Quantifying fecal shedding of *Mycobacterium avium* ssp. *paratuberculosis* from calves after experimental infection and exposure

Caroline S. Corbett, Herman W. Barkema, and Jeroen De Buck¹

Department of Production Animal Health, Faculty of Veterinary Medicine, University of Calgary, Calgary, AB, Canada, T2N 4N1

ABSTRACT

Johne's disease, a chronic enteritis caused by *Mycobacterium avium* ssp. *paratuberculosis* (MAP), causes large economic losses to the dairy industry worldwide. Fecal shedding of MAP contaminates the environment, feed, and water and contributes to new infections on farm, yet there is limited knowledge regarding mechanisms of shedding, extent of intermittent shedding, and numbers of MAP bacteria shed. The objectives were to (1) compare (in an experimental setting) the frequency at which intermittent shedding occurred and the quantity of MAP shed among pen mates that were inoculated or contact-exposed (CE); and (2) determine whether an association existed between inoculation dose and quantity of MAP shed. In the first experiment, 32 newborn Holstein-Friesian bull calves were allocated to pens in groups of 4, whereby 2 calves were inoculated with a moderate dose (MD; 5×10^8 cfu) of MAP and 2 calves acted as CE. Calves were group-housed for 3 mo, fecal samples were collected and cultured, and culture-positive samples were quantified. In the second experiment, 6 calves were inoculated with either a low (LD) or high (HD) dose of MAP (1×10^8 or 1×10^{10} cfu, respectively), and fecal samples were collected for 3 mo and cultured for detection of MAP. The amount of MAP was quantified using direct extraction (DE) of DNA from fecal samples and F57-specific quantitative PCR. In experiment 1, the average amount of MAP in all culture-positive samples did not differ between MD and CE calves. In experiment 2, when comparing inoculation doses, LD calves had the lowest proportion of MAP-positive culture samples and HD had the highest, but no difference was detected in the average quantity of MAP shed. This study provided new information in regards to Johne's disease research and control regarding shedding from various inoculation doses and from CE animals; these data should inform future trials and control programs.

Key words: paratuberculosis, fecal shedding, quantity, calf

INTRODUCTION

Johne's disease (JD), caused by *Mycobacterium avium* ssp. *paratuberculosis* (MAP), is a chronic enteritis primarily affecting ruminants and resulting in substantial losses to dairy industries worldwide due to decreased milk production, increased risk of culling, and decreased slaughter value (McKenna et al., 2006; Smith et al., 2016). Although most MAP-infected animals are culled before reaching the clinical stage of the disease, subclinically infected animals contribute to the infectious load in the herd through fecal shedding (Tiwari et al., 2006; Weber, 2006; Marcé et al., 2011). Johne's disease prevention and control programs are largely based on decreasing MAP transmission within herds, as there is currently no effective vaccine for the prevention of infection or treatment for infected animals (McKenna et al., 2006; Garry, 2011; Sorge et al., 2011). The primary route of MAP transmission is fecal–oral through the ingestion of contaminated milk, water, feed, and contact with the contaminated environment due to fecal shedding (Harris and Barletta, 2001; Tiwari et al., 2006; Slater et al., 2016). Although ingestion of shed bacteria is the main cause of new infections, very little is known about the mechanisms of shedding, the amount of MAP shed, or the frequency of intermittent shedding, all of which contribute to animals traditionally being classified as strictly shedders or nonshedders (Kralik et al., 2011, 2014; Mitchell et al., 2012; Münster et al., 2013; Mortier et al., 2014; Koets et al., 2015). However, as more research adds to our current understanding of shedding, further insights can be made. Recently, it has been accepted that MAP shedding can be intermittent, due to passive (pass-through) or active infection, and shedding quantities can be roughly estimated based on the number of colony-forming units following culture on solid media or time to detection; however, these quantification methods have not been standardized and do not result in absolute quantification, as 1 cfu may not be formed by a single bacterium (Pradhan et al., 2011).

