Comparison of commercial DNA extraction kits and quantitative PCR systems for better sensitivity in detecting the causative agent of paratuberculosis in dairy cow fecal samples

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ABSTRACT

Mycobacterium avium ssp. *paratuberculosis* (MAP) causes ruminant paratuberculosis (Johne's disease) worldwide. Oral-fecal contamination is the most important mode of transmission of paratuberculosis, so eradicating MAP-shedding animals could prevent disease propagation. Fecal culture, a well-known method for MAP diagnosis, requires costly specialized media and a long incubation time that sometimes ends in disappointing bacterial contamination. To facilitate the efforts of control programs, we evaluated the performance of direct fecal quantitative PCR (qPCR) assays for their sensitivity and robustness for MAP detection. Commercial kits use different strategies for extracting DNA, combined with qPCR systems, to detect the presence of MAP in fecal samples. In this study, we compared the sensitivity of 3 commercially available DNA extraction kits (A, B, and C) combined with 2 qPCR systems (T and V) for the detection of MAP in infectious cows. A total of 49 dairy cows from 5 herds were sampled twice a year for 3 yr and diagnosed using fecal culture and ELISA. Eight replicates of their fecal samples from the first sampling were tested using each DNA extraction method and qPCR detection system. Although all 3 of the commercial DNA extraction kits have been previously described as very efficient for the diagnosis of paratuberculosis, kit B provided the highest sensitivity. Indeed, 89% of the cows declared positive for paratuberculosis by both fecal culture and ELISA were identified with kit B, whereas only 23 and 43% of the cows were identified with kits A and C, respectively. Interestingly, kit B was able to detect some low-MAP shedders. The qPCR detection system also played a critical role: system T yielded qPCR with the highest sensitivity. The results of this study suggest that DNA extraction kit B

combined with detection system T provides the best amplification of MAP DNA from fecal samples with the highest sensitivity and specificity. Although 1 DNA extraction and qPCR analysis should be adequate to confirm that an animal with diarrhea or other signs of paratuberculosis is positive, detecting low shedders at the highest sensitivity should include repetitive testing. This study demonstrates the importance of repetitions using the most appropriate method for extracting DNA from fecal samples, combined with a compatible qPCR system for identifying MAP-shedding animals.

Key words: bovine paratuberculosis, diagnosis, quantitative PCR, fecal DNA extraction kit

INTRODUCTION

Mycobacterium avium ssp. *paratuberculosis* (MAP) is the pathogen responsible for paratuberculosis (Johne's disease) worldwide. Paratuberculosis has been described mainly in ruminants, although many species have been infected (Beard et al., 2001; Kennedy and Benedictus, 2001), and it is characterized by a slow progression to chronic granulomatous enteritis (Clarke, 1997; Stabel, 1998). During the subclinical stage, which lasts 4 to 6 yr, animals show almost no signs of the disease, but intermittent shedding of MAP in feces may occur. As the disease progresses, various symptoms appear, including chronic diarrhea, loss of milk production, decreased fertility, and even mastitis (Chiodini et al., 1984; Gonda et al., 2007). No treatment is currently available for paratuberculosis (Whan et al., 2001; Fecteau and Whitlock, 2011).

The increasing prevalence of infected animals as a result of modern farming has elevated bovine paratuberculosis to a global concern. Johne's disease is responsible for great economic losses in the livestock industry. In Canada, \$15 million to \$90 million is lost each year (Chi et al., 2002; McKenna et al., 2006). Oral-fecal contamination is the most important mode of paratuberculosis transmission, so eradicating MAP-

Received April 30, 2016.

Accepted October 4, 2016.

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shedding animals could prevent disease propagation. Detecting MAP in herds is therefore of the utmost importance, but precise diagnosis remains unsatisfactory and represents a limiting factor in preventing the disease and eradicating MAP.

Common methods for the detection of MAP include ELISA and fecal culture. Faster and cheaper than fecal culture, ELISA has high specificity but lack sensitivity (Whitlock et al., 2000; Dargatz et al., 2001; Salgado et al., 2007). Historically, Johne's disease was diagnosed in cattle mainly by direct culture of MAP from feces (Collins, 1996; Paolicchii et al., 2003; Clark et al., 2008). Unfortunately, this reference method is labor-intensive and often compromised by bacterial contamination. For this reason, it is gradually being replaced by molecular approaches. Analysis by PCR has been introduced as an alternative to compensate for the cost, time consumption, and lack of sensitivity in diagnosis (Taddei et al., 2004; Bögli-Stuber et al., 2005; Herthnek and Bolske, 2006). For this molecular approach, the DNA extraction method must be robust and efficient, and the detection system must be sensitive and specific to limit false-negative results.

