Evaluation of bulk tank milk PCR and bulk tank milk modified ELISA tests for the detection of paratuberculosis at the herd level in goat and sheep dairies in Ontario, Canada

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ABSTRACT

Early identification of dairy goat herds and dairy sheep flocks infected with *Mycobacterium avium* ssp. *paratuberculosis* is important for controlling this infection and minimizing economic losses. The objective of this study was to evaluate 2 bulk tank milk (BTM) paratuberculosis tests (PCR and modified ELISA) as potential herd-level tests. These tests were compared with the results obtained from testing 20 randomly selected lactating animals per farm (>2 yr) with an individual animal test (fecal culture, fecal PCR, serum ELISA, and milk ELISA). The study was conducted using 29 dairy goat herds and 21 dairy sheep flocks in Ontario, Canada, visited between October 2010 and August 2011. The sensitivity of the BTM PCR was poor in both the dairy goat herds (0.0%) and dairy sheep flocks (25.0%), but exhibited 100% specificity in both species. In comparison, the BTM modified ELISA demonstrated higher sensitivity. In goats, sensitivity ranged from 33.3 to 34.8% when fecal culture and PCR were the reference tests, respectively (specificities were both 100%), and 71.4 to 87.5% when the milk and serum ELISA, respectively, were the reference tests (specificities were 86.4 and 95.2%). The BTM modified ELISA in dairy sheep demonstrated comparable sensitivities, but lower specificities. When fecal culture and PCR were the reference test, sensitivities were 50.0 and 46.7%, respectively (specificities were 77.8 and 83.3%). The sensitivities when the milk and serum ELISA were the reference tests were 87.5 and 72.7%, respectively (specificities were 92.3 and 100%). Fecal PCR was the only individual animal test to identify significantly more farms as positive than the BTM PCR and modified ELISA test in both species. Therefore, whereas the

BTM modified ELISA may provide an organization or control program with a high level of confidence that a BTM-positive farm is actually positive (high positive predictive value), if a producer wishes to increase the odds that a positive farm will test positive, so as not to miss an infection, then sampling and testing 20 animals with fecal PCR will better meet that objective.

Key words: bulk tank milk, dairy goats, dairy sheep, paratuberculosis

INTRODUCTION

The province of Ontario produces 85% of Canada's dairy goat milk (45.9 million liters) and is home to over 50% of the country's goats (Canadian Dairy Information Centre, 2016). The province also produces about two-thirds of the country's sheep milk (~2.5–3 million liters per year). The majority of milk from both species is used to make cheese. Paratuberculosis, or Johne's disease, is a chronic wasting disease caused by infection with *Mycobacterium avium* ssp. *paratuberculosis* (MAP) and presents an economic challenge to domestic ruminant dairy industries, including sheep and goat dairies. Recent work indicates it is a common production-limiting disease in the Ontario dairy goat and sheep industries (herd-level prevalences of 86 and 68%, respectively; Bauman et al., 2016). Currently, no voluntary paratuberculosis control programs exist for either small ruminant species in Ontario or Canada.

Control programs in other species are not based solely on test and cull initiatives (NRC, 2003) due to the poor sensitivity of many commercial tests for detecting MAP infection at the individual level and the inconsistent shedding of bacteria in feces and immune response. Therefore, control programs also include on-farm management practices to interrupt the transmission of MAP between animals (e.g., removing animals from their dams at birth and feeding heat-treated colostrum to young stock, or raising them without exposure to the manure of adult animals; Bastida and Juste, 2011), but

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such control measures are expensive, time-consuming, labor-intensive, and require long-term commitment. Producers are unlikely to voluntarily make herd-level changes unless they are confident that their herd or flock has the disease. Therefore, a herd-level test that will accurately classify herds or flocks as infected and that will serve as a useful tool to measure the progress or efficacy of control measures is needed.

Bulk tank milk (BTM) has been evaluated in dairy cattle as a potential herd-level paratuberculosis testing strategy as it is easy and convenient to acquire (Department of the Environment Food and Rural Affairs, 2009) (Pillai and Jayarao, 2002; Bosshard et al., 2006; Beaver et al., 2016; Pesqueira et al., 2017). Bulk tank milk paratuberculosis tests published to date have been based on bacterial culture, PCR, and ELISA (Stabel et al., 2002; Favila-Humara et al., 2010). Milk culture is not a practical surveillance tool due its relatively high cost, lengthy delay (3–12 mo) for results to be returned, and low sensitivity (Stabel et al., 2002). Both PCR testing (Stabel et al., 2002; Beaver et al., 2016) and a modified ELISA (**mELISA**) protocol (Wilson et al., 2010) have shown potential for herd-level testing due to their quick turn-around time (24–48 h) and relatively low cost. However, more information is needed on the ability of these 2 tests to identify MAP-infected herds generally and small ruminants specifically. The objective of our study was to evaluate BTM PCR and the BTM mELISA paratuberculosis tests in dairy goat herds and dairy sheep flocks to determine if they identified more paratuberculosis-infected farms than individually testing 20 lactating animals using fecal culture, fecal PCR, serum ELISA, or milk ELISA.

