Detection of *Mycobacterium avium* subspecies *paratuberculosis* in tie-stall dairy herds using a standardized environmental sampling technique and targeted pooled samples

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Abstract

Mycobacterium avium ssp. *paratuberculosis* (MAP) is the etiologic agent of Johne's disease, a chronic contagious enteritis of ruminants that causes major economic losses. Several studies, most involving large free-stall herds, have found environmental sampling to be a suitable method for detecting MAP-infected herds. In eastern Canada, where small tie-stall herds are predominant, certain conditions and management practices may influence the survival and transmission of MAP and recovery (isolation). Our objective was to estimate the performance of a standardized environmental and targeted pooled sampling technique for the detection of MAP-infected tie-stall dairy herds. Twenty-four farms (19 MAP-infected and 5 non-infected) were enrolled, but only 20 were visited twice in the same year, to collect 7 environmental samples and 2 pooled samples (sick cows and cows with poor body condition). Concurrent individual sampling of all adult cows in the herds was also carried out. Isolation of MAP was achieved using the MGIT Para TB culture media and the BACTEC 960 detection system. Overall, MAP was isolated in 7% of the environmental cultures. The sensitivity of the environmental culture was 44% [95% confidence interval (CI): 20% to 70%] when combining results from 2 different herd visits and 32% (95% CI: 13% to 57%) when results from only 1 random herd visit were used. The best sampling strategy was to combine samples from the manure pit, gutter, sick cows, and cows with poor body condition. The standardized environmental sampling technique and the targeted pooled samples presented in this study is an alternative sampling strategy to costly individual cultures for detecting MAP-infected tie-stall dairies. Repeated samplings may improve the detection of MAP-infected herds.

Résumé

Mycobacterium avium *ssp.* paratuberculosis *(MAP) est l'agent étiologique de la maladie de Johne, une entérite chronique contagieuse des ruminants et responsable d'importantes pertes économiques. Plusieurs études, la plupart réalisées dans des grands troupeaux en stabulation libre, ont démontré que la technique de culture de prélèvements de l'environnement est appropriée pour la détection des troupeaux infectés par MAP. Dans l'est du Canada où prédominent les petits troupeaux en stabulation entravée, certaines conditions et pratiques de régie pourraient avoir un impact sur la survie, la transmission et l'isolement de MAP. Notre objectif était d'estimer la performance d'une technique standardisée de culture de prélèvements de l'environnement combinée à des échantillons groupés ciblés pour la détection des troupeaux laitiers en stabulation entravée infectés par MAP. Vingt-quatre troupeaux (19 infectés et 5 non infectés) ont été enrôlés, mais seulement 20 troupeaux ont été visités 2 fois dans la même année pour y prélever 7 échantillons de l'environnement et 2 échantillons groupés (vaches malades et vaches maigres). Des échantillons individuels de toutes les vaches dans le troupeau ont été également prélevés. L'isolement de MAP a été réalisé en utilisant le milieu de culture MGIT ParaTB et le système de détection BACTEC 960. Globalement, MAP a été isolée dans 7 % des cultures de l'environnement. La sensibilité de la technique était de 44 % (IC 95 % : 20 % à 70 %) en combinant le résultat des 2 visites et de 32 % (IC 95 % : 13 % à 57 %) en utilisant aléatoirement le résultat d'une seule visite. La meilleure stratégie d'échantillonnage était la combinaison des échantillons de la fosse, de l'écureur, du groupe de vaches malades et du groupe de vaches maigres. La technique standardisée de prélèvements de l'environnement combinée aux échantillons groupés ciblés présentée dans cette étude est une alternative économique à la culture individuelle pour détecter des troupeaux laitiers infectés par MAP. La répétition des prélèvements pourrait contribuer à améliorer la détection des troupeaux infectés par MAP.*

