Applied and Environmental Microbiology

Relationship between Presence of Cows with Milk Positive for Mycobacterium avium subsp. paratuberculosis-Specific Antibody by Enzyme-Linked Immunosorbent Assay and Viable M. avium subsp. paratuberculosis in Dust in Cattle Barns

Susanne W. F. Eisenberg, Ruj Chuchaisangrat, Mirjam Nielen and Ad P. Koets

Appl. Environ. Microbiol. **2013, 79(18):5458. DOI: 10.1128/AEM.01187-13.**
Published Ahead of Print 21 June 2013.

AEM
Journals.ASM.org

Relationship between Presence of Cows with Milk Positive for *Mycobacterium avium* subsp. *paratuberculosis*-Specific Antibody by Enzyme-Linked Immunosorbent Assay and Viable *M. avium* subsp. *paratuberculosis* in Dust in Cattle Barns

Susanne W. F. Eisenberg,[a] Ruj Chuchaisangrat,[a] Mirjam Nielen,[a] Ad P. Koets[a,b]

Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands[a]; Division of Immunology, Department of Infectious Disease and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands[b]

Paratuberculosis, or Johne's disease, in cattle is caused by *Mycobacterium avium* subsp. *paratuberculosis*, which has recently been suspected to be transmitted through dust. This longitudinal study on eight commercial *M. avium* subsp. *paratuberculosis*-positive dairy farms studied the relationship between the number of cows with *M. avium* subsp. *paratuberculosis* antibody-positive milk and the presence of viable *M. avium* subsp. *paratuberculosis* in settled-dust samples, including their temporal relationship. Milk and dust samples were collected in parallel monthly for 2 years. *M. avium* subsp. *paratuberculosis* antibodies in milk were measured by enzyme-linked immunosorbent assay (ELISA) and used as a proxy for *M. avium* subsp. *paratuberculosis* shedding. Settled-dust samples were collected by using electrostatic dust collectors (EDCs) at six locations in housing for dairy cattle and young stock. The presence of viable *M. avium* subsp. *paratuberculosis* was identified by liquid culture and PCR. The results showed a positive relationship (odds ratio [OR], 1.2) between the number of cows with ELISA-positive milk and the odds of having positive EDCs in the same airspace as the adult dairy cattle. Moreover, the total number of lactating cows also showed an OR slightly above 1. This relationship remained the same for settled-dust samples collected up to 2 months before or after the time of milk sampling. The results suggest that removal of adult cows with milk positive for *M. avium* subsp. *paratuberculosis*-specific antibody by ELISA might result in a decrease in the presence of viable *M. avium* subsp. *paratuberculosis* in dust and therefore in the environment. However, this decrease is likely delayed by several weeks at least. In addition, the data support the notion that *M. avium* subsp. *paratuberculosis* exposure of young stock is reduced by separate housing.

Paratuberculosis, or Johne's disease, is caused by *Mycobacterium avium* subsp. *paratuberculosis* and is an important bacterial infection in the dairy industry. In an infected herd, the disease may cause a decrease in milk production, chronic diarrhea, and weight loss despite good appetite in infected cows (1).

The generally accepted transmission route of paratuberculosis in cattle is the fecal-oral route. Transmission does occur by ingestion of *M. avium* subsp. *paratuberculosis*-contaminated milk, water, and feed products, as well as uptake from the environment (2). Since *M. avium* subsp. *paratuberculosis* can be identified in mammary gland tissues and milk may become contaminated during milking, milk and colostrum can contain the pathogen and cause transmission from adult cows to susceptible calves (3). Shedding of *M. avium* subsp. *paratuberculosis* in colostrum was found to be higher than in milk (4). However, in a recent study, no increased infection risk for calves fed *M. avium* subsp. *paratuberculosis*-positive colostrum compared to calves fed *M. avium* subsp. *paratuberculosis*-negative colostrum could be identified (5). This might be due to the fact that it is difficult to prevent the exposure of calves to environmental transmission routes after colostrum administration. In addition, intrauterine transmission of *M. avium* subsp. *paratuberculosis*, which is called vertical transmission (6, 7), was reported to occur frequently enough to hamper control programs (8). Further studies identified calf-to-calf transmission as the horizontal-transmission route (9). It was shown recently that dust in dairy barns contains viable *M. avium* subsp. *paratuberculosis* and that under experimental conditions the respiratory tract can act as a portal of entry, leading to intestinal *M. avium* subsp. *paratuberculosis* infection as well, suggesting that dust uptake is an additional route of transmission (10–12). Due to the long incubation time of paratuberculosis, it is difficult to quantify the effect of each route on *M. avium* subsp. *paratuberculosis* transmission.

After infection, Johne's disease can be divided into three stages. Stage 1, shortly after infection of a young animal, is a long latent stage without detectable *M. avium* subsp. *paratuberculosis* excretion and humoral response (13). Detection of infection by fecal-antigen detection and serum or milk antibody enzyme-linked immunosorbent assay (ELISA) is often possible in the second stage, 2 to 5 years after the initial infection, when infected cows start shedding *M. avium* subsp. *paratuberculosis* into the environment and develop a humoral immune response that is also detectable in milk. Which of the two events occurs first is not clear. Animals develop clinical signs of Johne's disease in the third stage, with detectable humoral responses and high shedding of *M. avium* subsp. *paratuberculosis*. Therefore, infected animals in stage 1 probably contribute only slightly to bacterial transmission and

Received 12 April 2013 **Accepted** 18 June 2013
Published ahead of print 21 June 2013
Address correspondence to Susanne W. F. Eisenberg, S.W.F.Eisenberg@uu.nl.
S.W.F.E. and R.C. contributed equally to this work.

doi:10.1128/AEM.01187-13

contamination of the environment compared to those in stages 2 and 3.

