The association of detection method, season, and lactation stage on identification of fecal shedding in *Mycobacterium avium* ssp. *paratuberculosis* infectious dairy cows

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ABSTRACT

Mycobacterium avium ssp. *paratuberculosis* (MAP) is the causative organism of Johne's disease. Although fecal culture is considered the standard diagnostic test, the long incubation times, costs, and intermittent shedding of MAP hinder efficient screening programs based on culture results. The primary objectives of this study were to determine the detection ability of solid culture, broth culture, and real-time PCR (qPCR) for MAP in fecal samples and to assess how shedding patterns of MAP in feces vary with lactation stage and season. This knowledge could improve the use of these diagnostic assays in Johne's management programs. For this study, 51 MAP-infectious cows from 7 Atlantic Canadian dairy farms had fecal samples collected monthly over a 12-mo period. Samples were analyzed for MAP bacterial load via solid culture, broth culture, and qPCR. For all fecal samples, 46% [95% confidence interval (CI): 40 to 51%] were positive by solid culture, 55% (95% CI: 50 to 60%) by broth culture, and 78% (95% CI: 73 to 82%) by qPCR. Sensitivity of qPCR was numerically higher in the dry and postpartum lactation periods, and qPCR detection in summer and fall was 85% of that in winter and spring. Furthermore, culture-determined moderate or light shedding categories generally corresponded to qPCR cycle threshold values <35, but heavy shedding categories corresponded to qPCR values <29. Direct fecal qPCR is a MAP detection method that is quick and less costly than culture techniques, and it avoids the use of decontamination steps that could decrease numbers of bacteria in a sample below the detection limit. This study indicates that, for known MAP-positive cows, fecal qPCR had high sensitivity of MAP detection, thereby supporting the use of direct fecal qPCR as part of a Johne's herd control program.

Key words: Johne's disease, feces, real-time polymerase chain reaction, culture

INTRODUCTION

In dairy cattle, Johne's disease, or paratuberculosis, is an important production-limiting disease leading to financial losses for dairy farmers. This economic impact is related to decreased milk production (Lombard et al., 2005), reproductive losses (Johnson-Ifeorlundu et al., 2000), mortality, culling (Ott et al., 1999), and increased veterinary costs (Benedictus et al., 1985). Caused by the organism *Mycobacterium avium* ssp. *paratuberculosis* (MAP), the disease manifests as chronic enteritis, with an incubation period extending from 2 to 10 yr, depending on the infective dose (Whittington and Sergeant, 2001). During the preclinical stage, from initial infection typically as calves, to the time of manifested clinical signs, most often as adults, bacteria can be intermittently shed from infected cows and silently spread throughout a farm (Fecteau and Whitlock, 2010). Adult cows with clinical symptoms of MAP can shed 10^6 to 10^8 cfu/g of feces (Jørgensen, 1982; Whittington et al., 2000). However, a calf can become infected with MAP by ingesting only 50 to 10^3 cfu per calf (Gilmour et al., 1965; Chiodini, 1996), making the volume of MAP-contaminated feces required to infect a calf extremely low.

Johne's disease status has commonly been tiered into 3 categories: cows that carry the MAP bacterium without detectable shedding (infected), cows that are shedding at time of diagnostic testing (infectious), and cows that exhibit clinical signs of the disease (affected) (Nielsen and Toft, 2008). Efficient diagnostic strategies for identification of infected cows and monitoring of infectious and affected cows can enhance best management practices to reduce transmission of MAP (Nielsen and Toft, 2006). Because asymptomatic MAP-infected cows can intermittently shed bacteria in their feces, a negative fecal detection test result does not imply that the cow is paratuberculosis free, but rather that the cow was not shedding the organism at the time of testing or that the concentration of bacteria in the sample was below the diagnostic detection limit. Stressful events, including parturition, can increase MAP shedding and

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precipitate the onset of clinical signs of Johne's disease (Sweeney, 2011).

Despite the intermittent shedding of MAP in preclinical phases of disease, fecal culture is considered the primary reference standard (Bölske and Herthnek, 2010). Unfortunately, MAP culture incubation times can take up to 7 wk on broth media and 16 wk on solid media. Furthermore, the slow growth of MAP necessitates the use of decontamination techniques, including antimicrobials, to decrease any faster-growing, competitive bacteria and other organisms. Whereas growth in liquid media is quicker than on solid media, a disadvantage of this technique is that the results are only indirectly quantitative (Whittington, 2010). The Trek broth system (Trek/Thermo Scientific, Oakwood Village, OH) uses a pressure detection system to signal positivity, and the days to positive can be used as an estimate of MAP concentration in the fecal sample (Whittington, 2010). In comparison, growth on solid media such as Herrold's egg yolk medium (**HEYM**) with mycobactin J, although requiring longer growth times and being more labor intensive, does allow for direct quantitation by a count of colony-forming units (Whittington, 2010). Acid-fast stain can be used to confirm that culture isolates have typical morphology. However, PCR of the isolated bacteria provides stronger evidence to confirm MAP over other mycobacteria.