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Introduction

Johne's disease is an incurable, chronic, and contagious enteritis of ruminants caused by *Mycobacterium avium* ssp. *paratuberculosis* (MAP). The disease causes significant economic losses related to reduced milk production, premature culling, increased replacement costs, and decreased slaughtered carcass weight (1–3). This intracellular bacterium, which invades the immune cells of the gastrointestinal tract, has also been linked to Crohn's disease in humans (4–6). Recent studies have led to increased concern about the zoonotic potential of MAP (7–9). Fecal-oral contamination is the main route of MAP transmission (10) and contact between calves and the feces of adult cows is the most important risk factor (11). Young calves are the most susceptible to MAP infection (12). They are prone to becoming infected by ingesting colostrum or milk from infected animals (13) or contaminated water or food (10). As excretion and clinical signs are observed at an older age (14), shedder cows are the main infectious source of environmental contamination. The ability of MAP to survive in the environment for up to 11 mo contributes to the perpetuation of infection in dairy herds (15,16).

Environmental sampling is one of the testing procedures recommended for control programs to assess MAP status in dairy herds (17). The evidence suggests that environmental sampling is a cost-effective method for determining infection status in previously untested dairy herds (18). The technique is simple, less expensive than individual tests, and does not require handling of individual animals. Some studies have compared the performance of environmental culture (EC) with that of individual fecal culture (IFC) and enzyme-linked immunosorbent assay (ELISA) (individual milk and serum samples) for detecting MAP-infected herds (19–22). Other studies have evaluated the correlation between EC and within-herd prevalence (WHP) based on IFC (23–25). In 1 study conducted on California dairy farms, no significant difference was observed among the 3 testing methods (EC, IFC, and ELISA) in terms of the proportions of herds correctly identified as infected (19). Another study found a highly significant relationship between EC and IFC (20). The sensitivity of EC for detecting MAP infection at the herd level has been estimated at between 40% and 81% (21,23,24). Specificity has been estimated to be close to 99% (25).

Environmental sampling has been evaluated mostly in large free-stall dairy herds in the United States (19–21,23) and recently in western and Atlantic Canada (22,25). Eastern Canada (the region east of Manitoba) is home to about 50% of Canada's dairy herds, most of which are small tie-stall herds. In the province of Quebec, the average herd size is 57 cows per farm and 92% of the herds are housed in tie-stall barns (26). Because of specific management practices and conditions in this part of the country, this area provides an interesting regional data set for evaluating the environmental sampling technique. Manure management practices in tie-stall barns differ from those in the large free-stall facilities typical in the United States and western Canada. These characteristics, combined with eastern Canada's humid continental climate, may influence the survival, transmission, and recovery of MAP in various environmental sampling areas.

The purpose of this study was to estimate the performance of a standardized environmental and targeted pooled sampling technique for identifying MAP-infected tie-stall dairy herds. Secondary objectives were to describe the distribution of MAP in the environment of tie-stall dairy herds in Quebec and to find the best sampling strategy for detecting MAP-infected tie-stall dairy herds.

Materials and methods

Study design and sample size

A cross-sectional study was designed to evaluate a standardized environmental and targeted pooled sampling method in tie-stall dairy herds. The source and target populations were, respectively, the dairy herds enrolled in the Quebec Voluntary Paratuberculosis Prevention and Control Program (QVPPCP) and Quebec dairies. A convenience sample of 24 tie-stall dairy herds was purposively selected based on historical MAP status. That sample included 19 MAP-infected herds (see case definition in next paragraph) and 5 non-infected herds. Additional inclusion criteria were the owner's willingness to participate, tie-stall configuration, regular veterinary herd health visits, access to electronic records, and no drastic changes in the farm system, e.g., a change to free stall, in the year before the study began.

Case definition

A herd was considered infected for the purpose of our analysis if MAP was cultured from at least 1 sample (IFC or EC) during the 24 mo before the study began or during the study period itself. The specificity of the bacteriologic culture was assumed to be 100% (17). A herd was considered negative if it had 2 negative results with EC (sampled in a 12- to 18-mo interval) and no clinical animals (persistent diarrhea and loss of body weight and normal appetite) during the 24 mo before the study began.

Sample collection

Initially, 20 herds were visited in summer 2011 (June 20 to August 23). These herds were visited again in fall 2011 (October 3 to November 24) and 4 additional herds were enrolled, for a total of 24 herds. The samples were analyzed at the Laboratoire d'épidémiosurveillance animale du Québec in Saint-Hyacinthe, Quebec. Upon reception and within 24 h of collection, the fecal samples were stored at −80°C until they were analyzed.