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¹Corresponding author: jdebuck@ucalgary.ca

Additionally, shedding was originally thought to occur only in adult cows but it is now recognized that calves can begin shedding MAP as early as 2 wk after exposure (van Roermund et al., 2007; Mortier et al., 2014; Wolf et al., 2015). Due to the increasing knowledge base on shedding, new insights can be gained from further research into the specific patterns of intermittent shedding and quantification of these fecal shedding events.

Dose and method of infection affect the frequency of MAP shedding; however, gaps in our knowledge remain regarding the quantity of fecal shedding and its relation to shedding on farm (Mitchell et al., 2012, 2015; Mortier et al., 2014). Consensus recommendations on doses and methods of inoculation for consistent experimental infection are available, but it is unclear to what extent these doses relate to natural exposure, leading to varying recommendations based on the outcome of interest (Hines et al., 2007; Begg and Whittington, 2008). Consequently, doses for infection in transmission trials have ranged from 4×10^4 to 10^{10} cfu, potentially affecting shedding intervals, immune responses, and degree of tissue infections (van Roermund et al., 2007; Eisenberg et al., 2011; Mitchell et al., 2012; Santema et al., 2012; Mortier et al., 2014).

Although a recent meta-analysis examined shedding patterns in experimental studies (Mitchell et al., 2012), information is lacking regarding the amount of MAP and the consistency of the quantity shed by inoculated and naturally exposed animals, as well as the effect of shedding animals on fecal shedding (passive or active) of pen mates (Barkema et al., 2017). Therefore, the aims of the current study were to (1) compare the frequency at which intermittent shedding occurred and the quantity of MAP shed over the course of group housing between inoculated and contact-exposed calves; and (2) determine the effects of inoculation dose on the quantity of MAP shed by calves over the course of 3 mo.

MATERIALS AND METHODS

The study was conducted using fecal samples collected in 2 independent calf MAP challenge experiments. Fecal samples in experiment 1 were used to compare frequency of shedding (based on culture) and quantities of MAP shed among inoculated and exposed (naturally infected) calves. All samples collected in experiment 1 were cultured, and only culture-positive samples were quantified. Selected fecal samples from 5 calves in experiment 1 and all fecal samples from calves in experiment 2 were used to quantify fecal shedding and quantity related to dose of inoculation. Fecal samples were categorized as either “pass-through shedding” of the inoculum (samples collected within the first 7 d af-

ter inoculation) or as “active shedding” (those collected after the first 7 d following inoculation); there were 5 samples per calf in each category.

Experiment 1

Study design, calf collection and care, and sample collection were as described previously (Corbett et al., 2017). Briefly, 32 newborn Holstein-Friesian bull calves were collected from 13 dairy farms in Alberta, Canada, that had tested negative for MAP for over 4 yr using 6 environmental samples and 1 of the following: bacteriological culture of 60 individual fecal samples tested as pooled samples into groups of 5, individual milk ELISA of the whole milking herd, or serum ELISA of the entire herd. Calves were group-housed in 7 experimental pens in a biosecurity level 2 facility, based on birth order. Each pen contained 4 calves, 2 of which were inoculated with a moderate dose (**MD**) and 2 of which were contact-exposed (**CE**) calves. An additional 4 calves acted as uninoculated control calves. Inoculum was prepared as described (Corbett et al., 2017). Briefly, a strain obtained from a clinical case (cow 69) in Alberta was used for inoculation. Following culture of the inoculum in 7H9 (Thermo Fisher Scientific, Waltham, MA)/mycobactin J (Allied Monitor, Fayette, MO)/OADC (Thermo Fisher Scientific) liquid broth from a frozen first-passage stock, 2 calves (2 wk of age) in each pen were inoculated orally with a moderate dose of 2.5×10^8 cfu on 2 consecutive days. At least 2 wk after inoculation (range: 14 to 21 d following inoculation), the CE calves were added to the group pen (to allow for pass-through of the inoculum), and the MD and CE calves were group-housed for 3 mo. The MD calves were euthanized after 3 mo of group housing, and the CE calves were euthanized after an additional 3 mo of individual housing. Control calves were group-housed throughout the study and were the last to be euthanized.

