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Detection of spatial and temporal spread of *Mycobacterium avium* subsp. *paratuberculosis* in the environment of a cattle farm through bio-aerosols

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ABSTRACT

Environmental samples were collected to investigate the spatial and temporal spread of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in a dairy cattle barn before and after the introduction of two groups of MAP-shedding animals. Samples collected off the floor of the barn reflected the moment of sampling whereas samples collected by microfiber wipes at a minimal of 3 m height contained the accumulated settled dust over a 3-week period. Samples were analysed by IS*900* qPCR for the presence of MAP DNA and by culture for viable MAP bacteria. MAP DNA was detected in a large number of sites both before and after introduction cattle. MAP DNA was detected inside the barn in floor and dust samples from cubicles and slatted floors and in settled dust samples located above the slatted floors and in the ventilation ridge opening. Outside the barn MAP DNA was detected by PCR in samples reflecting the walking path of the farmer despite hygiene measures. No viable MAP was detected before the introduction of shedder cattle. Three weeks later viable MAP was found inside the barn at 7/49 locations but not outside. Fifteen weeks later viable MAP was also detected in environmental samples outside the barn. In conclusion, introduction of MAP shedding cattle lead to widespread contamination of the internal and external environment of a dairy barn, including the presence of viable MAP in settled dust particles suggesting potential transmission of MAP infection through bio-aerosols.

1. Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) causes chronic granulomatous enteritis in cattle, where infection is considered to occur by the faeco-oral route. Infection usually occurs in animals younger than a year but bacterial excretion and clinical disease (if it ensues) are not usually evident for some years. Infection with MAP causes production loss in dairy production around the world. In addition, the possible relation with Crohn's disease in humans makes paratuberculosis in dairy herds a public health issue (Chamberlin et al., 2001; Naser et al., 2004; Schwartz et al., 2000).

The true prevalence of MAP positive dairy farms is difficult to determine due to the low sensitivity of diagnostic tests. In the Netherlands herd prevalence was estimated to be between 31% and 71% (Muskens et al., 2000). Since 1942, bovine paratuberculosis eradication programs were introduced in the Netherlands based on different tests but all with the purpose of identifying and culling test-positive animals (Benedictus, 1984; Kalis et al., 1999). This strategy was unsuccessful because transmission of MAP is possible before the infection can be diagnosed with the available tests (Benedictus, 1984). Subsequently introduced vaccination schemes decreased the number of clinical cases but eradication was not

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achieved because of the continued shedding of MAP by vaccinates. In 2000 the Dutch voluntary 'Paratuberculosis Program Netherlands' (PPN) was implemented as supported by the JohnSSim model (Groenendaal et al., 2003). The crucial step of the PPN was to separate calves and adults as soon as possible after birth and to improve management measures in order to minimize contact between infectious and susceptible age groups on a farm.

This practice is based upon two generally accepted assumptions. The first assumption is that the environment becomes contaminated by concurrent presence of faecal shedders. Secondly, it is the assumption that the spatial separation of young livestock and adult cows, in combination with hygiene measures, was sufficient to reduce contact of young stock with infectious faeces. These assumptions were based on the belief that MAP organisms multiply and survive only in the tissue of susceptible animals, and that they did not survive for long outside the host. However, it has been shown that MAP can persist for several months in the environment after shedding (Jorgensen, 1977; Whittington et al., 2004). With a long persistency in the environment, and continued shedding of bacteria, accumulation in the environment may occur. It has been demonstrated that dried faeces can also become airborne with the resultant dust containing viable bacteria although this has until now not been demonstrated for MAP (Green et al., 2006; Predicala et al., 2002; Wilson et al., 2002). Corner et al. (2004) hypothesised that young calves could become infected by ingestion or even by inhalation of accumulated dust. This would require dust contaminated with viable MAP to accumulate in the farm environment and reach infective levels. The spatial distribution and level of contamination would determine the risk of exposure and infection of susceptible animals. This phenomenon could play a significant role in MAP transmission, including decreasing the efficacy of calf isolation to minimize exposure to MAP, but has so far received little attention.

The first objective of this study was to detect and assess viability of MAP in environmental samples. The second objective was to study the environmental contamination over time after shedders were introduced into a cleaned dairy barn and thus provide information about the temporal and spatial spread of MAP on the farm.

2. Materials and methods

2.1. Experimental farm

A Dutch dairy barn with 80 bed stalls in a loose housing system with 4 open water troughs, a central corridor with feed bunk and overhead ridge ventilation was selected. The farm was destocked for 10 days before the current experiment. MAP status of animals which left the farm before the experiment was unknown. All the manures were immediately removed after destocking and the barn was cleaned with a high-pressure cleaner. The slurry pit was emptied. After high pressure cleaning surfaces were allowed to dry for 6 days before cows were introduced. In total 61 confirmed heavy and medium MAP shedders were identified on commercial dairy farms and purchased. Standard diagnostic absorbed ELISA (Pourquier-ELISA, Institut Pourquier, Montpellier, France) was used for identification of the MAP infected animals and MAP shedding was classified based on time-to-positive by the para-JEM® culture method with Ziehl Neelsen (ZN) stain and IS*900* PCR confirmation by the Dutch Animal Health Service (GD, Deventer) following instructions as provided by the manufacturer (TREK Diagnostic Systems, Cleveland, OH, USA). The cows arrived in two groups. First 19 heavy and medium shedders were housed in one section of the barn (light grey area, Fig. 1). Another 41 were introduced 3 weeks later to the remainder of the barn. Cows were non-lactating during the experiment. The use of animals was approved by the Ethical Committee for Animal Experiments of Intervet/Schering-Plough Animal Health.

On the farm a strict biosecurity protocol was maintained. The barn could only be entered through a hygiene barrier where boots and cotton overalls were provided. A second hygiene barrier required a second layer of plastic overalls, gloves, mouth protection and boots before the animal section with the slatted floors could be entered.