The PCR-based molecular techniques can detect MAP genetically, and the cycle threshold (**Ct**) values obtained can be used to estimate the quantity of MAP DNA present in the sample, with lower Ct values corresponding to higher MAP DNA concentration (Bölske and Herthnek, 2010). More recently, attempts have been made to categorize Ct values in reference to counts (cfu) obtained via solid culture techniques (Leite et al., 2013). Methods for direct PCR and culture isolate confirmation with PCR have been developed for feces, milk, colostrum, and tissue samples. Real-time quantitative PCR (**qPCR**), specifically, has 3 main advantages for MAP detection. First, qPCR is very rapid compared with culture. Additionally, the method does not require decontamination steps, thereby avoiding the inherent reduction in MAP concentration caused by that process. Finally, qPCR can detect dead as well as viable bacteria, which is helpful when fresh samples cannot be processed immediately as some bacteria may be killed during freezing (Bölske and Herthnek, 2010). Although the insertion element *IS900* has classically been the most common sequence targeted, the single copy gene *hspX* is another unique MAP-specific sequence (Ellingson et al., 1998; Bölske and Herthnek, 2010) and is used in the Tetracore real-time PCR kit for Johne's detection (Tetracore, Rockville, MD). By using a sequence unique for MAP, specificity is comparable to

that of culture techniques and can reach 100% (Leite et al., 2013). However, qPCR sensitivity for MAP depends upon additional factors including technique, bacterial concentration within the sample, bacterial loss during processing or storage, and reference standard accuracy (Bölske and Herthnek, 2010).

Because no treatment or cure is currently available for Johne's disease (Whitlock, 2010), a management-based control program is important for producers wishing to decrease the introduction and spread of MAP within a herd. Management programs have 2 main goals: decreased transmission to calves and heifers within infected herds and prevention of disease introduction into herds (Whitlock, 2010). Vaccines can be used concurrently with control measures, and in some cases, particularly for small ruminants, have been shown to be a cost-effective means for controlling clinical signs of Johne's disease, especially decreased milk production (de Lisle, 2010). However, in North America, use of the vaccine in dairy control programs is strictly controlled, primarily due to interference with bovine tuberculosis skin testing (Patton, 2011), as well as potential false-positive results in paratuberculosis immune tests (de Lisle, 2010; Tewari et al., 2014). Vaccines can benefit control programs by delaying and decreasing clinical signs and fecal shedding (Tewari, et al., 2014). However, they do not prevent infection in herds and can result in large granuloma reactions at inoculation sites (de Lisle, 2010). In the fourth National Animal Health Monitoring System (NAHMS) study of the US dairy industry (NAHMS, 2008), 5.0% of dairy herds incorporated vaccination in their Johne's control programs.

Consequently, once MAP has been detected within a dairy herd, management and biosecurity are the primary means of control. Because fecal shedding and subsequent ingestion is a major transmission mode, manure management, particularly in the maternity pens and among dry cows, becomes a priority. Pithua et al. (2013) observed that the use of individual calving pens in Midwestern US dairy herds reduced the subsequent risk of MAP infection for these calves compared with those born in group-calving pens, which underwent variable manure management protocols. Although this effect decreased over time, they recommended the use of individual calving pens, in which strict manure hygiene can be implemented.

Unfortunately, difficulty in identifying MAP-positive cows can impair biosecurity-based management programs. As a result, the use of more than one diagnostic method has often been suggested, particularly combining immune-based and culture methods, as they are based on different target conditions. This concept could also apply to culture and molecular techniques (Pinedo

et al., 2008). Understanding patterns of MAP shedding detected by the most commonly used diagnostic tests can lead to their use in a more time- and cost-efficient manner. Seasonal and lactation stage shedding patterns have been assessed previously but with conflicting results. One study did not find an association between ambient temperature (season) and detection of MAP by fecal culture (Strickland et al., 2005). However, in another study, increased MAP growth was detected in fecal samples collected during winter (November through March) (Crossley et al., 2005). A third study from New Zealand by Norton et al. (2010) found a higher fecal culture detection rate in spring (October). This corresponded to the calving period in that industry, so the effect of season could not be separated from a potential effect of stage of lactation. A knowledge gap exists regarding within-season and within-lactation fecal MAP shedding patterns for culture and molecular methods, especially broth culture and qPCR. However, such knowledge could enhance the efficient use of fecal diagnostic tests for Johne's disease detection by controlling for variations in fecal shedding patterns related to season or lactation stage.