Environmental samples — A set of 7 environmental samples and 2 pooled samples was collected from sick cows and cows with poor body condition by 2 members of the research group during each herd visit using a standardized technique (Table I). The sampling area was documented with photographs and videos in order to standardize the procedure throughout the study and record the precise sampling sites for each farm.

The 7 environmental samples came from 4 sites on each farm: 3 locations (gutter, manure pit, and heifers' area) were sampled twice and the 4th location (the boots of the farm owner or the sampler) was sampled once. A composite sample of about 20 g of manure from each site was made for the 4 locations.

Additionally, 2 pooled samples were collected from 3 to 5 cows purposively chosen from 2 categories: sick cows and cows with body condition scores (BCSs) lower than 3. For the sick cow group, the

Table I. Environmental and targeted pooled samples for identifying tie-stall dairy herds infected with MAP

Environmental and targeted pooled samples	Description	Number of samples per visit
Manure pit	Samples taken more than 10 cm deep	2
Gutter	At the end of the barn but before the manure pit	2
Heifers' pen	Composite samples from 4 different surfaces at the site	2
Boots	Samples scraped from the soles of boots at the end of each visit but before going to the manure pit	1
Sick cows	Pool of 3 to 5 cows affected by any disease but never having tested positive for paratuberculosis	1
Cows with poor body condition	Pool of 3 to 5 cows with a body condition score lower than 3 on a scale of 1 to 5	1
Total		9

owner selected cows that were affected by any disease, but had never been diagnosed as positive for paratuberculosis. For the group with poor body condition, the sampler chose cows with a BCS lower than 3 (using a scale of 1 to 5). An individual fecal sample of about 20 g was taken from the rectum of each selected cow, using a single-use veterinary glove without lubricant. At the farm, equivalent amounts of feces were gathered from each individual sample into their respective pools and homogenized as described previously (27). Briefly, the feces were mixed with a wooden tongue depressor by means of 10 vertical stirs from the bottom to the top, followed by 10 clockwise stirs, and 10 counter-clockwise stirs. Duplicates from each pool were stored in 2 plastic containers for transport to the laboratory.

Individual fecal and blood samples — From each herd, all cows older than 24 mo that had calved at least once were tested during each visit. An individual fecal sample of about 20 g was taken using a single-use veterinary glove without lubricant. Also, a single blood sample per cow was collected from the coccygeal vein in an 8-mL vacutainer tube without anticoagulant (Becton, Dickinson, Mississauga, Ontario). Blood samples were centrifuged and aliquots of serum were stored at −20°C until ELISA analysis.

MAP culture

Environmental and individual fecal samples were processed following the manufacturer's recommendations (Becton, Dickinson, Sparks, Maryland, USA) and the US Department of Agriculture (USDA) (28). The MAP was isolated using the MGIT Para TB culture media and the BACTEC 960 detection system (Becton, Dickinson) at the Laboratoire d'épidémiosurveillance animale du Québec in Saint-Hyacinthe, Quebec, which is a USDA-certified laboratory.

An initial 3-day decontamination was carried out on the samples. Initially, 2 ± 0.2 g of feces was diluted into 17.5 mL of sterile distilled water and allowed to settle at room temperature for 30 min. Then 2.5 mL of the supernatant was transferred aseptically to a 50-mL tube with 2.5 mL of 15% yeast extract and 0.2 mL of 10% sodium pyruvate. This solution was mixed briefly and incubated for 90 min at 36 ± 1°C. For each fecal sample preparation, 0.3 mL of sterile 5% malachite green solution was added to a solution of 25 mL of sterile half-strength brain heart infusion (BHI) medium and 0.9% hexadecylpyridinium chloride (HPC). Finally, all 5.2 mL of the feces-germination mix was added to the BHI-HPC solution to complete a 30 mL decontamination suspension, which was vortexed briefly and incubated overnight (18 to 24 h) at 36 ± 1°C.