MATERIALS AND METHODS

This study received approval from the University of Guelph Research Ethics Board (10JN004) for human participants and the University of Guelph Animal Care Committee (10R057) for use of animals.

Sampling

This study was part of a larger cross-sectional study conducted between October 2010 and August 2011. The purpose of the original study was to determine the herd-level and within-herd prevalence of paratuberculosis in dairy goat herds and dairy sheep flocks in the province of Ontario and to evaluate the performance of 7 individual- and 2 herd-level diagnostic tests. Greater detail on herd and animal sampling and sample size determination are available (Bauman et al., 2016). Briefly, the study sample size was determined by using

a formula to estimate proportions (Dohoo, et al., 2009), resulting in a need to select 29 dairy goat herds and 20 dairy sheep flocks. The number of animals sampled per farm was determined to be 20 through a combination of budget and logistics.

Dairy goat herds were selected from a government list (OMAFRA, 1990) using randomized, stratified sampling based on herd size. Dairy sheep flocks were selected from an incomplete government list and from contacting all licensed milk processors from across the province for their list of suppliers. All farms were visited once, at either the morning or evening milking. During that time, 20 lactating does or ewes over the age of 2 yr were randomly selected regardless of appearance or BCS and sampled for feces, blood, and milk, as described previously (Bauman et al., 2016). For the randomization procedure, the herd or flock size was obtained before visiting along with information on how the goats were grouped for milking in the parlor (e.g., grouped by age or stage of lactation). We stratified the sampling by group, weighted by size (e.g., if group 1 and 2 each contained 25% of the herd we selected 5 from each group, and if the third group represented 50% of the herd we selected 10 animals from that group). We used a random number generator to select the given number of animals within each group.

In goat herds and in the sheep flocks that used a bulk tank to store milk, the BTM was agitated postmilking for at least 30 min, and then a 50-mL sample was taken. In sheep flocks that collected and stored milk in pails, the individual pails were agitated using a sterile straw for each pail, and a 10-mL sample from each bucket was removed. The individual samples were then pooled in a separate sterile container, agitated again with a sterile straw, and, from the pooled sample, a 50-mL sample was taken. All milk samples were stored in plastic vials containing a 1.5-mg tablet of the preservative bronopol (2-bromo-2-nitro-propane-1,3-diol) that were provided by the milk testing laboratory (CanWest DHI, Guelph, ON). The vials were inverted until the tablet dissolved and the milk was a uniform color. All biological samples (feces, blood, and milk) were collected by the same investigator on all farms, stored at 4 to 8°C during transit, and submitted to the respective laboratories within 12 to 18 h.

Laboratory Testing

Fecal Culture, Fecal PCR, Serum and Milk ELISA. The individual animal samples originally underwent testing using 7 tests. The results of 4 of the tests are presented in this manuscript: fecal culture, fecal PCR, serum IDEXX ELISA, and milk IDEXX

ELISA (IDEXX, Westbrook, ME). Briefly, feces underwent a 3-d decontamination period before being cultured using the BD BACTEC MGIT 960 Mycobacterial detection system (Becton Dickinson, Franklin Lakes, NJ) and BACTEC MGIT Para TB medium (modified Middlebrook 7H9 with mycobactin J) according to manufacturer's instructions (https://www.finddx.org/wp-content/uploads/2016/02/mgit_manual_nov2006.pdf). Samples in the BACTEC system are typically incubated for up to 49 d before being deemed negative. In our study, the incubation period was extended to 240 d for all samples to allow for the potential growth of slower-growing strains of MAP (type I), which can be more commonly detected in sheep than other species. Confirmation of culture-positive samples was done by acid-fast staining of colonies and PCR confirmation using the *hspX* gene (Culture Confirmation Protocol, MAP Extraction System; Tetracore, Rockville, MD).

Real-time PCR was also performed on the feces before decontamination using the Two Gram MAP Extraction and Mini Beadbeater protocols and the VetAlert Johne's Real-Time PCR kit (Tetracore). Samples that reached fluorescence with a cycle count (Ct) ≤ 42.0 were considered positive for MAP as per kit instructions (<https://www.tetracore.com/animal-kits/info.html>).

The serum and milk were tested using IDEXX Mycobacterium Paratuberculosis Antibody Test Kit as directed by the manufacturer (<https://www.idexx.com/en/livestock/livestock-tests/ruminant-tests/idexx-paratuberculosis-screening-ab-test/>). IDEXX provided 2 different recommendations for cutoff values for small ruminants, 1 for Europe (serum ≥ 0.55 ; milk ≥ 0.3) and 1 for North America (serum ≥ 0.7 ; milk ≥ 0.4). The European values, which are lower and used to screen for infection, were used in the current study.