Regular fecal cultures probably give the most accurate information about the level of *M. avium* subsp. *paratuberculosis* shedding by an individual animal, but unfortunately, it is an expensive and time- and labor-consuming method. ELISA of milk is less expensive and much quicker and therefore is often used routinely to determine the *M. avium* subsp. *paratuberculosis* infection status of cows and herds (14). Since a positive correlation between fecal shedding and a positive *M. avium* subsp. *paratuberculosis* ELISA outcome in animals in infection stage 2 or 3 has been reported, an ELISA of milk for *M. avium* subsp. *paratuberculosis*-specific antibody can be used as a less costly and time-consuming proxy for fecal shedding and therefore for the contribution to environmental contamination (15, 16). *M. avium* subsp. *paratuberculosis* is present in the environment in manure storage areas, shared alleyways, soils, and lagoon samples from dairy barns (17–19). Previous studies also found that *M. avium* subsp. *paratuberculosis* can survive in manure storage areas and remain in pasture soil for more than 200 days after the removal of *M. avium* subsp. *paratuberculosis*-infected animals (20–22). Furthermore, in commercial dairy herds containing animals with milk positive for *M. avium* subsp. *paratuberculosis*-specific antibody by ELISA, *M. avium* subsp. *paratuberculosis*-positive dust samples were detected (11). However, there is limited information on the dynamics of *M. avium* subsp. *paratuberculosis*-positive dust in the environment of dairy herds.

The main objective of this longitudinal study on commercial dairy farms was to study the relationship between the number of cows with milk positive for *M. avium* subsp. *paratuberculosis*-specific antibody by ELISA and the presence of *M. avium* subsp. *paratuberculosis* in settled-dust samples, including their temporal relationship. It was hypothesized that the presence of more positive cows tested by ELISA of milk for *M. avium* subsp. *paratuberculosis*-specific antibody would generally lead to more settled-dust samples containing viable *M. avium* subsp. *paratuberculosis*. However, the start of *M. avium* subsp. *paratuberculosis* shedding by cows with milk positive for *M. avium* subsp. *paratuberculosis*-specific antibody by ELISA and the time needed for dust formation likely both influence this temporal relationship.

MATERIALS AND METHODS

Farms. Eight farms with a known *M. avium* subsp. *paratuberculosis* history were enrolled in this study. All the farms were situated in the northern part of The Netherlands. Paratuberculosis status was identified by using the data from the Intensive Paratuberculosis Program ($n = 3$) or the Bulk Milk Quality Assurance Program (BMQAP) ($n = 5$) (23, 24). All farms were categorized as high *M. avium* subsp. *paratuberculosis* prevalence farms according to the results from both of the above-mentioned programs. Information about the farm layout and basic farm characteristics was collected. The farms were visited every 4 weeks for a 2-year period in order to collect dust and milk samples in parallel.

Milk samples. Test day (TD) milk samples were collected routinely by CRV (Arnhem, The Netherlands), transported to the Faculty of Veterinary Medicine (Utrecht University, Utrecht, The Netherlands), and stored at −20°C until they were processed, with a maximum of 3 weeks. The Pourquier ELISA (IDEXX Europe B.V., Hoofddorp, The Netherlands) was performed according to the manufacturer's manual. To determine the number of animals positive by ELISA of milk for *M. avium* subsp. *paratuberculosis*-specific antibody, a sample-to-positive (*S*/*P*) ratio of 40 was used as a cutoff value.

Dust samples. Dust samples were collected by using electrostatic dust collectors (EDCs) consisting of electrostatic microfiber wipes (Zeeman, Alphen a/d Rijn, The Netherlands) that were replaced in the same week as TD milk sampling (10). EDCs were attached to metal holders, which were installed at six fixed locations in the housing for dairy cattle and young stock. Sampling locations were chosen at 2-m height to ensure only settled dust was collected, destruction by cows was prevented, and interference with routine management actions of the farmers was avoided. Dust preparation before analysis was performed as described previously, and *M. avium* subsp. *paratuberculosis* detection was performed using a liquid culture technique, according to the protocol for para-JEM automated *M. avium* subsp. *paratuberculosis* culturing provided by Trek Diagnostic Systems (Cleveland, OH, USA) (10). All samples were incubated for 42 days regardless of whether they showed a positive signal before day 42 and were tested by IS*900* real-time PCR after 42 days to confirm the presence of *M. avium* subsp. *paratuberculosis*. Samples were designated "viable *M. avium* subsp. *paratuberculosis* positive" if the PCR showed a positive signal with a specific melting peak (10).

Statistical analysis. Descriptive statistics explaining the characteristics of farms participating in this study were performed with the SPSS statistical software package (version 20). Further statistical analyses were performed using the R statistical software package (version 2.15.2, 2012; R Development Core Team). To explain the relationship between the presence of *M. avium* subsp. *paratuberculosis* in dust samples and the number of cows with milk positive for *M. avium* subsp. *paratuberculosis*-specific antibody, a logistic regression for grouped data was performed based on the following equation: logit $Yi = (\beta_0 + b_0) + (\beta_1 + b_1) \times N_{pos} + \beta_2 \times N_{milking\ cows} + \beta_3 \times$ season, where β_0 is an intercept, b_0 is a random intercept, β_1 is a coefficient of N_{pos}, b_1 is a random slope, β_2 is a coefficient of $N_{milking\ cows}$, β_3 is a coefficient of season, N_{pos} is the number of cows with milk positive for *M. avium* subsp. *paratuberculosis*-specific antibody by ELISA at TD, $N_{milking\ cows}$ is the total number of cows in milk at TD, and season is the effect of the season (winter [as the reference] or summer [May to September]).

