# Factors Influencing the Isolation of *Mycobacterium Avium* Subsp. *Paratuberculosis* from Bovine Fecal Samples

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# Factors influencing the isolation of *Mycobacterium avium* subsp. *paratuberculosis* from bovine fecal samples

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**Abstract.** A modified procedure was used for culture of *Mycobacterium paratuberculosis* (Mptb) from bovine feces. Bovine fecal samples were decontaminated with NaOH, exposed to a mixture of oxalic acid and malachite green, incubated in a mixture of neomycin and amphotericin B. Decontaminated specimens were inoculated onto modified Löwenstein-Jensen medium. Specimens processed by high-speed centrifugation showed growth earlier than specimens prepared by low-speed centrifugation. However, the overall number of positive cultures at 16 weeks was not different for the 2 methods. When infected dairy herds were sampled 4 times at 6-month intervals and culture-positive cows were culled, the prevalence of infected cattle declined over time. After selective culling, the cattle left in the herds shed low numbers of Mptb, which explains why it took longer for cultures to become positive. No heifers younger than 11 months were culture positive, but heifers 13–14 months of age were more frequently culture positive than were heifers of any other age. The 16-week culture period is needed with this method to detect cattle shedding low numbers of Mptb. High-speed centrifugation of samples does not increase the efficiency of identification of animals shedding Mptb.

Paratuberculosis in cattle is an infectious disease caused by *Mycobacterium paratuberculosis*, more correctly designated *Mycobacterium avium* subsp. *paratuberculosis* (Mptb).[12] The disease is widespread in The Netherlands and causes significant economic losses.[3,9,17,19] Control programs for paratuberculosis are based on early culling of infected animals and improvement of animal husbandry to prevent further spread of Mptb infections within the herd. Yet results have been disappointing, mainly because diagnostic procedures based on complement fixation test, intradermal Johnin test, and Ziehl-Neelsen staining of fecal smears have been inadequate,[4] and because farmers have not consistently carried out husbandry measures aimed at limiting infection transmission.[2,3] Vaccination has also been tried as a means of controlling the disease but has not led to the expected decline in the infection rate.[9,13] Strategies to eradicate the disease are based on the effective detection and removal of infected animals.

Culture of feces from infected cows is more sensitive than serologic tests and allows earlier detection of infected cattle. Fecal culture methods vary among countries and among laboratories.[4–6,11,14,21,22] In Europe, the most commonly used method[1] includes an additional decontamination step with NaOH to prevent fungal growth, because the use of acids only leads to high contamination rates. The use of an antimicrobial mixture has been introduced as a third decontamination step.[10] The growth media have been modified further to include an antimicrobial mixture to help control contamination. In 1993, this modification was adopted by the Animal Health Service in The Netherlands as a routine test for bovine paratuberculosis. However, there are no published studies that compare this culture method with the method most commonly used in the USA.[23] The incubation times of different methods vary considerably,[23] and long culture periods increase sensitivity.[22] Culture of fecal samples for 8 weeks has been advocated,[10] but there are no data available for longer incubation periods. Centrifugation is thought to improve the isolation of Mptb,[14] and high-speed centrifugation has been used during specimen processing.[7] However, both the longer culture period and the high-speed centrifugation increase costs.

The purposes of the present study were 1) to evaluate the method used by the Animal Health Service (The Netherlands) as a tool in an eradication program, 2) to evaluate the effect of the incubation period on the detection of Mptb, and 3) to quantify the effect of low-speed (1,000 × *g*) as compared with high-speed (3,000 × *g*) centrifugation on the isolation of Mptb.

## Materials and methods

*Animals.* The study was carried out with 6 dairy herds in the northern part of the Netherlands. The herds were selected on the basis of a history of clinical paratuberculosis: each herd had at least 1 culture-confirmed case of paratuberculosis in the last 2 mo before collection of samples for the present study. Cows were defined as female animals > 24 mo of age: heifers were 6–24 mo of age. The animals were

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of the Dutch Friesian breed, but the breed has a high percentage of Holstein in it. The farmers were advised to cull culture-positive animals promptly.