BTM Tank Testing. All milk samples underwent testing at the CanWest DHI milk laboratory (Guelph, ON, Canada). Samples were stored at approximately 4°C until testing could be performed (within 30 d). For PCR testing, 50-mL milk samples were centrifuged at $3,900 \times g$ for 30 min at 4°C after mixing with 100 μ L of Tween20 biomedical detergent (Sigma-Aldrich, St. Louis, MO). After removing the milk fat layer and decanting the liquid fraction as per Wilson et al. (2010), the pellet was resuspended in 1 mL of PBS-EDTA mixture, centrifuged at $18,400 \times g$ for 10 min at room temperature and the liquid aspirated. The pellet was then bead-beaten using zirconia/silica beads in 250 μ L of PBS for 2 min and then centrifuged an additional 10 min ($18,400 \times g$ at room temperature). The top 200 μ L of supernatant then underwent DNA extraction using the DNeasy blood and tissue kit (Qiagen, Valencia, CA), according to manufacturer's recommendations

(<https://www.qiagen.com/ca/resources/resourcedetail?id=6b09dfb8-6319-464d-996c-79e8c7045a50&lang=en>), and was stored at -20°C until batches of 5 to 10 could be processed. Samples were subsequently thawed and underwent amplification and analysis using the real-time PCR MPara-teQ milk assay (AntelBio, Lansing, MI). This assay is based on a DNA sequence in the IS900 element and was performed according to manufacturer's instructions (<https://www.northstarcooperative.com/diagnostic-services/>) using the LightCycler 2.0 (Hoffman-La Roche Ltd., Mississauga, ON, Canada). With this quantitative PCR method, samples were considered positive if the Ct was < 41.0 .

The BTM mELISA was performed using the ELISA IDEXX Mycobacterium Paratuberculosis Antibody Test Kit with a modified protocol (Wilson et al., 2010). In summary, the BTM samples were inverted gently 3 times until well mixed, then diluted at a lower ratio with test diluent (1 part milk:1.5 parts diluent) than is used for testing of individual milk samples (1:2). The samples were then processed according to manufacturer's instructions as for individual milk samples with the following modifications: the incubation time of the sample and the conjugate was doubled (i.e., 1 h), the dilution of the conjugate was lowered (i.e., 1:67), and the length of the color development stage was doubled (Wilson et al., 2010). Samples were designated positive or negative by the sample-to-positive ratio (S/P value). The cutoff ≥ 0.1 , as stipulated by the manufacturer, was used to designate herds or flocks as positive. All laboratory personnel were blinded to the results from the other tests.

Statistical Analysis

Test results for each farm were stored in Microsoft Excel 2016 (Microsoft Corp., Redmond, WA), and statistical analyses were performed using StataSE 14.0 (StataCorp LP, College Station, TX). The sensitivity and specificity of the BTM PCR, and mELISA tests were estimated against the dichotomous herd-level result of randomly testing 20 animals per farm. A herd or flock was classified as positive if one or more of the tested animals was positive on the individual animal test.

McNemar's tests were used to evaluate the marginal homogeneity between the different test combinations. When the frequencies of the discordant pairs were less than 10, the exact binomial *P*-values were reported; if the frequencies were ≥ 10 the McNemar's Chi-squared statistic was reported with its associated *P*-value (Petrie and Watson, 2006). The marginal proportions were deemed significantly different from one another at $P < 0.05$.

RESULTS

Goats

A total of 580 lactating dairy goats (20 per farm) were sampled from 29 dairy goat herds (Bauman et al., 2016) and provided individual animal-level results (feces and serum). Mean herd size of the sampled farms was 284 goats (SD = 258.6) and ranged in size from 64 to 1,350. On the day of BTM sampling, milk from an average of 7 milkings (3.5 d) was present in the dairy goat bulk tanks. Twenty-three goat herds (79.3%) had at least 1 animal test positive on fecal culture, 24 had at least 1 positive on fecal PCR (82.8%), 8 had at least 1 positive with the serum ELISA (27.6%), and 7 had at least 1 positive goat with the milk ELISA test (24.1%) (Table 1). Bulk tank milk PCR and mELISA results were available for all 29 goat herds.

The mean score of the all the mELISA tests was 0.084 (range = -0.01 to 0.48). Using the cutoff of 0.1, 8 goat farms had a positive BTM mELISA test with a mean score of 0.276 (range = 0.14–0.48). The sensi-

tivity for this BTM test was highest when the serum ELISA was the reference test [sensitivity = 87.5% (95% CI = 47.3–99.7)], whereas specificity was highest when either fecal culture or fecal PCR was the reference test (specificity = 100.0%). Sensitivities for the other 3 reference tests ranged between 33.3 and 71.4% (Table 2). The positive predictive value for the BTM mELISA was 100% when either fecal test was used as reference and <50% for the serum and milk ELISA.

When comparing the BTM mELISA results to the herd-level results of sampling 20 goats, McNemar's chi-squared statistic was used for the fecal tests because the discordant frequencies were all ≥ 10 ; however, the exact binomial *P*-values were used for the serum and milk ELISA. The 2 fecal tests (culture and PCR) detected significantly more MAP-positive herds than the BTM mELISA test (Table 3); however, the confidence intervals were very wide. The serum and milk IDEXX tests did not significantly identify more animals as positive compared with the BTM mELISA (Table 3).

