# Papers

# *Mycobacterium avium* subspecies paratuberculosis in bioaerosols after depopulation and cleaning of two cattle barns

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**Settled dust samples were collected on a commercial dairy farm in the Netherlands with a high prevalence of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) (barn A) and on a Dutch experimental cattle farm (barn B) stocked with cattle confirmed to be MAP shedders. Barns were sampled while animals were present, after both barns were destocked and cleaned by cold high-pressure cleaning, and after being kept empty for two weeks (barn A) or after additional disinfection (barn B). MAP DNA was detected by IS*900* real-time PCR and viable MAP were detected by liquid culture. MAP DNA was detected in 78 per cent of samples from barn A and 86 per cent of samples from barn B collected while animals were still present. Viable MAP was detected in six of nine samples from barn A and in three of seven samples from barn B. After cold high-pressure cleaning, viable MAP could be detected in only two samples from each barn. After leaving barn A empty for two weeks, and following additional disinfection of barn B, no viable MAP could be detected in any settled dust sample.**

PARATUBERCULOSIS, or Johne's disease, is a chronic wasting disease of cattle characterised by diarrhoea and weight loss in clinical cases. It is caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) and is mainly transmitted directly by the faecal-oral route from adult cattle shedding the pathogen to susceptible calves, or indirectly via contaminated colostrum, milk and roughage (Sweeney 1996). To prevent direct contact of susceptible animals with infective material, preventive management measures such as hygiene, cleaning and separation of different age groups are advised. Sensitivity analysis in a simulation study showed that these recommendations are a crucial component of the paratuberculosis control programme in the Netherlands (Groenendaal and others 2003).

However, few data are available to support the assumption that these management measures effectively reduce or prevent exposure of susceptible animals to MAP in the environment of dairy barns. The effect of these management practices on the seroprevalence of paratuberculosis on dairy farms has been previously evaluated in field studies. For example, comparison of seropositive and seronegative herds in the Netherlands showed that no association could be established between the results of serological tests and the application of preventive management measures on dairy farms (Muskens and others 2003). In California, USA, a study involving 21 dairy herds supported the importance of not feeding unsaleable milk to calves and preventing exposure of susceptible animals to the manure of adult cows in order to maintain a low MAP prevalence, but no influence of calf rearing on the prevalence of MAP could be detected (Tavornpanich and others 2008). Another study in Michigan, USA, identified cleaning calf hutches after each use as a protective measure against paratuberculosis transmission, but other calf-rearing practices were not found to be important (Johnson-Ifearulundu and Kaneene 1998). The effect of commonly recommended management practices on MAP seroprevalence in those studies varied, suggesting that the transmission route of paratuberculosis is still not completely understood. Additionally, it is known that MAP can survive for several months in the environment and that it can spread by bioaerosols on dairy farms (Whittington and others 2004, Eisenberg and others 2010b). These data suggest that the environment may play a role in MAP transmission and that cleaning of the environment in barns should therefore involve more steps than simply removing faeces and contaminated litter. Little is known about the effectiveness of cleaning and disinfection of commercial dairy barns in decreasing the environmental burden of MAP. It is rare for dairy barns in the Netherlands to be completely empty due to the year-round ongoing milk production cycle.

This paper describes the situation of two MAP-infected dairy herds – a commercial dairy herd and an experimental herd – that were culled and restocked. Both events occurred independently, so sampling methods were not standardised, but in both cases, dust was collected before the animals were removed, after high-pressure cleaning of the barn and before the introduction of new cattle. The effect of two different cleaning methods on the reduction of MAP in settled dust in the cattle barns is reported. The efficacy of cleaning was determined by IS*900* real-time PCR and by liquid culture of settled dust samples.