2.2. Sampling procedures

The samples analysed for this study were collected on three occasions—before the introduction of the first group of MAP shedders (T0), 3 weeks later immediately before the introduction of the second group of cows (T1) and 12 weeks later (T2).

Samples were classified into 3 categories—inside floor dust (dust, saw dust or manure), inside settled dust (airborne dust well above animal level) and outside floor dust (ground or floor surface dust/debris outside the biosecurity entry points). All sampling locations were specified on a map of the farm to allow repeated sampling at the same locations. Samples were collected in triplicates unless specified. Inside floor dust samples were collected from the cubicles (Fig. 1, locations 13–15), the slatted floors (Fig. 1, locations 10–12) and the corridor (Fig. 1, locations 8/9). Samples were also collected from the water troughs at T1 and T2 (empty at T0). Outside the barn a range of locations on the concrete walking path taken by the farmer were sampled (Fig. 1, locations 17–23). Initial floor dust samples (T0) were taken inside and outside with a vacuum cleaner (Miele De Luxe 246i, Gütersloh, Germany) using special single use vacuum bags (150 mm × 73/38 mm, micron rating 25 μm; article 12513139, Vacuum Bag, Allied Filter Fabrics PTY Limited) fitted inside the nozzle to minimize the risk of cross-contamination of samples. After dust collection at a spot the vacuum bag with contents was placed in a sterile 50 ml tubes and stored at −20 °C.

After restocking with cows, samples from the cubicles (sawdust) and from the slatted floors (manure) were collected from the specified locations in 50 ml tubes using a single disposable wooden spatula per location. Sample collection from the corridor and outside the barn was continued by vacuum cleaning. From each of the 4 water troughs, 1 l of water from the surface was collected and dust and particulate matter was concentrated by centrifugation (10 min at 2000 × *g*).

Accumulated settled dust samples were collected by electrostatic wipes (22 cm × 28 cm, Zeeman, Alphen a/d

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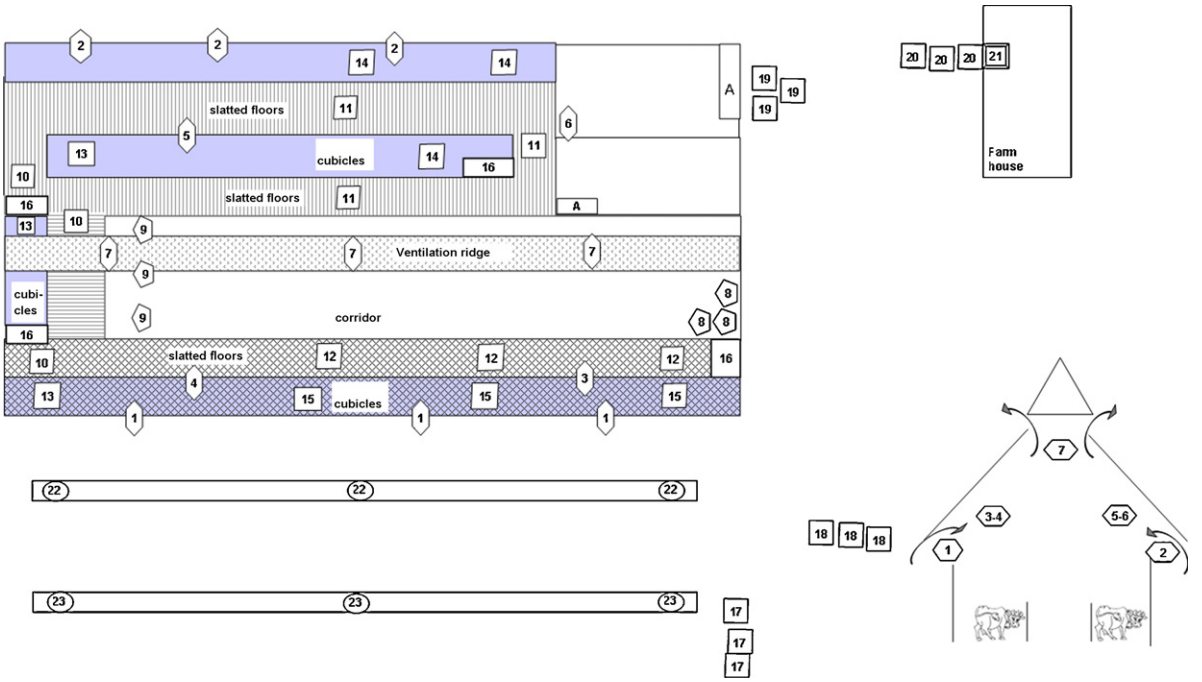

Fig. 1. Illustration of the experimental farm showing the different sample locations (each consisting of 3 sample spots) inside and outside the barn. Arrows represent the air movement for ventilation; A: hygiene barriers; *Inside settled dust samples*: (1) air inlet left, (2) air inlet right, (3–6) above slatted floor (3 m high), (7) ridge ventilation (10 m high); *Inside floor dust samples*: (8/9) corridor, (10–12) slatted floors, (13–15) cubicles, (16) waterers; *Outside floor dust samples*: (17–18) yard, (19) barn entrance, (20) farm house entrance, (21) doormat, (22) silage pit wall (1 m high) close to barn, (23) silage pit wall (1 m high) far away from barn; sampling location are represented by a hexagon for settled dust collection. The checked area where cows were present at T1.

Rhijn, The Netherlands) hanging for a 3-week period inside the air inlets (Fig. 1, locations 1/2), above the slatted floors (Fig. 1, locations 3–6) and inside the ventilation ridge (Fig. 1, location 7). Dust from the air inlet was collected by wiping 0.5 m^2 of the air inlet opening at each side of the barn. Circular microfiber wipes (radius = 7 cm) in petri dishes were placed at 3 m height to collect dust above the slatted floors. Dust from the exhaust airflow was collected by a wipe, stretched on a wire frame, placed in the open ridge of the roof at 10 m height. Samples collected above the slatted floors were collected in fourfold replicates.

