Association between *Mycobacterium avium* subspecies *paratuberculosis* infection and milk production in two California dairies

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ABSTRACT

The association between *Mycobacterium avium* ssp. *paratuberculosis* (MAP) and milk production was estimated on 2 California dairies using longitudinal data from 5,926 cows. Both study herds had moderate MAP seroprevalence, housed cows in freestalls, and had Johne's disease control programs. Cow MAP status was determined using both serum ELISA and fecal culture results from cows tested at dry-off and from whole-herd tests. Potential confounders were evaluated based on a causal diagram. Mixed models with 2 functions (splines) for days in milk (DIM) representing milk production pre- and postpeak used in similar studies were further modified to use each cow's observed DIM at peak and lactation length. Cows that were seropositive produced 2.5 kg less 4% fat-corrected milk (FCM) per day than their seronegative herdmates. In addition, cows that were fecal-culture positive by liquid culture and confirmed by PCR produced 2.2 kg less 4% FCM per day than their fecal-culture negative herdmates. The decrease in milk production in MAP test-positive compared with test-negative cows started in the second lactation. A switch in MAP status in either ELISA or fecal culture results from positive to negative had no significant association with milk production. Modified DIM functions that used the observed DIM at peak had better model fit than another function that assumed a fixed peak at 60 DIM. Cows that tested positive for MAP on serum ELISA or fecal culture produced less milk than cows that tested negative, and the association between MAP and milk production was not confounded by mastitis, elevated somatic cell counts, or uterine or metabolic cow conditions.

Key words: *Mycobacterium avium* ssp. *paratuberculosis*, fat-corrected milk, mixed-effect regression model, causal diagram

INTRODUCTION

Mycobacterium avium ssp. *paratuberculosis* (MAP) infection is subclinical in most dairy cows although decreased milk production may be evident before removal of the cow from the herd or the onset of clinical Johne's disease (Ott et al., 1999). Although most studies have found a statistically significant association between MAP test-positive status and decreased milk production (Kudahl et al., 2004; Lombard et al., 2005; Gonda et al., 2007; Raizman et al., 2007), a few have shown contrary findings (McNab et al., 1991; Johnson et al., 2001), or an initial increase in milk production in latent and low-shedding animals before a decrease in milk production as these cows became high shedders (Smith et al., 2009). A nationwide US study reported a 304-kg decrease in milk production per lactation in MAP-infected compared with noninfected cows (Gonda et al., 2007). Another study reported a 1,364-kg decrease in milk production per lactation in strong seropositive cows compared with seronegative cows (Lombard et al., 2005). Other studies on dairies from individual states reported a range varying from a 376-kg decrease in milk production per lactation (Nordlund et al., 1996) to an increase in milk production of infected cows during their first 100 DIM (Wilson et al., 1993). Such variability in estimates may be attributable to study design differences, the diagnostic criteria for defining a MAP-infected cow, the selected measure of milk production, and the statistical model used to estimate differences in milk production including adjustment for potential confounders.

Traditionally, a milk yield summary measure for an entire lactation such as 305-d mature equivalent (ME 305) has been used in cross-sectional or case-control studies to compare milk production between MAP test-positive and test-negative cows (Johnson et al., 2001; Lombard et al., 2005; Raizman et al., 2007). A standardized measure such as ME 305 provides a parity-adjusted estimate of milk production and summarizes test-day (TD) records and incomplete lactations. However, several issues need to be considered when modeling effects

Received August 2, 2009.

Accepted November 11, 2009.

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of a pathogen on milk production using ME 305. First, in records less than 155 DIM, the mature-equivalent herd average is used when estimating the cow's ME 305 (Wiggins, 1980; Wiggins and Dickinson, 1985), and this value may under- or overestimate a cow's production in low- or high-producing herds, respectively. Second, ME 305 does not provide information on the shape of the lactation curve. For these reasons, estimates of the magnitude of the association between MAP and milk production using TD milk yields from a longitudinal study design may be more accurate than estimates using ME 305.