*Samples.* Fecal samples were collected from the rectum of each animal 4 times at 6-mo intervals. The samples were collected using disposable plastic rectal examination gloves without lubrication gel. After the fecal sample was collected, the gloves were tied up and identified with preprinted self-adhesive labels that showed the bar code for each cow, as recorded in the Dutch Identification and Registration system.[18] Samples were kept at 4 C during transport and processed for isolation of Mptb within 24 hr after arrival at the laboratory.

*Culture medium.* Modified Löwenstein-Jensen (LJ) medium[10] was prepared as follows. A mixture of 55.8 g of LJ base[a] dissolved in 900 ml distilled water and 18 ml glycerin was autoclaved (121 C) for 15 min. After the solution was cooled to 30 C, 20 ml of a sterile solution of pyruvate (0.48 g/ml), 2.4 ml mycobactin,[b] and 14.4 ml of an antimicrobial stock solution were added. The mycobactin was prepared by addition of 1.0 ml of 95% ethanol to each of 3 vials, the contents of which were pooled. The antimicrobial stock solution was prepared by combining 1.8 g of cycloheximide in 6.0 ml of acetone, 0.48 g of chloramphenicol in 6.0 ml ethanol, and 2.4 ml of sodium penicillin G (200,000 units/ml). These solutions were prepared weekly. A whole egg suspension (about 30 eggs) was made by soaking eggs (purchased from retail outlets) in 70% ethanol, aseptically harvesting the contents, and mixing them in a sterile blender. The egg homogenate was filtered through sterile surgical gauze to remove clumps, and 1,500 ml was added to warm (not hot) medium with thorough mixing. The total volume of the medium was 2,400 ml, and the final concentrations of critical components were 0.25 mg/ml malachite green (component of the LJ base), 0.0075 ml/ml glycerin, 4.0 mg/ml pyruvate, 2.0 μg/ml mycobactin, 0.75 mg/ml cycloheximide, 0.2 mg/ml chloramphenicol, 200 units/ml sodium penicillin G, and 62.5% whole egg homogenate. Glass tubes (16 mm diameter × 15 cm) were filled with 7 ml of medium, which was allowed to solidify at an angle such that the surface of the medium extended three-fourths up the tube. The tubes of medium were incubated at 90 C for 2 hr and then stored upright at 4 C for a maximum of 1 mo.

*Decontamination reagents.* Three solutions were used in the decontamination procedure: 1) 4% NaOH in sterile water, 2) a mixture of oxalic acid[b] and malachite green (OA-MG), and 3) a mixture of neomycin[c] and amphotericin B[d]. The OA-MG solution was prepared by mixing 50 g oxalic acid with 40 ml of a 2.5% solution of malachite green and 960 ml of sterile water, yielding final concentrations of 5 mg/ml oxalic acid and 1 mg/ml malachite green. The antibiotic mixture was prepared by combining 500 mg amphotericin B (10 ml of Fungizone 50 mg/ml), 2.0 ml of neomycin solution (25 mg/ml), and 988 ml of sterile physiologic saline solution, yielding final concentrations of 50 mg/ml amphotericin B and 0.5 mg/ml neomycin. This antibiotic mixture was prepared fresh each week and stored at 4 C.

*Decontamination procedure.* Fecal samples were decontaminated as described previously.[1,10] As further modified, the proportion of feces to NaOH was lowered, and the entire fecal sample mixed with the NaOH solution was used for centrifugation. The additional step involved resuspension in amphotericin B and neomycin instead of saline with overnight incubation at room temperature. Five milliliters of feces, weighing approximately 2 g (1.8–2.2 g), was thoroughly mixed with 8 ml of 4% NaOH in a pestle and mortar for 5–10 sec, depending upon the consistency of the feces. The mixture was poured into a sterile 10-ml plastic centrifuge tube, which was then placed on its side in a mechanical rotary shaker and mixed for 15 min. The sample was centrifuged (15 min at 1,000 × *g*), and the supernatant was poured off. Five milliliters of OA-MG solution was added to the pellet, which was resuspended using a sterile wooden stick. This suspension was placed in a mechanical rotary shaker for 15 min. The mixture was then centrifuged (15 min at 1,000 × *g*), and the supernatant was poured off. Four milliliters of antibiotic solution was added to the tube, and the pellet was resuspended using a sterile wooden stick and vortexed. The mixture was then allowed to stand overnight at room temperature (20 C).