Fecal matter is generally considered a difficult sample type for the molecular detection of microbes, because of the presence of PCR inhibitors and the abundance of other microorganisms, which dilute the presence of MAP genome equivalents (**Ge**) in the DNA extract. A wide selection of commercial kits is available for MAP detection, and it is reasonable to expect that their efficacy could differ. Different studies have shown that commercial DNA extraction methods, which include different sample treatments, greatly affect PCR results (Monteiro et al., 1997; Kabir, 2004) in terms of the sensitivity and specificity of MAP detection (Chui et al., 2004; Amaro et al., 2008; Leite et al., 2013; Radomski et al., 2013). Concerning the specificity of the assay, several molecular reference markers for MAP detection have been described. In the present study, we compared the sensitivity of 3 commercial DNA extraction kits using 2 different MAP-specific probes, *HspX* and *ISMAP02*. The purpose of this study was to evaluate combinations of different commercial systems for MAP detection of in artificially spiked versus naturally infected fecal samples from dairy cattle.

MATERIALS AND METHODS

All animal procedures were carried out according to the Canadian Council on Animal Care guidelines for institutional animal use, and ethical approval for the study was obtained from the Agriculture and Agri-Food Canada Animal Ethics Committee (protocol 362).

Sampling

Fecal and blood samples were collected every 6 mo between December 2013 and December 2014 from Holstein cows ($n = 528$) in 5 commercial tie-stall-housed dairy herds. The selected animals were lactating cows that were more than 24 mo old at first sampling. Fecal samples were taken per rectum, and a clean glove was used for each cow. Because MAP is better preserved at -80°C (Raizman et al., 2011), samples were kept cold at 4°C for a maximum of 24 h until they were aliquoted and then stored at -80°C as recommended (Whitlock and Rosenberger, 1990; Khare et al., 2008). Concurrent individual blood samples were also collected in dry tubes for serum collection (BD Biosciences, Mississauga, ON, Canada). Within a few hours of sampling, the tubes were centrifuged at $1,800 \times g$ at 4°C for 15 min, and sera were collected and then stored at -80°C until analysis by ELISA. We used a selection of 49 lactating cows for which we had 3 paired data sets and that were not culled during the sampling period to compare the extraction kits.

Diagnosis

Fecal samples from the first and last sampling ($n = 1,056$) were sent to the Laboratoire d'épidémiologie animale du Québec (Saint-Hyacinthe, QC, Canada). Aliquots were kept frozen at -80°C until diagnosis was made. Isolation of MAP was achieved using BD MGIT ParaTB culture medium and the Bactec MGIT 960 system, as described previously (Arango-Sabogal et al., 2016). We performed an acid-fast bacilli stain using the TB Fluorescent Stain Kit M (Fisher Scientific, Ottawa, ON, Canada). Positive samples were confirmed by real-time PCR [TaqMan MAP (Johne's) Reagents; Applied Biosystems, Foster City, CA]. Serum samples from all individuals were processed using the Idexx Pourquier MAP antibody test kit (Idexx Laboratories Inc., Westbrook, ME) according to the manufacturer's instructions (https://www.idexx.com/pdf/en_us/livestock-poultry/map-ab-test-protocol.pdf). Optical density values were transformed to a sample-to-positive ratio, as described previously (Collins, 2002). Samples with a ratio $\geq 55\%$ were considered positive. In parallel, a panel of samples from 6 animals in each of the shedding groups—that is, animals shedding high, moderate, or low amounts of MAP—was confirmed by culture, as described previously (Whitlock and Rosenberger, 1990). Three DNA extraction kits were tested on the high shedders (>100 cfu per tube), moderate shedders (10–100 cfu per tube), and low shedders (<10 cfu per tube) for comparison. The status of the cows was considered paratuberculosis-positive when

detection by fecal culture or ELISA was positive in at least 1 of the sampling periods.

