Environmental contamination with *Mycobacterium avium* subspecies *paratuberculosis* within and around a dairy barn under experimental conditions

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ABSTRACT

To establish environmental contamination in and around a dairy barn, cows shedding *Mycobacterium avium* ssp. *paratuberculosis* (MAP) were housed in a freestall barn. Fecal samples were collected 15 times at 3-wk intervals, and samples of all animals were cultured by using the Trek Diagnostic Systems culture system (Cleveland, OH) to quantify levels of MAP shedding. In parallel, air and floor dust samples were collected inside and outside the experimental farm and analyzed by *IS900* real-time PCR for the presence of MAP DNA. Inside the barn, MAP was detected with equal frequency in samples directly contaminated with feces compared with air dust samples above animal level and in dust samples of the corridor. Dust samples collected within the barn were positive more frequently than outside samples, with exception of the outside sample from the farmer's doormat. The risk of MAP exposure was distributed evenly within the dairy barn. Additionally, footwear should be considered as a high-risk fomite for dispersion of dust-related MAP outside the barn. Prevention of MAP exposure in youngstock may require housing of youngstock in separate barns as an additional management measure.

Key words: *Mycobacterium avium* ssp. *paratuberculosis*, environment, exposure, cattle

INTRODUCTION

Paratuberculosis or Johne's disease is a chronic granulomatous enteritis caused by *Mycobacterium avium* ssp. *paratuberculosis* (**MAP**). Calves are infected by uptake of MAP during the first months of life (Larsen et al., 1975). Paratuberculosis has a considerable eco-nomic impact on dairy industry due to decreased milk production, higher culling rates, and decreased slaughter values in affected herds (Johnson-Ifearulundu et al., 1999). The potential involvement of MAP in Crohn disease suggests a public health issue as well (Schwartz et al., 2000; Naser et al., 2004).

Infections with MAP are common, having an estimated herd prevalence >50% in Europe (Nielsen and Toft, 2009). Because no cure exists for the disease, control programs focus on prevention of transmission to reduce disease impact. These programs are partly based on the phenomenon of age resistance, which has been described for this infection (Windsor and Whittington, 2010). Management measures aim to improve biosecurity especially around calving and youngstock rearing, often combined with a test-and-cull strategy in adult cows. Follow-up of herds enrolled in such control programs never shows complete disease eradication, but disease prevalence is decreased within enrolled herds (Wells et al., 2008; Ferrouillet et al., 2009; Collins et al., 2010).

Modeling approaches indicate that cow-calf transmission of MAP is the most important transmission route through either cow contact or an environment contaminated by feces of adult cows (Groenendaal et al., 2002; Marcé et al., 2011). However, calves born to MAP-shedding cows or calves fed with colostrum positive for MAP DNA did not show increased infection risk in recent field studies (Pithua et al., 2010, 2011). These findings suggest the presence of other, currently unidentified, routes of transmission. Intrauterine transmission and calf-to-calf transmission were classified to be of lower importance as long as opportunities for postnatal infection, such as cow-calf transmission through contaminated feces or colostrum and the environment, are not controlled (Whittington and Windsor, 2009). Recently, the presence of MAP in bioaerosols was documented and suggested as a possible route for within-farm transmission (Eisenberg et al., 2010a, 2011a).

The objective of this longitudinal study was to investigate spatial spread of MAP in an environment with steady MAP contamination by shedder cows. The contamination of different locations inside and outside the barn with MAP was used to describe the risk of exposure over time.

MATERIALS AND METHODS

Experimental Farm

The study was conducted in a Dutch dairy farm building with 80 bed stalls in a freestall housing system with 4 open waterers, a central corridor with feed bunk, and ridge ventilation, as described previously (Eisenberg et al., 2010b). The farm was destocked and cleaned with a high-pressure cleaner, and the manure pit was emptied before the current experiment began. Confirmed MAP-shedding cows (n = 45; Dutch Animal Health Services, Deventer, the Netherlands) were purchased and dried-off for the experiment.

For welfare reasons, cows with clinical paratuberculosis characterized by diarrhea, extensive weight loss, and edema were removed from the study population during the experiment. The study was conducted from July 2007 to June 2008, and was approved by the local Ethical Committee for Animal Experiments.