*Medium inoculation procedure.* After the suspension had settled overnight, there was a lower layer of particulate matter and an upper layer of clear green solution. The clear upper layer just above the pellet was removed with a Pasteur pipette. Three or 4 drops of this solution were then distributed evenly over the surface of each of 4 tubes of modified LJ culture medium (all containing mycobactin). The inoculated tubes were loosely capped and left to stand for 24 hr at room temperature (20 C) such that the agar surface was horizontal to permit maximum absorption of the inoculum onto the surface of the medium. The tubes were tightly sealed with a rubber stopper, placed in racks, and incubated for 26 wk at 37 C. The tubes were inspected at 8, 9, 10, 11, 12, 14, 16, and 26 wk for evidence of growth. Colonies thought to be Mptb on the basis of their appearance and slow growth rate were stained by acid-fast stain. A positive culture was defined as having 1 or more small, smooth to slightly rough, white to slightly yellow bacterial colonies of small, acid-fast rod-shaped bacteria. The number of colonies of Mptb in each tube was recorded.

*Effect of centrifugation speed.* A second sample was collected from 59 animals that were identified as positive by fecal culture. This sample was divided into 2 parts and each part was processed identically except for the centrifugation speeds, which were either 1,000 × *g* or 3,000 × *g*, both for 15 min.

*Data analysis.* The results obtained with low- and high-speed centrifugation were compared using the McNemar test.[20] Statistical significance was declared at $P = 0.05$. Results of cultures from samples collected in spring and fall and of age-dependent cultures were compared using chi-square analysis and contingency tables.

## Results

A total of 2,989 bovine fecal samples were cultured for Mptb. One hundred fifty (5.0%) animals sampled were positive for Mptb during the 2-year period. Of the 11,956 cultures (2,989 samples × 4 tubes medium/sample), 842 (7.04%) were contaminated by normal

**Table 1.** Age of the cattle and number (percentage) of *M. paratuberculosis*-positive fecal cultures at the first herd sampling.

| Age (yr) | No. cattle tested | No. (%) positive |
|---|---|---|
| <1 | 76 | 1 (1) |
| 1 | 160 | 5 (3) |
| 2 | 151 | 8 (5) |
| 3 | 106 | 13 (1) |
| 4 | 74 | 15 (1) |
| 5 | 45 | 12 (2) |
| 6 | 31 | 4 (1) |
| 7 | 25 | 5 (2) |
| >7 | 21 | 2 (9) |
| Total | 689 | 65 (9) |

**Table 2.** Age of heifers and number (percentage) of *M. paratuberculosis*-positive fecal cultures at 4 herd samplings.

| Age (mo) | No. cattle tested | No. (%) positive |
|---|---|---|
| <11 | 51 | 0 |
| 11 + 12 | 92 | 2 (2.2) |
| 13 + 14 | 102 | 6 (5.9) |
| 15 + 16 | 88 | 1 (1.1) |
| 17 + 18 | 118 | 2 (1.7) |
| 19 + 20 | 159 | 3 (1.9) |
| 21 + 22 | 131 | 2 (1.5) |
| 23 + 24 | 242 | 5 (2.1) |
| Total | 983 | 21 (2.1) |

bovine fecal microflora. Only 4 (0.13%) samples were lost due to contamination of all 4 cultures after 8 weeks of incubation. All culture-positive animals were promptly culled from the herd, except 3 heifers in 1 herd that were sampled a second time, again with positive results.

*Effect of animal age.* The effect of the age of animals on the number of positive cultures was examined by using the first samples collected in all 6 herds (Table 1). The age-specific prevalence rose to 26.9% in the group of cows that were 5 years old. Fecal cultures were negative for Mptb in all heifers younger than 11 months. The heifers 13–14 months of age had a significantly higher percentage of positive cultures (5.9%) than did either the younger and older heifers (≤2.2%, $P < 0.01$; (Table 2). The shedding rate was low, with 86% of cattle shedding <10 colony-forming units (CFUs). This effect of shedding at a young age was consistent with the finding that 5 older animals (4–7 years) were positive at the fourth sampling period after being negative at the 3 earlier sampling periods.