All samples were analysed according to the diagnostic procedure shown in Fig. 2 using techniques described below (Sections 2.3–2.7).

2.3. Samples for IS900 qPCR validation

Purified MAP DNA (316 F) isolated from a liquid culture was used as a positive control and Milli-Q ultrapure water (Milli-Q; Millipore Corporation, Billerica, MA, USA) was used as an analytical blank in the IS*900* qPCR.

The specificity of the IS*900* qPCR was evaluated using 5 dust samples from a city house, 5 samples from clean packed saw dust, and 5 faecal samples from a certified paratuberculosis negative herd – Status 10, PPN – (Benedictus et al., 2000). In addition, IS*900* qPCR products of 9 different positive environmental samples from the present study were sequenced and compared to the NCBI DNA data bank by 'BLAST' to confirm IS*900* as the target of the primers (Altschul et al., 1997).

The DNA detection limit for viable MAP was determined using a serial tenfold dilution of cultured MAP with concentrations of the equivalent of 10^9–10^1 colony forming units (CFU).

The detection limit for purified MAP DNA was determined using a serial tenfold dilution of pure MAP DNA with concentrations ranging from the equivalent of 10^9–10^1 CFU of viable MAP.

The detection limit for MAP DNA IS900q PCR when applied to faecal samples was determined using a faecal sample originating from a cow from a certified paratuberculosis negative herd which was spiked with cultured MAP bacteria. A serial tenfold dilution of cultured MAP with concentrations of the equivalent of 10^9–10^1 CFU was added to 0.5 g of faeces.

2.4. Sample preparation

All samples were stored at −20 °C and processed within 10 weeks according to a method described previously with slight modifications (Jaravata et al., 2006). A total of 35 ml Milli-Q was added to 0.5 g of a sample, either dust, manure, sawdust, soil, spiked faeces or to a microfiber wipe. After vortexing, the samples were allowed to settle for 30 min. The supernatant was transferred to a 50 ml tube and centrifuged at 3400 × *g* for 20 min. The supernatant was discarded with the exception of 150 μl and the pellet was resuspended by vortexing. The sample was pipetted in to a 2 ml micro-centrifuge tube and centrifuged at 14,000 × *g* for 3 min. The supernatant was discarded and the pellet

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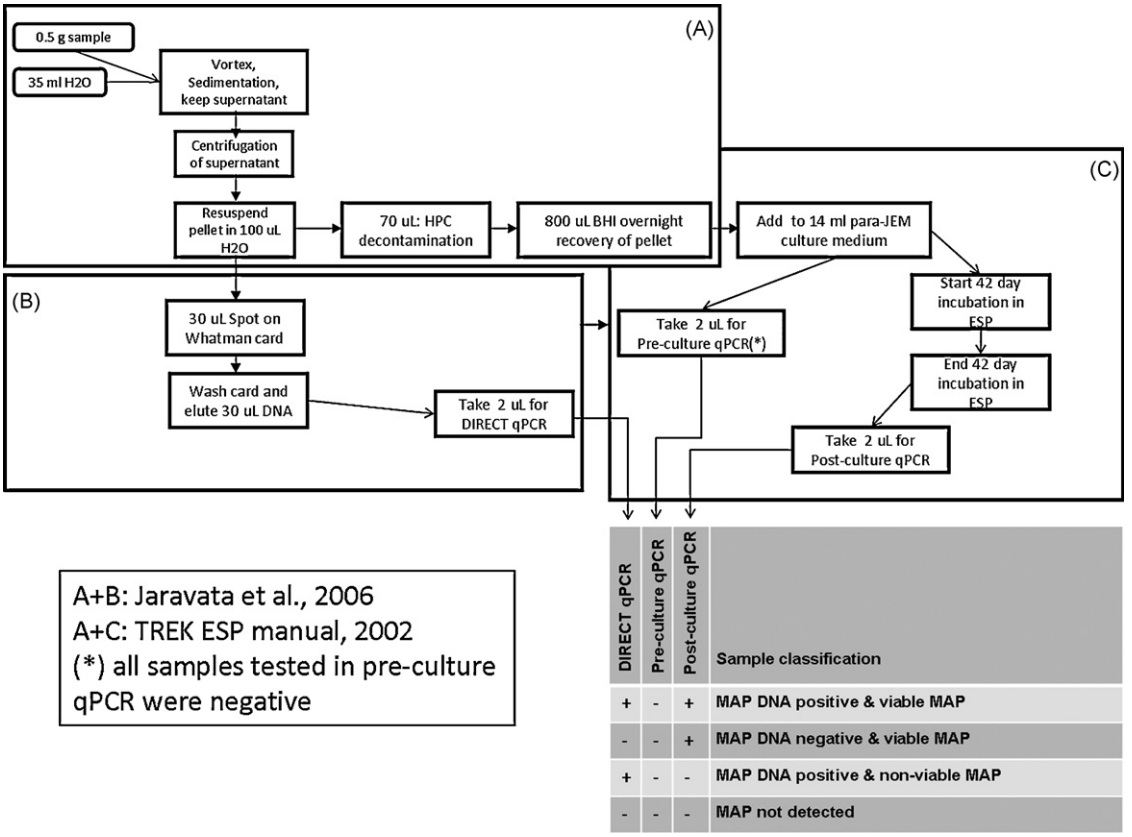

Fig. 2. All environmental samples in this study were analysed according to this diagnostic procedure scheme to distinguish between the presence of MAP DNA and viable MAP in the sample.

was resuspended in 100 μl Milli-Q. 30 μl of this sample were used for DNA extraction, the other 70 μl were used for MAP culturing.