The use of a cutoff of 0.05 for the BTM mELISA was also explored to determine how this affected test

Table 1. Paratuberculosis test results for 29 Ontario dairy goat herds comparing a bulk tank milk (BTM) modified ELISA (mELISA) to the number of goats testing positive on individual animal tests after testing 20 randomly selected animals per farm¹ [% positive of the 20 animals randomly selected (1/20 = 5%) in parentheses]

Farm	Herd size	BTM mELISA ²	FC	FPCR	SI	MI
1	64	0	1 (5)	4 (20)	0	0
2	75	0.01	2 (10)	0	0	2 (10)
3	75	0.22	5 (25)	7 (35)	1 (5)	1 (5)
4	125	0.01	0	5 (25)	0	0
5	140	0	3 (15)	0	0	0
6	140	0.05	8 (40)	2 (10)	0	0
7	140	-0.01	0	1 (5)	0	0
8	150	-0.01	3 (15)	5 (25)	0	0
9	150	0	1 (5)	3 (15)	0	0
10	160	0.4	5 (25)	3 (15)	2 (10)	1 (5)
11	200	-0.01	3 (15)	6 (30)	0	0
12	200	0.38	4 (20)	4 (20)	1 (5)	0
13	200	0	0	2 (10)	0	0
14	200	0	0	0	0	0
15	250	0	0	4 (20)	0	0
16	250	0.09	2 (10)	3 (15)	0	0
17	250	0.06	8 (40)	6 (30)	0	0
18	250	0.01	0	1 (5)	0	0
19	255	0	2 (10)	2 (10)	1 (5)	0
20	260	-0.01	11 (55)	1 (5)	0	0
21	350	0.02	5 (25)	2 (10)	0	0
22	300	0	1 (5)	0	0	1 (5)
23	300	0.01	6 (30)	8 (40)	0	0
24	300	0.48	12 (60)	5 (25)	2 (10)	1 (5)
25	350	0.01	1 (5)	0	0	0
26	400	0.22	7 (35)	4 (20)	1 (5)	1 (5)
27	440	0.23	5 (25)	4 (20)	0	0
28	900	0.14	6 (30)	6 (30)	1 (5)	0
29	1350	0.14	4 (20)	8 (40)	1 (5)	1 (5)

¹FC = fecal culture; FPCR = fecal PCR; SI = serum IDEXX ELISA (IDEXX, Westbrook, ME); MI = milk IDEXX ELISA.

²Bolded numbers indicate positive bulk tank results using cut-off = 0.01.

Table 2. Sensitivity and specificity of the bulk tank milk modified ELISA (IDEXX, Westbrook, ME) paratuberculosis test using 4 different individual animal reference tests (fecal culture, fecal PCR, serum, and milk ELISA) in 29 dairy goat herds in Ontario, Canada¹

Reference test (%)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Cutoff 0.1				
Fecal culture	34.8 (16.4–57.3)	100.0 (54.1–100.0)	100.0 (63.1–100.0)	28.6 (11.3–52.2)
Fecal PCR	33.3 (15.6–55.3)	100.0 (47.8–100.0)	100.0 (63.1–100.0)	23.8 (8.2–47.2)
Serum IDEXX ELISA	87.5 (47.3–99.7)	95.2 (76.2–99.9)	87.5 (47.3–99.7)	95.2 (76.2–99.9)
Milk IDEXX ELISA	71.4 (29.0–96.3)	86.4 (65.1–97.1)	62.5 (24.5–91.5)	90.5 (69.6–98.8)
Cutoff 0.05				
Fecal culture	47.8 (26.8–69.4)	100.0 (54.1–100.0)	100.0 (63.1–100.0)	33.3 (13.3–59.0)
Fecal PCR	45.8 (25.6–67.2)	100.0 (47.8–100.0)	100.0 (71.5–100.0)	27.8 (9.7–53.5)
Serum IDEXX ELISA	87.5 (47.3–99.7)	81.0 (58.1–94.6)	63.6 (30.8–89.1)	94.4 (72.7–99.9)
Milk IDEXX ELISA	71.4 (29.0–96.3)	72.7 (49.8–89.3)	45.5 (16.7–76.6)	88.9 (65.3–98.6)

¹PPV = positive predictive value; NPV = negative predictive value.

performance. The lower cutoff increased the test sensitivity of the BTM mELISA slightly when either of the fecal reference were used without altering the 100.0% specificity. When the serum and milk ELISA tests were the reference tests, the sensitivity of the BTM mELISA

remained unchanged but we did note a reduction in test specificity (Table 2). At this cutoff, the fecal tests still detected a significantly greater number of positives ($P < 0.0001$) than the mELISA; however, we still found no significant difference between the serum and milk ELISA and the BTM mELISA test ($P > 0.2$) (Appendix, Tables A1 and A2). No goat herds tested positive on BTM PCR (sensitivity = 0.0%; specificity = 100.0%); therefore, no further statistical analysis of the PCR results was performed.