The next day, this decontaminated suspension was centrifuged for 30 min at 900 × *g*. The supernatant was gently poured off. Then, 1 mL of an antibiotic brew (vancomycin at 100 μg/mL, nalidixic acid at 100 μg/mL, and potency-adjusted amphotericin B at 25 μg/mL) was added to the pellet. The suspension was incubated overnight (18 to 24 h) at 36 ± 1°C. Also, 1.5 mL of an additive cocktail was added to each MGIT Para TB culture tube (Becton, Dickinson). The additive cocktail contained Para TB supplement (bovine albumin, catalase, casein, oleic acid; Becton, Dickinson), egg yolk enrichment, sterile water, and antimicrobials (2.5% vancomycin, 2.5% nalidixic acid, and 1% amphotericin B). These tubes were stored in a safety cabinet at room temperature for 18 to 24 h.

On the third day of the fecal sample processing, the concentrated specimen suspension was mixed by swirling and 0.1 mL was inoculated into the MGIT Para TB culture tubes before they were introduced into the BACTEC 960 detection device for incubation at 37°C for a maximum of 56 d. The additive cocktail used for the environmental samples processed in 2011 included a higher concentration of nalidixic acid (10 times more). From January 2012 onward, a single additive cocktail, which included an additional 1% ceftriaxone, was used for processing all the samples, both individual and environmental.

The tubes that gave a positive signal before 42 d were always put back in the device for further incubation. The tubes flagged as positive between 42 to 56 d of incubation were incubated for an additional 72 h at 36 ± 1°C and an acid-fast bacilli stain was done using the TB Fluorescent Stain Kit M (Fisher Scientific, Ottawa, Ontario). Positive samples were confirmed by real-time polymerase chain reaction (PCR) [*Taq*Man MAP (Johne's) Reagents; Applied Biosystems, Foster City, California, USA]. The results of previous tests were interpreted according to USDA recommendations (28). Samples were identified as MAP-positive if they were flagged in the system and confirmed by both the acid-fast bacilli stain and the real-time PCR.

ELISA

Sera were processed using the IDEXX Pourquier MAP antibody test kit (IDEXX Laboratories, Westbrook, Maine, USA) according to the manufacturer's instructions. Optical density (OD) values were transformed into sample-to-positive (S/P) ratios as described previously (29). Samples with an S/P ratio of 55% or greater were considered positive. As suggested previously (30), a herd was declared ELISA-positive if the serum within-herd prevalence (WHP) was 2%

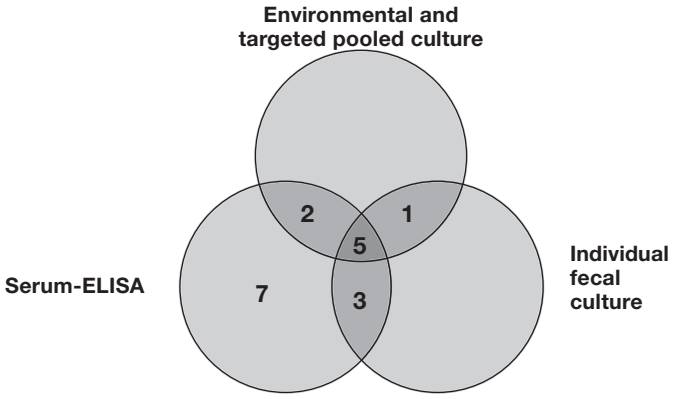

Figure 1. Of 24 herds, identification of those herds positive for MAP at either sampling using 3 detection methods.

or greater, given that the specificity of ELISA relative to fecal culture has been estimated at 98% to 99% (31–33).

Statistical analysis

Statistical analyses were carried out with the SAS software (Version 9.3; SAS Institute, Cary, North Carolina, USA). Descriptive statistics for the individual and environmental samples were conducted to characterize the distribution of positive results. The Wilcoxon rank test was used to compare the WHP by ELISA and IFC and the number of positive ECs between the 2 visits. The percentage and confidence interval of infected herds detected by the tests used in the study were calculated for a single visit and for 2 visits to assess the impact of repetitive samplings. For the herds visited twice, 1 sampling was randomly chosen for the assessment of a single visit. The association between the number of positive ECs and fecal WHP was evaluated with the Chi-square test. This test was also used to compare the number of infected herds confirmed by each diagnostic test. The percentage of positive EC, IFC, and ELISA samples per visit was compared using the Z test. All results were considered significant if $P < 0.05$.

