberculosis* DNA positive at each studied time. The number of *M. avium* subsp. *paratuberculosis* cells ( $1.96 \times 10^4$ ) at 18 months after destocking is noteworthy. One of six silage samples was positive, with a low number of *M. avium* subsp. *paratuberculosis* cells. Two fecal samples of roe deer (*Capreolus capreolus*) collected on the farm were negative (Table 1).

The average number (geometric mean) of *M. avium* subsp. *paratuberculosis* cells in positive samples at the beginning (0 months) and after destocking (average of months 12, 18, and 24) decreased, respectively, from  $3.09 \times 10^3$  to  $3.02 \times 10^2$  ( $P < 0.01$ , unpaired *t* test). The geometric mean numbers of *M. avium* subsp. *paratuberculosis* cells in positive samples collected in the cattle barn ( $8.81 \times 10^2$ ) and calf barn ( $9.8 \times 10^2$ ) are not significantly different ( $P > 0.05$ , unpaired *t* test). No differences in the geometric mean numbers of *M. avium* subsp. *paratuberculosis* cells in positive organic ( $3.61 \times 10^2$ ) and cobweb ( $9.36 \times 10^2$ ) samples was seen ( $P > 0.05$ , unpaired *t* test).

**Distribution of *M. avium* subsp. *paratuberculosis* outside the farm studied by culture and qPCR.** A total of 36 environmental samples from outside the farm, e.g., from the field midden, field, and pond, were collected during the study period, and 16 (44%) of these were positive for *M. avium* subsp. *paratuberculosis* DNA. However, no sample was positive by culture. *M. avium* subsp.

*paratuberculosis* DNA was isolated from the soil, remains of manure, vegetables originating from the field, or the field midden and also from the pond (Table 2). A trend toward a decrease in proportions of positive samples over time was observed ( $P < 0.05$ , chi-square test for trend), but differences between the separate time periods were not statistically significant ( $P > 0.05$ , Fisher's exact test). The geometric mean numbers of *M. avium* subsp. *paratuberculosis* cells at individual times of 12, 18, and 24 months were  $5.03 \times 10^2$ ,  $8.76 \times 10^2$ , and  $5.43 \times 10^2$ , respectively; differences between time periods were not statistically significant ( $P > 0.05$ , Kruskal-Wallis test) (Table 2).

## DISCUSSION

Although *M. avium* subsp. *paratuberculosis* infection has been studied for many decades, no adequate information about the survival of *M. avium* subsp. *paratuberculosis* and the persistence of its DNA in different environments after the destocking of a dairy cattle herd exists. In the present study, we focused on monitoring *M. avium* subsp. *paratuberculosis* contamination in a farm environment before and after the removal of an infected herd and subsequent high-pressure cleaning and decontamination with chloramine B and ammonium as well as lime application in adjacent places around barns. We examined areas occupied by adult cows that were the source of infection on the farm and also the places thought to harbor low levels of *M. avium* subsp. *paratuberculosis*, such as the calf barn or mow and feeding rooms. We were able to detect *M. avium* subsp. *paratuberculosis* by culture in only one sample obtained from the waste pit before destocking of the infected herd although the prevalence in the herd at this time was estimated to be approximately 75.6%. The environmental positiv-

ity as determined by qPCR on the farm at this time was found to be high and reached 81% (Table 1). A similar situation was observed by Berghaus et al. (2), who determined that the prevalence of paratuberculosis among cows was proportional to the environmental contamination by *M. avium* subsp. *paratuberculosis*. Since *M. avium* subsp. *paratuberculosis* excretion into the environment via the feces of infected cows is common, environmental sampling was also proposed as a convenient method for estimating prevalence in the herd (2, 29, 32).

Early diagnosis of *M. avium* subsp. *paratuberculosis* infection is crucial for preventing transmission in a herd. It is recommended that massive shedders as well as low shedders be removed (21). For this, it is necessary to have a sensitive and rapid method for *M. avium* subsp. *paratuberculosis* detection. qPCR assays have been shown, in comparison with liquid and solid cultures, to be suitable and sensitive for fecal as well as environmental samples (1, 3, 15). Moreover, the qPCR method applied in this study has been previously compared to culture on solid HEYM (18), and a predictive model for *M. avium* subsp. *paratuberculosis* detection that demonstrates the probability of culture-positive samples according to the number of cells per gram of feces was designed. In this model, when the number of cells per gram is  $10^4$ , there is only a 40% probability of *M. avium* subsp. *paratuberculosis* isolation by culture on HEYM. It was documented that cultivation in liquid medium produced better results than growth in solid medium; unfortunately, this method is not up and running in our laboratory. The lower sensitivity of cultivation may relate to the effects of the decontamination method as well as the absorption of the pathogen to some particles and consequent discarding during processing. Further, loss of *M. avium* subsp. *paratuberculosis* viability may be associated with storing of the sample. Aly et al. (1) demonstrated a loss of viability due to freezing and thawing; on the other hand, minimal loss of DNA was observed.

The existence of a false-positive reaction in qPCR may also be considered, but due to the negative results of isolation control and tested specificity of qPCR in our previous study (39), we eliminated this possibility.