Sample Collection

Every 3 wk, all cows present were sampled. Fecal samples were obtained directly from the rectum of individual cows using a clean glove for each sample. Samples were individually processed for fecal culture as described in the section below.

Additionally, every 3 wk, environmental samples were collected. All environmental sampling locations were specified on a map of the farm to facilitate repeated sampling at the same locations over time. All environmental sampling and analysis was done in triplicate per location as described previously (Eisenberg et al., 2010b). In short, floor dust samples of the corridor (n = 2 locations), yard (n = 6 locations), and doormat (n = 1 location) were collected by cleaning 1 m^2 of surface using a vacuum cleaner (Miele De Luxe 246i, Gütersloh, Germany) and special vacuum cleaner bags (150 mm × 73/38 mm, micron rating 25; article 12513139, VacAllied Filter Fabrics Pty Ltd., Hornsby, NSW, Australia). Cubicles (n = 3 locations) and slatted floors (n = 3 locations) were sampled using wooden spatulas. Settled dust accumulated over a 3-wk period was collected with electrostatic wipes (Zeeman textielSupers, Alphen a/d Rijn, the Netherlands) at the air inlets (n = 2 loca-

tions), above the slatted floors (n = 4 locations), and in the ventilation ridge (n = 1). One liter of water was collected from each waterer (n = 4) and concentrated by centrifugation at 3,400 × g for 20 min until the pellet could be suspended in 1 mL. Then, the sample preparation protocol described below was followed.

Sample Preparation

All environmental samples were processed as described previously (Eisenberg et al., 2010b). In short, each sample was suspended in Milli-Q water (Millipore Corp., Billerica, MA). After vortexing, the sample was allowed to settle and the supernatant was centrifuged for 20 min at 3,400 × g. The pellet was suspended by vortexing and subsequently centrifuged in a microcentrifuge tube for 3 min at 14,000 × g. The supernatant was discarded and the pellet was suspended in 100 μL of Milli-Q water. Forty microliters of this suspension was used for DNA extraction.

Extraction of DNA was performed by using the Elute Micro Card (Whatman, Clifton, NJ). The cards were dried overnight and 2 punches were collected with a biopsy punch (4 mm) and processed according to the manufacturer's instructions. Two microliters of the eluate was used as template in the real-time PCR (MiQ, Bio-Rad, Hercules, CA). Primers targeting an internal sequence of the MAP-specific *IS900* gene were used for the real-time PCR protocol (Hruska et al., 2005; Eisenberg et al., 2010b); the protocol consisted of 45 cycles. Samples were indicated as positive for MAP DNA in the case of a melting peak between 93°and 94°C, and the software-determined cycle threshold (**Ct**) value was recorded. Samples without the desired melting peak were indicated as negative for MAP DNA and were assigned a Ct value of 45. The Ct values of triplicates were averaged to increase precision. However, for statistical analysis, averaged results were transformed into a binomial outcome [MAP presence yes (average Ct ≤44) vs. no (average Ct = 45)] and presented as proportions of positive locations.

Fecal samples were analyzed in the para-JEM automated MAP culturing system (Trek Diagnostic Systems) according to the manufacturer's protocol. The presence of MAP in culture medium was confirmed by real-time PCR for *IS900* (see above). Time to detection (**TTD**) was used as a measure to quantify MAP in fecal samples. The maximum TTD was 42 d and was considered to indicate low shedding if the presence of MAP was confirmed by PCR, whereas a shorter TTD indicated higher shedding. Samples with a negative PCR result were considered to contain no viable MAP.

Table 1. Overview of the number and proportion of *Mycobacterium avium* ssp. *paratuberculosis* (MAP)-positive samples found in environmental samples as determined by IS900 real-time PCR samples collected over 15 time periods in a Dutch experimental dairy farm

MAP-positive samples	Settled dust samples			Inside floor samples			Outside floor samples	
	Air inlet	Above slatted floors	Ventilation ridge	Cubicles/ slatted floors	Corridor	Waterer	Doormat	Farm yard
Number (no./total)	23/30	55/60	14/15	81/90	20/30	6/60	6/15	4/90
Percentage	77	92	93	90	67	10	40	4

Management Actions

A strict biosecurity protocol was implemented on the farm with a 2-step hygienic barrier. In short, the use of farm-owned boots and cotton coveralls was required when entering the barn (first step), and a second layer of plastic disposable coveralls and different boots had to be put on when entering the animal section (second step). Additionally, masks, caps, and latex gloves had to be worn inside the barn. Plastic coveralls and gloves had to be discarded before leaving the animal section, and animal-section boots had to be disinfected before being changed for farm-owned boots. Before personnel left the barn, farm-owned boots had to be disinfected as well.