EDCs positive for viable *M. avium* subsp. *paratuberculosis* were grouped per TD within a farm and showed a binomial distribution. Only EDCs placed in barns with dry and lactating dairy cows were included as a dependent variable (EDC_{in}). One farm (farm 6) moved to a new location during the study and was split into two different farms (Tables 1 and 2). Variables b_0 and b_1 represent a random farm and a random time within the variable farm. Akaike's information criterion (AIC) was used to determine the best-fitting model (25). An odds ratio (OR) was calculated as exponentiation of β from the models (26).

To study the temporal relationship between the number of cows with milk positive for *M. avium* subsp. *paratuberculosis*-specific antibody by ELISA and the number of settled-dust samples positive for viable *M. avium* subsp. *paratuberculosis*, the analysis was also performed with a time lag for ELISA-positive cows. The number of ELISA-positive cows was shifted forward and backward in time compared to the EDC observation. This procedure was done to test if it was possible to identify whether cows start contributing to environmental *M. avium* subsp. *paratuberculosis* contamination before or after becoming ELISA positive. Dust sample results were linked to the ELISA outcomes of a previous or future TD, as shown in Fig. 1 in five separate models. The default model was rerun on sub-data sets with equal observations in order to be able to compare AIC values among the 5 models (see Table 4). A model was considered to fit the data better if it had a lower AIC value (an AIC difference of ≥2).

RESULTS

Dairy farm characteristics. Dairy barn floor plans and the number of separate buildings differed between the farms. On two farms, all age groups were housed within one barn. On six farms, young stock and dairy cows were housed in different buildings; however, two of those farms had the youngest age group housed in the same barn as dairy cows, whereas only the older young stock

TABLE 1 Characteristics of the 8 participating farms at the beginning of the study and results of the 2-year observation period

Farm	Rolling yr avg			Median no. of cows tested (minimum;maximum)	Median no. of cows positive by ELISA of milk (minimum;maximum)	Median no. of EDCs positive for viable *M. avium* subsp. *paratuberculosis* (minimum;maximum)
	Herd size	305-day milk (kg/cow)	Age (yr)			
1	97	8,465	4.03	83 (76;101)	13 (1;17)	1 (0;5)
2	120	8,965	4.02	104 (85;117)	3 (0;9)	0 (0;2)
3	91	8,773	4.07	77 (68;84)	4 (0;9)	0 (0;2)
4	122	7,028	4.09	111 (88;125)	7 (1;13)	2 (0;4)
5	153	7,156	5.00	132 (128;161)	7 (2;13)	0 (0;3)
6[a]	98	10,354	4.10	86 (65;103)	0 (0;3)	0 (0;2)
7	101	9,736	4.07	89 (78;102)	7 (1;12)	1 (0;4)
8	114	9,364	4.03	95 (86;103)	1 (0;3)	0 (0;2)

[a] Farm moved to another location halfway through the study.

(>2 weeks old) were housed separately. During the summer period, five farms kept their dairy cattle inside, whereas the other three farmers let their cows graze outside in the daytime.

One farm moved halfway through the study to a different location, housing the lactating cows in a new barn. Dry cows and young stock were housed in one building at the new location; however, the building was not newly built. The separations between groups of animals were comparable in the two locations.

Farm characteristics and the basic data from test results are summarized in Table 1. Herd sizes varied between 97 and 153 adult cows, with the 305-day milk production varying between 7,028 and 10,354 kg/cow.

Details on the number of ED complements in various farm buildings (EDC_{in}, in the same building as the adult cattle; EDC_{out}, in a building with young stock only) and the numbers of viable *M. avium* subsp. *paratuberculosis*-positive EDCs in various farm buildings are provided in Table 2.

Correlation between the results of ELISA of milk for *M. avium* subsp. *paratuberculosis*-specific antibody and viable *M. avium* subsp. *paratuberculosis* in dust. The results of longitudinal ELISA of milk for *M. avium* subsp. *paratuberculosis*-specific antibody (the number of positive cows) and viable *M. avium* subsp. *paratuberculosis* dust (the number of positive EDCs) are shown in Fig. 2a to h. Almost all EDCs containing viable *M. avium* subsp. *paratuberculosis* were found in barns where adult dairy cattle were housed (EDC_{in}). However, in all but one farm, EDCs in housing for young stock were found to be positive at least once during the sampling period, although they were located in separate buildings where no adult dairy cows were present. Farms 6 and 8 had a low incidence of viable *M. avium* subsp. *paratuberculosis*-positive EDCs and a low number of cows with milk positive for *M. avium* subsp. *paratuberculosis*-specific antibody by ELISA. After the movement of farm 6, EDCs both within the same airspace as the adult dairy cattle (EDC_{in}) and in the separate youngstock barn (EDC_{out}) were negative.

The season did not have an effect on the relationship between the number of ELISA-positive cows and the number of positive EDCs placed in the same airspace.

The grazing period (in farms 4, 5, and 8) also did not positively influence the model. Based on the AIC, the variable "season" was removed. The final generalized linear mixed models of the five hypotheses are shown in Table 3.