Analysis of longitudinal milk production data such as TD milk yields is possible through mixed-effects regression models (**MRM**; Hedeker and Gibbons, 2006). Such models account for the natural hierarchy of TD milk yields nested within lactations, within cows, and within herds (Jiang, 2007). The characteristic shape of a lactation curve, an increase in milk production followed by a more persistent postpeak decrease in milk production, may be modeled using various statistical techniques including piece-wise linear regression (Bennedsgaard et al., 2003; Macciotta et al., 2005; Liu et al., 2006; Silvestre et al., 2006). A piece-wise MRM assuming a 60 DIM peak and 305-d lactations has been used to estimate the differences in TD milk production between MAP antibody test-positive and test-negative cows (Kudahl et al., 2004; Nielsen et al., 2009). However, not all cows have 305-d lactations or peak at the same time because of breed, management practices, and nutrition differences. For example, large dairy herds commonly use bST, which may increase the lactation period and delay the peak (Dunlap et al., 2000). A 2-piece MRM using each cow's observed lactation curve as defined by the cow's lactation length and DIM at peak may improve model fit and hence, predict milk production with less error. In Danish herds, Kudahl et al. (2004) reported a minimum loss of 2.7 kg of 4% FCM per 1 unit increase in serum ELISA optical density (**OD**) with an exponential increase in milk loss as cows' ELISA scores increase (Kudahl et al., 2004). Nielsen et al. (2009) reported a decrease in ECM production of Danish herds 300 d before they tested positive by milk ELISA. Such longitudinal studies estimate the magnitude of the association between MAP and milk production; however, fundamental differences between Danish and California herds including cow breeds, nutrition, herd size, and management practices such as use of bST, and culling practices may limit the generalizability of Danish estimates to California dairies.

Estimated differences in milk production between MAP test-positive and test-negative cows may depend on whether an antibody-detection test, an organism-detection test, or both are used for classification of

MAP infection status of cows. For example, Beaudeau et al. (2007) reported a decrease in TD milk yield ranging from 1.6 to 7.2 kg in French dairy cows depending on the MAP diagnostic test. In addition, a few studies attempted to adjust estimates for clinical diseases that may confound the association between MAP and milk production (Johnson et al., 2001; Raizman et al., 2007; Smith et al., 2009) yet such associations have not been outlined in a causal diagram, which may help justify their inclusion or exclusion (Dohoo et al., 2003). The objective of this longitudinal study was to estimate the magnitude of the association between MAP and milk production in 2 California dairy herds adjusted for potential confounders identified through a causal diagram approach.

MATERIALS AND METHODS

Study Herds

Two central California dairies with routine MAP testing at dry-off as part of an ongoing Johne's control program initiated in January 2003 were enrolled in the study. Dairy 1 (1,676 Holsteins) and dairy 2 (3,577 Jerseys) have moderate seroprevalence of MAP of 3.5% and 4.5%, respectively. Dairy 1 used bST on milking cows after 100 DIM, whereas dairy 2 used bST after 80 DIM. On both dairies, adult milking cows were fed monensin (**MN**) starting in November 2004. Dairy Herd Improvement records from March 2002 to March 2007 for cows on dairy 1 and to October 2008 for cows on dairy 2 were downloaded from DHI-Plus (DHI-Provo, Provo, UT) and Dairy Comp 305 (Valley Ag Software, Tulare, CA) software, respectively, into a relational database (Microsoft Access 2007, Microsoft Corp., Redmond, WA). Only cows that had their first calving on the study dairies were included; hence, lactation records of older cows that may have been introduced before implementation of a Johne's disease control program were excluded. Cow records included identification (**ID**), birth date, dam ID, sire ID, lactation, calving date, dry date, and monthly TD information such as milk yield, SCC, percentage fat, and percentage protein. Calving date was transformed to the categorical variable calving season (**CALVSEAS**) made up of 4 levels: January to March (reference level), April to May, June to August, and September to December. Four percent FCM was computed and used in all analyses (Tyrrell and Reid, 1965). Health events for cows on dairy 1 were recorded on paper and entered by hand into the relational database, whereas those for cows in dairy 2 were downloaded from Dairy Comp 305. Health events entered into the database included mastitis (**MAS**) and whether a cow ceased milk production in

one or more udder quarters because of chronic mastitis (**3TT**), metritis (**ME**), retained placenta (**RP**), milk fever (**MF**), displaced abomasum (**DA**), and ketosis (**KE**). Because of the possibility of different diagnostic criteria between herds and over time by different veterinarians, MAS and 3TT were combined into a single event for udder related infections (**MA**). Similarly, ME and RP were combined into uterine conditions (**UC**) and MF, DA, and KE were combined into metabolic conditions (**MC**).