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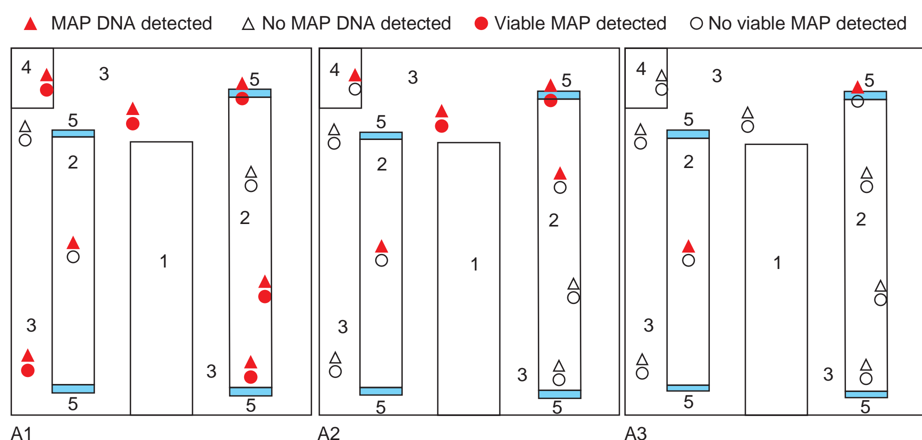


FIG 1: Schematic map of commercial dairy barn A with all sampling locations for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) indicated. Samples were collected while cows were present in the barn (A1), after high-pressure cleaning (A2) and after the barn had been left empty for 14 days (A3). 1 Feeding corridor, 2 Cubicles, 3 Slatted floors, 4 Inside roofing above the milking parlour, 5 Water troughs

## Materials and methods

### Farms

The barns on the commercial dairy farm (barn A) and the experimental farm (barn B) were similar Dutch dairy free-stall barns with cubicles, open water troughs and a central corridor with a feed bunk and ridge ventilation.

Barn A housed 130 dairy cows with a high prevalence (30 per cent) of MAP shedders, as determined by liquid culture by the Dutch Animal Health Service (GD, Deventer). During this study, the entire herd A was culled due to low performance and high disease incidence. After culling, the farmer removed faeces and bedding material from the barn, cubicles, slatted floors and corridor. Next, the floors, walls and roof beams that could be reached from the ground were high-pressure washed with water by the farmer. Following cleaning, the barn was kept empty and allowed to dry for two weeks.

In barn B, 45 non-lactating cows that were confirmed MAP shedders (100 per cent prevalence, determined by liquid culture by GD, Deventer) were housed over a period of 12 months. During these 12 months, cows that developed clinical paratuberculosis, as defined by diarrhoea, extensive weight loss and oedema, were culled. At the end of the 12-month period, the barn was destocked. The experiment was approved by the Ethical Committee for Animal Experiments of Intervet/Schering-Plough Animal Health, Boxmeer, the Netherlands. After destocking, the farmer removed all faecal material and bedding from the barn, cubicles, slatted floors and corridor. Subsequently, all surfaces including the walls, air inlets and roof beams that could be reached from the ground were high-pressure washed with water. Following high-pressure cleaning, the barn was disinfected by a professional cleaning agency with a commercial disinfectant containing didecyldimethylammonium chloride, glutaraldehyde and formaldehyde (Halacid; Veip).

### Sample collection and preparation

All sampling locations were specified on a map of the farm to allow repeated sampling of the same locations. Sampling locations were at least 2.5 m above the floor, and sampling was performed three times during the summer. The method of collection of settled dust samples was as previously described in detail by Eisenberg and others (2010b), with slight modifications in barn A.

In barn A, samples were collected on roof beams located above the slatted floors, the cubicles and the corridor, distributed evenly throughout the whole barn to cover the entire area (nine locations) (Fig 1). These locations were included in the cleaning process after removal of the cows. At the first sampling, settled dust was collected by wiping the defined location of the roof beams with electrostatic dust collectors (EDC) (Zeeman) while the cows were still present (sampling A1). Three days after removal of all the dairy cows and high-pressure cleaning of the barn (including the sampling locations), the farm was visited again. The sampling locations were clean on the basis of visual inspection. The sampling locations were wiped with EDCs to collect the second sample (A2) and clean EDCs were then placed on the locations to collect settling dust. After two weeks, the EDCs with the accumulated settled dust were collected, and this constituted the third sampling (A3).

