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Clinical disease and stage of lactation influence shedding of *Mycobacterium avium* subspecies *paratuberculosis* into milk and colostrum of naturally infected dairy cows

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ABSTRACT

Mycobacterium avium ssp. *paratuberculosis* (MAP) is the causative agent of Johne's disease (JD). One mode of transmission of MAP is through ingestion of contaminated milk and colostrum by susceptible calves. The objective of this study was to determine if the amount of MAP shed into the milk and colostrum of infected cows was affected by severity of infection as well as the number of days in milk (DIM). Milk was collected over the 305-d lactation period from naturally infected cows in the asymptomatic subclinical (n = 39) and symptomatic clinical (n = 29) stages of disease, as well as 8 noninfected control cows. All milk samples were assayed for MAP by culture on Herrold's egg yolk medium and either BACTEC 12B (Becton Dickinson, Franklin Lakes, NJ) or *para*-JEM (Thermo Fisher Scientific, Trek Diagnostic Systems Inc., Cleveland, OH) liquid medium, and by direct PCR for the *IS900* target gene. *Mycobacterium avium* ssp. *paratuberculosis* was detected in 3.8, 4.1, and 12.6% of milk samples collected from cows with subclinical JD after culture in Herrold's egg yolk medium, liquid medium, and direct PCR, respectively. The frequency of MAP positivity increased to 12.9, 18.4, and 49.2% of milk samples collected from cows with clinical JD by these same methods, respectively. None of the milk samples collected from control cows was positive for MAP by any detection method. Viable MAP was primarily isolated from milk and colostrum of subclinically and clinically infected cows collected in early lactation (DIM 0–60), with negligible positive samples observed in mid (DIM 60–240) and late (DIM 240–305) lactation. This study demonstrates that shedding of MAP into milk is affected by infection status of the cow as well as stage of lactation, providing useful information to producers to help break the cycle of infection within a herd.
Key words: *Mycobacterium avium* ssp. *paratuberculosis*, milk, lactation, shedding

INTRODUCTION

Johne's disease (**JD**), caused by *Mycobacterium avium* ssp. *paratuberculosis* (**MAP**), results in significant annual economic loss to the dairy industry, primarily due to reduced milk production and premature culling (Ott et al., 1999). Though primarily transmitted through ingestion of contaminated feces, MAP also can be transmitted through milk and colostrum, as well as in utero (Chiodini et al., 1984). As JD progresses, MAP can disseminate throughout the body and has been isolated from milk, supramammary lymph nodes, and lymph fluid from the udder (Sweeney et al., 1992; Chiodini, 1996; Khol et al., 2012).

Milk and colostrum containing MAP can be consumed by neonates and young stock that are most susceptible to MAP infection (Windsor and Whittington, 2010). Calves that are fed maternal colostrum are more likely to become infected with MAP than those that are fed colostrum replacer or pasteurized colostrum, indicating that raw colostrum and milk may be the initial point of exposure for neonates (Stabel, 2008; Pithua et al., 2009). Furthermore, if producers feed pooled milk or colostrum, they are increasing the likelihood of transmitting MAP to an even larger number of calves. Unfortunately, milk and colostrum replacers are an expensive alternative to feeding pooled milk and colostrum.

Little is known about the factors that influence the amount of MAP shed into milk. The excretion of MAP may be influenced by the stage of infection, with the highest shedding occurring in the clinical stages of JD and very little or even undetectable amounts of MAP shed in early stages (Sweeney et al., 1992). Days in milk also may be correlated with the amount of MAP