The results of the models testing the different time relationships showed that the number of cows with milk positive for *M. avium* subsp. *paratuberculosis*-specific antibody by ELISA per TD was positively related to the outcome "number of viable *M. avium* subsp. *paratuberculosis*-positive EDCs inside the dairy barn detected per TD" (Table 3). In all hypotheses, this explanatory variable was highly significant ($P < 0.001$), with ORs of approximately 1.2 for all models.

Shifting the variable "number of cows with milk positive for *M. avium* subsp. *paratuberculosis*-specific antibody by ELISA" in time did not change the OR of the number of EDCs inside the dairy barn positive for viable *M. avium* subsp. *paratuberculosis* detected

TABLE 2 Numbers of EDCs positive for viable *M. avium* subsp. *paratuberculosis* in the same indoor airspace as adult cattle and EDCs outside the buildings of adult cattle

Farm	No. of EDC_{in}	Median no. of EDC_{in} positive for viable *M. avium* subsp. *paratuberculosis* (minimum;maximum)	Total no. of EDC_{in} positive for viable *M. avium* subsp. *paratuberculosis*	Median no. of EDC_{out} positive for viable *M. avium* subsp. *paratuberculosis* (minimum; maximum)	Total no. of EDC_{out} positive for viable *M. avium* subsp. *paratuberculosis*
1	5	1 (0;4)	35	0 (0;1)	3
2	2	0 (0;2)	6	0 (0;2)	5
3	6	0 (0;2)	12		
4	5	2 (0;4)	43	0 (0;1)	2
5	2	0 (0;2)	9	0 (0;2)	6
6	4	0 (0;2)	4	0 (0;0)	0
6[a]	5	0 (0;0)	0	0 (0;0)	0
7	6	1 (0;4)	30		
8	4	0 (0;1)	4	0 (0;1)	2

[a] Farm moved to another location halfway through the study.

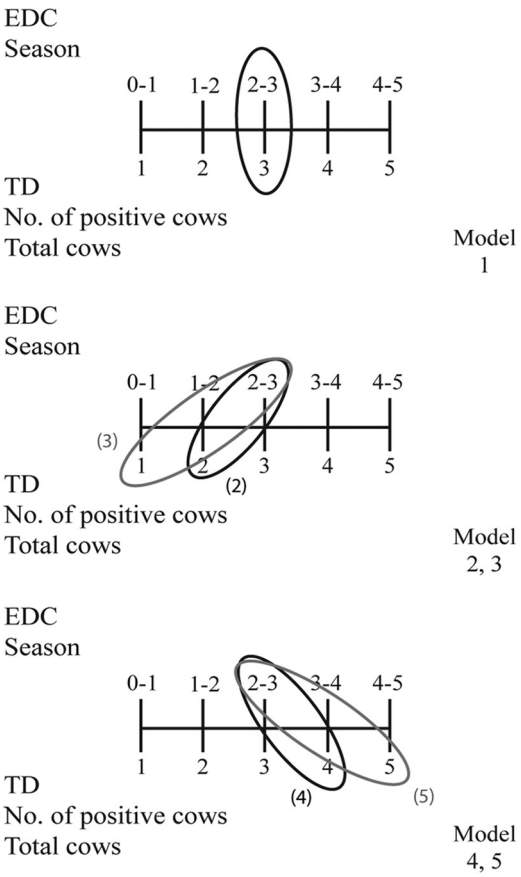

FIG 1 Different relationships tested between the ELISA results and the number of positive EDCs to investigate what number of *M. avium* subsp. *paratuberculosis* milk ELISA-positive cows per TD sampling was most related to EDCs positive for viable *M. avium* subsp. *paratuberculosis*. The EDC results were modeled in relation to milk samples from the same TD (1) and at −4 weeks (2), −8 weeks (3), +4 weeks (4), and +8 weeks (5). The numbers at the top of the scales represent periods of dust sampling, while the numbers on the lower side of the scales represent milk sampling. The ovals represent the shifting of the number of the ELISA-positive cows forwards and backwards in time relative to the EDC observation. The model numbers are listed below the scale in parentheses (next to the relevant oval).

per TD substantially. The variable "'total cows in milk" showed an OR for each hypothesis slightly above 1. Shifting ELISA results back in time led to an insignificant result for the latter variable in hypotheses 2 and 3. For models 4 and 5, with ELISA results shifted forward in time, the variable "total cows in milk" remained significant, with an OR similar to that of model 1. Interestingly, three out of four shifted models had a higher AIC value than model 1; the exception was model 4, in which dust samples were linked to the ELISA-positive cows of a TD 4 weeks later (Table 4).

DISCUSSION

Since earlier studies showed that the presence of *M. avium* subsp. *paratuberculosis* in environmental samples can be useful to estimate the herd prevalence, it was hypothesized that a similar association exists between the number of cows with milk positive for *M. avium* subsp. *paratuberculosis*-specific antibody by ELISA in a herd and the presence of viable *M. avium* subsp. *paratuberculosis* in settled dust (17, 18, 27, 28). Therefore, the association between the number of cows with milk positive for *M. avium* subsp. *paratuberculosis*-specific antibody by ELISA in a herd and the presence of viable *M. avium* subsp. *paratuberculosis* in settled dust was studied for a 2-year period. Descriptive statistical analysis of the current study indicated that most positive dust collectors shared the same indoor airspace with adult cattle. Therefore, the data support the idea that there is a relationship between the presence of cows shedding *M. avium* subsp. *paratuberculosis* and EDCs positive for viable *M. avium* subsp. *paratuberculosis* when they are located in the same indoor airspace as adult cattle. However, some farms with separately housed young stock sporadically had viable *M. avium* subsp. *paratuberculosis*-positive EDCs even inside the separate barns, but much less frequently than inside dairy herd barns. This suggests that when calves are housed separately but in the same airspace and sharing the same environment, they are not shielded from airborne contact with *M. avium* subsp. *paratuberculosis*, which disperses inside the barn, as shown previously, and transmission might occur even though animals are not in close contact (10, 11, 29).