Results

Herd characteristics

The 24 herds were located in 4 regions of Quebec, Canada (Bas-Saint-Laurent, Capitale-Nationale, Montérégie, and Centre-du-Québec). Median herd size was 59 lactating cows (95% CI: 48 to 65), ranging from 30 to 211 cows. In 16 herds, the cows were exclusively Holstein, in 1 herd the cows were exclusively Jersey, and in 7 herds, more than 1 breed was also present (Holstein and Jersey in 6 herds and Holstein and Brown Swiss in 1 herd). The mean age of the cows sampled at the beginning of the study was 4.5 y (2 to 14 y). The apparent WHP ranged from 0% to 28% for IFC and from 0% to 31% for ELISA. The number of positive EC samples per herd ranged from 0 to 7. The proportion of culled animals during the study for the herds that were sampled twice was 13% on average (2% to 27%).

When combining sampling results from both seasons, out of the 24 enrolled herds, 17 MAP-infected herds (according to the initial MAP status) were found positive by at least 1 of the detection methods used (Figure 1). According to the initial MAP status, 5 MAP-infected herds were found positive by all 3 tests. Although 7 herds were found positive only by ELISA, 1 of these herds was presumed to be non-infected based on our case definition.

Among the MAP-infected herds according to the initial MAP status ($n = 19$), 17 were detected as positive during the study by at least 1 of the detection methods used (Table II). Overall, the number of infected herds confirmed during either visit was 8 out of 19 for EC, 9 out of 19 for IFC, and 16 out of 19 for ELISA. More infected herds were confirmed by ELISA than by the other tests ($P = 0.01$). The different combinations of test results for a single visit or for both visits of MAP-infected herds are presented in Table III. When the results from 2 different visits were combined, the percentage of infected herds detected by environmental sampling was 44% (95% CI: 20% to 70%) and 32% (95% CI: 13% to 57%) when the results of only 1 random herd visit were considered, although the difference between 1 and 2 herd visits was not significant ($P = 0.5$). Among infected herds, more herds were found positive at both samplings based on individual tests (IFC and ELISA) compared to EC (Table III). At the individual level, 14 cows were IFC-positive at both samplings. Six cows that were IFC-negative in the summer were found to be IFC-positive in the fall.

Individual samples

A total of 3100 samples was tested from 1844 adult cows sampled [summer only ($n = 172$), fall only ($n = 416$), and both seasons ($n = 1256$)]. The MAP pathogen was cultured from 21 IFC samples in the summer (1.5%; 95% CI: 0.9% to 2.2%) and from 24 IFC samples in the fall (1.4%; 95% CI: 0.9% to 2.1%). In total, 35 cows (2.5%; 95% CI: 1.7% to 3.4%) were seropositive in the summer and 49 in the fall (2.9%; 95% CI: 2.2% to 3.9%) (Table II). The proportion of positive samples per visit was not significantly different for IFC ($P = 0.8$) or ELISA ($P = 0.4$).

Environmental samples

Overall, MAP was recovered from 29 out of 392 environmental cultures (EC) carried out during the study (7%; 95% CI: 5% to 11%) from 8 positive farms. In the summer, MAP was cultured from 12 out of 177 ECs (7%; 95% CI: 4% to 12%) from 5 infected herds. In the fall, 17 out of 215 ECs were positive (8%; 95% CI: 5% to 12%) from 5 infected herds. We did not observe a difference between the percentages of positive environmental samples per sampling period ($P = 0.8$).