Therefore, the primary objectives of the study were to determine the sensitivity (**Se**) of 3 fecal diagnostic methods (solid culture, broth culture, and qPCR) and to assess the effects of season and lactation stage on MAP detection. Secondary objectives included use of parallel **Se**, agreement evaluation among the diagnostic methods, and the association between **Ct** values for qPCR and shedding patterns determined by culture techniques.

MATERIALS AND METHODS

Farm and Cow Selection

For this study, 7 known MAP-positive dairy farms were purposively selected from a companion project assessing MAP herd prevalence in dairy farms in New Brunswick, Nova Scotia, and Prince Edward Island (Lavers et al., 2013). In that study, MAP culture-positive cows were identified following twice-yearly herd monitoring with pooled fecal culture. Each pool comprised 5 cows, which were individually cultured if the pool was positive. The farms recruited for our study were selected based on the number of these MAP culture-positive cows as well as proximity to our laboratory to facilitate monthly sample collection. Three of the selected farms were located in Prince Edward Island and 4 were located in New Brunswick. Farm prevalence ranged from 3 to 15%, and herd size ranged from 83 to 490 cows per herd. From the 7 farms, 51 MAP-infectious Holstein

cows were recruited in total, with a range of 2 to 15 cows recruited per farm.

For this study, the target condition was a MAP-infectious cow that was actively shedding the organism at the time of fecal testing. Cows were considered to be MAP-infectious at the start of the study if they were positive by fecal culture (confirmed with acid-fast stain and qPCR) a minimum of 1 time within a 1-yr period before the start of this study (case definition), as determined from the companion project (Lavers et al., 2013). Therefore, for the remainder of this study, the term "MAP-infectious" is used to identify these cows. Age, parity, and lactation stage were recorded for each cow at each sampling time.

Sample Collection

All animal protocols were preapproved by the Animal Care Committee at the University of Prince Edward Island. Individual fecal samples were collected from these 51 MAP-infectious cows using individual, clean rectal sleeves without lubrication, and samples were placed into individual, clean plastic specimen jars. Sampling was performed monthly for up to 12 consecutive months (during the period from July 2010 to December 2011) per cow or until removal from the herd. Postpartum fecal samples were collected by the farmers within 14 d of calving. All samples were frozen for long-term storage at -80°C until processing could be completed at the Maritime Quality Milk laboratory (Charlottetown, PEI, Canada), which is USDA-accredited for fecal culture and qPCR techniques.

Laboratory Procedures

Solid Culture. Solid cultures were performed on HEYM slants supplemented with mycobactin J and amphotericin B, nalidixic acid, and vancomycin (Becton, Dickinson and Co., Sparks, MD), and quantitative counts (cfu) were performed on positive cultures. Procedures used partly followed methods described by Stabel (1997). Briefly, 3 g of thawed fecal sample was added to 30 mL of half-strength brain-heart infusion (**BHI**) broth with 0.9% hexadecylpyridinium chloride monohydrate (**HPC**) and vortexed. The suspension was allowed to settle for 30 min at room temperature, after which 15 mL of the supernatant was transferred to a 50-mL polypropylene tube and then centrifuged at $1,700 \times g$ for 20 min at room temperature. The supernatant was subsequently discarded. The remaining pellet was vortexed with 30 mL of the 0.9% HPC-BHI solution and then incubated overnight at 37°C . The sample was subsequently centrifuged at $1,700 \times g$ for

20 min at room temperature. One hundred microliters of 0.9% saline solution was added to the pellet and the solution vortexed. One hundred microliters of this decontaminated, resuspended sample was inoculated onto the HEYM slant. The tube was incubated for 24 h horizontally at a slight incline at 37°C with the cap loose to allow for drying, after which the cap was secured tightly and the tube placed vertically in a rack. The tube was subsequently incubated at 37°C for 84 d, with weekly examinations under a dissecting microscope for counts until >100 cfu per tube was observed, or until growth of competitive organisms covered the medium so that no MAP colonies were distinguishable. At this point, the sample was labeled as contaminated.

Broth Culture. Broth cultures were performed using the Trek ESP culture system II (Trek/Thermo Scientific, Oakwood Village, OH) to obtain semiquantitative days to positive counts. This is a pressure detection system in which MAP growth is signaled by decreased pressure in the medium bottle. Briefly, 2 g of thawed fecal sample was placed in 35 mL of sterile water and placed on an automatic shaker for 10 min followed by 30 min of sedimentation at room temperature. Afterward, 5 mL from the top one-third of the solution was placed in 25 mL of 0.9% HPC-BHI solution and incubated overnight at 37°C. The following day, the sample was centrifuged at $1,500 \times g$ for 20 min and the supernatant discarded. One milliliter of an antibiotic brew consisting of 18.5 mg/mL BHI, 975 $\mu\text{L/mL}$ deionized water, 100 $\mu\text{g/mL}$ vancomycin, 100 $\mu\text{g/mL}$ nalidixic acid, and 50 $\mu\text{g/mL}$ amphotericin B was added to the pellet, vortexed, and again incubated overnight at 37°C. Subsequently, 2.5 mL of a solution of the kit's Growth Supplement (1 mL), Egg Yolk Supplement (1 mL), and Antibiotic Supplement (0.5 mL) was added to a Para-JEM broth bottle (Thermo Scientific, Nepean, ON, Canada) along with 1 mL of the decontaminated sample. The bottle was then incubated in the Trek incubator for a maximum of 49 d or until a positive pressure signal was detected and confirmed as described below.