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shed into milk and colostrum, because the volume and composition varies greatly throughout the 305-d lactation. Effects of milk volume and composition on the diagnosis of MAP infection using the milk ELISA have been observed (Nielsen and Toft, 2012). A commercial ELISA was used to measure MAP antibodies in over 1 million milk samples collected from cows enrolled in the Danish paratuberculosis control program over a 31-mo period. Results demonstrated that DIM was a significant factor in the identification of positive cows and that early stage lactation samples at 1 to 2 DIM are more likely to test positive, suggesting that dilution factors may be responsible for a loss in sensitivity of detection (Nielsen and Toft, 2012; Zervens et al., 2013). Further testing demonstrated that colostral immunoglobulins were not found to contribute to false-positive reactions in the ELISA, and measurement of colostral antibody improved the odds of an animal testing positive for MAP infection by 130-fold compared with samples taken at 4 DIM (Zervens et al., 2013). Although the measurement of milk antibodies is quite different than quantifying the shedding of MAP into the milk, these results suggest that DIM may affect the sensitivity of detection. The objectives of this study were to determine if a correlation exists between stage in JD, DIM, and the amount of MAP shed into milk and colostrum of naturally infected dairy cows.

MATERIALS AND METHODS

Milk Collection

Milk was collected for 91 complete 305-d lactation cycles over a period of 12 yr from 76 Holstein dairy cows well characterized for JD. Lactation cycles were stratified across infection status of cows as follows: asymptomatic cows with subclinical disease (n = 39) and symptomatic cows with clinical signs of disease (n = 29), and noninfected control cows (n = 8). Cows were stratified into infection groups by monitoring fecal shedding of MAP by culture and PCR as previously described (Khalifeh et al., 2009). By definition, clinical cows were shedding more than 100 cfu/g of feces and presented with weight loss and intermittent diarrhea. Subclinically infected cows were shedding less than 10 cfu/g of feces and were asymptomatic. The noninfected control cows were characterized by repeated negative fecal culture performed semi-annually over a 3- to 5-yr period. In addition, these animals were negative for production of antibodies specific for MAP and IFN-γ performed during that period.

Due to the long-term collection period, we were able to obtain samples from 2 lactation periods from 13 of the 76 cows and 3 lactation periods from 1 of the 76 cows. Milk was collected into sterile containers after thorough cleaning of the teats on d 1, 3, 7, 14, 21, 28, 90, 180, and 305 after calving. Milk was immediately frozen and stored at −80°C until processing. Animals used in this study were housed at the National Animal Disease Center (**NADC**, Ames, IA). All procedures performed on the dairy cows were approved by the Institutional Animal Care and Use Committee (NADC).

Positive and Negative Control Milk Preparation

Milk was obtained from a noninfected healthy cow at the NADC. Milk from the cow was hand stripped into sterile collection containers after thorough disinfection of the udder and teats with iodine teat dip and 70% ethanol. Large volumes of milk were collected at one time and aliquots were dispensed into tubes and stored at −80°C until use. This milk was used for the negative control and also artificially spiked to a final concentration of 10^6 cfu/mL with MAP strain 167 (obtained from a field isolate at the NADC) for the positive control.

Decontamination of Milk with N-Acetyl-L-Cysteine-NaOH

Each sample of milk was thawed overnight at 4°C. The samples were lightly shaken to ensure a homogeneous sample. A 20-mL aliquot of the milk was transferred to a sterile container centrifuged at 5,600 × *g* for 30 min at 4°C. The whey layer was discarded, and the cream and pellet layers were retained. The cream and pellet layers were resuspended in PBS (Sigma-Aldrich, St. Louis, MO) to bring the final volume to 5 mL. The samples were decontaminated with 5 mL of a solution containing sterile 1.45% sodium citrate, 3.0% sodium hydroxide, and 0.5% *N*-acetyl-L-cysteine (NALC-3.0% NaOH; Sigma-Aldrich) as previously described (Bradner et al., 2013). The NALC component of the solution was added immediately before use. The samples were inverted to mix, and incubated for 15 min at 22°C. Immediately after incubation, 15 mL of PBS was added to dilute the NaOH. Samples were centrifuged at 5,600 × *g* for 30 min at 4°C, the aqueous layer was discarded, and the cream and pellet were resuspended in 1 mL of PBS for inoculation of the media.

Media for Recovery of MAP

Initially, BACTEC 12B liquid medium [Becton Dickinson (**BD**), Franklin Lakes, NJ] and Herrold's egg yolk (**HEY**) agar medium (BD), were used to culture MAP from milk samples. However, the BACTEC 12B medium was discontinued by the company midway through the experiment and *para*-JEM medium (Thermo Fisher