Although *M. avium* subsp. *paratuberculosis* contamination of the environment found by cultivation was very low and although we were not able to demonstrate a reduction in live *M. avium* subsp. *paratuberculosis* cells at different time periods after the destocking and decontamination of the farm, we were able to observe a significant decrease in *M. avium* subsp. *paratuberculosis* DNA before and during the time after destocking when the qPCR method was applied. Although a declining trend in the amounts of *M. avium* subsp. *paratuberculosis* DNA was noted over time, *M. avium* subsp. *paratuberculosis* DNA was detected in cobwebs and organic substrate (floor scraping) localized in the building 24 months after destocking and decontamination of the farm (Table 1). The high sensitivity of qPCR and a failure to fully eliminate *M. avium* subsp. *paratuberculosis* DNA after destocking and high-pressure cleaning were also documented by Eisenberg et al. (7). They demonstrated that more than 50% of samples from a barn (after destocking and high-pressure cleaning) were positive using a qPCR method.

A crucial preventative measure on farms is the separation of calves from the adults and their feces to minimize *M. avium* subsp. *paratuberculosis* transmission. It has been demonstrated that calves are most susceptible to infection with *M. avium* subsp. *paratuberculosis*, but *M. avium* subsp. *paratuberculosis* shedding and clinical signs appear in adult animals. That is why the most

commonly contaminated areas on farms are cow alleyways and sites of manure storage (29, 41). We detected *M. avium* subsp. *paratuberculosis* DNA in 82% of the samples from the cattle barn during the studied period (Table 1). In contrast to our expectations, we also found a high number of positive samples in the calf barn area. The difference in proportions of *M. avium* subsp. *paratuberculosis* DNA-positive samples and average number of *M. avium* subsp. *paratuberculosis* cells between cattle barn and calf barn was not statistically significant; 82% of cattle barn samples were positive, with an average of  $8.81 \times 10^2$  cells, and 55% of calf barn samples were positive, with an average of  $9.80 \times 10^2$  cells. *M. avium* subsp. *paratuberculosis* DNA was also found at sites without any animals, such as the mow and feed preparation rooms. The *M. avium* subsp. *paratuberculosis* DNA-positive samples originated from cobwebs as well as organic substrate (Table 1). In contrast to these results, other authors described the isolation of live *M. avium* subsp. *paratuberculosis* from the calf area, feed, and water; however, compared to samples from flooring and manure storage, this detection was minor only (29, 41).

Due to the presence of high-shedding animals in the cow barn, we assume that distribution to the calf barn was through contaminated boots, instruments, or dust. Our results also indicate the importance of *M. avium* subsp. *paratuberculosis* spreading by aerosol (presence of *M. avium* subsp. *paratuberculosis* in cobweb samples) within the farm (Table 1). This is supported by the studies of Eisenberg et al. (7), who described the spreading of *M. avium* subsp. *paratuberculosis* through bio-aerosols after the introduction of an infected shedding cow. They were able to detect live *M. avium* subsp. *paratuberculosis* in the floor and in settled dust in the barn and later also in the floor dust outside the barn. As the feces of the calf were not tested, contamination caused by *M. avium* subsp. *paratuberculosis* shedding by the calf cannot be ruled out (32, 43).

Composting is generally viewed as a way to decrease pathogen concentrations; however, most studies of pathogen dynamics in compost did not report complete elimination of all pathogens. Wastewater lagoons were estimated as one of the most common areas to be contaminated with *M. avium* subsp. *paratuberculosis* and have been described to be more contaminated than manure or alleyways (2, 32). Our study also confirmed a high range of contamination of the waste pit; *M. avium* subsp. *paratuberculosis* DNA was found in each examined sample from the waste pit at each time up to 547 days, when  $1.96 \times 10^4$  cells/g of sample were detected (Table 1). Grewal et al. (10) were able, using qPCR, to detect *M. avium* subsp. *paratuberculosis* DNA in liquid manure up to day 175 at 20 to 25°C, but *M. avium* subsp. *paratuberculosis* cells were isolated by culture during the studied period only up to 56 days. Jorgensen (13) observed that a drastic reduction in *M. avium* subsp. *paratuberculosis* survival in cattle slurry occurred over the first 7 days but that *M. avium* subsp. *paratuberculosis* cells were isolated by culture for up to 252 days at 5°C. Therefore, the practice of spreading manure on fields and open access to middens may contribute to the possible transmission of different pathogens including *M. avium* subsp. *paratuberculosis* (40).

In this study, we analyzed samples from the midden (remains of filed manure) as well as from a field that was previously fertilized with contaminated manure (soil, upper parts of plants, and their roots) and subsequently limed. Further, we examined samples from an adjacent pond. We did not detect culturable *M. avium* subsp. *paratuberculosis* in any of these samples during the studied period. In contrast, Whittington et al. (44, 46) observed

the survival of *M. avium* subsp. *paratuberculosis* in soil and water sediment for approximately 1 year, and Salgado et al. (36) detected viable *M. avium* subsp. *paratuberculosis* in the upper part of the soil 21 months after the application of manure to the top of 90-cm soil columns. Differences in physical, chemical, and other conditions, e.g., nutrient availability and competition with other microorganisms present in the soil, may interact and affect pathogen persistence. Whittington et al. (45) reported a 99% decrease in *M. avium* subsp. *paratuberculosis* isolation in soil samples mixed with contaminated feces. This was explained by the adsorption of bacteria to soil particles and the later removal of these particles by sedimentation during sample processing. This reduction was two orders of magnitude less than that from feces.