Table 3. Paratuberculosis test results from 29 dairy goat farms: comparison of bulk tank milk modified ELISA (IDEXX, Westbrook, ME) results to herd-level results of 1 or more goats testing positive on fecal culture, fecal PCR, serum ELISA, or milk ELISA, in Ontario, Canada

Item	Bulk tank milk modified ELISA		Total
	Positive	Negative	
Fecal culture ¹			
Positive	8	15	23
Negative	0	6	6
Total	8	21	29
Fecal PCR ²			
Positive	8	16	24
Negative	0	5	5
Total	8	21	29
Serum IDEXX ELISA ³			
Positive	7	1	8
Negative	1	20	21
Total	8	21	29
Milk IDEXX ELISA ⁴			
Positive	5	2	7
Negative	3	19	22
Total	8	21	29

¹Odds ratio (OR) = ∞ (95% CI: 3.6– ∞); McNemar's $\chi^2 = 15.0$; $P < 0.0001$.²OR = ∞ (95% CI: 3.9– ∞); McNemar's $\chi^2 = 16.0$; $P < 0.0001$.³OR = 1.0 (95% CI: 0.0–78.5); $P = 1.0000$.⁴OR = 0.7 (95% CI: 0.1–5.8); $P = 1.0000$.

Sheep

As reported previously, the target sample size of 20 dairy sheep flocks was exceeded by visiting 21 flocks (Bauman et al., 2016). Individual animal results (feces, serum, and milk) were available for 397 lactating dairy sheep. Mean herd size of the sampled farms was 135 sheep (SD = 211.7), ranging in size from 25 to 1,000. Seven sheep farms (33.3%) stored milk in buckets immediately after milking, and the milk was subsequently frozen. Sampling of these farms therefore represented a composite of 1 milking only. Fourteen sheep farms stored milk in bulk tanks. On the day of BTM sampling, there was an average of 7 milkings (3.5 d) in the tank.

Twelve sheep flocks (57.1%) had at least 1 animal test positive on fecal culture, 15 flocks had at least 1 positive on fecal PCR (71.4%), 11 flocks had at least 1 positive with the serum ELISA (47.6%), and 8 flocks had at least 1 positive with the milk ELISA (38.1%; Table 4). Bulk tank milk mELISA results were avail-

Table 4. Paratuberculosis test results for 21 Ontario dairy sheep flocks comparing a bulk tank milk (BTM) modified ELISA (mELISA) to the number of sheep testing positive on individual animal tests after testing 20 randomly selected animals per farm¹ (% of the 20 animals sampled who tested positive in parentheses)

Farm	Herd size	BTM mELISA ²	BTM PCR (Ct)	FC (n = 20)	FPCR (n = 20)	SI (n = 20)	MI (n = 20)
1	25	0.02	0	1 (5%)	1 (5%)	0	0
2	30	0.08	0	0	1 (5%)	1 (5%)	0
3	35	0.01	0	0	0	1 (5%)	0
4	50	0.02	0	0	3 (15%)	0	0
5	50	0.24	0	1 (5%)	3 (15%)	1 (5%)	3 (15%)
6	50	0.08	0	2 (10%)	0	0	0
7	55	0.15	0	0	0	1 (5%)	1 (5%)
8	60	0.10	0	0	1 (5%)	1 (5%)	1 (5%)
9	65	0.03	0	0	0	1 (5%)	0
10	70	0.00	0	3 (15%)	5 (25%)	0	0
11	70	0.07	NR ³	0	0	0	0
12	75	0.05	0	2 (10%)	9 (45%)	0	0
13	80	0.59	0	2 (10%)	10 (50%)	1 (5%)	0
14	85	0.51	0	2 (10%)	3 (15%)	1 (5%)	3 (15%)
15	90	1.62	1 (38.49)	2 (10%)	10 (50%)	3 (15%)	2 (10%)
16	100	0.01	0	0	1 (5%)	0	1 (5%)
17	100	0.01	NR	4 (20%)	0	0	0
18	200	0.01	0	3 (15%)	4 (20%)	0	0
19	200	0.02	0	0	2 (10%)	0	0
20	350	0.27	1 (39.94)	3 (15%)	2 (10%)	2 (10%)	6 (30%)
21	1000	1.51	1 (33.35)	5 (25%)	4 (20%)	3 (15%)	2 (10%)

¹Ct = count threshold; FC = fecal culture; FPCR = fecal PCR; SI = serum IDEXX ELISA (IDEXX, Westbrook, ME); MI = milk IDEXX ELISA.

²Bolded numbers indicate positive bulk tank results.

³NR = no result.

able from all 21 sheep flocks; however, 2 sheep flock BTM samples had inadequate volume to complete the PCR testing after the mELISA was performed due to handling errors. As such, only 19 PCR results were available for sheep.