The number of ELISA-positive cows detected in farms throughout varied the study (2a to h), because the Dutch Milk Quality Assurance Program started during the sampling period (24). Removal of ELISA-positive animals from a herd is thought to result in a decrease in *M. avium* subsp. *paratuberculosis* shedding into the environment, because of the positive correlation of antibody titers and fecal shedding in infected cows (15, 16). This concept is incorporated in Johne's disease control programs, as well (30, 31). However, in this study, no such direct effect was observed. In a previous study on dust in housing for cattle, lower dust concentrations were detected during the night than during the day, which was correlated with the activity patterns of the animals (32). A similar pattern was expected to be observed during grazing season in this study. Since the total dust production was not measured, no effect of grazing could be determined. However, a change in EDCs positive for viable *M. avium* subsp. *paratuberculosis* was not detected in that period, which might be because cows on only 3 out of 8 farms were grazing outside during daytime. Furthermore, on those three farms, the cows were kept inside the barn during milking and at night, giving them several hours to create and circulate dust inside the barn due to their movement even during grazing season, reducing the effect.

It was hypothesized that a higher number of cows with milk positive for *M. avium* subsp. *paratuberculosis*-specific antibody by ELISA in a dairy barn leads to a higher number of EDCs positive for viable *M. avium* subsp. *paratuberculosis*. The model results confirmed a positive relationship between the two parameters when EDCs were placed in the same airspace as adult dairy cattle. Moreover, herd size (incorporated in the model as "total cows in milk at TD") also had a similar positive relationship to EDCs positive for *M. avium* subsp. *paratuberculosis*, as was shown in previous studies where herd size was positively correlated with seroprevalence in dairy farms (33, 34). Comparison of AIC values between model 1 and the other models did not indicate that there is a strong temporal relationship between the number of cows with milk positive for *M. avium* subsp. *paratuberculosis*-specific antibody by ELISA and the presence of viable *M. avium* subsp. *paratuberculosis* on EDCs (Table 4). This was also indicated by the stable OR estimates (Table 3).

Since this study was conducted over a 2-year period, seasonality was examined but was not determined to have any relationship with the presence of viable *M. avium* subsp. *paratuberculosis* in