*Effect of centrifugation.* Of the samples taken from the 59 animals that were previously culture positive, 43 and 46 samples were culture positive after low- and high-speed centrifugation procedures, respectively. Forty samples were positive with both methods. After 9 weeks, 73% of the high-speed centrifugation samples were positive compared with 46% of the low-speed centrifugation samples ($P < 0.001$). After 10 weeks, the proportions of positive samples were 78% and 63%, respectively ($P < 0.05$; Fig. 1). However, after 12 weeks of culture, the proportions of positive cultures for the low- and high-speed centrifugation procedures, 73% and 80%, respectively, were not different ($P = 0.32$). Samples that were culture positive after both high- and low-speed centrifugation tended to have a higher number of CFUs after high-speed centrifugation ($P = 0.08$; Table 3). There was no difference in the detection of low shedders (1–10 CFUs) between high- and low-speed centrifugation, with 24 and 28 cattle, respectively. The longer it took before a culture became Mptb positive, the fewer CFUs were found, irrespective of the speed of centrifugation ($P < 0.001$). Because no samples were contaminated, the effect of centrifugation speed on contamination could not be evaluated.

*Effect of culling of Mptb-positive animals on the results of fecal culturing.* The proportion of Mptb-culture-positive animals in each herd (prevalence) at the start of the investigation ranged from 5.6% to 16.5% ($x = 9.7\%$; Table 4). The prevalence of Mptb was significantly lower at all 3 subsequent sampling periods in both cows and heifers. Compared with the cultures prepared from the first samples collected, fecal cultures prepared from the successive herd samples took longer to become positive (Fig. 2). This effect was most pronounced at 8–12 weeks of culture and when the first and last herd samples were compared. However, after 16 weeks of culture the cumulative percentage of positive cultures was the same for all 4 herd samples. In the period between 12 and 16 weeks of incubation, 43 of 149 low shedders (<10 CFUs) were found. Thus, 29% of the low shedders were missed at the first sampling period, and 83%, 24%, and 100% were missed at the second, third, and fourth sampling periods, respectively, when the cultures were incubated for 12 weeks only. Samples collected in the fall (sampling periods 1 and 3) became positive sooner than did samples collected in the spring (sampling periods 2 and 4). At 10 weeks of incubation, 52% of the samples collected in the fall became positive compared with only 11% of the samples collected in the spring. After 12 weeks of incubation, these figures were 74% and 11%, respectively ($P < 0.0001$). No difference was found in the number of CFUs between the samples collected in the fall and in the spring ($P > 0.1$).

## Discussion

The results show that the Jørgensen culture method is a helpful tool in an eradication program for paratuberculosis. The technique is laborious because of the 3-step approach to decontamination. The 30-minute incubation with NaOH is critical because a longer period