Data Analysis

Data were analyzed using Excel 2003 (Microsoft Corp., Redmond, WA) and the SPSS statistical software package (version 16.0.1, SPSS Inc., Chicago, IL). Times to detection were used as a semiquantitative measure for the amount of MAP shed per cow (Kim et al., 2002). The contamination of the environment with MAP per period was estimated using the TTD of fecal samples collected from all cows present at a sampling moment.

Environmental samples were grouped per location: cubicle/slatted floor, water and corridor samples (named inside floor samples), and yard and doormat samples (named outdoor floor samples). Air samples were referred to as settled dust samples. For each triplicate, presence of MAP was assessed as a binomial outcome (MAP DNA yes/no). Presence of MAP per location was evaluated by considering proportions of positive PCR results per location. The Ct values were used for a semiquantitative comparison of MAP in settled dust samples in the environment as described previously (Eisenberg et al., 2010b). Normality of both fecal samples (TTD) and environmental settled dust samples (Ct) was visually checked by quantile-quantile (QQ) plot. Analysis of variance with sampling moment as factor was used to test differences in both mean TTD and mean Ct values over time. Differences in propor-

tion positive samples between locations (Table 1) were compared by χ^2 and Fisher exact tests, where $P < 0.05$ was considered statistically significant.

RESULTS

Fecal MAP Shedding

The median TTD of the shedding cows per sampling moment shows that high amounts of MAP were shed into the environment continuously (Figure 1). The number of cows decreased during the study period by 20%, and the number of shedding animals varied over time between 73 and 93%. No statistical significant difference in mean TTD between sampling moments was found.

Environmental Contamination

An overview of MAP DNA detected in environmental samples is given in Table 1. A large number of settled dust samples located above the slatted floors and in the ventilation ridge were positive for MAP. The level of MAP DNA in settled dust samples was at a constant level during the study period. Only sampling number 2 was significantly different from other sampling moments (Figure 2).

We detected MAP with equal frequency in samples from air inlets compared with settled dust samples collected above animal level (Table 1). Presence of MAP in samples from slatted floors and cubicles was also equally common. The contamination of the corridor samples was not significantly different compared with that of cubicles and slatted floors or the settled dust samples. Only a small number of the water samples collected within the barn tested positive for MAP during the study period. No statistically significant difference in MAP presence between inside locations could be detected, with exception of the waterers.

Outside the barn, most environmental samples tested negative for MAP DNA, which was significantly different from the inside samples. The doormat of the farmhouse was contaminated significantly more frequently than was the farmyard.
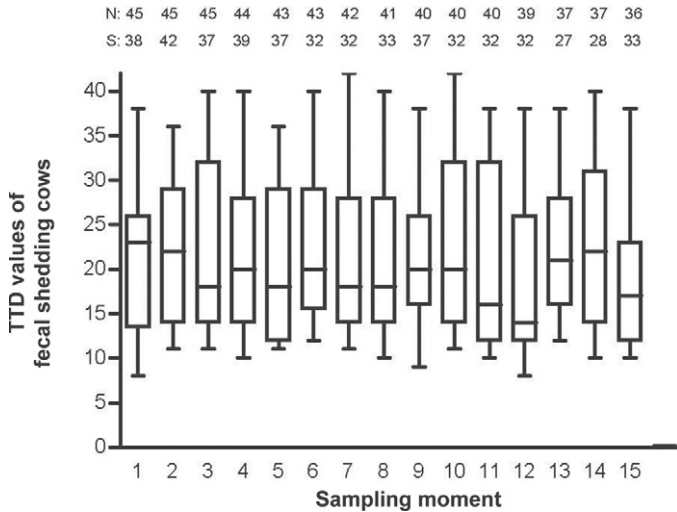
N: 45 45 45 44 43 43 42 41 40 40 40 39 37 37 36
S: 38 42 37 39 37 32 32 33 37 32 32 32 27 28 33