All samples were collected as described previously (Corbett et al., 2017). Briefly, individual fecal samples were collected 3 times per week, starting on the first day of group housing. Additional fecal samples were collected from 5 MD calves on d 1 to 4, 7, and 14 after inoculation. Fecal samples were aliquoted into 3 separate containers; one was cultured as described below within 1 wk after collection, and the others were frozen at -80°C pending further testing.

Experiment 2

Study design and sample collection were as described by Mortier et al. (2014); however, only fecal samples collected from calves that were inoculated at 2 wk of

age with additional samples on d 1 to 4 were used (3 of 5 calves in each dose group). Briefly, 6 newborn Holstein-Friesian bull calves, originating from dairy farms that were negative for MAP based on similar herd infection testing described for experiment 1, were inoculated (2 wk of age) with either a low dose (**LD**, 5×10^7 cfu) or a high dose (**HD**, 5×10^9 cfu) of MAP on 2 consecutive days, using the same MAP strain as in experiment 1. Calves were housed individually in strictly separated pens. Fecal samples were collected on d 1 to 4 after inoculation; weekly on d 7, 14, 21, 28; and monthly on d 57 and 91. All samples were cultured and then frozen at -80°C until direct extraction (**DE**) and PCR detection of MAP as described below. All samples positive by direct extraction were used for quantification, regardless of their culture status.

Culture and Direct Extraction of MAP

Fecal samples collected on d 1 to 4, 7, 14, 21, 28, 57, and 91 following inoculation from 5 MD calves of experiment 1, and all samples from 3 LD and 3 HD calves from experiment 2 were used. All fecal samples were cultured as described (Mortier et al., 2014; Corbett et al., 2017). Briefly, 2 g of fecal sample was disinfected in 0.9% hexadecylpyridinium chloride in half-strength brain-heart infusion broth at 37°C overnight following centrifugation of each sample. The pellet was resuspended in a mixture of antibiotic solution (**AS**; para-Jem, Trek Diagnostic Systems, Cleveland, OH), water, and full-strength brain-heart infusion and incubated at 37°C overnight, and 1 mL was added to paraJEM culture bottles and incubated at 37°C for 49 d. Following incubation, DNA was extracted as described (Forde et al., 2012), and a duplex quantitative real-time (**qPCR**) targeting the single copy, MAP-specific F57 region with an internal amplification control (**IAC**) was done (Slana et al., 2008). Based on fecal culture and qPCR results, MAP culture-positive fecal samples from the MD calves were selected to undergo direct extraction of DNA from samples. All fecal samples from LD and HD calves underwent both culture and direct extraction. Fecal samples were thawed and direct extraction performed using a MagMAX total nucleic acid isolation kit (Applied Biosystems, Carlsbad, CA) following the manufacturer's instructions, with modifications as described (Forde et al., 2013). Briefly, 0.3 g of fecal material was used, and a bead beater was used to release DNA. The final lysate product was then used for F57-specific duplex qPCR with an IAC, using the same protocol as for PCR confirmation after culture.

qPCR Quantification and Verification

Duplex qPCR targeting the MAP-specific F57 region and an IAC was performed with primers and probes identical to those described previously (Slana et al., 2008) after direct extraction of fecal samples or after MAP culture. Amplification conditions for qPCR were as follows: 50°C for 2 min, 95°C for 20 s to allow for initial denaturation, and then 42 cycles of 95°C for 30 s and 59°C for 30 s. Eluate from direct extraction was run in duplicate, with the lowest cycle threshold (**CT**) value from both runs retained for quantification.