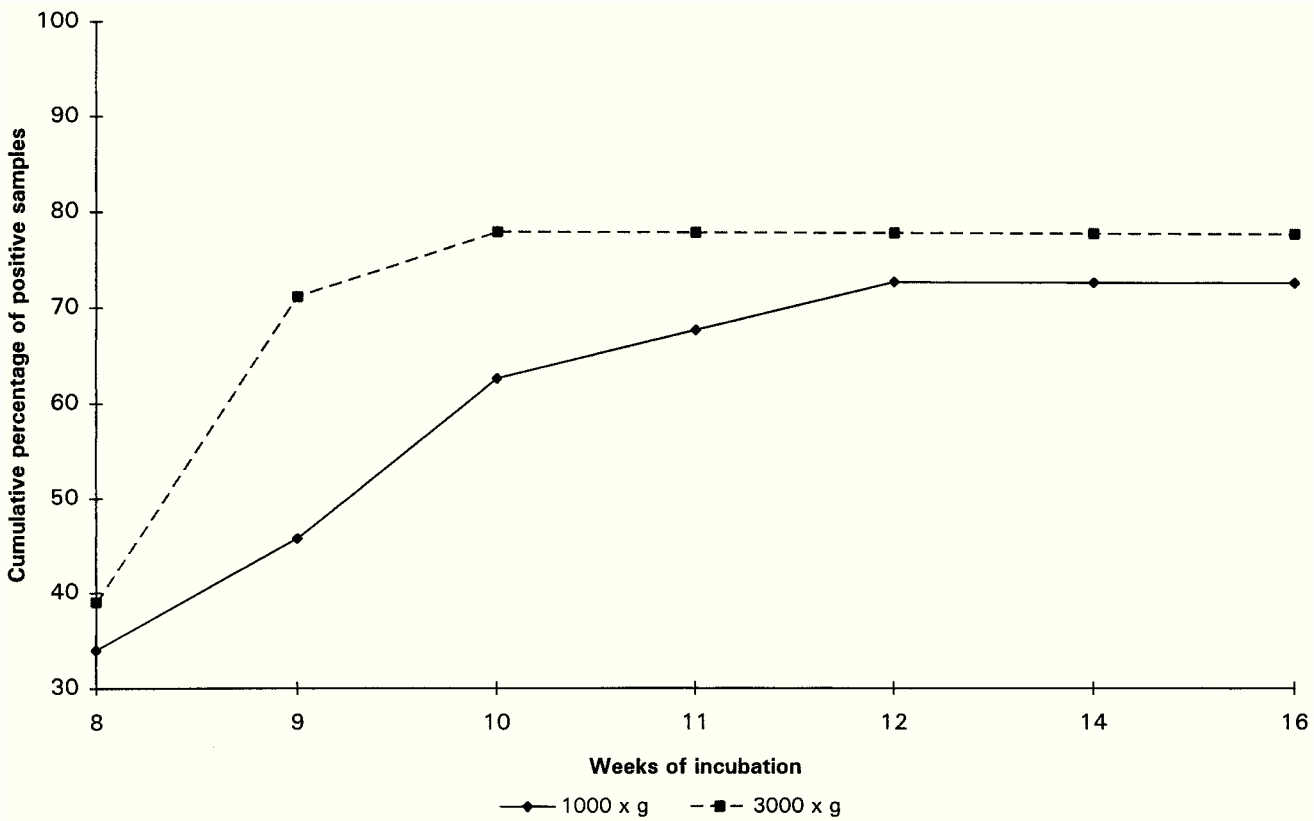


**Figure 1.** Cumulative percentage of *M. paratuberculosis*-positive bovine fecal samples and incubation time after preparation of samples by low-speed (1,000 × *g*) and high-speed (3,000 × *g*) centrifugation.

leads to killing of Mptb and a shorter period leads to a higher contamination rate.[1] The pestle and mortar may introduce auxiliary contaminants; it has been replaced by a stomacher. The use of mycobactin-containing culture tubes means that mycobactin dependency cannot be assessed. Although this demonstration of mycobactin dependency is not really necessary for Mptb isolates from cattle, positive cultures are now confirmed by (IS900) PCR tests.

Herd prevalence decreased over time in both cows and heifers. The largest decrease in herd prevalence occurred after the first round of testing, when culture-positive cows were culled. The 3 culture-positive cows at the third round of testing were the culture-positive heifers detected during the second round of testing, which had not been culled by the farmer. This finding however is not the only explanation for the high percentage of culture-positive animals in the third round of testing. The results of the second round were influenced by the culling of the animals found positive at the first round, and the majority of the remaining infected cows took >6 months to excrete a detectable number of CFUs. This finding could be an argument for prolonging the period between herd tests. The standard procedure in the eradication programs is to culture feces from all cows >24 months of age at 12-month intervals. If farmers wish to eradicate paratuberculosis aggressively, the recommended procedure is

**Table 3.** Effect of centrifugation speed on yield of *M. paratuberculosis* colonies from bovine fecal cultures.

| | No. samples | |
|---|---|---|
| CFUs | 1,000 × *g* | 3,000 × *g* |
| 1–10 | 28 | 24 |
| 10–50 | 5 | 12 |
| 50–100 | 2 | 3 |
| >100 | 8 | 7 |
| Total | 43 | 46 |