Direct qPCR. Direct qPCR methods followed the procedures listed for the VetAlert Johne's Real-Time PCR kit (Tetracore, Rockville, MD), targeting the *hspX* gene. Briefly, 2 g of thawed fecal sample was added to 35 mL of sterile distilled water, vortexed, placed on an automatic shaker for 15 min, and incubated at room temperature for 30 min. The top 20 mL of the solution was placed in a new 50-mL centrifuge tube and centrifuged at $2,500 \times g$ for 10 min at room temperature. The supernatant was discarded, and 1 mL of $1 \times$ Tris-EDTA buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) was added to resuspend the pellet.

The DNA extraction was then performed as follows: 1 mL of the solution was added to a disruption tube containing sterile glass beads, vortexed, and beaten (Mini-Beater 8, BioSpec Products, Bartlesville, OK) at 4,800 rpm for 5 min. The sample was then centrifuged for 10 min at $16,000 \times g$, the supernatant transferred to a new 2-mL microcentrifuge tube, 100 μL of the kit's Nucleic Acid Buffer (NAB) added, and inverted 5 times to mix. The sample was then centrifuged at $1,200 \times g$ for 3 min and the supernatant discarded; then, 560 μL of the kit's Binding Buffer was added to resuspend the pellet, and the tube was incubated at room temperature for 10 min. Subsequently, 560 μL of 100% ethanol was added to the tube, which was then vortexed. Part of the sample (630 μL) was added to a spin column, placed in a new collection tube, and centrifuged at $5,200 \times g$ for 1 min. This same step was repeated with the remaining sample. For all the following steps involving the spin column, a new collection tube was used. The kit's Wash buffer A (500 μL) was added and centrifuged at $5,200 \times g$ for 1 min. Then, the kit's Wash buffer B (500 μL) was added and centrifuged at $12,000 \times g$ for 3 min followed by centrifugation at $16,000 \times g$ for 1 min without buffers. The spin column was then placed into a microcentrifuge tube, 50 μL of deionized water was added, and the sample was left at room temperature for 1 min. The sample was then centrifuged at $5,200 \times g$ for 1 min, and the DNA eluent saved at 4°C for up to 48 h or stored at -20°C .

For the qPCR fluorogenic probe hydrolysis assay procedure, 22.5 μL of the kit's Master mix was added to a thermocycler reaction tube. To each sample tube, 2.5 μL of the eluted DNA was added. To another 2 tubes, the same amount of positive and negative control was added, respectively. The reaction tubes were then centrifuged briefly and subsequently loaded into the Cepheid SmartCycler II Thermocycler (Cepheid, Sunnyvale, CA). After an enzyme activation step at 95°C, a 2-step cycling reaction at 95°C and 62°C was used. Results were recorded as Ct values, with values considered positive if <42 Ct; the run was considered valid if the positive control values were between 20 and 26 Ct.

Culture Confirmation. All broth and solid culture results were confirmed by positive acid-fast stain. Any acid-fast results positive by either culture method were further confirmed with qPCR. For broth culture confirmation, the bottle was removed from the Trek incubator and placed in an automatic shaker for 5 min. One milliliter from the bottle was then placed in a disruption tube containing sterile glass beads, and the procedures followed for DNA extraction and qPCR test as outlined above for the direct qPCR procedures. For solid culture confirmation, the tube of medium was removed from

the incubator, and 2 or 3 colonies were collected with a sterile loop into a sterile centrifuge tube containing 1 mL of sterile 0.9% saline and vortexed. One milliliter of this mixture was then added to the disruption tube containing sterile glass beads, and the DNA extraction and qPCR test procedures as outlined above were followed.

Statistical Analysis

Statistical analysis was performed using STATA/IC (version 12; StataCorp LP, College Station, TX). A P -value of 0.05 was chosen as a cut-off for statistical significance. Proportions of positive culture results were analyzed overall and at categorized shedding levels. For solid culture, low shedding was set to <10 cfu per culture tube, moderate shedding from 10 to 50 cfu per tube, and high shedding >50 cfu per tube (Crossley et al., 2005). For broth culture, high shedding was set to <21 d to a positive change-in-pressure signal, moderate shedding between 21 and 28 d, and low shedding >28 d (Shin et al., 2000, 2001).