The mean score of the all the mELISA tests was 0.257 (range = 0.01–1.62). Using the cutoff of 0.1, 8 sheep farms had a positive BTM mELISA test with a mean score of 0.624 (range = 0.10–1.62). The sensitivity and specificity for this BTM test was highest when the milk ELISA was the reference test [sensitivity = 87.5% (95% CI = 47.3–99.7)]. Sensitivities for the other 3 reference tests ranged between 46.7 and 72.7% (Table 5). Specificity was highest when the serum ELISA was the reference test (100.0%).

When comparing the BTM mELISA results to the herd-level results of sampling 20 sheep, the exact binomial *P*-values were reported for all 4 tests because the discordant frequencies were all <10. The only test result that was significantly different from the BTM mELISA test was the fecal PCR test (*P* = 0.0391; Table 6). The odds of a positive flock testing positive for MAP were 8 times higher when 20 animals per flock were tested using fecal PCR than with the BTM mELISA; however, the confidence interval was very wide (95% CI = 1.1–355.0).

The use of a cutoff of 0.05 for the BTM mELISA was also explored to determine how this affected test performance. It increased the test sensitivity of all tests except for the milk ELISA, which remained unchanged. However, this lower cutoff value caused a decrease in specificity in all 4 tests (specificity range = 50–70%) (Table 5). At this cutoff, fecal PCR no longer detected a significantly greater number of positive farms (*P* = 0.5078; Appendix, Tables A1 and A2).

Three sheep flocks had positive BTM PCR test results. All 3 BTM PCR-positive sheep farms were also positive with the mELISA; the farms with the 2 lowest cycle threshold values (33.35 and 38.49) also had the 2 highest BTM mELISA scores (1.51 and 1.62), respectively. All of the PCR-positive farms machine-milked the ewes and used bulk tanks to store milk. The sensitivity and specificity of the BTM PCR test was 25.0% (95% CI = 5.5–57.2) and 100.0% (95% CI = 66.4–100.0) when any of the individual animal tests were used as the reference.

DISCUSSION

Without evaluating how well paratuberculosis BTM tests perform, it becomes difficult to interpret the validity of prevalence studies based on these tests. As

Table 5. Sensitivity and specificity of the bulk tank milk modified ELISA (IDEXX, Westbrook, ME) paratuberculosis test using 4 different reference tests (fecal culture, fecal PCR, serum and milk ELISA) in 21 dairy sheep flocks in Ontario, Canada¹

Reference test (%)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Cutoff 0.1				
Fecal culture	50.0 (21.1–78.9)	77.8 (40.0–97.2)	75.0 (34.9–96.8)	53.8 (25.1–80.8)
Fecal PCR	46.7 (21.3–73.4)	83.3 (35.9–99.6)	87.5 (47.4–99.7)	38.5 (13.9–68.4)
Serum IDEXX ELISA	72.7 (39.0–94.0)	100.0 (69.2–100.0)	100 (63.1–100.0)	76.9 (46.2–95.0)
Milk IDEXX ELISA	87.5 (47.3–99.7)	92.3 (64.0–99.8)	87.5 (47.3–99.7)	92.3 (64.0–99.8)
Cutoff 0.05				
Fecal culture	66.7 (34.9–90.1)	55.6 (21.2–86.3)	66.7 (34.9–90.1)	55.6 (21.2–86.3)
Fecal PCR	60.0 (32.3–83.7)	50.0 (11.8–88.2)	75.0 (42.8–94.5)	33.3 (7.5–70.1)
Serum IDEXX ELISA	81.8 (48.2–97.7)	70.0 (34.8–93.3)	75.0 (42.8–94.5)	77.8 (40.0–97.2)
Milk IDEXX ELISA	87.5 (47.3–99.7)	61.5 (31.6–86.1)	58.3 (27.7–84.8)	88.9 (51.8–99.7)

¹PPV = positive predictive value; NPV = negative predictive value.

BTM prevalence studies that have been published to date have used culture (Grant et al., 2001; Stabel et al., 2002), PCR (Grant et al., 2001; Corti and Stephan, 2002; Stabel et al., 2002; Muehlherr et al., 2003), and ELISA tests (van Weering et al., 2007), which all have previously demonstrated low sensitivity at the individual animal-level, concern exists that these studies underestimate the true level of MAP infection present

Table 6. Paratuberculosis test results from 21 dairy sheep farms: comparison of bulk tank milk modified ELISA results to herd-level results of 1 or more sheep testing positive on fecal culture, fecal PCR, serum ELISA, or milk ELISA, in Ontario, Canada

	Bulk tank		
Item	Positive	Negative	Total
Fecal culture ¹			
Positive	6	6	12
Negative	2	7	9
Total	8	13	21
Fecal PCR ²			
Positive	7	8	15
Negative	1	5	6
Total	8	13	21
Serum ELISA ³			
Positive	8	3	11
Negative	0	10	10
Total	8	13	21
Milk ELISA ⁴			
Positive	7	1	8
Negative	1	12	13
Total	8	13	21

¹Odds ratio (OR) = 3 (95% CI: 0.5–30.4); $P = 0.2891$.²OR = 8 (95% CI: 1.1–355.0); $P = 0.0391$.³OR = ∞ (95% CI: 0.4– ∞); $P = 0.2500$.⁴OR = 1 (95% CI: 0.0–78.5); $P = 1.0000$.