**Table 4.** Herd size and prevalence of *M. paratuberculosis* culture-positive cattle in the 6 study herds sampled at 4 time points.

| | No. | % Culture positive | | | |
|---|---|---|---|---|---|
| Herd no. | cattle | Sample 1 | Sample 2 | Sample 3 | Sample 4 |
| 1 | 98 | 6.1 | 6.4 | 1.1 | 0.0 |
| 2 | 215 | 8.8 | 5.2 | 0.9 | 0.7 |
| 3 | 322 | 10.9 | 3.2 | 2.7 | 3.5 |
| 4 | 70 | 11.4 | 4.4 | 0.0 | 3.0 |
| 5 | 79 | 16.5 | 1.3 | 9.1 | 2.3 |
| 6 | 124 | 5.7 | 2.8 | 6.2 | 0.0 |
| Mean | 151 | 9.7 | 3.9 | 3.3 | 1.6 |

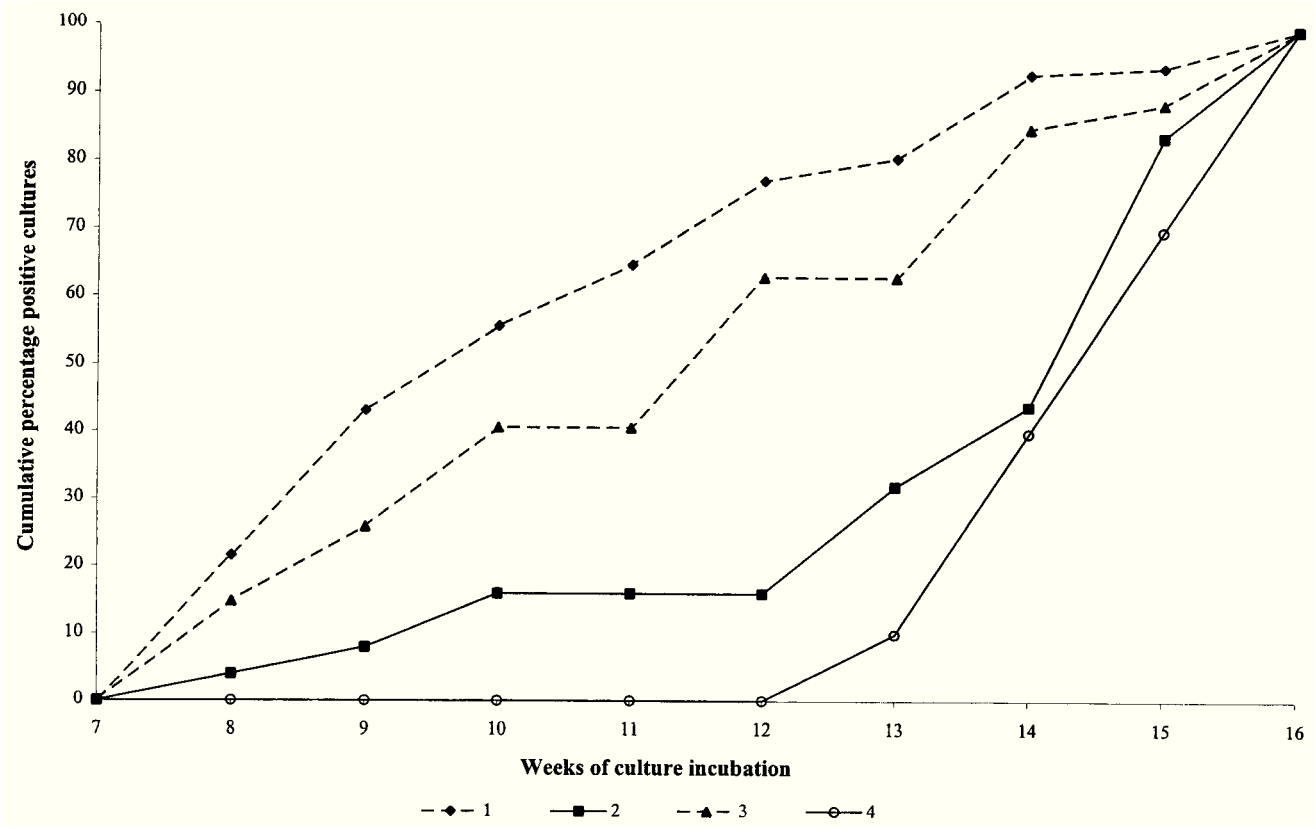


**Figure 2.** Cumulative percentage of *M. paratuberculosis*-positive bovine fecal cultures and incubation time for 4 samples collected at 6-month intervals.