Cultured fecal samples were considered MAP-positive when the CT value was <40 . All culture-positive samples from MD and CE calves and all samples collected from LD and HD calves were direct extracted for quantification. For direct extraction fecal samples, CT values from the qPCR were used to estimate relative amount of MAP present in each sample using a plasmid standard containing the F57 region cloned into pCR 2.1 cloning vector (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations and as described previously (Slana et al., 2008). Plasmid concentration was estimated using Qubit Fluorometric Quantification (Invitrogen), and 10-fold serially diluted 9 times. The F57 copy numbers from the direct extraction fecal samples were extrapolated from CT values, based on a plasmid standard curve generated from the plasmid standard dilutions to estimate the relative number of MAP bacteria per gram of feces per sample (Kralik et al., 2011).

A spiking experiment was conducted in triplicate as described previously (Kralik et al., 2011) to verify direct extraction and the quantification method. Briefly, a MAP culture suspension was prepared as described above for inoculation and quantified after a 10-fold serial dilution in broth by counting cells in a Neubauer chamber. Only the first 4 serial dilutions could be quantified using cell counting; therefore, dilutions were extrapolated based on the average of the first 3 serial dilutions. Seven serial dilutions of MAP cells were used to spike negative cattle fecal samples, with the number of cells ranging from approximately 5×10^8 to 10^1 . Following spiking, fecal samples underwent direct extraction as described above and quantification following F57-specific qPCR. The efficiency of direct extraction and quantification was determined as described (Kralik et al., 2011); briefly, the estimated recovered MAP per gram of feces was based on CT value and plasmid curve was divided by the theoretical input concentration (based on cell count) and multiplied by 100. Mean efficiency for each dilution was calculated.

Statistical Analyses

All statistical analyses were performed using Stata 11.2 (StataCorp LP, College Station, TX). For all analyses, $P < 0.05$ was considered significant. Normality was assessed using the Shapiro-Wilk test. The difference between quantities shed before and after group housing were compared using the Wilcoxon rank sum test. Differences between the mean MAP shedding quantity of MD and CE calves and differences between proportions of positive samples for each inoculation group were compared using a Kruskal-Wallis rank test. A regression was used to determine if there was a relationship between shedding quantities and days following group housing for MD and CE calves.

RESULTS

Fecal Culture Results (Experiment 1)

A total of 325 MAP culture-positive fecal samples of 1,176 were collected during group housing; 254 (78%) of these were from MD calves and 71 (22%) from CE calves. All calves had at least 2 MAP culture-positive fecal samples during the 3 mo of group housing. Although shedding was detected intermittently throughout group housing, a peak occurred between 24 and 40 d with $>50\%$ calves having MAP culture-positive fecal samples (Figure 1). Following this peak, the proportion of MAP culture-positive fecal samples decreased among inoculated samples. The proportion of all culture-pos-

Figure 1. Proportion of all fecal samples collected that were *Mycobacterium avium* ssp. *paratuberculosis* (MAP) culture-positive based on F57-specific quantitative real-time PCR. Culture-positive fecal samples from calves inoculated with a moderate dose (5×10^8 cfu) of MAP are represented by the dark gray bars and contact-exposed (not inoculated) calves are represented by light gray bars.

Table 1. Validation of the efficiency of direct extraction to quantify the total *Mycobacterium avium* ssp. *paratuberculosis* (MAP) DNA isolation from spiked cattle feces based on F57-specific quantitative real-time (q)PCR

Input of MAP cells ¹		Output of MAP cells ²		Efficiency ³ (%)
Mean ⁴	SE	Mean ⁴	SE	
4.8×10^8	1.7×10^7	2.1×10^8	3.5×10^7	44.3
5.3×10^7	5.0×10^6	2.6×10^7	7.3×10^6	50.2
3.5×10^6	9.8×10^5	4.4×10^6	2.6×10^6	107.2
4.5×10^5	5.0×10^4	6.0×10^5	3.0×10^5	122.3
4.5×10^4	5.0×10^3	2.4×10^4	7.9×10^3	56.5
4.5×10^3	5.0×10^2	9.3×10^3	4.9×10^3	187.0
4.5×10^2	5.0×10^1	7.8×10^2	7.8×10^2	181.4
4.5×10^1	5.0×10^0	0	0	0

¹Number of MAP cells (quantified using cell counting) used for spiking cattle feces.