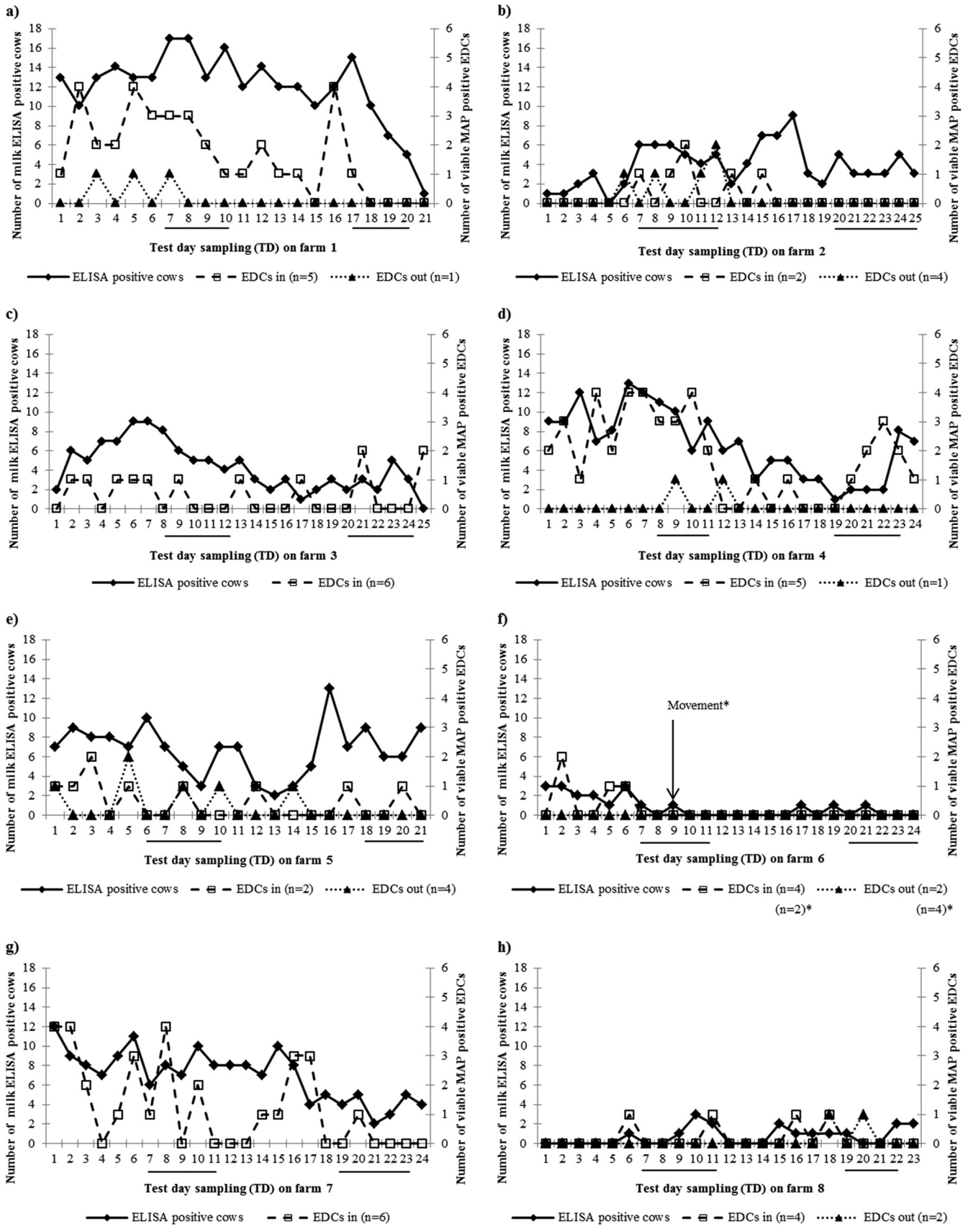

FIG 2 (a to h) Number of cows with milk positive for *M. avium* subsp. *paratuberculosis*-specific antibody (MAP) by ELISA (◆) and the number of EDCs positive for viable *M. avium* subsp. *paratuberculosis* detected per farm per TD. Farm 6 moved to a different location during the study (July 2010) (asterisk). Farms 3 (c) and 7 (g) had all EDCs under the same roof. Summer periods at each farm are underlined on the *x* axis.

TABLE 3 Parameter estimates derived from fitting the model of hypotheses 1 to 5 for EDCs inside barns with adult cows[a]

Model	Parameter	OR[b]	95% CI[c] (lower–upper)	Significance (*P*)
1	Intercept	0.012		<0.001
	No. of cows with milk positive for *M. avium* subsp. *paratuberculosis*-specific antibody by ELISA	1.267	1.198–1.340	<0.001
	Total no. of cows in milk	1.014	1.001–1.030	<0.05
2	Intercept	0.023		<0.001
	No. of cows with milk positive for *M. avium* subsp. *paratuberculosis*-specific antibody by ELISA (−4 weeks)	1.272	1.198–1.350	<0.001
	Total no. of cows in milk (−4 weeks)	1.009	0.994–1.024	>0.05
3	Intercept	0.024		<0.001
	No. of cows with milk positive for *M. avium* subsp. *paratuberculosis*-specific antibody by ELISA (−8 weeks)	1.239	1.167–1.315	<0.001
	Total no. of cows in milk (−8 weeks)	1.008	0.993–1.023	>0.05
4	Intercept	0.011		<0.001
	No. of cows with milk positive for *M. avium* subsp. *paratuberculosis*-specific antibody by ELISA (+4 weeks)	1.270	1.200–1.343	<0.001
	Total no. of cows in milk (+4 weeks)	1.016	1.002–1.031	<0.05
5	Intercept	0.009		<0.001
	No. of cows with milk positive for *M. avium* subsp. *paratuberculosis*-specific antibody by ELISA cows (+8 weeks)	1.257	1.192–1.327	<0.001
	Total no. of cows in milk (+8 weeks)	1.019	1.005–1.034	<0.01

[a] The farm parameter was modeled as a random effect.
[b] The OR was calculated as exp(β).
[c] CI, confidence interval.

dust. The sensitivity of the ELISA for *M. avium* subsp. *paratuberculosis*-specific antibody is not higher than 45%, which indicates that not all cows shedding *M. avium* subsp. *paratuberculosis* into the environment were identified by the test (14). In addition, dry dairy cows could not be tested by ELISA of milk samples for *M. avium* subsp. *paratuberculosis*-specific antibody in this study but could still shed *M. avium* subsp. *paratuberculosis* during the dry period. ELISA of serum could have been utilized for all cows or only dry cows, but the sensitivity of the test does not differ substantially from that of tests of milk samples, which would still mean that cows shedding *M. avium* subsp. *paratuberculosis* would be missed (14). Both the test characteristics and animal selection lead to an underestimation of *M. avium* subsp. *paratuberculosis*-positive cows.

In this study, the number of cows with milk positive for *M. avium* subsp. *paratuberculosis*-specific antibody by ELISA showed a positive relationship (OR, 1.2) with the odds of viable *M. avium* subsp. *paratuberculosis* in settled-dust samples, indicating that culling of *M. avium* subsp. *paratuberculosis*-positive cows reduces the levels of dust positive for *M. avium* subsp. *paratuberculosis*.

TABLE 4 Comparison of AIC values of different models testing the temporal relationship between the number of ELISA-positive cows and the number of settled-dust samples positive for viable *M. avium* subsp. *paratuberculosis*[a]

Data set	*n*	AIC for model: 1	2	3	4	5
1	187	239.6				
2	178	226.9	232.6			
3	169	220.5	221.0	231.3		
4	178	233.8			225.9	
5	169	214.8			218.6	218.1

[a] Dust sample results were linked to ELISA outcomes of a previous or future TD, as illustrated in Fig. 1. AIC values in the same row are compared.