Figure 1. Illustration of detected *Mycobacterium avium* ssp. *paratuberculosis* (MAP) shedding, the number of cows present (N), and the number of cows shedding at each day of sampling (S) in a Dutch experimental farm that housed confirmed MAP-shedding cows between July 2007 and June 2008. Cows were sampled individually every 3 wk. The total shedding level at each sampling was estimated by time to detection (TTD) determined by Trek liquid culture system (Trek Diagnostic Systems, Cleveland, OH) determined on a fecal sample of each individual cow. Medians and quartiles are displayed in the box. Upper and lower bars represent 97.5 and 2.5 percentiles, respectively.

DISCUSSION

Environmental sampling of fecal contaminated areas on a dairy farm such as milking parlor exits, lagoons, and common alleyways has been identified as a predictor for MAP herd infection status (Berghaus et al., 2006; Lombard et al., 2006). Sampling of these areas

has been proposed as an economical and useful tool in monitoring programs for identification and classification of MAP-positive herds. Environmental sampling has been shown to be less sensitive for the identification of herds with a low MAP prevalence (Smith et al., 2011; Donat and Schau, 2012). Because the environment of most MAP-positive herds is contaminated with MAP, it has been accepted as an important route of disease transmission (Fecteau et al., 2010; Lombard, 2011). Therefore, MAP control programs focus on the separation of calves from infectious adult cattle and their feces (Benedictus et al., 2008; Ferrouillet et al., 2009; Collins et al., 2010). However, MAP presence in dust and its role in transmission were not considered in those programs. Because recent studies have confirmed that viable MAP can be isolated in barn dust, not only fecal but also dust contamination of the environment should be considered when developing MAP control strategies (Eisenberg et al., 2010a,b).

The high MAP prevalence present on this experimental farm is not likely to occur on a commercial dairy herd but this study offered a unique opportunity to examine the dissemination of MAP in the environment of a herd. We determined the presence of MAP in various types of dust samples near MAP-shedding cows, indicating a potential risk of MAP exposure for calves.

Fecal shedding estimated by TTD was continuous throughout the entire study period. Reduction of the number of cows by 20% for welfare reasons did not significantly decrease MAP shedding, as characterized by the median TTD value within the remaining herd. Although TTD might not be a very precise measure to determine environmental contamination because of its semiquantitative nature, it does suggest that during the
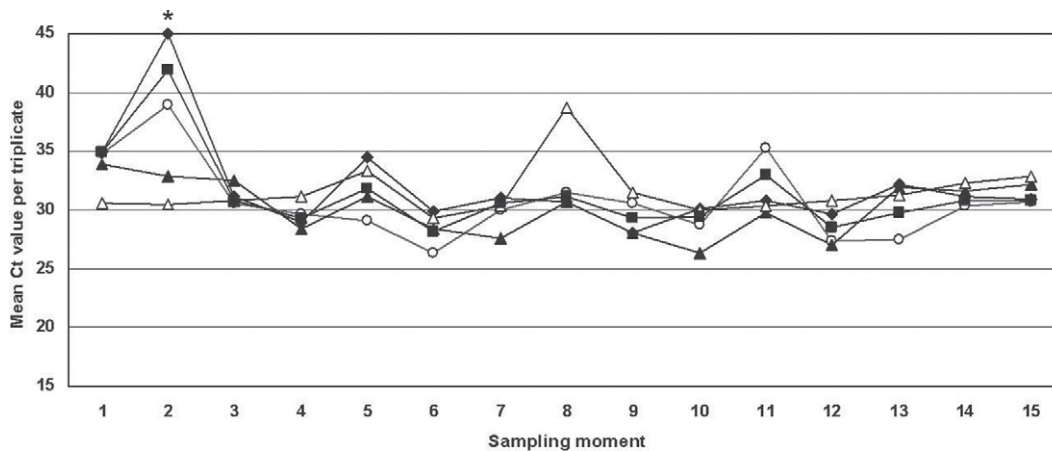

Figure 2. Results of the *Mycobacterium avium* ssp. *paratuberculosis* (MAP)-specific *IS900* real-time PCR expressed as mean cycle threshold (Ct) of triplicate settled dust samples collected at 4 locations above the slatted floors (○, ◆, ▲, ■) and in the ventilation ridge (△) in the experimental barn that housed MAP shedders. Locations were sampled 15 times for settled dust of a previous 3-wk period. * = time point significantly different from all others with the exception of timepoints 1 and 8.

study period the amount of MAP shed in the environment remained at a relatively constant level.