²Number of MAP cells recovered following direct extraction of DNA and quantification by F57-specific qPCR.

³Mean MAP DNA extraction and quantification efficiency, calculated as experimental output of MAP per gram of feces divided by the input concentration, multiplied by 100.

⁴Mean values from triplicate serial dilutions corresponding to amount of MAP per gram of feces.

itive fecal samples varied daily, ranging from 0 to 57% ($P < 0.01$; Figure 1).

Spiking Experiment: Quantification Verification

Mean efficiency for the direct extraction and quantification of MAP from spiked fecal samples was 93.6% (95% CI: 40.5–146.7), with no difference between the first 3 serial dilutions and the last 3 ($P = 0.44$; Table 1). The MAP quantified from the CT values following F57-specific qPCR were within 0.5 log of the spiked concentration and highly correlated ($r = 0.98$); therefore, direct extraction and quantification were further used to quantify fecal samples.

Quantification of MAP in Culture-Positive Fecal Samples in MD and CE

Of the 325 MAP culture-positive samples collected during group housing, 111 (34%) were positive for both culture and direct extraction, 8 from CE and 103 from MD calves (Figure 2). Quantification using the standard plasmid curve was applied to all 111 fecal samples that were MAP-positive for both culture and direct extraction. Average MAP shed in all positive samples tended to be higher for MD than for CE calves: 7.6×10^5 MAP per g (103 samples) and 3.4×10^6 MAP per g (8 samples), respectively ($P = 0.08$).

Mean MAP shed per gram of feces among MD calves was higher before group housing (2.8×10^6) than during group housing (1.3×10^5 ; $P = 0.003$); however, there was no difference among calves for average MAP per gram of feces during group housing ($P = 0.24$), and no difference in shedding quantities over time during

group housing for either MD or CE calves ($P = 0.32$ and $P = 0.50$, respectively; Figure 3).

Quantification of MAP in LD, MD, and HD

All fecal samples collected from the 3 calves in the LD group, 3 calves in the HD group, and 5 calves in the MD group were cultured and direct extracted with relative quantification (Figure 4). A lower proportion of samples of the LD calves were MAP culture-positive compared with samples from HD calves ($P = 0.04$); and the proportion of MD samples tended to be higher than the proportion of LD samples ($P = 0.09$), but did not differ from the HD samples ($P = 0.16$). Of the 136 MAP culture-positive samples, 74 (54%) were positive following direct extraction (Figure 4). Of all fecal samples collected from all 3 doses (251 samples), 98 (40%) samples were MAP-positive after direct extraction.

For all 3 doses, there was a higher average quantity of MAP shed during pass-through shedding (first 7 d after inoculation) than in the average of active shedding (all samples after the first week; Table 2). Quantities shed during pass-through versus active shedding did not differ among doses (Table 2).

DISCUSSION

Calves shed the highest quantity of MAP in fecal samples in the first 7 d after inoculation, regardless of dose, at levels higher than the average of all remaining samples collected over the next 3 mo. Shedding did not increase over time. The highest proportion of samples were MAP culture-positive at 24 to 40 d of group housing of calves. The proportion of MAP-culture positive

Figure 2. Fecal samples collected from group-housed calves that were included in the quantification study for shedding of *Mycobacterium avium* ssp. *paratuberculosis*. DNA was direct extracted (DE) from fecal culture-positive samples from calves inoculated at a moderate dose (MD; 5×10^8 cfu) and contact-exposed (CE) calves.

samples increased with increasing inoculation dose, and the average quantity of MAP shed by CE calves was similar to that of MD-inoculated calves. After the first week after inoculation, no differences were found between the 3 doses for quantity of MAP shed. Therefore, the primary difference between doses was in frequency, not quantity, of shedding.