DNA Extraction

We tested 3 commercially available kits for sample processing (A, B, and C) that had previously been studied by Dr. Stabel's group (who referred to them as kits A, E, and F; Leite et al., 2013). We tested them in 49 cows from different herds that contained negative and positive cows. We compared the sensitivity of MAP detection in the extracted samples using the same kit-specific PCR. All 3 methods included a bead-beating step, and only kits A and B included lysis buffer. For DNA binding, kit A used paramagnetic beads, whereas kits B and C used a column-based technology. Kit A was the certified MagMAX Total Nucleic Isolation Kit (Applied Biosystems) and was carried out using the MagMAX Express Magnetic Particle Processor (Applied Biosystems) according to the manufacturer's instructions (https://tools.thermofisher.com/content/sfs/manuals/fm_1840.pdf). Kit B was the ZR Fecal DNA MiniPrep (Zymo Research Corp., Irvine, CA). Kit C was the MAP Extraction System with the Two Grams Protocol (Tetracore Inc., Rockville, MD) for maximum sensitivity as recommended by the manufacturer (<http://tetracore.com/real-time-pcr-detection/johnes-disease/info/PLM-0048-4MAPEXtractionSystem.pdf>). The manufacturer of kit C also sells PCR reagents for MAP detection [quantitative PCR (qPCR) detection system V]. Elution was done with 50 μ L for kits A and C, but with 100 μ L for kit B, as recommended by the manufacturer. Extraction was done following the manufacturers' recommendations and protocols (<http://www.zymoresearch.com/downloads/dl/file/id/93/d6012i.pdf>). To analyze sample-to-sample variation and determine the sensitivity of the extraction system, each fecal sample from the 49 cows was extracted 8 times with the respective commercial kits, for a total of 392 extractions per kit. The protocols followed were based on the manufacturers' instructions. Briefly, all 3 kits mechanically disrupted the bacteria using bead beating. The concentration and purity of the 1,176 DNA extracts were quantified in each sample using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE), and the extracts were stored at -80°C until qPCR analysis. We assessed the integrity of DNA using agarose gel electrophoresis.

qPCR Analysis

Fecal extracts from the 49 cows were extracted 8 times with the 3 DNA kits (total of 1,176 extracts) and were analyzed using the 2 qPCR systems (sys-

tem V and system T), in parallel. The target gene for system V (VetAlert Johnes's Real-Time PCR; Tetracore Inc.) is *HspX*, and the target gene for system T (Taq-Man MAP Johnes's Reagents; Life Technologies, Foster City, CA) is *ISMAP02*. Both targets are specific for MAP (Ellingson et al., 1998; Stabel and Bannantine, 2005). We performed qPCR following each manufacturer's recommendations (https://tools.thermofisher.com/content/sfs/manuals/cms_086496.pdf), using 2.5 or 8 μ L of each extracted sample for systems V or T, respectively. For system V, the PCR program was 10 min at 95°C , and then 45 cycles of denaturation at 95°C for 15 s and annealing/extension at 62°C for 1 min. For system T, the PCR program was 2 min at 50°C and then heating at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and then 60°C for 45 s for annealing and extension. We prepared positive controls using DNA extracted from pure MAP strains. To compare the 2 detection systems, we increased the number of cycles for analysis with qPCR system T to 45. A test was considered positive with at least 1 cycle threshold (Ct) value <38 .

Detection Limit

We evaluated the detection limit using theoretical considerations as well as experimental evaluations. The theoretical detection limit was defined according to the manufacturer's protocol for each of the 3 DNA extraction kits. The procedures included different dilution steps and used different fractions of the working solution in their respective strategies, which are summarized in Table 1. To define the experimental detection limit of each kit, we used fecal samples from a healthy 6-yr-old cow from a paratuberculosis-free herd that had tested negative by ELISA and bacterial culture over a 2-yr period. The samples were spiked with different amounts of living MAP (strain 39382), ranging from 10 to 1 million bacteria per gram of fecal material. We used optical density to quantify MAP, because PCR and turbidity correlate well (Elguezabal et al., 2011). We measured the absorbance ($A_{600\text{nm}}$) of the bacterial suspension every week to assess bacterial growth. After 10 wk, cells were harvested, washed with sterile PBS (pH 7.4), and then resuspended in PBS. The suspension was passed through a 26-gauge needle to disperse clumps, and MAP concentration was evaluated by optical density as described elsewhere (Janagama et al., 2006; Shin et al., 2007; Zhu et al., 2008; Kralik et al., 2012; Plain et al., 2014). Spiked samples were then processed using each DNA extraction kit and analyzed by qPCR using detection kits T and V. In addition to our analysis of artificially spiked samples, we also studied feces from naturally infected cows. Fecal samples