Population averaged cow-level generalized estimating equation logistic models (Dohoo et al., 2009) were used to analyze whether season and lactation stage were predictive of solid culture, broth culture, or qPCR positivity. This approach, with an autoregressive within-cow correlation structure and robust standard errors, was chosen to best handle the repeated samples per cow. Separate models were built for each of the 3 dichotomous outcomes (solid culture, broth culture, qPCR). The model structure was

$$\text{logit}(p) = \text{intercept} + \text{season} \\ + \text{lactation stage} + \text{farm} + \text{age},$$

where p is the probability for a cow to be test-positive relative to the test under evaluation in that model; season is a categorical variable for the season of sample collection; lactation stage is a categorical variable for the cow's lactation stage at the time of sample collection; and farm and age are categorical variables analyzed as possible confounders and referring to the respective farm and age (yr) of the cow at the time of sample collection. Seasons were set to be July through September for summer, October through December for fall, January through March for winter, and April through June for spring to correspond to local weather patterns. Lactation stages analyzed were categorized as follows: <60 DIM, 60 to 100 DIM, 100 to 240 DIM, >240 DIM, and dry. Contrasts and linear comparisons of all significant predictors and any interactions were analyzed using Bonferroni adjustments of P -values to account for multiple comparisons.

Agreement between assays was calculated using the following tests: Cohen's kappa test for agreement beyond chance and the McNemar exact test for differences (significant outcome) between proportions of positive results from the testing methods (Dohoo et al., 2009). Sensitivity, which is the proportion of positive results in the known MAP-positive group of cows, was recorded along with a 95% CI. Conditional Se between tests was calculated, as well as parallel Se, when either or both of the tests being compared were positive (Dohoo et al., 2009).

Finally, the relationship between shedding level from each culture method and qPCR Ct values was also assessed. An additional mixed linear regression model (Dohoo et al., 2009), with random effects at the cow-level, was built using qPCR results for the continuous outcome and using an autoregressive within-cow correlation structure and robust standard errors as follows:

$$Y = \text{intercept} + \text{season} + \text{lactation stage} \\ + \text{farm} + \text{age} + u(\text{cow}) + \varepsilon,$$

where Y refers to the outcome of the positive (nonzero) qPCR Ct values; the predictors season, lactation stage, farm, and age are explained as for the previous models; and u and ε refer to the random effects. Transformations for the outcome were evaluated, but none were required for maintaining the assumption of linearity for the model. In addition, all relevant interactions for significant predictors were analyzed, as well as pairwise comparisons, using Bonferroni adjustments of P -values.

RESULTS

Descriptive Data

Samples from 51 cows from 7 farms were used for this study. Cows ranged from 2 to 9 yr of age (mean = 4.5 yr) and from first to seventh parity (mean = 2.9). In total, 395 samples were collected, with 13 of these samples from cows during their 14-d postpartum period and 28 from cows during their dry period (from the time of milking cessation to parturition). Analysis was limited to data from cows with <400 DIM (345 samples), to stay within a typical lactation length and because data were sparse above this threshold.

Sensitivity of Detection Methods

The Se for each of the 3 testing methods (solid culture, broth culture, and qPCR) for the 51 MAP-infectious cows over the full collection, postpartum, and dry periods are listed in Table 1. For solid cultures, 32 of the

Table 1. Sensitivity (%; 95% CI in parentheses if >40 observations available; no. of observations in brackets) of solid culture, broth culture, and real-time PCR (qPCR) for fecal samples collected monthly over a 12-mo period from 51 *Mycobacterium avium* ssp. *paratuberculosis* (MAP)-infectious cows from 7 herds

Item	Method ¹		
	Solid culture	Broth culture	qPCR
All samples	45.7 (40.1–51.2) [313]	54.8 (49.5–60.1) [345]	77.6 (73.2–82.0) [344]
Postpartum ²	33.3 [18]	47.6 [21]	81.0 [21]
Dry period	60.0 [11]	81.8 [11]	90.9 [11]

¹Solid = culture on Herrold's egg yolk medium slants with mycobactin J; broth = Trek ESP system (Trek/Thermo Scientific, Oakwood Village, OH); qPCR = VetAlert (Tetracore, MD) real-time PCR kit targeting the MAP-specific *hspX* gene.

²Feces collected up to 14 d postcalving.

345 samples were contaminated (9.3% contamination rate), leaving 313 samples available for analysis. For direct qPCR, 1 sample was lost, leaving 344 samples. Predicted confidence intervals are included for overall data collection, but not for the postpartum and dry periods because sample numbers were too low. For all fecal samples collected, qPCR had a significantly higher Se than either broth or solid culture. Numerically, dry-period samples had slightly higher Se than those in other periods, but numbers of samples available from dry cows was very limited.