Environmental sites — The MAP pathogen was isolated from 14% of the boot samples, 11% of the sick cow group samples, 9% of the manure pit samples, 8% of the gutter samples, 5% of the samples from the group with low BCSs, and 1% of the samples from the heifers' area. There were MAP-infected herds identified by the manure pit, gutter, and sick cow sites. From the gutter alone, 3 MAP-infected herds were detected (Table IV). If only 2 sites were sampled, the best sampling option was to combine the samples from the manure pit with either the samples from the sick cow group or the boots because 6 MAP-infected herds were detected with each combination. In order to detect all the herds found positive by EC during the study, a combination of a minimum of 4 sites was

Table II. Herd characteristics and WHP of MAP (estimated using IFC or serum ELISA) and number of positive environmental and targeted pooled cultures for 2 sampling seasons in 24 tie-stall dairy herds in Quebec

	Summer				Fall				Number
	Herd	WHP[b] (%)		Number of	Herd	WHP[b] (%)		Number of	of animals
Status[a]	size	IFC[c]	ELISA[d]	positive EC-TPS[e]	size	IFC[c]	ELISA[d]	positive EC-TPS[e]	sampled twice
+	179	1.1	0.6	2	178	0.6	1.7	0	165
+	38	0	0	1	45	0	**2.2**	0	37
+	52	3.6	**3.8**	2	55	3.6	**3.6**	6	44
+	35	28.6	**31.4**	6[e]	45	20.0	**17.8**	7	29
+	97	0	1.0	1	100	2.0	**3.0**	0	94
+	76	3.9	**3.9**	0	79	3.8	**6.3**	2	67
+	52	0	0	0	52	0	**3.8**	1	38
+	NS	NS	NS	NS	72	5.6	**2.8**	1	—
+	45	0	0	0	48	2.1	**2.1**	0	36
+	60	3.3	**3.3**	0	57	0	1.7	0	53
+	210	0	1.4	0	211	0	**4.2**	0	190
+	64	0	**3.1**	0	55	0	0	0	44
+	41	0	**9.8**	0	43	0	**9.3**	0	36
+	63	0	**3.2**	0	62	0	1.6	0	54
+	39	0	**2.6**	0	39	0	0	0	31
+	NS	NS	NS	NS	30	0	**3.3**	0	—
+	42	0	0	0	46	0	0	0	41
+	NS	NS	NS	NS	46	0	0	0	—
+	42	4.8	**4.8**	0	44	4.5	**2.3**	0	39
−	90	0	0	0[e]	92	0	0	0[e]	87
−	71	0	1.4	0	73	0	1.3	0	60
−	NS	NS	NS	NS	72	0	1.4	0	—
−	69	0	0	0	65	0	1.5	0	58
−	63	0	0	0	63	0	**3.2**	0	53

[a] Status: (+) Infected (−) Not infected.

[b] Within herd prevalence.

[c] Individual fecal culture.

[d] Herds were considered positive if ELISA WHP was ≥ 2% (bold characters).

[e] Environmental and targeted pooled cultures; 7 environmental and 2 targeted pooled samples (TPS) were taken during all herd visits except 3.

NS — Not sampled.

required (the manure pit, the sick cows, the cows with low BCSs, and either the gutter or the boots). When MAP was cultured from the boots, there was at least one other positive environmental sample on the farm.

Consecutive negative EC results — Among herds that tested negative by EC at both samplings, the odds of not detecting a cow shedding MAP tended to be greater [odds ratio (OR) = 5.4; 95% CI: 0.9 to 38.2; $P = 0.06$] compared to herds found positive by EC in at least 1 of 2 samplings.

Discussion

When the results of 2 visits were combined, the sensitivity of the standardized environmental and targeted pooled sampling technique proposed in this paper was within the range of the values reported in the literature (23–25). Recently, 2 Canadian studies reported a higher sensitivity of environmental sampling (68% to 71%) when it was conducted quarterly (22,25). It is expected that repeated samplings may increase the capacity of the environmental sampling technique to detect infected herds. On the other hand, herds with 2 consecutive negative EC results were more likely to have no cows shedding MAP. Even if a negative EC result does not guarantee that the farm is not infected, that result may indicate a negative or a low-prevalence herd (17). Repeated negative samplings may increase confidence that the farm has a very low prevalence or is MAP-negative.