Table 1. Comparison of the 3 DNA extraction kits (A, B, C) evaluated by quantitative PCR (qPCR) in feces artificially spiked with *Mycobacterium avium* ssp. *paratuberculosis* (MAP)¹

Fecal sample spiked with MAP (cfu/g)	qPCR system V			qPCR system T		
	A	B	C	A	B	C
No MAP	ND	ND	ND	ND	ND	ND
50	ND	ND	ND	ND	ND	ND
500	ND	ND	ND	39.21 (0.07) ^a	38.08 (0.74) ^a	36.86 (1.61) ^a
5,000	ND	ND	40.00 (0.36)	37.83 (2.26) ^a	35.03 (1.28) ^a	32.44 (0.15) ^a
50,000	43.47 (0.71) ^a	38.19 (0.96) ^b	37.87 (2.26) ^b	36.03 (0.58) ^a	28.25 (0.29) ^b	27.94 (0.09) ^b

^{a,b}Mean cycle threshold values within a row with different superscripts differ ($P < 0.05$).

¹The coefficient of variation is shown in parentheses. ND = not detectable.

from 6 high-, medium-, and low-shedding cows were extracted with the 3 extraction kits and compared by qPCR using either detection kit T or V (with assays performed in triplicate) to assess their practical sensitivity.

We estimated Ge in the suspensions of MAP by calculating the molecular weight of MAP described previously (Li et al., 2005). The concentration of MAP in the suspension from 5 different strains isolated from clinical cows (gift from Jeroen De Buck, University of Calgary, Calgary, AB, Canada) was confirmed by both culture (direct evaluation) and qPCR. Standard curves using serial dilutions of MAP DNA revealed the expected Ct values for different amounts of MAP Ge (Figure 1). The concentration of the working MAP suspension with an optical density of 0.7 at 600 nm was confirmed to be 2×10^9 to 2.5×10^9 cfu/mL. We evaluated genomic DNA and DNA quality using NanoDrop spectrophotometry, and the detection limit of qPCR systems T and V using serial dilutions.

Statistical Analysis

Results are expressed as means $\pm$ standard deviations. Significant differences between the kits were calculated using the GLIMMIX procedure for generalization of our results in a linear model, followed by a Tukey's test. Values of $P < 0.05$ were considered significant.

RESULTS

Sensitivity of MAP Detection in Spiked Feces

Extractions were performed using feces that had been artificially contaminated with a specific number of MAP units, from 50 to 50,000 cfu/g of feces. The DNA extracted with the different kits were then analyzed in combination with qPCR system V or T. Overall, MAP detection was better using system T rather than system V (Table 1). Coupled with system V, kit C performed the best with artificially infected samples,

detecting feces spiked with 5,000 cfu/g, but it showed late Ct values of 40. At 50,000 cfu/g, all kits permitted MAP detection in combination with qPCR system V, but sensitivity differed greatly: kits B and C presented similar Ct values of 38.19 ± 0.96 and 37.87 ± 2.26 , respectively, whereas the sensitivity of kit A was poor, with very late PCR detection (43.47 ± 0.71). With qPCR system V, the average performance of kits B and C was 38 and 49 times better, respectively, than that of kit A, based on a 2-fold PCR amplification. When the DNA extraction kits were used with system T, their sensitivity was improved. Kits A, B, and C could detect MAP in feces spiked with 500 cfu/g. At this level of spiking, we observed no significant difference in Ct values, but kit C would have appeared better if not for the larger variation among replicates (Table 1). At 50,000 cfu/g, a marked gap appeared between kit A and kits B and C (220- and 273-fold differences, respectively). At 50 cfu/g, the signal was lost, and none of the kits permitted MAP detection.

