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

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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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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 after 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