One study did not find positive ECs when within-herd prevalence (WHP) was 2% or less (34). In the present study, positive ECs were observed in herds without positive IFCs, as previously reported (21,25). Intermittent fecal shedding may explain the absence of concurrent positive IFCs in the infected herds that tested positive by EC (35). Another hypothesis is that shedder cows had been culled before the herd visit and MAP remained in the environment of the farm. One study suggested that EC may be a measure of the persistency of MAP on farms even if no individual cows are positive by concurrent IFC, indicating that some environmental contamination remains despite the reduction in prevalence (34). Because of the bacterium's

Table III. Combinations of test results for either a single visit or 2 visits to detect MAP in tie-stall dairy herds in Quebec

Number of samplings	Sampling option (Number of infected herds[a])	Number of positive herds[b]			Sensitivity (CI)		
		EC-TPS	IFC	ELISA	EC-TPS	IFC	ELISA
Single sampling	Summer (16)	5	6	9	31 (11 to 59)	38 (15 to 65)	56 (30 to 80)
	Fall (19)	5	8	12	26 (9 to 51)	42 (20 to 67)	63 (38 to 84)
	One random sampling[c] (19)	6	7	9	32 (13 to 57)	37 (16 to 62)	47 (24 to 71)
Two samplings[d]	Summer or fall[e] (16)	7	8	14	44 (20 to 70)	50 (25 to 75)	88 (62 to 98)
	Summer and fall[f] (16)	2	5	5	13 (2 to 38)	31 (11 to 59)	31 (11 to 59)

[a] Infected herd: MAP was cultured from at least 1 sample (IFC or EC) for 30 mo including the duration of the study period.
[b] Herds were considered positive through environmental culture and targeted pooled sampling (EC-TPS) strategy and individual fecal culture (IFC) if at least 1 positive sample was obtained. For ELISA, herds were considered positive if within-herd prevalence (WHP) was ≥ 2%.
[c] Nineteen infected herds had available results in at least 1 sampling season. From the herds visited twice, 1 sampling was randomly chosen.
[d] Sixteen infected herds had available test results for both seasons.
[e] Herds meeting the criteria for a positive herd according to each diagnostic test, either in summer or fall.
[f] Herds meeting the criteria for a positive herd according to each diagnostic test, in both summer and fall.

Table IV. Distribution of MAP-positive sites on 8 farms tested by environmental and targeted pooled sampling

EC-TPS positive herds[a]	Number of positive samples by site[b]						Summer			Fall		
							Number of positive	Within-herd prevalence		Number of positive	Within-herd prevalence	
	MP	G	H	B	S	P	EC-TPS[a]	IFC[c]	ELISA	EC-TPS[a]	IFC[c]	ELISA
A	0	1	0	1	0	0	2	1.1	0.6	0	0.6	1.7
B	1	0	0	0	0	0	1	0	0	0	0	2.2
C[d]	2	2	0	2	2	0	2	3.6	3.8	6	3.6	3.6
D[d]	4	4	1	2	1	1	6	28.6	31.4	7	20.0	17.8
E	0	0	0	0	0	1	1	0	1.0	0	2.0	3.0
F	0	0	0	1	1	0	0	3.9	3.9	2	3.9	6.3
G	1	0	0	0	0	0	0	0	0	1	0	3.8
H	0	0	0	0	1	0	NS	NS	NS	1	5.6	2.8
Number of herds detected by site	4	3	1	4	4	2						

[a] Positive herds by environmental culture and targeted pooled sampling (EC-TPS) strategy in either summer or fall.
[b] MP — Manure pit; G — gutter; H — heifers' area; B — boots; S — sick cows group; P — group of cows with poor body condition.
[c] IFC — Individual fecal culture.
[d] Positive farms both in summer and fall.
NS — Not sampled.

ability to survive in the environment for up to 11 mo under optimal conditions, EC has the potential to detect MAP in herds even after infected animals have been culled or in the presence of intermittent shedding. This is a strong advantage of any technique used at any particular point in time.