Sensitivity of the Extraction Kits in Natural MAP Shedders

We assessed the detection of cows shedding MAP at different levels using the 3 DNA extraction kits combined with qPCR system V or T (Figure 2). In high-shedding cows, MAP was detected by all DNA extraction kits coupled with system V or T. We obtained Ct values that were significantly lower, by 8 to 10, using system T. The performance of kits B and C was similar; kit A reported the lowest sensitivity using both detection systems. In medium shedders, system T also showed the best qPCR sensitivity, detecting MAP in all 6 cow replicates (6/6) using kits A and B, with mean Ct values of 35.86 and 31.91, respectively. For kit C, however, only system V could detect moderate shedders, and only at high Ct values. System V performed better (6/6) with its companion extraction kit C; detection was positive for only 1/6 replicates with kit A and 4/6 with kit B. Even though system V detected MAP, its

sensitivity was very low, with Ct values close to 40. In low shedders, system V could not detect MAP in any of the kit extracts. When kit A was used to detect MAP in the low-shedding cows, we observed no signal at all. Overall, we observed no detection in low shedders using system V. In contrast, combining kit C with system T and kit B with system T permitted the detection of 1/6 and 3/6 replicates, with mean Ct values of 38.29 and 38.16, respectively.

Range of PCR Sensitivity Analysis

We evaluated the number of Ge of detectable MAP using qPCR system T. We performed the extraction of DNA from different MAP strains (A1-42, A1-75, A1-

76, A1-92, and A1-139; Figure 1), and we evaluated the capacity to detect MAP by qPCR using kit T with serial dilutions ranging from 0.001 to 10 pg of genomic DNA. We then recorded the Ct values and associated them with their corresponding number of MAP genome copies based on the molecular weight evaluated at 3.7×10^9 . All strains except A1-75 presented similar results in terms of minimal Ct values for detecting 1 genome, with average Ct values ranging from 35.10 to 36.88 for 0.005 pg of MAP. The detection of strain A1-75 always resulted in a difference of 2 in Ct values compared with the slopes of the other strains. Interestingly, this isolated strain is the bison type (Ahlstrom et al., 2016). Repeated detections of the serial dilution points were 100% successful for all strains down to 1 MAP genome

Figure 1. Detection of different strains of *Mycobacterium avium* ssp. *paratuberculosis* (MAP) by quantitative PCR (qPCR). Bacterial genomic DNA was extracted from 5 strains of MAP (A1-042, A1-075, A-076, A1-092, and A1-139). Serial dilutions of known amounts of DNA corresponding to known quantities of genome equivalents (Ge) were quantified by qPCR. Each sample quantification was repeated 6 times. Detection rates are presented as percentages.

Figure 2. Detection of *Mycobacterium avium* ssp. *paratuberculosis* (MAP) by quantitative PCR (qPCR; systems V and T) in feces extracted with different commercial kits (A, B, C) from cows grouped according to their shedding level. Samples from 6 different animals that were shedding different levels of MAP were grouped as follows: high shedders (>100 cfu per tube), medium shedders (10–100 cfu per tube), and low shedders (<10 cfu per tube). Detections were repeated 6 times for each sample. When replicates did not permit positive detection, the number of positive tests is indicated. ND = not detectable (used when no detection was reported for all replicates). Error bars represent standard errors.

copy. At 0.2 genome copy, we observed 50% sample detection. The signal was lost below that level (not detectable; Figure 1).

Comparison of DNA Extraction Kits in Commercial Herds

We used the 3 DNA extraction kits in combination with qPCR system T on 49 cows from different herds. These cows had been previously diagnosed by fecal culture and ELISA and classified based on their results: positive only by fecal culture, positive only by serum ELISA, or double-positive (positive by both tests). We tested 8 aliquots of the same feces sample from each cow and recorded the number of positive extracts for the respective DNA extraction kit (Table 2). We then classified the cows according to the score obtained in the 8 repeated detections. Kits A, B, and C detected MAP in the double-positive group (7 cows) at rates of 43%, 100%, and 29%, respectively. It should be noted that the 2 double-positive cows detected with kit C had only 2/8 and 1/8 positive PCR replicates, respectively. In the fecal-culture-positive group (2 cows negative by serum ELISA), the detection rates of kits A and C were null; kit B generated a steady diagnosis for 1 of

the 2 cows (8 positive repetitions) but no detection for the second cow. Detections made in the ELISA-positive group were less successful for kits A and C: only 1 of the 15 ELISA-positive cows was detected in more than half of the repetitions for each kit, with both scores being 7/8. Kit A could detect a second cow, but with low sensitivity (2/8), and that kit's overall success rate was 14%, representing 2 of 15 cows. Kit B performed well by diagnosing 7 cows (47%), with more than half of the repetitions detected. It should be noted that from the 14 cows that were negative by both fecal culture and ELISA in the first sampling, we obtained at least 1 positive result in further sampling a few months later. This "false-negative" group of cows was distinguished from "healthy" cows at first sampling and can be found in Table 2 under the "false negative" group (fecal-culture-negative and ELISA-negative). The highest detection rate was obtained with kit B, at 29% detection. Concerning these subclinical cows, kit C detected only 1 cow with low sensitivity (2/8); kit B was more sensitive (8/8) for detecting this cow. Kit A failed to detect any subclinical cows. With respect to the 11 healthy cows, no kit had any detection for this group. Overall, kit B performed better than the others. Of the 34 cows found positive by fecal culture, serum ELISA, or both