2.5. DNA extraction

DNA extraction was performed as described previously with slight modifications (Jaravata et al., 2006). In short, each dissolved sample was pipetted onto an Elute® microcard (Whatman, Clifton, NJ). The cards were dried overnight in closed petri dishes to avoid contamination by settled dust. Using a biopsy punch (4 mm) 2 disks from each microcard were placed in a single 1.5-ml micro-centrifuge tube and processed according to the manufacturer's instructions. The disks were vigorously washed twice for 5 s in 1.5 ml of Milli-Q. After removing the wash water, 30 μl Milli-Q was added and the micro-centrifuge tube was incubated in a heating block at 95 °C for 30 min. The samples were pulse vortexed after 15 min and again at the end of the incubation period. The disks were removed immediately and 2 μl of the eluate were used as a template in the IS*900* qPCR (MiQ, Biorad®, Hercules, USA).

2.6. IS900 qPCR

Primers targeting an internal sequence of the IS*900* insertion element were used (Hruska et al., 2005). The described protocol was adapted to a real-time PCR protocol as follows: 10 μl SYBR®Premix Ex Taq™ (TaKaRa Bio Inc., USA), 1 μl P3N (GGG TGT GGC GTT TTC CTT CG), 1 μl P4N (TCC TGG GCG CTG AGT TCC CT), 6 μl Milli-Q and 2 μl of sample template was added to a total 20 μl/reaction. MyiQ 96-well single-colour real-time PCR (Biorad®, Hercules, USA) plates were used. Samples were tested in duplicate wells. With each run a technical positive control (pure MAP DNA), a technical negative control (Milli-Q) and tenfold serial dilutions of a positive spiked faecal samples (ranging from 10^9 to 10^6 CFU/g faeces) were tested. The PCR conditions were a hot start at 95 °C for 3 min, 45 cycles consisting of denaturation at 95 °C for 10 s, annealing at 64 °C for 10 s, and amplification at 72 °C for 10 s. Fluorescence was recorded after each amplification step. A melting cycle consisted of heating to 95 °C for 1 min, 65 °C for 1 min and a 67 times repeated increase of 0.5 °C up to 98 °C for 10 s. The fluorescent signal was monitored up to 98 °C. DNA with a melting peak of 93–94 °C was accepted as consistent with standard MAP DNA IS*900*. This IS*900* qPCR was used on all environmental samples (direct qPCR) and on culture media pre- and post-incubation to check for the presence of viable MAP.

2.7. para-JEM® MAP culturing

The protocol for para-JEM® automated MAP culturing provided by TREK Diagnostic Systems (Cleveland, OH, USA) was used with the following modifications. A 70 μl aliquot

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of the washed sample (see Section 2.4) was decontaminated overnight in 350 μl of half-strength brain heart infusion (BHI) with 0.9% cetylpyridinium chloride (HPC) at 35 °C. The next day, the micro-centrifuge tube was centrifuged for 20 min at 3000 × *g*. The pellet was resuspended in 800 μl of half-strength BHI after discarding the supernatant. The para-JEM® bottle was prepared according to the protocol provided by the manufacturer. Before the bottle was incubated a sample was taken for IS*900* qPCR analysis (pre-incubation IS*900* qPCR). Following instructions provided by the manufacturer, all cultures were tested with IS*900* qPCR to confirm growth of MAP.

Pilot specificity experiments with numbers of environmental samples from the test farm frequently yielded growth according to para-JEM® software but were confirmed negative for MAP on IS*900* qPCR. As a result, the para-JEM® culturing with time-to-detection could not be used as a semi-quantitative method but was used as a binary viability indicator only. All samples were incubated for 42 days when all samples were checked with IS*900* qPCR for MAP confirmation. Environmental samples were considered to be positive for viable MAP only if the pre-incubation IS*900* qPCR was negative (i.e. MAP below the IS*900* qPCR detection limit) and the post-incubation IS*900* qPCR showed a positive signal with a specific melting peak consistent with MAP i.e. 93–94 °C. Note that originally a quantitative analysis of bacterial CFU based on time-to-positive was aimed for in this study. It turned out that it could only be used as a binary indicator (positive/negative) of viability at 42 days.

2.8. Data analysis

Data were analysed with the SPSS statistical software package (version 12.0.1). Analysis was performed for each sampling point. Threshold cycle (TC) values were considered as crude estimates of the amount of MAP in collected samples (Heid et al., 1996). The intra-plate variation (analytical error) was determined from the TC values of duplicate analyses at T2. TC values were corrected for inter-plate variation using the variation coefficient of the dilution series and the positive control. Sampling error of the environmental samples was determined on triplets per location at T2 and expressed as the coefficient of variation (CV = standard deviation/mean × 100%).

Spatial and temporal variation of MAP DNA concentration was analysed using Cox proportional hazard regression to model a time to event dependent association (survival analysis). The PCR run was modelled as study time and the TC as the time at which an event occurred. The event was defined as the TC at which the fluorescence exceeded the threshold limit prior to 45 cycles and satisfied a melt peak between 93 and 94 °C. All samples without a specific PCR signal (event) were censored and were assigned to TC 45. The hazard ratio (HR) can be interpreted as the probability of a MAP DNA positive sample in the environment at T1 or T2 relative to a reference environment, defined as T0 for temporal analysis and varying locations for spatial analysis. Difference in viable MAP proportions between T0, T1 and T2 was tested by McNemar's χ^2. Samples from the water trough (location 16) were only used to investigate the spatial variation because at T1 and T2 they contained no water prior to the arrival of the cattle.

3. Results

3.1. Specificity and detection limit of the IS900 qPCR

The dust samples from a city house, samples from clean packed saw dust, and faecal samples from a certified paratuberculosis negative herd were all negative in the IS*900* qPCR, indicating high specificity. Sequence analyses of all 9 PCR products of IS*900* qPCR positive environmental samples confirmed the specific amplification of the intended MAP IS*900* fragment.