However, removal of adult cows with milk positive for *M. avium* subsp. *paratuberculosis*-specific antibody did not directly result in a decrease in the presence of *M. avium* subsp. *paratuberculosis* in dust samples collected in the environment. These data suggest that control measures to reduce contamination of the environment, like culling of detectable infected animals and purchasing substitute animals from farms known to be free of *M. avium* subsp. *paratuberculosis* infection, might have a substantial delayed effect on environmental *M. avium* subsp. *paratuberculosis* presence, likely because of a saturated environment in farms where *M. avium* subsp. *paratuberculosis* infection is endemic (35–37). In addition, separation of calves from adult cattle but within the same airspace seems not to be effective in preventing contact with and uptake of settled dust with viable *M. avium* subsp. *paratuberculosis* via exploration of the environment or via respiration of calves. Although the latter route has been shown to be effective only under experimental conditions so far, it should be taken into account when advising farmers about *M. avium* subsp. *paratuberculosis* control measures (12). Housing young stock in a different barn than dairy cows reduces exposure to dust positive for *M. avium* subsp. *paratuberculosis* and at the same time may enhance the efficacy of other *M. avium* subsp. *paratuberculosis* infection management measures, because the risk of accidental contact with infectious material is further decreased. However, due to the limited knowledge about the contributions of different transmission routes to *M. avium* subsp. *paratuberculosis* infection in a dairy herd, it is difficult to quantify the effect on *M. avium* subsp. *paratuberculosis* infection when exposure to infective dust is reduced.

In conclusion, a positive relationship was found between the number of cows with milk positive for *M. avium* subsp. *paratuberculosis*-specific antibody by ELISA and dust samples positive for viable *M. avium* subsp. *paratuberculosis* on commercial dairy farms. This relationship remained the same for accumulated dust

samples collected up to 2 months before or after the milk sampling, indicating that a change in the number of cows with milk positive for *M. avium* subsp. *paratuberculosis*-specific antibody by ELISA does not lead directly to a change in the environmental burden of *M. avium* subsp. *paratuberculosis*. Moreover, to effectively reduce the exposure of young calves to *M. avium* subsp. *paratuberculosis*, they should be housed in separate barns with no adult dairy cattle present, which might support the efficacy of *M. avium* subsp. *paratuberculosis* control programs in reducing the prevalence in dairy herds.

ACKNOWLEDGMENTS

We thank the farmers for the opportunity to sample on their farms. We are grateful to Marina Bouman and Jeroen Hoeboer, who helped with the collection and analysis of the milk and dust samples. We also thank Hans Vernooij for helping with the statistical analysis of the data.

The study was financially supported by Stichting Mesdag-zuivelfonds NLTO (Leeuwarden, The Netherlands).