Environmental sampling was performed in 2 ways: collection of settled dust (providing information over the 3-wk interval) and collection of floor samples (measuring contamination at that specific time; Eisenberg et al., 2010b). On locations with direct fecal contact (cubicles and slatted floors), we detected a large number of MAP-positive samples, as expected (Smith et al., 2011). Interestingly, in settled dust samples (reflecting exposure through the air), MAP was also present at a high frequency, similar to that of samples collected in the cubicles and slatted floors. These findings indicate that MAP particulates become airborne easily and pose a potential exposure risk for youngstock housed within the same barn as shedder cows. After the positive cows had been housed in the barn for several months, continuous contamination of settled dust samples seemed independent of the number of positive cows present, indicating saturation of the environment in this particular high-prevalence setting (Figure 2).

Determination of MAP presence in environmental samples was performed using a real-time PCR. A positive outcome indicates presence of MAP DNA but not whether the MAP are viable. Earlier studies compared MAP detection by direct PCR on environmental samples and by liquid culture (Eisenberg et al., 2010a,b). Results of both methods were similar, especially in more heavily contaminated environments, indicating that detection of MAP DNA is a valid proxy for viable MAP contamination in these samples.

Although MAP levels in dust detected on this experimental farm might be higher and therefore easier to detect compared with those likely to be found on commercial dairy farms, the current findings confirm that environmental samples collected within the same building as dairy cows more likely contain MAP (Eisenberg et al., 2010a). Whereas oral uptake of MAP from an environment contaminated with feces or accumulated dust might depend on specific behaviors or activities, uptake of MAP by inhalation is likely to occur continuously. Respiratory MAP uptake has recently been identified as a potential point of entry leading to MAP infection in calves under experimental conditions (Eisenberg et al., 2011a).

Although the presence of MAP in the corridor was influenced by the cleaning practices of the farmer, MAP was detected only slightly less frequently in corridor samples compared with cubicle, slatted floor, and settled dust samples. Basic hygiene measures therefore did not seem suitable to effectively reduce MAP contamination within the barn. To remove MAP-contaminated dust from the inside of a barn environment, a combination of regular high-pressure cleaning followed by a disinfection step has been reported to be useful (Eisenberg et al, 2011b).

We found MAP in only a few water samples. Contamination probably occurred directly with fecal material or by dust settling on the water surface. The drinking activity of cows would dilute such fecal material and dust, and the farmer replaced visibly contaminated water. The probability of detecting contaminated water at a single sampling moment was therefore low in this situation.

Detection of MAP was significantly less frequent outside the barn compared with inside the barn. We did not assess emission of dust from the barn in detail. High dilution of airborne contamination from the barn combined with high biosecurity levels apparently contained MAP mostly within the barn. These results seem consistent with earlier reports on commercial dairies, where no MAP was detected in youngstock housing separated from the dairy herd (Eisenberg et al., 2010a). To what extent airborne transmission between barns is possible could only be established by using more refined sampling methods with lower limits of detection. Outside the barn, MAP was present significantly more often in the doormat samples than in other environmental samples, highlighting the difficulty in preventing spread of MAP via footwear, despite an implemented two-step hygienic barrier, in which boots had to be changed before entering the barn and a second time when entering the animal section. This finding should be kept in mind when developing a walking routine between different buildings on a commercial dairy farm, where biosecurity measures might be less strict.

CONCLUSIONS

Detecting MAP with similar frequency at different locations throughout the barn documents that the risk of MAP exposure for calves housed in the same barn as dairy cows may be high, even without direct contact between cows and calves. Furthermore, this study confirms earlier findings on commercial dairy farms where MAP was detected within the building of the adult dairy cows. Therefore, housing youngstock in separate barns should be considered as an additional management practice in Johne's control programs.

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