Frequency of fecal shedding detected by culture was highest among HD calves, followed by MD, LD, and the CE group, consistent with findings from previous trials in which dose and type of exposure (inoculation or exposure) had a direct effect on frequency of shedding (Mitchell et al., 2012; Mortier et al., 2014). Direct extraction was performed on all MAP culture-positive samples; however, fewer positive samples were detected by this method. Although direct extraction allows for estimation of MAP present in a fecal sample, low amounts of MAP that may be detected via the culture method due to growth during incubation may not be detected using the direct extraction technique. Calves inoculated with a lower dose, resulting in fewer culture-positive samples, may shed less MAP, resulting in more samples negative by direct extraction (due to decreased detectability). Low shedding quantities among calves in all dose groups resulted in samples negative by direct extraction in 46% of culture-positive fecal samples, which may result in overestimation of quantities of MAP shed, particularly among LD and CE calves. Additionally, low concentrations of MAP in fecal samples that can be detected by samples negative

by direct extraction may be overestimated using the quantification method, as indicated by spiking validation in this experiment and others (Kralik et al., 2011) and potentially resulting in a false plateau of shedding for all doses. Regardless, the proportion of culture-positive samples detected in each dose group clearly indicated that a higher dose caused more frequent fecal shedding events than lower doses.

Although culture may be a more sensitive technique to identify MAP-positive and MAP-negative fecal samples, direct extraction allows for estimation of the relative quantity of MAP present in fecal samples (Kralik et al., 2011). The average amount of MAP shedding after the first week following inoculation did not differ among the 3 doses; however, these amounts were lower than those in the first week after inoculation. These differences between the amount of MAP detected in the first week following inoculation and subsequent shedding events may be indicative of the difference between pass-through shedding of the inoculum and active shedding following infection (Sweeney et al., 1992; Hines et al., 2007). Although a minimum infection dose for MAP is not known, it has been suggested that 1.5×10^6 cfu is sufficient to reliably detect infection in tissues, with doses as low as 10^3 cfu able to cause infection (Sweeney et al., 2006; Begg and Whittington, 2008). Based on quantification results from the current study, shedding would be sufficient to cause infection on farm and could have major implications, given that a shedding level $>10^4$ cfu/g feces is extremely high. “Super

Figure 3. Fecal shedding quantities of *Mycobacterium avium* ssp. *paratuberculosis* (MAP) based on MAP-specific F57 gene copies for calves during 3 mo of group housing (starting on d 0). Dark bars indicate log MAP copies per gram of feces quantified for calves inoculated at a moderate dose (MD; 5×10^8 cfu). Multiple calf samples on the same day are indicated by white horizontal lines across the dark bars. Light bars indicate log MAP copies per gram of feces quantified for contact-exposed (CE) calves.

Figure 4. Fecal samples collected from calves inoculated with a low dose (LD, 1×10^8 ; $n = 3$), moderate dose (MD, 5×10^8 cfu; $n = 5$), or high dose (HD, 1×10^{10} ; $n = 3$) of *Mycobacterium avium* ssp. *paratuberculosis*, with a breakdown of culture and direct extraction (DE) results for each dose.

shedders” are defined as those animals shedding 1×10^4 cfu/g of feces; however, it should be noted that the relative quantification method uses copy number based on the single copy gene F57, which might give a more accurate estimation of the number of MAP in a sample compared with colony-forming units (Whitlock et al., 2005). Additionally, the minimum threshold at which direct extraction and relative quantification can reliably detect F57 is reported to be 1.0×10^4 (Kralik et al., 2011), and although results from the spiking experiment indicate that lower numbers can be detected with qPCR, detection and quantification of MAP may be more difficult in low-shedding samples collected from the calves because of the low quantities and the unknown mechanisms of shedding (Koets et al., 2015).