to culture feces from all heifers and cows >12 months of age at 6-month intervals. The benefit of this test-and-cull program in combination with improved animal husbandry was first evident 2 years after the start of the program, when culture-positive heifers were no longer found. Thus, calves born after the onset of the program were better protected than those born before the program started. A control program based on culture of feces from heifers alone is not recommended because it is essential to eliminate as many sources of infection as possible, and the majority of the fecal shedders start shedding mycobacteria at an older age. The oldest cow in the present study started shedding at 7 years of age. However, the culture of fecal samples from heifers is advisable to detect the fecal shedders as soon as possible, although these heifers shed low numbers of infective organisms.

Elimination of the more heavily infected animals from herds over the study period left mostly low shedders in the herd, which meant that longer culture times were needed to detect the low numbers of Mptb. These low numbers probably were due to the active pursuit of paratuberculosis control over approximately a 2-year period. The seasonal influence on the culture period cannot be explained by a difference in the number of colonies between the positive samples collected in the fall and the spring. Possible influences of the diet or the calving period in the fall in The Netherlands need further investigation. Although the results for high-speed and low-speed centrifugation (Fig. 1) suggest that a 12-week incubation period is enough, the results for all 4 herd sampling times show that a 16-week incubation period is needed to detect low shedders (Fig. 2). At 16 weeks of incubation, 29% of the low shedders (<10 CFUs) were detected, leading to a 41% increase in rate of detection of low shedders. This prolongation of the incubation period is especially important when farmers adopt a test-and-cull strategy.

The prevalence of Mptb-positive animals at first sampling increased with the animals' age. Animals as young as 13–14 months of age can shed Mptb, as has been previously described for both experimentally and naturally infected cattle.[15,16] This pattern supports observations[16] that shedding occurs in an early phase of bovine paratuberculosis, followed by a phase of ''silent'' infection not detectable by fecal culture. In the final phase, when the infection becomes progressive, consistent fecal shedding again occurs. These data further suggest that it may be more fruitful than previously thought to test heifers. Shedding of Mptb by 13–14-month-old heifers also raises the question of the risk of potential horizontal transmission when shed-

ding heifers are housed in groups under less than optimal sanitary conditions. In an epidemiologic study of 26 dairy herds with paratuberculosis, general farm sanitation, newborn calf care, grower calf care, and bred heifer care were significantly associated with the herd prevalence of paratuberculosis.[8]

The sensitivity of the culture technique can be increased by extending the culture period from 8 to 16 weeks. After elimination of culture-positive animals after the first sampling, a culture period of 8 or 12 weeks for subsequent samplings led to an even lower test sensitivity. A culture period of 16 weeks was sufficient to detect all culture-positive animals. Centrifugation at a higher speed did not influence the overall detection of Mptb in cultures after 16 weeks of incubation. The high-speed centrifugation did, however, yield higher numbers of Mptb in the inoculum, as evidenced by the earlier detection of positive cultures. A disadvantage of high-speed centrifugation is that fewer specimens can be processed because of the smaller capacity of high-speed rotors and because its takes longer for the centrifuge to stop. The observed contamination rate of 7.04% is considered typical and acceptable for culture methods for Mptb isolated from bovine feces.[14,24]

The fecal culture method used in this study allows detection of low numbers of Mptb in bovine fecal samples. Although the method is somewhat laborious and thus expensive, the advantages afforded by early detection of infected heifers and high diagnostic specificity make the technique appropriate for routine use in paratuberculosis control or eradication programs. To achieve better international standardization of culture methods for Mptb, the techniques used in different countries and laboratories should be compared.

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b. Allied Monitor, Fayette, MO.
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d. Squibb, Apothecom, Princetown, NY.

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