in their populations. This concern is validated by the results of the current study. The apparent herd-level prevalence based on BTM PCR would have been 0% in Ontario dairy goat herds and 14.6% in Ontario dairy sheep flocks (based on similar methodology and results from the United Kingdom and Switzerland; Grant et al., 2001; Muehlherr et al., 2003), which is markedly lower than the actual true herd- and flock-level prevalences of 83.0% (95% CI = 62.6–98.1) and 66.8% (95% CI = 41.6–91.4), respectively, based on individual animal testing (Bauman et al., 2016). Hence, the BTM IS900 PCR test in our study has poor sensitivity [0.0% in goats and 25.0% in sheep (reference test – fecal culture)] and its use would result in a marked underestimation of the herd- and flock-level prevalence.

The performance of BTM PCR in these small ruminant populations is disappointing as 2 previous studies involving IS900 and dairy cows had reported sensitivities of 77 and 100%, respectively (Stabel et al., 2002; Favila-Humara et al., 2010). The low sensitivity in this study may indicate, overall, that a low amount of shedding of MAP occurs directly in goat and sheep milk at the BTM level, that udder hygiene is sufficient to minimize environmental contamination, or that some component of small ruminant milk inhibits the performance of the PCR test. Whereas the detection threshold of the IS900 test could also play a role in the low sensitivity of this test (limit of detection in milk is ~ 0.6 cfu/mL; Donaghy et al., 2011), it is able to detect a lower amount than either the f57 (~ 6.2 cfu/mL; Donaghy et al., 2011) or *hspX* tests (~ 8.23 cfu/mL; Beaver et al., 2016). During the preparation of the BTM samples in

our study, the fat fraction was discarded to be consistent with the methodology of a previous study (Wilson et al., 2010). Future studies could investigate the role of retaining the fat portion of goat and sheep milk plays in the performance or sensitivity of a BTM PCR test, as MAP can be present in the cream layer as well (Gao et al., 2005; Herthnek et al., 2008).

Paratuberculosis tests based on IS900 have historically demonstrated imperfect specificity due to the presence of this sequence in other non-MAP mycobacteria (Bosshard et al., 2006; Slana et al., 2008). However, false positives were unlikely to have been an issue in our study, as the dairy sheep flocks that did test positive on BTM PCR also had the highest BTM mELISA scores and the 3 highest prevalences of ewes test positive on fecal culture (20–25%).

Beaver et al. (2016) noted that some dairy cattle farms with high MAP BTM antibodies still managed to test negative on PCR, indicating there are likely some farms implementing rigid udder hygiene practices that manage any environmental MAP contamination below the detectable threshold. Further investigation of the link between specific cleaning protocols on MAP-positive farms with negative PCR tests will yield valuable information that can be used to continue to reduce the zoonotic risk of consuming raw milk.

We are not aware of any published studies of the use of the BTM mELISA for MAP in small ruminants. The same laboratory methodology was used in a study of BTM in US dairy cattle (Wilson et al., 2010), which reported a herd-level sensitivity of 34% and specificity of 100%, which are comparable to the sensitivity and specificity estimates for dairy goats reported here (33.3–34.8% and 100%, respectively) when a fecal reference test was used. The BTM mELISA performed slightly better in dairy sheep flocks, with sensitivities of 46.7 to 50.7% (fecal culture and PCR as reference tests, respectively). It would be expected that the mELISA would demonstrate higher sensitivity for identifying farms with affected animals (those with detectable circulating antibodies in their serum or milk). This hypothesis holds true in our study (Tables 2 and 5), increasing sensitivities to 87.5% (serum ELISA as reference test) and 71.4% (milk ELISA as reference test) in dairy goats and 72.7% (serum ELISA as reference test) and 87.5% (milk ELISA as reference test) in dairy sheep. Although some variation would be anticipated in test performance based on animal species due to the volume of milk routinely produced (cows ~25 L/d, goats ~3.5 L/d, sheep ~1.5 L/d), creating a dilution effect, other factors (e.g., season) may also play a role in test performance. Both sheep and goats are seasonal breeders, which results in larger volumes of milk in the

spring and early summer (along with many animals in early lactation) and reduced production in late fall and winter (many animals in later stage lactation). The samples in our study were collected throughout the year to attempt to represent all seasons.

Sensitivity was also slightly increased when the cutoff for the BTM mELISA was reduced from ≥ 0.1 to ≥ 0.05 (Tables 2 and 5); however, this increase was at the expense of a reduction in specificity across all 4 reference tests in dairy sheep and in the ELISA tests in dairy goats. Whereas the BTM ELISA has been evaluated as a continuous variable previously (Beaver et al., 2016), the small sample size in the present study and lack of range of data prevented this from being a useful exercise.