Shedding levels from the MAP-infectious cows are listed in Table 2. Because criteria for each shedding category differ by culture method, comparisons from the data were made only within culture method rather than between culture methods. Therefore, within solid culture results, more cows shedding >50 cfu were identified, but within broth culture results, fewer cows with heavy shedding were identified, as cultures typically required >21 d for a positive signal.

Detection Patterns Across Seasons and Lactation Stages

The generalized estimating equation model analyses revealed no significant association between test Se (the probability of an average MAP-infectious cow

testing positive) and lactation stage with either culture technique. For direct qPCR, lactation stage was significantly associated ($P < 0.01$), especially with inclusion of the dry-period category, in which higher MAP detection was observed. Seasonal patterns in test results with direct qPCR were significant as an unconditional association ($P < 0.05$), but borderline significant ($P = 0.09$) when considering the confounding effects of lactation stage and farm. We found no statistically significant interactions between season and lactation stage. The probability of the test being positive in the summer and fall seasons was 85% of the probability of the test being positive in the winter and spring seasons. However, after Bonferroni adjustment of P -values due to multiple comparisons, no significant differences were detected between seasons or lactation stages despite the raw data trends, as some categories had very low sample counts. Predicted probabilities for positive qPCR results within lactation stages by season are shown in Figure 1.

For solid culture of MAP, increased failure of the decontamination procedures to eliminate competitive organisms on the medium in the summer and fall months led to more samples being labeled as contaminated during those periods. The mean contamination rate was 12.5% within the 206 samples from the summer and fall seasons, compared with 4.2% within 139 samples from

Table 2. Proportion (%; no. of observations in parentheses) of solid culture and broth culture outcomes across shedding levels for fecal samples collected monthly over a 12-mo period from 51 *Mycobacterium avium* ssp. *paratuberculosis* infectious cows from 7 herds

Culture ¹	Negative	Shedding level ²		
		Low	Moderate	Heavy
Solid	54.3 (170)	15.0 (47)	7.0 (22)	23.6 (74)
Broth	45.2 (156)	29.9 (103)	9.0 (31)	15.9 (55)

¹Solid = culture on Herrold's egg yolk medium slants with mycobactin J; broth = Trek ESP system (Trek/Thermo Scientific, Oakwood Village, OH).

²Shedding levels for solid culture were defined as low (<10 cfu per culture tube), moderate (10 to 50 cfu), high (>50 cfu) (Crossley et al., 2005). Those for broth culture were low (>28 d to a positive signal), moderate (21 to 28 d), high (<21 d) (Shin et al., 2000, 2001).

Figure 1. Predicted probability (proportion) for positive direct real-time PCR (qPCR; VetAlert, Tetracore, MD) across lactation-stage categories by season for fecal samples collected monthly over a 12-mo period from 51 *Mycobacterium avium* ssp. *paratuberculosis* infectious cows from 7 herds.

the winter and spring seasons. However, the effects of season ($P = 0.22$) and lactation stages ($P = 0.48$) were not significant for contaminated samples.

Comparison Among Detection Methods

The McNemar exact test (Table 3) indicated that in all 3 test comparison combinations, proportions of positive results obtained were significantly different between tests, with qPCR being most sensitive and solid culture being least sensitive. Despite the higher Se of broth over solid culture, the kappa coefficient between the 2 culture-based methods was approximately twice as high as that between qPCR and either culture method. Use of qPCR in parallel testing with culture

techniques did not improve Se substantially over use of qPCR alone. However, conditional Se (data not shown) of qPCR varied from 58.1% if broth culture was negative to 93.7% if broth culture was positive, whereas the Se of broth culture varied from 15.6 to 66.3% if qPCR was negative or positive, respectively.

Patterns in qPCR Values Across Shedding Levels

Mean Ct values were also noted according to the corresponding growth pattern in broth or solid medium (Table 4). For all fecal samples in which either solid or broth culture as well as qPCR results were positive, our study indicated a decreasing trend in qPCR Ct value with increasing MAP shedding. More specifically, on average, a Ct value of <35 cycles was obtained when culture results were positive, with a Ct value of <29 cycles, on average, corresponding to heavy shedding levels with both culture techniques. In addition, the mean Ct value in summer was 31.3 (95% CI: 30.0 to 32.7) compared with 33.1 (31.9 to 34.2) in fall, 33.9 (32.7 to 35.1) in winter, and 33.8 (32.4 to 35.3) in spring. The mixed linear model for positive qPCR results revealed a significant age $\times$ season interaction, but number of observations was small for some groups. Pairwise comparisons between seasons indicated that this trend was significant (using Bonferroni adjusted P -values) between summer and fall ($P < 0.01$) and between summer and winter ($P = 0.01$). All other season comparisons were nonsignificant.