Table 2. Evaluation of paratuberculosis diagnosis by quantitative PCR in commercial herds using different DNA extraction methods¹

DNA extraction method	No. of replicates that tested positive ²	Double-positive (7)	Fecal-culture positive (2)	ELISA-positive (15)	Fecal-culture-negative and ELISA-negative (14)	Healthy (11)
Kit A	0/8	4 (57%)	2 (100%)	13 (86%)	14 (100%)	11 (100%)
	<4/8	0	0	1 (7%)	0	0
	>4/8	3 (43%)	0	1 (7%)	0	0
	Total detection	43%	0%	14%	0%	0%
Kit B	0/8	0	1 (50%)	8 (53%)	10 (71%)	11 (100%)
	<4/8	0	0	3 (20%)	3 (22%)	0
	>4/8	7 (100%)	1 (50%)	4 (27%)	1 (7%)	0
	Total detection	100%	50%	47%	29%	0%
Kit C	0/8	5 (71%)	2 (100%)	14 (94%)	13 (92%)	11 (100%)
	<4/8	2 (29%)	0	0	1 (7%)	0
	>4/8	0	0	1 (6%)	0	0
	Total detection	29%	0%	6%	7%	0%

¹Cows found positive by both fecal culture and serum ELISA were called “double-positive.” Cows found positive by culture only were called “fecal-culture positive”; these cows had negative results by serum ELISA at the first sampling. The cows in the ELISA-positive group had no positive fecal culture results.

²Each sample was analyzed in 8 repetitions; results are grouped according to the number of replicates that tested positive in each category.

at first sampling, 12 were found positive by qPCR (system T) over more than 4/8 replicates, meaning that kit B detected significantly more positive cows than kit A ($P < 0.05$) or kit C ($P < 0.01$).

DISCUSSION

We evaluated 3 fecal DNA extraction procedures coupled with 2 qPCR systems for the identification of subclinical paratuberculosis in cows from herds with low prevalence. Our results contrast with the theoretical detection limit of each kit (Table 3). Kit C should have outperformed the other kits because of the larger

amount of feces used in its protocol, considering the theoretical number of bacteria that could likely be found in the volume required for 1 qPCR assay. At first glance, the theoretical performance of kit C appears better, given that kit C should be able to obtain 1 MAP Ge per PCR reaction from a sample containing 20 cfu/g of feces, whereas fecal samples extracted using kit A or B would require almost 500 or 200 cfu/g, respectively, to reach a similar Ge sensitivity. However, 50% of the 24 cows identified as positive by serum or fecal culture or both at the first sampling were detected in more than half of the repetitions (>4/8) by kit B, compared with 17% by kit A and 4% by kit C (Table 2).

Table 3. Theoretical sensitivity (in genome equivalents) of the extraction techniques¹

Artificially spiked feces (cfu/g)	MAP extraction kit A		MAP extraction kit B		MAP extraction kit C	
	In total extract (50 µL)	In qPCR rxt (8 µL) ²	In total extract (100 µL)	In qPCR rxt (8 µL) ²	In total extract (50 µL)	In qPCR rxt (2.5 µL) ³
0	0.0	0.0	0.0	0.0	0.0	0.0
10	0.1	0.0	0.7	0.1	10.8	0.5
100	1.2	0.2	6.7	0.5	108.1	5.4
1,000	11.6	1.9	66.7	5.3	1,081.1	54.1
10,000	116.1	18.6	666.7	53.3	10,810.8	540.5
100,000	1,161.1	185.8	6,666.7	533.3	108,108.1	5,405.4
1,000,000	11,610.6	1,857.7	66,666.7	5,333.3	1,081,081.1	54,054.1

¹According to the manufacturer’s protocol, a fraction of the initial volume of fecal sample is processed and eluted in a final recommended volume specified in the protocol. The volume equivalence of the fecal material is evaluated to 1 mL/g of feces. The final dilution factor of the respective kits was evaluated as suggested to 0.039 for kit A, 0.444 for kit B, and 0.541 for kit C. MAP = *Mycobacterium avium* ssp. *paratuberculosis*; qPCR = quantitative PCR; rxt = reaction.