The IS*900* qPCR had a detection limit of approximately 10^5 CFU-equivalents of MAP/g faeces (Fig. 3) whereas for MAP DNA and MAP bacteria dilutions the detection limit was equivalent to 10^1 CFU of MAP/ml of culture (results not shown).

3.2. Detection of MAP DNA in environmental samples (direct qPCR)

The analytical and sampling CV's were 26% and 20% respectively. The difference between duplicates and between sample spots per location was less than 1 TC. Note that TC values are expressed on an negative logarithmic scale where reducing the TC by 1 represents a doubling of CFU-equivalents i.e. a lower TC value represents a greater amount of MAP DNA in the initial sample.

The percentage of positive concordant duplicate tests performed on samples was highest at T2 whereas the percentage of discordant samples at T2 was lowest for inside settled dust samples.

A high proportion of T0 tests on inside floor (59%) and inside settled dust (79%) and a low proportion of outside floor dust tests (21%) were positive for MAP DNA in the direct IS*900* qPCR (Table 1).

Analysis of TC values showed significant decreases in TC values (higher DNA concentration) inside the barn than outside the barn at all time points ($p < 0.05$; HR: T0 3.6; T1 8.5; T2 5.1; data not shown). At locations 1 and 2 significantly lower TC values were found between T0 and T2 ($p < 0.05$; HR 14.7; Fig. 4). At locations 3–7 no temporal changes in concentration were observed (Fig. 4). Inside floor dust samples showed a strong tendency to decreasing TC values at T1 compared to T0 ($p = 0.065$, HR 1.6) and T2 ($p < 0.05$, HR 2.6) compared to T0. The concentrations of MAP DNA in outside floor dust did not change significantly over time.

At T1 there were no significant differences observed between the wipes hanging above the cows and those hanging above the unstocked part of the barn (results not shown). In contrast, the floor samples showed a significant difference between stocked and unstocked section of the barn ($p < 0.05$; HR 3.3). At T1 all water samples were MAP DNA negative; three out of 4 watering troughs (sample 16) contained MAP DNA at T2.

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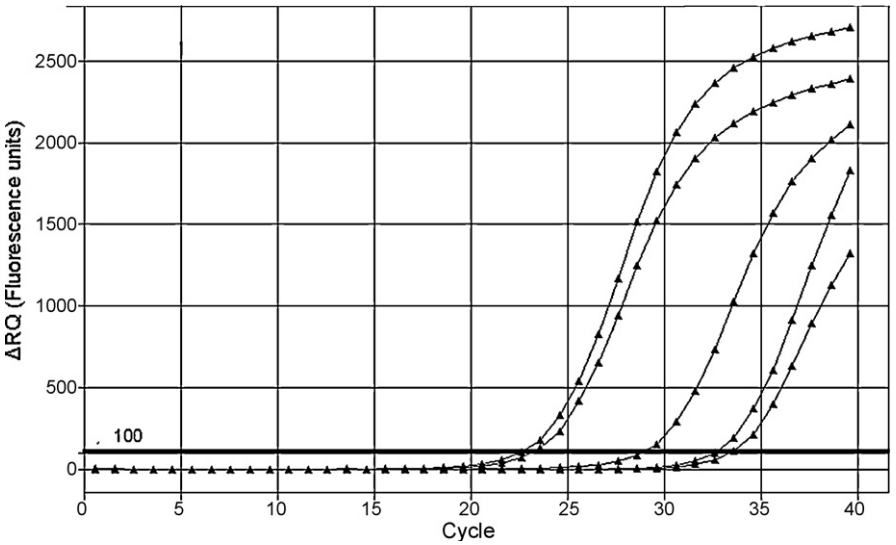

Fig. 3. IS*900* qPCR results of serial tenfold dilutions of *M. paratuberculosis* spiked faeces. The detection limit of the IS*900* qPCR was 10^5 CFU/g faeces. From left to right 10^9–10^5 CFU/g faeces. The threshold was set at 100. The event was defined as TC at the fluorescence exceeded the threshold limit less than 45 cycles with a melt peak between 93 and 94 °C.

3.3. Viability of MAP in environmental samples

For environmental samples at T0, T1 and T2 the pre-incubation IS*900* qPCR on the culture medium with added sample was negative. Viable MAP was detected in culture medium after 42 days of incubation from inside floor and settled dust samples only at T1 and T2, after animals shedding MAP were introduced onto the farm (Table 2). Cultures were viable MAP positive from outside floor samples only at T2. One water trough contained viable MAP at T1 while all 4 water troughs contained viable MAP at T2.

Detection frequencies of viable MAP by culture for all sites combined increased significantly from T0 (0%) to T2 (63/66 spots positive, 95%; Fig. 5). At T1, 8% inside settled dust and 21% inside floor sampling spots, located on both sides of the barn, were culture positive, indicating viable MAP. Viable MAP detection from the inside floor samples at T1 (21%) differed significantly from T0 (0%).

Table 1
Overview of number of cows present at the different sampling times, the number of locations and spots sampled, the results of the environmental samples analysed by direct IS*900* qPCR and the descriptive statistics of the TC values. Samples 1–2 and 3–7 of the settled dust were analysed separately because of non-sampling of 3–7 at T0.