Diagnostic Testing

Beginning in March 2003, cows on both dairies were routinely tested for MAP at dry-off at the California Animal Health and Food Safety (CAHFS) Laboratories (Fresno and Tulare) using serum ELISA (HerdCheck, Idexx Laboratories Inc., Westbrook, ME) and liquid fecal culture (**FC**; ESP para-JEM culture system II, Trek Diagnostic Systems Inc., Sun Prairie, WI) followed by acid-fast staining and PCR confirmation as described previously (Kim et al., 2004; Berghaus et al., 2006). In addition, whole-herd tests using the same serum ELISA were conducted on dairy 1 in 2004 and on dairy 2 in 2003, 2007, and 2008. A cow's MAP status was based on an ELISA value using OD readings and was reported as sample-to-positive (**S/P**) ratio, where $S/P = (\text{sample OD} - \text{negative control OD}) / (\text{positive control OD} - \text{negative control OD})$; and a dichotomous (positive or negative) fecal culture status.

Relational Database

Health events were linked to TD milk records such that a health event that occurred between 2 monthly TD was linked to the most recent TD. In contrast, a cow's first ELISA or FC result (in its first lactation) was linked to its subsequent TD milk records until the second ELISA or FC result, in which case the latter was linked to its subsequent TD milk records, and so on. In addition, for first-lactation cows, an ELISA or FC result of a sample collected on or before 305 DIM was linked to its previous first-lactation TD milk records. Such a sample collected while the cow is milking is representative of TD milk records within the industry-standard lactation length of 305 d. For example, consider a cow with 2 ELISA results, the first S/P value of 0.05 from a whole-herd test collected at 300 DIM of its first lactation, and the second S/P value of 0.40 during its first dry-off. The S/P of 0.05 was linked to the cow's first-lactation TD milk records between DIM 1 and 300, and all subsequent TD milk records until its dry-off ELISA result, after which the S/P of 0.40 was linked to the subsequent TD milk records in its second

Figure 1. Causal diagram for a confounder: the primary association of interest, the effect of exposure X on outcome Y, is confounded by variable Z. Variable Z has to be associated with the exposure and outcome, the latter causally to confound the primary association of interest. A causal association is depicted with a single-headed arrow, and a noncausal association is depicted with a 2-headed arrow.

lactation. Had the first ELISA result been for a sample collected after 305 DIM it would have been linked only to the subsequent TD milk records and until the dry-off ELISA result.

Confounders and Intermediate Factors

A confounder has to be associated with the exposure and outcome, the latter causally (Figure 1; Szklo and Nieto, 2000). Confounders distort the association between exposure and outcome and may result in incorrect interpretations if not taken into account. Parity is a confounder of the association between MAP and milk production because parity is associated with testing positive for MAP in an infected animal and is causally associated with an increase in milk production. A cow's immune status may vary from calving to dry-off (either because of stress or aging) and hence, DIM may be associated with a cow's MAP test result; at the same time DIM has a causal effect on volume of milk produced and thus may be a confounder. Similarly, feeding MN is associated with MAP serostatus (Hendrick et al., 2006) and is causally associated with increased milk production (Phipps et al., 2000). Hence, MN is a confounder of the association between MAP and milk production.

In contrast, intermediate factors are on the causal pathway between an exposure and an outcome; hence, the effect of exposure on the outcome can be mediated directly or indirectly through an intermediate factor (Figure 2). Such direct and indirect effects of exposure on outcome are not necessarily distinct and may overlap and therefore, separate estimates of either can be biased (Rothman et al., 2008). Although the association between a factor such as MA, SCC, ME, RP, MF, DA, or KE, and decreased milk production may be causal and thus, attains one of the criteria of confounding, the association and its direction between any of these factors and MAP is not known. For example, if an event such as mastitis, causally associated with

Figure 2. Causal diagram for an intermediate factor: the primary association of interest, the effect of exposure X on outcome Y, may be mediated directly (solid line) and indirectly (dashed line) through variable U, an intermediate factor. A causal association is depicted with a single-headed arrow.