²DNA extraction kits A and B were associated with qPCR system T. The volume used with this method was 8 µL.

³The performance (sensitivity) of DNA extraction kit C was calculated with its companion qPCR system using 2.5 µL. Because detection was totally lost using 8 µL of the extract’s kit C (data not shown), it was nonsensical to report the theoretical yield with 8 µL. The theoretical yield was calculated using 2.5 µL, the volume used in kit C’s companion qPCR system, system V.

These DNA extraction kits had been tested previously by another group (Leite et al., 2013). In that study, kit C also performed the most poorly.

These results also differed from those of the spiked samples, for which differences were not significant for low-MAP samples (500 cfu/g) (Table 1). The reasons for the discordant results from the spiked and naturally infected samples, particularly with kit C, are not clear. It is possible that decantation in the pretreatment step for fecal matter affects sensitivity. The MAP bacterium may be bound within the naturally infected fecal matter in a structure that is decanted more easily than dispersed MAP in spiked samples. Clumps of MAP are largely reduced when the MAP culture is passed through the needle to measure the turbidity of the bacterial suspension and to determine its concentration before spiking. Although the 3 DNA isolation kits use bead beating for cell disruption, a method previously described as improving MAP detection (Odumeru et al., 2001), decantation may reduce sensitivity with naturally infected feces, unlike with spiked samples. The kit B procedure does not have a decantation step, instead using direct bead beating of the fecal matter followed by column-based DNA purification, which explains the success of this strategy. Combined with qPCR system T, which uses the multicopy *ISMAP02*-specific target, kit B provided the highest sensitivity and perfect specificity. The detection limit with system T was confirmed using 0.2 to 1 Ge, corresponding to 0.0005 to 0.001 pg of MAP, and was found to be approximately 37 to 39 Ct (Figure 1); that DNA quantity is in keeping with the quantity reported in another study (Plain et al., 2014). In the artificially spiked feces, all 3 DNA extraction kits coupled with system T performed equivalently in the detection of MAP at a signal corresponding to the detection limit by real-time PCR (approximately 37 to 39 Ct).

As reported by Leite et al. (2013), better DNA yield does not correlate with better detection. Although fecal samples extracted with kit C gave the highest DNA concentrations, ranging from about 350 to 470 ng/ μ L (Supplementary Table S1; <https://doi.org/10.3168/jds.2016-11384>), kit C performed poorly in low shedders (Figure 2) and infectious cows (Table 2). This lack of sensitivity may not be attributable to the inhibitory effect of high amounts of DNA, because inhibition in real-time PCR is observed with more than 10 μ g of DNA (Radomski et al., 2013). The poor performance of kit C could be explained by the low DNA integrity on gel electrophoresis (Supplementary Figure S1; <https://doi.org/10.3168/jds.2016-11384>), or by the presence of PCR inhibitors, given that this is a problem usually associated with fecal material (Monteiro et al., 1997; Thornton and Passen, 2004). Fecal suspensions are of-

ten diluted in most protocols to decrease the concentrations of PCR inhibitors, but this strategy undeniably reduces PCR sensitivity. An easy way to estimate the presence of inhibitors is to run dilutions of the sample. A series of 1/10 dilutions of the spiked fecal samples revealed that the expected 3.3-Ct difference between the crude 2.5- μ L samples and the 1/10 samples was reduced to 1 Ct with both systems T and V. This finding suggests the presence of PCR inhibitors that were not found with dilutions of the kit A or B extracts (data not shown). Even though the protocol for kit C starts with 2 g of feces, this kit's performance was unsatisfactory, because only 22% (2/9) of the fecal-culture-positive cows were detected in these naturally infected samples.