On the other hand, using qPCR we detected *M. avium* subsp. *paratuberculosis* DNA in 44% of the examined samples from the field and field midden as well as from the pond. In spite of the decline in *M. avium* subsp. *paratuberculosis* DNA detection over time, the qPCR method revealed IS900 gene copies 24 months after the destocking of infected herd from the farm and subsequent manure application on the field and manure storage. *M. avium* subsp. *paratuberculosis* DNA has been revealed not only in soil but also in upper parts of plants and their roots. In our previous study we detected *M. avium* subsp. *paratuberculosis* DNA in the roots of plants and soil samples (20 cm below the soil surface) 15 weeks after natural exposure to mouflon feces on one mouflon farm (30). This is in agreement with other studies, in which slow *M. avium* subsp. *paratuberculosis* movement in soil and adsorption to soil particles under *in vitro* conditions were demonstrated (31, 36).

Manure application together with rainfall may also pose a risk in terms of surface runoff, and in this way the pathogen may be transmitted to water environments. Pickup et al. (28) found that more than 60% of river water samples from an area with infected cows tested positive for IS900 genes, indicating the presence of *M. avium* subsp. *paratuberculosis*. Similarly, in the present study, we also detected *M. avium* subsp. *paratuberculosis* DNA in three out of nine samples from a pond close to a fertilized field. *M. avium* subsp. *paratuberculosis* survival in this environment may be supported by persistence in protists, as was documented by Mura et al. (26), who proved the presence of *M. avium* subsp. *paratuberculosis* in *Acanthamoeba polyphaga* for up to 4 years.

qPCR positivity may be caused by the presence of viable culturable *M. avium* subsp. *paratuberculosis* cells, nonviable *M. avium* subsp. *paratuberculosis* cells, viable but nonculturable *M. avium* subsp. *paratuberculosis* cells, or by residual DNA. It has been documented that short DNA fragments can persist in soil and other environments for extended periods of time despite the presence of DNA-degrading enzymes. These DNA fragments can be partially protected against enzymatic degradation due to the DNA adsorption to soil particles, which depends mainly on clay proportion, pH, and the length of the fragments (27, 33, 34). Romanowski et al. (35) demonstrated the persistence of plasmid DNA in soil for weeks and even for months after its release from the cell. England et al. (8) detected a *Pseudomonas aureofaciens* gene in soil 4 weeks after inoculation of the cell lysates. However, in contrast to these reports, Young et al. (48) showed that PCR amplification of a specific gene is directly correlated to the presence of viable cells. DNA from dead *Mycobacterium bovis* cells was not detectable in soil for more than 10 days, and when free DNA was introduced into soil, the detection time was only approximately 3 days.

Viable but nonculturable bacteria or dormant bacterial states were observed in many bacterial species including mycobacteria (19, 23, 37). This dormant state is characterized by drastically decreased metabolic activity and enhanced resistance to physical and chemical factors. Some studies suggest that a dormant state also exists in *M. avium* subsp. *paratuberculosis*. This suspicion is supported by observations that *M. avium* subsp. *paratuberculosis* in response to starvation stress produces a large array of specific proteins which are linked with the dormancy state in other bacteria (11, 46). Recently, Lamont et al. (20) described spore-like structures in 1-year-old broth cultures of *M. avium* subsp. *paratuberculosis*. These spore-like structures survived exposure to heat, lysozyme, and proteinase K and upon germination in a well-established bovine macrophage model displayed enhanced infectivity.

In conclusion, although we did not isolate *M. avium* subsp. *paratuberculosis* by culture after the destocking of an infected herd and following application of decontamination methods, we cannot be sure if *M. avium* subsp. *paratuberculosis* DNA detected for up to 24 months after destocking of the infected herd came from viable but nonculturable *M. avium* subsp. *paratuberculosis* cells or from dead cells or was simply residual DNA. The low probability of *M. avium* subsp. *paratuberculosis* isolation by culture compared to qPCR detection was described in our previous study (18). Our present study illustrates the difficulty with interpretation of qPCR results and highlights the possibility of underestimation of culture results from the environment. Since the infection doses for different animal species are unknown and since it is not clear if repeated small amounts of *M. avium* subsp. *paratuberculosis* cells can cause infection, it is necessary to take into account qPCR results that are usually more sensitive than culture methods. Furthermore, *M. avium* subsp. *paratuberculosis* aerosol transmission should also be considered when prevention measures are applied on a farm. From these results, the question also arises of how long after the removal of an infected herd is it possible to stock new animals and if it is safe to apply the manure of infected cows on a field.

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