milk production (outcome), leads to reduced immune responsiveness to concurrent infections with pathogens such as MAP (exposure), and hence results in MAP fecal shedding and a positive MAP culture result, then mastitis confounded the association between MAP and milk production. Adjustment for mastitis status or a surrogate such as SCC is then warranted, unless SCC is an imperfect surrogate, which may lead to residual confounding (Rothman et al., 2008). In contrast, if MAP infection is immunosuppressive and increases the risk of infections such as mastitis and subsequently results in lower milk production, then the association between MAP and milk production was mediated in part through mastitis. In this case, the data analyst should not adjust for mastitis or SCC. Similar hypotheses about the association between MAP and SCC have been proposed by Baptista et al. (2008) and can be applicable to the association between MAP and mastitis. Similarly, metritis or retained placenta may act as confounders or intermediate factors of the association between MAP and milk production. Likewise, metabolic diseases may result in a negative energy balance, stress, and lowered immunity against MAP and hence, confound the association of interest. Alternatively, MAP infection may reduce a cow's capability to absorb nutrients leading to metabolic diseases and decrease in milk production. In the latter scenario, the association between MAP and milk production was mediated through negative energy balance and hence, metabolic conditions should not be adjusted for. Factors that are associated with either the exposure or outcome only, such as number of times milked and ration fed, need not be adjusted for, provided they are similar in the different study herds.

Causal diagrams are based on current knowledge and results of other studies, which can be inconclusive in terms of the association between MAP and milk production. In the present study, we constructed a causal diagram for a model assuming MA, UC, and MC as intermediate factors (Figure 3A) and another for a model considering the same factors as confounders (Figure

Figure 3. Causal diagram for the association between *Mycobacterium avium* ssp. *paratuberculosis* (MAP) and milk production, and their possible association with monensin, DIM, and parity as confounders (solid lines). Mastitis, uterine, and metabolic conditions are shown in A) as possible intermediate factors on the causal pathway (dashed lines) and in B) as confounders (solid lines).

3B) as suggested in similar circumstances (Weng et al., 2006).

Statistical Analysis

Descriptive Statistics. Total number of ELISA and FC results matched to monthly DHI records and their lactations, and cows were summarized by herd. The mean number of ELISA and FC tests per cow in each herd, the number of cows that switched ELISA or FC status, and the number of cows that tested positive by ELISA, FC, or both was calculated for each dairy.

Two-Piece Mixed-Effects Regression Models. The magnitude of the association between MAP and milk production was estimated using a 3-level MRM

with a random lactation intercept, nested within a random cow intercept, nested within a random herd intercept. A cow's MAP status was time-varying, and estimates of difference in milk production were reported per unit increase in test result (S/P ratio) or a change from MAP-negative to MAP-positive status for ELISA and FC, respectively. A switch in MAP ELISA status (**ELISAswitch**) was also included in the analyses and defined as a positive test result followed by a negative result using the manufacturer's recommended S/P ratio cut-off of 0.25. Similarly, a switch in fecal culture status (**FCswitch**) was included in the analyses and defined as a switch from a positive to a negative test result.

In all models, the shape of the lactation curve was modeled using a 2-piece linear function for DIM similar to that described by Bennedsgaard et al. (2003) but modified to use each cow's peak milk production and its lactation production. The function resulted in 2 splines, an initial increase in milk production from calving to the cow's lactation-specific observed peak (**PK**) followed by a decrease in milk production to the end of lactation (dry-off) and were generated by transforming DIM to 2 variables: DIM pre-peak (**DIMprePK**) and DIM post-peak (**DIMpostPK**). For TD before or on a cow's PK DIM, DIMprePK was calculated as (DIM - DIM at PK)/DIM at PK, and DIMpostPK = 0. For TD on or after a cow's PK DIM, DIMpostPK was calculated as (DIM - DIM at PK)/(total DIM - DIM at PK), and DIMprePK = 0. The resulting splines meet at a knot equivalent to the observed cow's lactation peak. In addition, DIMprePK² and DIMpostPK² were added to model the curvilinear shape of each spline and retained in the model if the Akaike information criterion (**AIC**) showed improved model fit, where $AIC = -2 \text{ natural logarithm (likelihood)} + 2 \times \text{model degrees of freedom}$. Models with a lower AIC had a better fit. Random coefficients for DIMprePK and DIMpostPK were also used at the lactation level to account for variability between cows in pre- and post-peak milk production, respectively, and make up the random DIM slope. Fixed effects for herd, lactation, DIMprePK, and DIMpostPK were forced into all models given their biological and curve-modeling importance. Similarly, ELISA and FC were forced into the model as explanatory variables.