In barn B, samplers were placed in the air inlets, above the slatted floors and in the ventilation ridge (seven locations) (Fig 2). EDCs for the first measurement (sampling B1) were placed three weeks before the animals were culled, and were exchanged for clean EDCs after the animals had been removed and high-pressure cleaning had been done. Three weeks after high-pressure cleaning, the EDCs were collected (B2). EDCs for the third sampling were placed before disinfection of the barn, when the locations were clean on visual inspection, and collected four weeks later (B3).

All settled dust samples were processed according to the method described by Eisenberg and others (2010b). Briefly, the dust in the EDC was washed out using ultrapure water (Milli-Q; Millipore). After settling, the ultrapure water was removed into a clean 50 ml tube and the particles were accumulated by centrifugation. The supernatant was discarded and the pellet was resuspended in 100 µl ultrapure water, of which 40 µl was used for DNA extraction and 60 µl was used for MAP culture. For DNA extraction, Elute microcards (Whatman) were used. The cards were dried overnight and two punch biopsies were collected into a microcentrifuge tube and processed according to the manufacturer's instructions. A 2 µl aliquot of the eluate was used as a template for the real-time PCR (MiQ; Bio-Rad).

### Sample analysis

Real-time PCR targeting an internal sequence of the IS*900* gene was used as previously described by Eisenberg and others (2010b). The results were assessed as a binominal outcome (MAP DNA present: yes or no). A technical positive control (purified MAP DNA) and a technical negative control (ultrapure water) were included in each run. Evidence of a specific melting peak between 93 and 94°C was a positive indicator of MAP DNA. The real-time PCR was used directly on all environmental samples to detect MAP DNA and was also used to confirm the presence of viable MAP in culture medium after the 42-day incubation period.

The protocol for para-JEM(r) automated MAP culturing provided by TREK Diagnostic Systems was used to prepare the dust samples for liquid culture, and all the samples were incubated for 42 days. Following incubation, all culture samples were tested by real-time PCR for the presence of MAP as previously described (Eisenberg and others 2010b). Growth of MAP in culture medium was confirmed if the PCR showed a positive signal with a specific melting peak, and these samples were interpreted as viable MAP-positive.

### Statistical analysis

Due to the semiquantitative nature of the analytical tests used in this case report, data were assessed as a binominal outcome (MAP detected: yes or no) and are presented as proportions of positive locations. Due to the differences in sampling on the two farms, intervention effects could be compared only within and not between farms. Findings at samplings A2 and A3 were compared with the situation at A1 by the Cochran test, and similarly, findings at samplings B2 and B3 were compared with the situation at B1. Differences with $P<0.05$ were considered significant.

## Results

Figs 1 and 2 show the results of analysis of the samples collected in barns A and B while the cows were present, after high-pressure cleaning, and after leaving the barn empty without and with disinfection, respectively.



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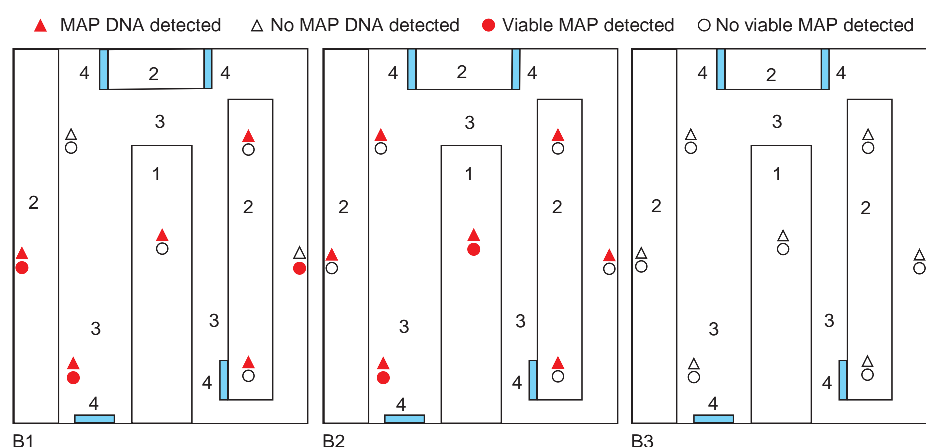