The sensitivity of kit A for artificially spiked feces was consistent with the theoretical value, with a detection limit of 500 cfu/g (Table 1). Indeed, the theoretical MAP load of the starting feces that gives a Ct value of almost 38, which corresponds to a minute amount or fraction of Ge (Figure 1), ranged from 100 to 1,000 cfu/g. Thus, the theoretical sensitivity and the observed detection limit from artificially spiked samples using kit A were concordant. However, the performance of this kit with naturally infected samples was very poor, given that it detected only 33% of the fecal-culture-positive samples (3/9). In addition, the magnetic bead disruption with kit A also provided the poorest Ct values, detected only 1/6 moderate shedders, and failed to detect low shedders (Figure 2). Even though the best DNA quality was achieved using a magnetic bead purification system, the beads may limit the amount of DNA that magnetically bond, a situation that is unfavorable for low-MAP-abundant samples. To obtain additional information, we evaluated the performance of the extraction kits with a range of different amounts of MAP in spiked samples. The Ct gap between the 10-fold different concentrations should be 3.3, but was almost 1 for kit A (39.21 ± 0.07 vs. 37.83 ± 2.26 vs. 36.03 ± 0.58). In contrast, both kits B and C performed better with low-abundant MAP levels in the artificially spiked feces, given an approximately 3-Ct difference between 1/10 MAP dilutions (Table 1). Kits B and C were thus better able to quantify the bacterial load or shedding level in feces.

In our experiments, the sensitivity of detection dropped radically when DNA samples were analyzed using qPCR system V. This system was initially designed for use with kit C and performed better with its companion kit for moderate shedders (Figure 2), as previously described (Leite et al., 2013). For high shedders, the combination of kit C with its companion system V could not achieve results as sensitive as those obtained with qPCR system T (lower Ct values). This gap in performance could be due to the specific qPCR target,

given that *HspX* (system V) is represented by 1 single copy per Ge, whereas *ISMAP02* (system T) is found in 6 to 8 copies (Stabel and Bannantine, 2005; Kralik et al., 2011). That trend was also confirmed in naturally contaminated fecal samples. The low scores (<8/8) for positive detection in low-shedding cows reflect the probability of a low-abundant event around Ct values of 38. This phenomenon of rare-event probability is observed when the likelihood of detection decreases below 100%, in agreement with a previous report (Plain et al., 2014). It is difficult to determine whether Ct values around 38 are due to passive-through, the phenomenon of MAP being ingested from contaminated feed and then being detected in the feces without causing disease (Kralik et al., 2014). However, we observed PCR detection of positive cows around Ct values of 38, corresponding to 1 Ge for non-bison strains. To date, very few studies have evaluated the effect of repetitions on the sensitivity of the PCR system. Considering the high number of low shedders, this fact suggests that repetitions and an appropriate DNA extraction kit combined with most sensitive qPCR systems should improve the detection of subclinical cows.

The identification of shedding cows is essential for preventing the spread of paratuberculosis in herds. However, only 38% of true-positive cows can be detected by culture in the first sampling because of low shedding (Whitlock et al., 2000). It was quite obvious that kit B performed the best overall, detecting 7 of the 9 culture-positive cows with a PCR score >4/8, as well as 5 cows that tested negative at first culture but were found positive at a later stage. Interestingly, the sensitivity of kit B might permit the detection of paratuberculosis-positive cows in the early subclinical stage (not advanced enough for the disease to be detectable by ELISA or fecal culture). Direct fecal qPCR represents a good alternative for the diagnosis of herds. However, choosing the right DNA extraction kit associated with a compatible specific and sensitive PCR technology is mandatory. Our results show that kit B is highly recommended over the others for DNA extraction from naturally infected fecal samples and is compatible with qPCR system T. We observed that the use of repetitions improved sensitivity, and this has been observed elsewhere (Plain et al., 2014). Although 1 test should be adequate for confirmation that an animal with intermittent diarrhea or other signs associated with the disease is positive, detecting low shedders with the highest sensitivity should include repetitive DNA extractions. When these recommendations are combined, the quality of diagnosis in herds should improve, and the prevention of the transmission of paratuberculosis would be more effective.

ACKNOWLEDGMENTS

This study was funded by Agriculture and Agri-Food Canada (Project AAFC-1101/J000079). A special thank-you is given to the veterinarians and dairy producers for their valuable collaboration. We thank Émilie Constant and Catherine Thibault (Agriculture and Agri-Food Canada, Sherbrooke Research and Development Center) for their technical assistance and Olivia Labrecque of the Laboratoire d'épidémiologie animale du Québec for the MAP culture. The authors confirm that they had no conflict of interest regarding any aspect of this study.

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