REFERENCES

1. **Sweeney RW.** 1996. Transmission of paratuberculosis. Vet. Clin. North Am. Food Anim. Pract. **12:**305–312.
2. **Lombard JE.** 2011. Epidemiology and economics of paratuberculosis. Vet. Clin. North Am. Food Anim. Pract. **27:**525–535.
3. **Sweeney RW, Whitlock RH, Rosenberger AE.** 1992. Mycobacterium paratuberculosis cultured from milk and supramammary lymph nodes of infected asymptomatic cows. J. Clin. Microbiol. **30:**166–171.
4. **Streeter RN, Hoffsis GF, Bech-Nielsen S, Shulaw WP, Rings DM.** 1995. Isolation of Mycobacterium paratuberculosis from colostrum and milk of subclinically infected cows. Am. J. Vet. Res. **56:**1322–1324.
5. **Pithua P, Godden SM, Wells SJ, Stabel JR.** 2011. Evaluation of the risk of paratuberculosis in adult cows fed Mycobacterium avium subsp paratuberculosis DNA-positive or -negative colostrum as calves. Am. J. Vet. Res. **72:**1456–1464.
6. **McQueen DS, Russell EG.** 1979. Culture of Mycobacterium paratuberculosis from bovine foetuses. Aust. Vet. J. **55:**203–204.
7. **Sweeney RW, Whitlock RH, Rosenberger AE.** 1992. Mycobacterium paratuberculosis isolated from fetuses of infected cows not manifesting signs of the disease. Am. J. Vet. Res. **53:**477–480.
8. **Whittington RJ, Windsor PA.** 2009. In utero infection of cattle with Mycobacterium avium subsp. paratuberculosis: a critical review and meta-analysis. Vet. J. **179:**60–69.
9. **van Roermund HJW, Bakker D, Willemsen PTJ, de Jong MCM.** 2007. Horizontal transmission of Mycobacterium avium subsp. paratuberculosis in cattle in an experimental setting: calves can transmit the infection to other calves. Vet. Microbiol. **122:**270–279.
10. **Eisenberg SW, Nielen M, Santema W, Houwers DJ, Heederik D, Koets AP.** 2010. Detection of spatial and temporal spread of Mycobacterium avium subsp. paratuberculosis in the environment of a cattle farm through bio-aerosols. Vet. Microbiol. **143:**284–292.
11. **Eisenberg SW, Koets AP, Hoeboer J, Bouman M, Heederik D, Nielen M.** 2010. Presence of Mycobacterium avium subsp. paratuberculosis in environmental samples collected on commercial Dutch dairy farms. Appl. Environ. Microbiol. **76:**6310–6312.
12. **Eisenberg SW, Koets AP, Nielen M, Heederik D, Mortier R, De Buck J, Orsel K.** 2011. Intestinal infection following aerosol challenge of calves with Mycobacterium avium subspecies paratuberculosis. Vet. Res. **42:**117.
13. **Cocito C, Gilot P, Coene M, de Kesel M, Poupart P, Vannuffel P. Paratuberculosis.** 1994. Clin. Microbiol. Rev. **7:**328–345.
14. **Collins MT, Wells SJ, Petrini KR, Collins JE, Schultz RD, Whitlock RH.** 2005. Evaluation of five antibody detection tests for diagnosis of bovine paratuberculosis. Clin. Diagn. Lab. Immunol. **12:**685–692.
15. **Nielsen SS, Ersboll AK.** 2006. Age at occurrence of Mycobacterium avium subspecies paratuberculosis in naturally infected dairy cows. J. Dairy Sci. **89:**4557–4566.
16. **Nielsen SS, Grohn YT, Enevoldsen C.** 2002. Variation of the milk antibody response to paratuberculosis in naturally infected dairy cows. J. Dairy Sci. **85:**2795–2802.
17. **Smith RL, Schukken YH, Pradhan AK, Smith JM, Whitlock RH, Van Kessel JS, Wolfgang DR, Grohn YT.** 2011. Environmental contamination with Mycobacterium avium subsp. paratuberculosis in endemically infected dairy herds. Prev. Vet. Med. **102:**1–9.
18. **Berghaus RD, Farver TB, Anderson RJ, Jaravata CC, Gardner IA.** 2006. Environmental sampling for detection of Mycobacterium avium ssp. paratuberculosis on large California dairies. J. Dairy Sci. **89:**963–970.
19. **Lombard JE, Wagner BA, Smith RL, McCluskey BJ, Harris BN, Payeur JB, Garry FB, Salman MD.** 2006. Evaluation of environmental sampling and culture to determine Mycobacterium avium subspecies paratuberculosis distribution and herd infection status on US dairy operations. J. Dairy Sci. **89:**4163–4171.
20. **Jorgensen JB.** 1977. Survival of Mycobacterium paratuberculosis in slurry. Nord. Vet. Med. **29:**267–270.
21. **Lovell R, Levi M, Francis J.** 1944. Studies on the survival of Johne's bacilli. J. Comp. Pathol. **54:**120–129.
22. **Cook KL, Britt JS.** 2007. Optimization of methods for detecting Mycobacterium avium subsp. paratuberculosis in environmental samples using quantitative, real-time PCR. J. Microbiol. Methods. **69:**154–160.
23. **Weber MF, Groenendaal H, van Roermund HJW, Nielen M.** 2004. Simulation of alternatives for the Dutch Johne's disease certification-and-monitoring program. Prev. Vet. Med. **62:**1–17.
24. **Weber MF, Nielen M, Velthuis AG, van Roermund HJ.** 2008. Milk quality assurance for paratuberculosis: simulation of within-herd infection dynamics and economics. Vet. Res. **39:**12.
25. **Akaike H.** 1973. Information theory and an extension of the maximum likelihood principle, p 267–281. *In* Petrov BN, Csaki F (ed), Second International Symposium on Information Theory. Akademiai Kiado, Budapest, Hungary.
26. **Dohoo I, Martin W, Stryhn H.** 2010. Veterinary Epidemiologic Research, 2nd ed, p 395–426. Transcontinental, Prince Edward Island, Canada.
27. **Raizman EA, Wells SJ, Godden SM, Bey RF, Oakes MJ, Bentley DC, Olsen KE.** 2004. The distribution of Mycobacterium avium ssp. paratuberculosis in the environment surrounding Minnesota dairy farms. J. Dairy Sci. **87:**2959–2966.
28. **Pillars RB, Grooms DL, Kaneene JB.** 2009. Longitudinal study of the distribution of Mycobacterium avium subsp. paratuberculosis in the environment of dairy herds in the Michigan Johne's disease control demonstration herd project. Can. Vet. J. **50:**1039–1046.
29. **Eisenberg SWF, Nielen M, Hoeboer J, Rutten V, Heederik D, Koets AP.** 2012. Environmental contamination with Mycobacterium avium subspecies paratuberculosis within and around a dairy barn under experimental conditions. J. Dairy Sci. **95:**6477–6482.
30. **Collins MT, Eggleston V, Manning EJ.** 2010. Successful control of Johne's disease in nine dairy herds: results of a six-year field trial. J. Dairy Sci. **93:**1638–1643.
31. **Ferrouillet C, Wells SJ, Hartmann WL, Godden SM, Carrier J.** 2009. Decrease of Johne's disease prevalence and incidence in six Minnesota, U. S. A., dairy cattle herds on a long-term management program. Prev. Vet. Med. **88:**128–137.
32. **Hartung S, Seedorf J.** 1999. Characterization of airborne dust in livestock housing and its effect on animal and environment, p 140–152. Proceedings of the International Symposium on Dust Control in Animal Production Facilities, Aarhus, Denmark. Horsens, Bygholm, Denmark.
33. **Muskens J, Elbers AR, van Weering HJ, Noordhuizen JP.** 2003. Herd management practices associated with paratuberculosis seroprevalence in Dutch dairy herds. J. Vet. Med. B **50:**372–377.
34. **Wells SJ, Wagner BA.** 2000. Herd-level risk factors for infection with Mycobacterium paratuberculosis in US dairies and association between familiarity of the herd manager with the disease or prior diagnosis of the disease in that herd and use of preventive measures. J. Am. Vet. Med. Assoc. **216:**1450–1457.
35. **McKenna SL, Keefe GP, Tiwari A, VanLeeuwen J, Barkema HW.** 2006. Johne's disease in Canada part II: disease impacts, risk factors, and control programs for dairy producers. Can. Vet. J. **47:**1089–1099.
36. **Cetinkaya B, Erdogan HM, Morgan KL.** 1997. Relationships between the presence of Johne's disease and farm and management factors in dairy cattle in England. Prev. Vet. Med. **32:**253–266.
37. **Johnson-Ifearulundu YJ, Kaneene JB.** 1998. Management-related risk factors for M. paratuberculosis infection in Michigan, U. S. A., dairy herds. Prev. Vet. Med. **37:**41–54.