FIG 2: Schematic map of experimental barn B with all sampling locations for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) indicated. Samples were collected while cows were present in the barn (B1), after high-pressure cleaning (B2) and after the barn had been left empty after additional disinfection (B3). 1 Feeding corridor, 2 Cubicles, 3 Slatted floors, 4 Water troughs

Before any intervention in barn A, seven of nine dust samples were positive for MAP DNA, while six of seven samples from barn B were positive. In these samples, viable MAP was present in six of nine and three of seven samples, respectively. After high-pressure cleaning, the number of MAP DNA-positive dust samples decreased in barn A to five of nine, but in barn B all seven samples were MAP DNA-positive. Viable MAP could still be detected in both barns, at two locations. The reduction after high-pressure cleaning in viable MAP-positive locations in barn A was significant ($P<0.05$).

After barn A had been left empty for two weeks, the number of MAP DNA-positive locations decreased significantly to two positive locations ($P<0.05$). Leaving the barn empty combined with an additional disinfection step also significantly reduced the number of MAP DNA-positive locations in barn B ($P<0.05$): MAP DNA was no longer detected at any location in barn B. The number of viable MAP-positive locations decreased significantly after the third intervention in both barns, to zero in each barn ($P<0.05$).

## Discussion

Little information has been published about the efficacy of cleaning and disinfection on dairy farms in general, and even fewer data are available about its efficacy in decreasing the environmental burden of MAP caused by bioaerosols on commercial dairy farms.

Over the past decade, several authors have presented evidence that MAP can be found in the environment of dairy farms (Raizman and others 2004, Berghaus and others 2006) and that it can survive for a prolonged period of time in the environment (Berghaus and others 2006). The authors recently reported that MAP is detectable in bioaerosols on dairy farms (Eisenberg and others 2010a, b). These facts indicate that in order to reduce the environmental burden of MAP, removal of manure as well as effective cleaning and disinfection of the environment may be needed.

The main route of transmission of MAP between infectious cattle and susceptible calves is via the direct uptake of faecal material. Infection studies have shown that oral inoculation is very effective. Preventing direct contact between calves and adult cattle reduces the prevalence of MAP on a farm but does not lead to elimination of the disease, indicating that there are other uncontrolled modes of transmission (Benedictus and others 2008). Transmission via bioaerosols might be a possible route, considering the presence of viable MAP in dust (Eisenberg and others 2010a) and the presence of MAP in tracheobroncheal and retropharyngeal lymph nodes (Pavlik and others 2000). Dust is continuously produced in animal housing by movement of the animals, and consists of skin, hair, dried faecal material, feed and bedding material that can spread throughout the barn by air movements (Collins and Algers 1986, Eisenberg and others 2010b).