Sampling	Inside settled dust			Inside floor dust			Outside floor dust		
	T0	T1	T2	T0	T1	T2	T0	T1	T2
No. of cows present	0	19	60	0	19	60	0	19	60
No. of locations sampled	2	7	7	8	8	8	7	7	7
No. of spots sampled	6	25	23[a]	24	24	24	19	19	19
Total tests (samples tested in duplicate)	12	50	46	48	48	48	38	38	38
% Positive (no.)	58 (7)	82 (41)	76 (35)	79 (38)	71 (34)	79 (38)	21 (8)	18 (7)	29 (11)
IS*900* qPCR duplicates of spots									
% Positive concordant	50 (3)	68 (17)	74 (17)	75 (18)	63 (15)	75 (18)	11 (2)	6 (1)	16 (3)
% Negative concordant	33 (2)	4 (1)	22 (5)	17 (4)	20 (5)	17 (4)	68 (13)	68 (13)	58 (11)
% Discordant	17 (1)	28 (7)	4 (1)	8 (2)	17 (4)	8 (2)	21 (4)	26 (5)	26 (5)

Sampling	Inside settled dust				Inside floor dust			Outside floor dust		
Descriptive statistics TC values	Loc.	T0	T1	T2	T0	T1	T2	T0	T1	T2
IS*900* qPCR test results										
Mean TC	1–2	39	39	33	39	36	33	43	44	42
	3–7	–	35	35	6	6	7	4	3	6
SD TC	1–2	5	5	1						
	3–7	–	5	7						

loc.: sampling locations. (–) no samples collected.
[a] Wipes of 2 spots were blown away.

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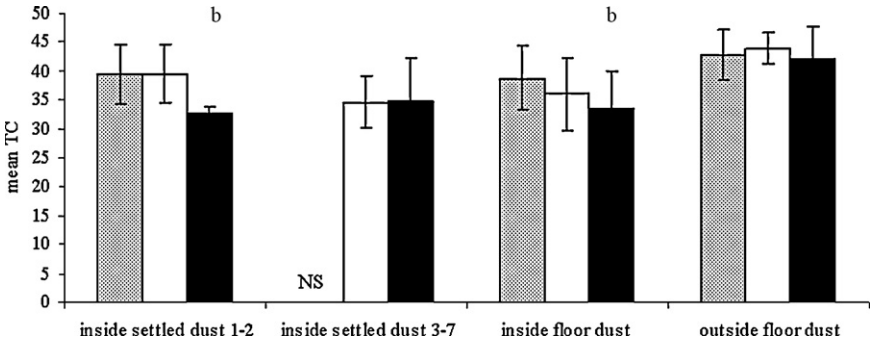

Fig. 4. The spatial and temporal spread of MAP DNA expressed as mean threshold cycles ±SD detected in environmental samples by direct IS*900* qPCR. Dotted bars: T0, blank bars: T1, black bars: T2. At T0 no cows, at T1 19 cows and at T2 61 cows were present at the farm. NS: no samples collected. b: Significant difference ($p < 0.05$) between time point T0 and T2. Note that a lower TC value represents a greater amount of MAP DNA in the initial sample.

Table 2
Comparison of direct IS*900* qPCR results and viable MAP cultured in para-JEM® medium on environmental samples.

Sampling	Inside settled dust			Inside floor dust			Outside floor dust		
	T0	T1	T2	T0	T1	T2	T0	T1	T2
Number of locations sampled	2	7	7	8	8	8	7	7	7
Number of spots sampled	6	25	23[a]	24	24	24	19	19	19
Number of spots culture positive (%)	0(0)	2 (8)	23 (100)	0 (0)	5 (21)	24 (100)	0 (0)	0 (0)	16 (84)
Number of spots IS*900* qPCR positive (%)	4 (67)	24 (96)	18 (78)	20 (83)	19 (79)	19 (79)	6 (32)	5 (26)	8 (42)
Agreement between IS*900* qPCR and para-JEM® per spot sampled									
PCR and culture positive	0%	8%	78%	0%	17%	79%	0%	0%	32%
Culture positive only	0%	0%	22%	0%	4%	21%	0%	0%	53%
IS*900* qPCR positive only	67%	88%	0%	83%	63%	0%	32%	26%	11%
IS*900* qPCR and culture negative	33%	4%	0%	17%	17%	0%	68%	74%	5%

[a] Wipes of 2 spots were blown away.

Direct IS*900* qPCR (MAP DNA) and viable MAP culture were not in agreement for all samples (Table 2). At T1 MAP presence was detected more often by direct IS*900* qPCR than in para-JEM® culture medium after 42 days of incubation. The numbers of concordant and discordant results are shown in Table 2b. Overall, 27% positive concordant, 22% negative concordant and 61% discordant (40% MAP DNA positive/viable MAP negative; 21% MAP DNA negative/viable MAP positive) samples were detected.

At T1 viable MAP was also detected in the water samples at the stocked side of the barn whereas the direct IS*900* qPCR was negative. At T2, 19 (79%) inside floor spots, 18 (78%) inside settled spots and 6 (32%) outside floor spots were positive in both test systems (Table 2b). Viable MAP

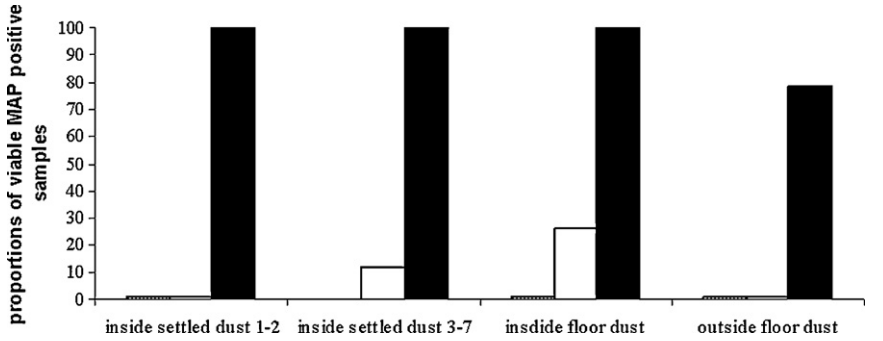

Fig. 5. The spatial and temporal spread of viable MAP expressed as proportions of viable MAP positive samples. Dotted bars: T0, blank bars: T1, black bars: T2. At T0 no cows were present at the farm. At T1 19 cows were present at the farm. At T2 61 cows were present at the farm. NS: no samples collected. a: Significant ($p < 0.05$) difference between time point T0 and T1; b: significant difference ($p < 0.05$) between time point T0 and T2; c: significant difference ($p < 0.05$) between time point T1 and T2.