DISCUSSION

As a costly production-limiting disease of adult dairy cows, Johne's disease is a major concern for dairy herds. However, current Johne's tests are inadequate to meet the dairy industry's needs for herd biosecurity and control programs. Therefore, understanding the ability to detect MAP in fecal samples with various

Table 3. Individual and parallel sensitivity comparisons and Cohen's kappa agreement between solid culture, broth culture, and real-time PCR (qPCR) for fecal samples collected monthly over a 12-mo period from 51 *Mycobacterium avium* ssp. *paratuberculosis* infectious cows from 7 herds

Test ¹			Sensitivity (%)			
1	2	No. ²	Test 1	Test 2	Parallel	Kappa
Solid	Broth	313	45.7 ^a	56.9 ^b	59.1	0.691
qPCR	Solid	312	78.8 ^a	45.8 ^b	80.1	0.321
qPCR	Broth	344	77.6 ^a	54.9 ^b	81.1	0.373

^{a,b,c}Sensitivities within a row with different superscripts differ ($P < 0.01$) using the McNemar exact test.

¹Solid = culture on Herrold's egg yolk medium slants with mycobactin J; broth = Trek ESP system (Trek/Thermo Scientific, Oakwood Village, OH); qPCR = VetAlert (Tetracore, MD) real-time PCR kit targeting the MAP-specific *hspX* gene.

²Number of observations when results from both tests were available concurrently.

Table 4. Mean (95% CI in parentheses; no. of observations in brackets) real-time PCR (qPCR) cycle threshold values corresponding to bacterial growth patterns in solid culture or broth culture for fecal samples collected monthly over a 12-mo period from 51 *Mycobacterium avium* ssp. *paratuberculosis* infectious cows from 7 herds¹

Culture	Negative	Shedding level ²		
		Low	Moderate	High
Solid	36.4 (30.0–42.7) [107]	34.5 (28.8–40.2) [44]	32.1 (27.1–37.1) [21]	26.5 (18.9–34.2) [74]
Broth	36.8 (29.7–43.9) [90]	34.3 (28.8–39.9) [94]	29.2 (20.7–37.7) [30]	26.6 (17.3–36.0) [53]

¹Solid = culture on Herrold's egg yolk medium slants with mycobactin J; broth = Trek ESP system (Trek/Thermo Scientific, Oakwood Village, OH); qPCR = VetAlert (Tetracore, MD) real-time PCR kit targeting the MAP-specific *hspX* gene.

²Shedding levels for solid culture were defined as low (<10 cfu per culture tube), moderate (10 to 50 cfu), high (>50 cfu) (Crossley et al., 2005). Those for broth culture were low (>28 d to a positive signal), moderate (21 to 28 d), high (<21 d) (Shin et al., 2000, 2001).

testing methods as well as patterns in MAP shedding can lead to efficient testing of animals for enhanced control programs.

Sensitivity of Detection Methods

The results of our study support the use of direct fecal qPCR as part of a Johne's herd management program, particularly for known infected herds. Data analysis for pathogen detection revealed a higher Se for qPCR compared with broth culture, with an even greater difference from solid culture, for fecal samples from MAP-infectious cows. This was in contrast to Alinovi et al. (2009), who previously reported qPCR (Tetracore) sensitivity of 60% and specificity of 97%. However, they reported qPCR accuracy (90%) comparable to the accuracy of solid (HEYM; 91%) and broth (Trek; 93%) culture results. The pattern observed in our data was most apparent in the qPCR results for feces collected during a cow's dry period and postcalving period. However, the numbers of observations during these periods are relatively small, reducing the statistical power of these analyses. Therefore, additional research with larger sample sizes is recommended to confirm the apparent results in these periods.

Detection Patterns Across Seasons and Lactation Stages

An advantage to qPCR that may explain the improved Se is the absence of decontamination steps that could decrease low numbers of bacteria in a sample and hinder detection via culture techniques (Bölske and Herthnek, 2010). In addition, qPCR is able to detect MAP regardless of the presence of competing organisms, because it detects MAP by a different biological principle (genetically) compared with culture methods. However, the presence of competing organisms can occasionally hinder the interpretation of culture results. Generally, a 5 to 15% contamination rate for fecal solid culture is expected (Collins and Manning, 2014). In our

data, solid culture of MAP in feces showed a pattern of increased failure of the decontamination procedures to eliminate competitive organisms on the medium, leading to more samples potentially being classified as contaminated in summer and fall (12.5%) than in winter and spring (4.2%). However, this rate can vary widely depending on the decontamination method used, type of medium, and the number of slants per sample (Whittington, 2010). Our overall contamination rate of 9.3% was within these bounds, as were the seasonal rates. However, overall low numbers of contaminated samples may have led to the decrease in statistical power to report the seasonal effect. Using qPCR, particularly in summer and fall, may eliminate the need for repetitive cultures.