Real-time IS*900* PCR is a quick method for detecting MAP DNA, but some limitations of this method have to be considered. The IS*900* insertion element has been detected in other mycobacteria, which decreases the specificity of the test (Englund and others 2002). The specificity of this real-time PCR for environmental samples has been verified, however, by sequencing positive samples (Eisenberg and others 2010b). Furthermore, detection of DNA does not give information about the number of viable bacteria in bioaerosols, since DNA of inactivated MAP might be detected as well. Only viable MAP in bioaerosols would be able to play a role in transmission. Culture does identify viable bacteria, and the specificity of culture has been estimated to be 100 per cent at individual animal level (Nielsen and Toft 2008). The liquid culture system used detects bacterial growth on the basis of metabolic activity and not exclusively on bacterial replication, as is the case for conventional plating on solid media. Quantification of samples in terms of the numbers of viable cells is therefore not as straightforward as for culture on solid media. In addition, the standard culture period of six weeks in liquid systems is considerably shorter than the 12 to 16 weeks required for solid media. With low numbers of bacteria, the liquid culture system is likely to be less sensitive than culture on solid media. This is indicated by positive PCR results on culture medium at the end of the standard culture period in the absence of a positive growth signal from the automated liquid culture system.

In farms with a low MAP prevalence, MAP could be detected in settled dust samples when two locations in the dairy barn were sampled (Eisenberg and others 2010a). In the present study, which focused on the possibility of removal of MAP from dust in dairy barns, seven or nine locations were sampled in the dairy barns only as a point of comparison for before and after the intervention. A significant reduction in MAP DNA-positive and viable MAP-positive locations could be detected, indicating adequate sample size.

Samplings A1 and B1 were used as the reference for each farm; between-farm comparison was not deemed appropriate. At barn B, settled dust was always collected for a period of three weeks, whereas at barn A, accumulated dust was collected. At the first sampling in barn A, this dust had probably been settling on these locations for longer than three weeks because no cleaning had been performed on farm A to remove dust in the environment before the cows were culled. High-pressure cleaning had some effect in reducing the presence of MAP DNA in barn A; in contrast, the number of MAP-positive locations in barn B (in which the animals had an extremely high prevalence of MAP) increased after high-pressure cleaning. This increase in MAP DNA-positive locations at sampling B2, to 100 per cent, could be explained by the possibility that the high-pressure cleaner disseminated MAP DNA from the cubicles and the slatted floors throughout the whole barn in aerosols (Böhm 1998, Elchos and others 2008). However, high-pressure cleaning reduced the number of viable MAP-positive locations in both barns. Using water to remove faeces and dust in the barns may have led to dilution of the amount of viable bacteria in the environment. In addition, the high-pressure cleaner might have caused mechanical damage to the bacteria and reduced their viability. No viable MAP could be detected in either barn after it had been kept empty for 14 days or had been additionally disinfected; however, in barn A, MAP DNA was still detectable. Incorporation of a disinfection step for the whole barn might be an expensive additional step for dairy farmers to take, but leaving the barn empty for some time after cleaning in the summer should be manageable.

This study shows that it is difficult to remove viable MAP-containing dust completely from the environment of cattle barns. Both types of thorough hygienic measures, high-pressure cleaning combined with disinfection or keeping the barn empty for two weeks, could achieve removal of viable MAP. However, on dairy farms, cleaning is performed less thoroughly due to the continuous presence of youngstock and cattle on the farm. When asked, dairy farmers stated



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that they aimed to completely clean the barn using a high-pressure cleaner once a year or every two years; however, they admitted to not often meeting this goal (Eisenberg and others 2010a). This is in contrast to pig and poultry farming, where an all-in/all-out system with a cleaning and disinfection protocol is maintained and strictly regulated by the Product Boards for Livestock, Meat and Eggs, Zoetermeer, the Netherlands, because a clean environment is considered important in reducing disease transmission between batches (Proux and others 2001, Gast and others 2004). In the present study, the MAP situation in the cubicles and slatted floors was not investigated. Assuming that the bacterial load of surfaces with direct faecal contact would be much higher, it would be even more difficult to achieve an effective reduction of bacteria and prevent the formation of new infective bioaerosols at the time of restocking.

In conclusion, this study suggests that the environmental burden of MAP can be effectively reduced by additional disinfection or by leaving the barn empty for two weeks beyond what is common in current Dutch dairy farming practice.

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