Once pass-through of the inoculum had occurred (7 d following inoculation) in group-housed MD calves, the quantity of MAP shed was consistent; however, the frequency at which MAP was detected in fecal samples was highly variable. In agreement with previous studies, inoculated calves shed more frequently than CE calves, and a higher dose increased the frequency of positive samples (Mitchell et al., 2012, 2015; Mortier et al., 2014). Although individual shedding patterns were intermittent, it is important to consider the cumulative effect on environmental contamination due to MAP fecal shedding in calves, as any shedding calf will contribute to environmental contamination, potentially leading to new infections of susceptible calves.

The main objectives of current JD prevention and control programs are to decrease the incidence of new MAP infections by decreasing transmission from infectious to susceptible calves and to decrease the prevalence of infection by preventing entry of infectious animals into the herd and identifying and removing infectious animals from the herd (McKenna et al., 2006; Garry, 2011; Cho et al., 2013). Currently, identification and removal of infectious animals within the herd focuses on cows that have calved at least once; however, based on results from this study and others, it may be necessary to begin fecal testing of young stock (Weber et al., 2010; Mortier et al., 2015; Wolf et al., 2015). Di-

rect extraction and qPCR targeting F57 may facilitate rapid identification of MAP-positive calves shedding on farm as well as quantification of shedding. However, repeated sampling may be needed and culture-based methods appear to be more sensitive, especially if low quantities of MAP are shed (Kralik et al., 2011, 2012). It is also important to consider that the results from this experimental study may not entirely reflect fecal shedding occurring in naturally infected animals in a herd; however, important insights can still be made to improve JD control programs especially regarding the effect of shedding calves. Control programs should include testing of environmental samples collected from areas that house young stock, because MAP-positive calves may not be detected due to the nature of intermittent shedding, and the environment may act as a reservoir of accumulated infectious material previously shed (Moravkova et al., 2012; Wolf et al., 2015; Corbett et al., 2017).

In conclusion, inoculum dose and method were associated with the frequency of shedding MAP in feces as detected by culture but not with quantity of MAP shed in the months following the first week after inoculation. Frequency of shedding increased with dose and shedding was more frequent in inoculated than in CE calves. Immediately after inoculation, MAP quantities were relative to the dose administered; however, later amounts of MAP shed per gram of feces were consistently between 9×10^4 and 2×10^5 during the first 4 mo, regardless of inoculum dose. This study provides important information regarding shedding quantities in calves inoculated with 3 doses of MAP, patterns of shedding among pen mates, and quantities of MAP shed in CE calves, allowing future experimental trials to implement this information in the decision-making process regarding inoculation doses. Additionally, understanding shedding patterns and quantities in naturally infected calves should improve JD control programs, allowing inclusion of effective calf sampling and the addition of young stock environmental sampling to better prevent new infections from occurring in MAP-positive herds.

Table 2. Quantity of *Mycobacterium avium* ssp. *paratuberculosis* (MAP) per gram of feces shed following inoculation with a low, moderate, or high dose for direct extraction (DE)-positive fecal samples based on F57 quantitative real-time PCR

Inoculation dose	No. of DE-positive samples	Mean MAP/g of feces for first 7 d after inoculation ¹ (pass-through)	Mean MAP/g of feces for remaining days (>7 d) after inoculation ¹ (active)
Low (1×10^8 cfu)	14	8.2×10^6 ^a	1.3×10^5 ^b
Moderate (5×10^8 cfu)	61	1.3×10^6 ^a	9.4×10^4 ^b
High (1×10^{10} cfu)	23	1.6×10^8 ^a	1.7×10^5 ^b

^{a,b}Means in rows and columns without a common superscript differ ($P < 0.05$).

¹Fecal samples were categorized as either pass-through shedding of the inoculum (samples collected within the first 7 d after inoculation) or as “active” shedding (those remaining after 7 d).

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