In our data, apparent patterns across seasons and lactation stages were observed for qPCR overall. Seasons with more similar climatic temperatures were also more similar in qPCR detection ability. In general, Se was higher in winter and spring, and Se was improved in the dry period. However, because of low numbers of observations within some of the categories for season and particularly lactation stage, statistically significant associations were not observed when accounting for multiple comparisons.

Although we did not find an association between season and culture detection of MAP, the association between season and qPCR MAP detection agreed with other studies looking at the effect of season on MAP growth in culture. Norton et al. (2010) observed higher MAP recovery in spring and indicated a potential correlation between season and lactation stage, particularly in seasonal calving. Crossley et al. (2005) reported a higher count (cfu in HEYM) during the winter months and in larger herd sizes. The authors of that study suggest that stressors could trigger increased MAP shedding in winter, including adverse weather conditions (Jørgensen, 1977), winter calving, herd management, and body condition. Although that study assessed culture detection of MAP, it is likely that some of these stressors could also explain the patterns seen within

our data. However, differing protocols make comparisons between this and other research studies difficult because of the varying definitions of herd or cow MAP status, MAP prevalence, test and decontamination protocols, sampling protocols, and herd sizes, among others. Therefore, further research is needed to assess seasonal patterns over a period of several years with more herds to increase statistical power. In addition to this, other important factors to analyze regarding MAP fecal shedding include the effect of housing, dietary changes, and herd management practices that expose youngstock to the manure of adult cattle, from farms of various levels of MAP prevalence and from cows within the dry and postpartum periods of lactation.

Comparison Among Detection Methods

The use of qPCR is based on a different biological principle (detection of the organism's genetic material) than culture methods. Therefore, Pinedo et al. (2008) suggest that parallel testing with both PCR and serum ELISA results in the best detection ability over other combinations of culture, PCR, and serum ELISA assays. Because of this, we were interested to determine whether the higher qPCR Se in our study was based on the detection of a different population of positive animals or better overall detection. To assess this, we examined parallel interpretation of the test with either culture method. Parallel use did not improve Se substantially over qPCR alone, indicating that qPCR was most sensitive by identifying MAP in additional cows to those detected with culture assays. The kappa value also indicated lesser agreement between qPCR and culture but greater agreement between culture methods.

Patterns in qPCR Values Across Shedding Levels

Comparison between solid and broth culture regarding degree of shedding is difficult, as criteria for each shedding category differ by culture method. Because qPCR Se was improved over culture Se in our data, especially in the dry period, it was also of interest to determine if a pattern in Ct ranges could be observed across shedding levels. As a benefit to control programs, the risk of environmental contamination and subsequent infection to calves could be indirectly calculated using qPCR Ct values as an indicator of the degree of fecal MAP shedding in dams without simultaneous culture testing. Our results indicated that if the sample yielded a positive broth or solid culture result, qPCR values were typically <35.0 Ct. Moderate and heavy shedding cows, as determined for each culture technique (>10 cfu for solid culture or <28 d to positive for broth culture) typically corresponded to <29.0 Ct.

In a recent study by Leite et al. (2013), a comparison was made between several different qPCR kits and gene sequences for MAP, including the Tetracore kit for both *IS900* and *hspX*. The authors found that qPCR Se and specificity depended on which kit was used and thereby the volume of DNA elution and which gene sequence was identified. For the Tetracore kit identifying the *hspX* gene, they found an Se of 57.1% for samples growing <10 cfu per tube, and these low shedders on average were detected with a Ct value of 36.2 (range 33.5 to 38.4). Heavy shedding, which they identified as >100 cfu per tube, had an Se of 100% for qPCR with an average Ct of 29.9 (range 26.5 to 35.1). Although in our study, we defined a heavy shedder as having >50 cfu per culture tube (Crossley et al., 2005), we did not find any difference in mean Ct value between the 74 observations >50 cfu and the 64 observations >100 cfu (26.5 Ct).

CONCLUSIONS

The primary objectives of our study were to assess the abilities of common fecal testing assays to accurately diagnose MAP-infectious cows and identify any patterns across seasons or lactation stages. Direct fecal qPCR is an invaluable MAP identification test, with greater sensitivity, faster processing times, and lower laboratory costs compared with culture methods. Our study highlighted a potential seasonal pattern with increased qPCR Se in winter and spring, as well as a benefit to using qPCR in months when culture contamination is more likely. Consideration of Ct values further extends the application of qPCR within MAP control programs, because lower Ct values are indicative of greater MAP bacterial load in a cow's feces. This approach allows prioritized management of high-shedding cows and could ultimately increase the success of herd MAP control programs.

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