Specificity values for the BTM mELISA were unexpectedly low in the dairy sheep population, which may more accurately reflect the performance of the reference tests or study methodology as opposed to true mELISA false positives. When a test is used as the reference or gold standard it is assumed to have perfect sensitivity and specificity; however, it is well known that the individual animal paratuberculosis tests all demonstrate low sensitivity and are therefore imperfect comparisons. This imperfection is also compounded by the variation in animal-level shedding of MAP and circulation of MAP antibodies. In addition, the study protocol of only testing 20 animals per farm does not ensure that a farm is correctly identified as positive or negative. Although all goats and sheep were selected randomly on farms to try and reduce the risk of missing a positive animal, the only way to eliminate this risk would be to sample all animals in the herd or flock. Wilson et al. (2010) demonstrated that the farms in their study with positive BTM mELISA tests all had a positive animal detected on the farm when they followed up with additional animal-level testing, suggesting the test may have close to 100% specificity at the 0.1 cutoff.

As the small ruminant industry struggles with low economic margins, the BTM mELISA was evaluated to see if it significantly identified more positive farms than the reference tests. If the reference tests performed better, they would yield the additional benefit of providing the producer with disease status knowledge on those 20 animals as well. In dairy goats, fecal culture and fecal PCR significantly detected more positive farms than did the BTM tests, whereas in dairy sheep only fecal PCR detected significantly more farms. As fecal culture is not practical beyond the research setting, randomly sampling and testing 20 animals per farm with fecal PCR would be a more accurate method of identifying positive herds or flocks.

CONCLUSIONS

The main objective of evaluating BTM test performance in our study was to determine which testing methodology provided greater benefit to producers, the cheaper, less-invasive BTM tests or individual testing of 20 animals on each farm. The answer depends on the goal of the stakeholder. For the producer who wants the more sensitive test and to minimize the chance animals would be misclassified as negative, using fecal PCR on 20 animals would be the method of choice. The sensitivity could likely be improved further by testing higher-risk animals demonstrating signs of muscle wasting. For milk processors and organizations concerned with the potential zoonotic and animal welfare effect of MAP, the BTM mELISA used at a 0.1 cutoff is a test that, when it identifies positive farms, shows a high probability of animals that are actively shedding MAP. These test conclusions should be interpreted in light of the relatively small size of our study and wide confidence intervals of the generated odds ratios. Ideally, these conclusions would be explored further using larger sample sizes. In the future, other methods of herd-level testing, such as environmental sampling in sheep and goats, could also be explored. However, small ruminant feces are inherently dry and PCR kits, such as that from Tetracore, state they do not perform optimally on dry feces; therefore, testing should likely be limited to bedding packs or manure storage areas.

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APPENDIX

Table A1. Paratuberculosis test results from 29 dairy goat farms: comparison of bulk tank milk modified ELISA (IDEXX, Westbrook, ME) results to herd-level results of ≥1 goat testing positive on fecal culture, fecal PCR, serum ELISA, or milk ELISA, in Ontario, Canada

Item	Bulk tank milk modified ELISA (cutoff 0.05)		Total
	Positive	Negative	
Fecal culture ¹			
Positive	11	12	23
Negative	0	6	6
Total	11	18	29
Fecal PCR ²			
Positive	11	13	24
Negative	0	5	5
Total	11	18	29
Serum IDEXX ELISA ³			
Positive	7	1	8
Negative	4	17	21
Total	11	18	29
Milk IDEXX ELISA ⁴			
Positive	5	2	7
Negative	6	16	22
Total	11	18	29

¹Odds ratio (OR) = ∞ (95% CI: 2.8–∞); McNemar's $\chi^2 = 12.0$; $P < 0.0001$.

²OR = ∞ (95% CI: 3.0–∞); McNemar's $\chi^2 = 13.0$; $P < 0.0003$.

³OR = 0.3 (95% CI: 0.0–2.53); $P = 0.3750$.

⁴OR = 0.3 (95% CI: 0.0–1.8); $P = 0.2891$.

Table A2. Paratuberculosis test results from 21 dairy sheep farms: comparison of bulk tank milk modified ELISA (IDEXX, Westbrook, ME) results to herd-level results of ≥1 sheep testing positive on fecal culture, fecal PCR, serum ELISA, or milk ELISA, in Ontario, Canada

Item	Bulk tank milk modified ELISA (cutoff 0.05)		Total
	Positive	Negative	
Fecal culture ¹			
Positive	8	4	12
Negative	4	5	9
Total	12	9	21
Fecal PCR ²			
Positive	9	6	15
Negative	3	3	6
Total	12	9	21
Serum IDEXX ELISA ³			
Positive	9	2	11
Negative	3	7	10
Total	12	9	21
Milk IDEXX ELISA ⁴			
Positive	7	1	8
Negative	5	8	13
Total	12	9	21

¹Odds ratio (OR) = 1 (95% CI: 0.2–5.4); $P = 1.0000$.

²OR = 2 (95% CI: 0.4–12.4); $P = 0.5078$.

³OR = 0.7 (95% CI: 0.1–5.9); $P = 1.0000$.

⁴OR = 0.2 (95% CI: 0.0–1.8); $P = 0.2188$.