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detection at T2 differed significantly from T0 and T1 at all locations (Fig. 5).

4. Discussion

The findings in the present study are the first to indicate that viable MAP can become airborne and may spread through dust. We used settled dust samples to detect MAP on a farm containing verified MAP-shedding animals. Until now MAP has only been measured in environmental samples directly contaminated by cattle faeces such as manure pit, soil and lagoon samples (Berghaus et al., 2006; Lombard et al., 2006; Raizman et al., 2004). In the present study viable MAP was detected in settled dust samples 3 m above the cows and in exhaust air near the ventilation ridge at 10 m height. Viable MAP was also detected outside the barn following the introduction of faecal shedders on the farm, despite strict hygiene protocols.

In this study the experimental farm was destocked and cleaned with a high-pressure cleaner before the baseline measurement. However, more than 50% of samples inside as well as >20% outside the barn were still detected positive for MAP DNA by direct IS*900* qPCR after cleaning. No sampling was undertaken prior to cleaning, hence the baseline contamination level was not determined. It remains unclear whether there had been viable MAP present immediately before cleaning or whether the DNA was long standing in the absence of viable MAP. No samples at T0 showed any growth during culturing, so it is likely that the signal was caused by the presence of residual DNA, either from intact but non-viable MAP or from DNA liberated after cell breakdown. High pressure cleaning with cold water probably led to decreased numbers of viable bacteria by mechanical removal and dilution, however it is also possible that is has some direct bactericidal activity due to osmotic or mechanical effects. Another explanation for the presence of DNA could be the degradation of MAP by DNases from other organisms which are also present in the environment (Whittington et al., 2004). The continued presence of MAP DNA illustrates the difficulties in achieving perfect cleaning of the barn before restocking. High pressure cleaning, while not routinely carried out in most commercial dairy farms, may be a useful addition to current hygiene measures in reducing viable MAP burden of dairy barns.

The IS*900* qPCR, detecting a specific repetitive DNA sequence for MAP (Collins et al., 1989; Green et al., 1989) was, as expected, highly sensitive (equivalent to 10 CFU) using MAP DNA as a template. Sensitivity in spiked faecal samples was markedly less (10^5 CFU/g). This was assumed to be similar to many of the environmental samples tested although the DNA sensitivity in dust samples may well be higher in the absence of faecal organic material. Interference by PCR inhibiting factors in the presence of organic material has been reported to lower sensitivity of the PCR on DNA isolated from spiked faeces (Wilde et al., 1990; Wilson, 1997). Direct IS*900* qPCR and culture were as expected not always concordant. In many samples MAP DNA was found without a positive culture result. The environmental concentration of MAP CFU-equivalents in many samplings was probably close to the detection limit of the direct IS*900* qPCR which can be concluded from the percentage of discordant duplicate results at T0 and T1 (inside samples) and also at T2 (outside samples). The presumed lower sensitivity of the direct IS*900* qPCR on environmental samples compared to pure DNA samples may have lead to underestimation of the number of true MAP positive samples at T0 and T1. The trend to increasing number of positive concordant samples over time (for both DNA and culture) can be interpreted as an increase of MAP DNA and viable MAP concentrations caused by the increased number of shedders. Since MAP can survive several months in the environment, accumulation of MAP by continuous shedding could also play a role (Jorgensen, 1977; Whittington et al., 2004). Since only viable MAP in the environment can play a role in paratuberculosis infection of young cattle the use of culture to identify viability of the bacteria is essential.

It was expected that MAP would be found preferentially where cows were present, in particular when the barn was partly stocked. Results indicated an increased likelihood of MAP DNA positive samples from cubicles and slatted floor in stocked areas compared to unstocked areas. In contrast, no significant difference could be found in the number of MAP DNA positive microfiber wipes hanging above the slatted floors at the empty side compared to the wipes hanging at the stocked side of the barn. Viable MAP was detected both at the stocked and unstocked side. When the barn was completely stocked with MAP shedders the probability of finding a MAP DNA positive floor dust location inside increased significantly compared to T0 and all inside samples contained viable MAP.

The presence of viable MAP in the water troughs can be explained by faecal contamination or by settling of dust on the water surface. Such contamination could lead to a spread of infection via water consumption.

The results of the environmental samples collected outside showed an increase in number of positive concordant direct IS*900* qPCR samples and a significant increase in the number of viable MAP positive samples over time. MAP is known to survive best in shaded and moist conditions but in this study the outside sampling locations were not in the shade nor moist (Whittington et al., 2004). Viable MAP positive samples were located at the barn entrance, at the entrance of the house and the doormat (results not shown), despite the standard twofold change of footwear and clothing when entering/leaving the barn. The distribution of viable MAP positive samples seemed related to the activity patterns of the farmer and movement of farm equipment. At time point T2 viable MAP was found in almost all sampling locations collected outside the barn. The proportion of sampling sites that were viable MAP positive decreased with distance away from the barn, which could indicate that the barn acted as point source of airborne MAP for the farm environment. Airborne spread of viable MAP leaving the barn through the ridge ventilation could therefore be a plausible explanation as well. Most likely a combination of all routes of dispersion occurred.

Since not only MAP DNA but also viable MAP could be detected in settled dust inside the barn these findings confirm that viable MAP can become airborne and spread

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with dust particles through the barn. The finding of MAP DNA and viable MAP outside the barn shows that even with a strict hygiene barrier as used on this farm, it is difficult to prevent MAP from spreading to the surroundings. To assess the role of viable MAP in dust in transmission of MAP infections more knowledge on MAP concentrations in dust and infective dosages for susceptible animals is needed. It should be determined if a single high dose is necessary for infection or if repeated ingestion of a low concentration of viable MAP can also lead to infection. A consequence of airborne spread of MAP may be that general hygiene measures and separation of dam and calf might be less effective in preventing new MAP infections than is commonly supposed.