Overall, no statistically significant difference was observed between the proportion of infected herds identified by EC, IFC, or ELISA. The absence of a statistically significant difference, however, could be a consequence of the low power of the study. In the present study, ELISA misclassified 1 non-infected herd as positive, according to our case definition. This herd may actually be an infected herd that our case definition failed to classify or a non-infected herd that ELISA misclassified due to a lack of specificity.

The choice of detection method depends on the objectives established by the owners or veterinarians. The environmental culture (EC) is the most cost-effective option to determine MAP-herd status (17,18). In the context of the QVPPCP, where the main objective is to detect high-prevalence herds (those herds with the most important economic losses), EC is the most appropriate sampling alternative. Screening a whole herd with individual tests (IFC or ELISA) is more invasive, expensive, and time-consuming than EC. Individual tests are more suitable for identifying infected animals within a MAP-infected herd. At the herd level, our study indicates that all tests (EC, IFC, and ELISA) give different results at different points of time. The demographic changes in the herds could explain such variability.

In the present study, MAP was cultured from the manure pit and the gutter, which is where manure accumulates in tie-stall farms. These locations are traditionally chosen as sampling sites because they have been proven to have a high sensitivity for detecting MAP-infected free-stall herds (19–21,36). The MAP pathogen was also

cultured from samples collected from boots, the sick cow group, cows with low BCS, and the heifers' area. While boot sampling was previously found to be a sensitive technique for detecting high-prevalence herds (37), it needs to be evaluated in low-prevalence herds as these authors suggest. Another study suggested including boots as an additional sample in the environmental sampling strategy (38). Although boot sampling (either from the owner or a researcher) is interesting, it is both recommended and expected that owners disinfect and/or change boots as they move from one area on the farm to another. This would be particularly important when different age groups are visited. As for researchers, the same rule should apply so they cannot become a potential risk of dissemination of a pathogen within a herd.

Sampling cows with low BCS was previously suggested for screening beef cattle herds for MAP (17). The pools proposed in our study may be collected by the veterinarian during a herd health visit with minimal additional animal handling. In contrast, a sample from the heifers' area does not seem to increase the sensitivity of the technique, as MAP was cultured from only 1 sample throughout the study. This finding demonstrates that young animals may be exposed to and shed MAP, as suggested in a previous study (39). This positive sample was collected from the herd with the highest prevalence (fecal WHP, ELISA WHP, and EC prevalence). Our study suggests that a combination of samples from the manure pit or the gutter, the sick cows, and/or the cows with low BCS may be an effective strategy to detect MAP-infected tie-stall dairy herds.

It has been suggested that the sensitivity of environmental sampling is expected to be higher in high-prevalence herds (14). Another study found that sensitivity may be close to 100% even in moderate-prevalence herds (when the WHP is 8% or greater) (5). Some factors may have affected the sensitivity of the environmental sampling technique in our study. The low-prevalence herds included were purposively selected from the QVPPCP list because of the owner's willingness to participate in research projects. It can be assumed that these producers are more aware of bovine paratuberculosis than producers who did not participate in the program. The exposure of QVPPCP herds to several years of veterinarian recommendations in order to control MAP infections may have contributed to the lower MAP prevalence. Additionally, in our study several herds that were initially considered to be infected turned out to be either negative or very low-prevalence herds. Possible reasons for this inconsistency may have been the culling of cows (either for paratuberculosis or other reasons) or the delay between the positive diagnosis classifying the herd as infected and the beginning of the study. If at the time of the positive test, the herd had low prevalence and biosecurity measures had been introduced to reduce transmission (as is supposed to be done for herds enrolled in the QVPPCP), it is very likely that the WHP decreased or at least remained at the same level. Although the sensitivity of EC tended to increase when 2 samplings were considered instead of 1, our sample size did not allow us to observe a significant difference.

In conclusion, the proposed standardized environmental and targeted pooled sampling technique was a useful diagnostic method for detecting the MAP-infected tie-stall dairy herds in this study. This inexpensive and non-invasive method detected mainly high-prevalence herds, but it did detect low-prevalence herds as well. Repeated samplings may increase sensitivity for detecting low-prevalence herds and confidence in a negative result. Our sampling strategy proposes that new sample types, compared to current methods, be included to improve MAP detection.

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