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References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Benedictus, G., 1984. Evaluation of the organized control of paratuberculosis in the province of Friesland. Tijdschr Diergeneeskd 109, 905–916.

Benedictus, G., Verhoeff, J., Schukken, Y.H., Hesselink, J.W., 2000. Dutch paratuberculosis programme history, principles and development. Vet. Microbiol. 77, 399–413.

Berghaus, R.D., Farver, T.B., Anderson, R.J., Jaravata, C.C., Gardner, I.A., 2006. Environmental sampling for detection of Mycobacterium avium ssp. paratuberculosis on large California dairies. J. Dairy Sci. 89, 963–970.

Chamberlin, W., Graham, D.Y., Hulten, K., El-Zimaity, H.M., Schwartz, M.R., Naser, S., Shafran, I., El-Zaatari, F.A., 2001. Review article: *Mycobacterium avium* subsp. *paratuberculosis* as one cause of Crohn's disease. Aliment. Pharmacol. Ther. 15, 337–346.

Collins, D.M., Gabric, D.M., De Lisle, G.W., 1989. Identification of a repetitive DNA sequence specific to *Mycobacterium paratuberculosis*. FEMS Microbiol. Lett. 51, 175–178.

Corner, L.A., Pfeiffer, D.U., Abbott, K.A., 2004. The respiratory tract as a hypothetical route of infection of cattle with Mycobacterium avium subspecies paratuberculosis. Aust. Vet. J. 82, 170–173.

Green, C.F., Gibbs, S.G., Tarwater, P.M., Mota, L.C., Scarpino, P.V., 2006. Bacterial plume emanating from the air surrounding swine confinement operations. J. Occup. Environ. Hyg. 3, 9–15.

Green, E.P., Tizard, M.L., Moss, M.T., Thompson, J., Winterbourne, D.J., McFadden, J.J., Hermon-Taylor, J., 1989. Sequence and characteristics of IS900, an insertion element identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis*. Nucleic Acids Res. 17, 9063–9073.

Groenendaal, H., Nielen, M., Hesselink, J.W., 2003. Development of the Dutch Johne's disease control program supported by a simulation model. Prev. Vet. Med. 60, 69–90.

Heid, C.A., Stevens, J., Livak, K.J., Williams, P.M., 1996. Real time quantitative PCR. Genome Res. 6, 986–994.

Hruska, K., Bartos, M., Kralik, P., Pavlik, I., 2005. *Mycobacterium avium* subsp. *paratuberculosis* in powdered infant milk: paratuberculosis in cattle—the public health problem to be solved. Vet. Med. 50, 327–335.

Jaravata, C.V., Smith, W.L., Rensen, G.J., Ruzante, J.M., Cullor, J.S., 2006. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine manure using Whatman FTA card technology and Lightcycler real-time PCR. Foodborne Pathog. Dis. 3, 212–215.

Jorgensen, J.B., 1977. Survival of *Mycobacterium paratuberculosis* in slurry. Nord. Vet. Med. 29, 267–270.

Kalis, C.H., Hesselink, J.W., Russchen, E.W., Barkema, H.W., Collins, M.T., Visser, I.J., 1999. Factors influencing the isolation of *Mycobacterium avium* subsp. *paratuberculosis* from bovine fecal samples. J. Vet. Diagn. Invest. 11, 345–351.

Lombard, J.E., Wagner, B.A., Smith, R.L., McCluskey, B.J., Harris, B.N., Payeur, J.B., Garry, F.B., Salman, M.D., 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.

Muskens, J., Barkema, H.W., Russchen, E., van Maanen, K., Schukken, Y.H., Bakker, D., 2000. Prevalence and regional distribution of paratuberculosis in dairy herds in The Netherlands. Vet. Microbiol. 77, 253–261.

Naser, S.A., Ghobrial, G., Romero, C., Valentine, J.F., 2004. Culture of *Mycobacterium avium* subspecies *paratuberculosis* from the blood of patients with Crohn's disease. Lancet 364, 1039–1044.

Predicala, B.Z., Urban, J.E., Maghirang, R.G., Jerez, S.B., Goodband, R.D., 2002. Assessment of bioaerosols in swine barns by filtration and impaction. Curr. Microbiol. 44, 136–140.

Raizman, E.A., Wells, S.J., Godden, S.M., Bey, R.F., Oakes, M.J., Bentley, D.C., Olsen, K.E., 2004. The distribution of *Mycobacterium avium* ssp. *paratuberculosis* in the environment surrounding Minnesota dairy farms. J. Dairy Sci. 87, 2959–2966.

Schwartz, D., Shafran, I., Romero, C., Piromalli, C., Biggerstaff, J., Naser, N., Chamberlin, W., Naser, S.A., 2000. Use of short-term culture for identification of *Mycobacterium avium* subsp. *paratuberculosis* in tissue from Crohn's disease patients. Clin. Microbiol. Infect. 6, 303–307.

Whittington, R.J., Marshall, D.J., Nicholls, P.J., Marsh, I.B., Reddacliff, L.A., 2004. Survival and dormancy of *Mycobacterium avium* subsp. *paratuberculosis* in the environment. Appl. Environ. Microbiol. 70, 2989–3004.

Wilde, J., Eiden, J., Yolken, R., 1990. Removal of inhibitory substances from human fecal specimens for detection of group A rotaviruses by reverse transcriptase and polymerase chain reactions. J. Clin. Microbiol. 28, 1300–1307.

Wilson, I.G., 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. 63, 3741–3751.

Wilson, S.C., Morrow-Tesch, J., Straus, D.C., Cooley, J.D., Wong, W.C., Mitlohner, F.M., McGlone, J.J., 2002. Airborne microbial flora in a cattle feedlot. Appl. Environ. Microbiol. 68, 3238–3242.

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