Based on the causal assumptions in Figure 3A, ELISAswitch, FCswitch, CALVSEAS, and the interaction between herd and lactation (**HerdLact**) were each added separately to model A and tested for statistical significance. Variables that were not significant or were borderline significant were compared with model A with and without them using the likelihood ratio test to test their significance as a group. A $P < 0.05$ was considered statistically significant. In addition, confounding by MN was assessed using the method of change in es-

timates (Mickey and Greenland, 1989; Rothman et al., 2008; Weng et al., 2009). A 10% or greater change in the estimate of the coefficient for ELISA or FC between the model with and without MN was used as evidence of confounding based on findings from simulation studies of confounder control (Maldonado and Greenland, 1993). In addition, the difference in the estimates of the effect of MAP from model A and B (with and without confounders, respectively) was reported to assess any substantial differences that may be considered confounding. Hence, both causal diagrams and the method of change in estimates were used to identify confounders (Weng et al., 2009).

Model A predicting Y_{ijkl} , the 4% FCM yield on the i th TD of the j th lactation of the k th cow in the l th herd is

$$\begin{aligned} Y_{ijkl} = & \beta_0 + \beta_1 \text{Herd}_l + \beta_2 \text{Lactation}_{jkl} + \beta_3 \text{HerdLact}_{lj} \\ & + \beta_4 \text{DIMprePK}_{ijkl} + \beta_5 \text{DIMpostPK}_{ijkl} \\ & + \beta_6 \text{DIMprePK}_{ijkl}^2 + \beta_7 \text{DIMpostPK}_{ijkl}^2 \\ & + \beta_8 \text{ELISA}_{ijkl} + \beta_9 \text{FC}_{ijkl} + \beta_{10} \text{ELISAswitch}_{kl} \\ & + \beta_{11} \text{FCswitch}_{kl} + \beta_{12} \text{CALVSEAS}_{jkl} + \beta_{13} \text{MN}_{ijkl} \\ & + z_{0l}^{\text{herd}} + v_{0kl}^{\text{cow}} + u_{0jkl}^{\text{lactation}} + u_{1jkl}^{\text{lactation}} \text{DIMprePK}_{ijkl} \\ & + u_{2jkl}^{\text{lactation}} \text{DIMpostPK}_{ijkl} + e_{ijkl}, \end{aligned}$$

where the intercept β_0 is the overall mean milk production at peak, β_1 to β_{13} represent the respective fixed effects ($X\beta$); z_{0l}^{herd} , v_{0kl}^{cow} , and $u_{0jkl}^{\text{lactation}}$ are the random herd, cow, and lactation intercepts, respectively; $u_{1jkl}^{\text{lactation}}$ and $u_{2jkl}^{\text{lactation}}$ are the random slope coefficients at the lactation level for DIM pre-peak and post-peak, respectively, and e_{ijkl} are the residual errors.

Random effects were assumed to be independent, identically distributed, and estimated by the empirical BLUP. The random effects for herd and cow were each assumed to be distributed univariate normal with means of 0 and variances σ_{herd}^2 and σ_{cow}^2 , respectively. The random effects at the lactation level, the lactation intercept $u_{0jkl}^{\text{lactation}}$, pre-peak slope $u_{1jkl}^{\text{lactation}}$, and post-peak slope $u_{2jkl}^{\text{lactation}}$ constitute the design vector $\mathbf{U}^{\text{lactation}}$ assumed to be distributed multivariate $N(0_{3 \times 1}, \Sigma_{\text{lactation}})$, where $\Sigma_{\text{lactation}}$ is a 3×3 unstructured variance-covariance matrix (6 parameters) to account for the correlation in sequential monthly milk measurements in a given lactation:

$$\begin{bmatrix} \sigma_{u(0)}^2 & \sigma_{u(0),u(1)} & \sigma_{u(0),u(2)} \\ \sigma_{u(0),u(1)} & \sigma_{u(1)}^2 & \sigma_{u(1),u(2)} \\ \sigma_{u(0),u(2)} & \sigma_{u(1),u(2)} & \sigma_{u(2)}^2 \end{bmatrix}.$$

The residual errors e_{ijkl} were assumed to be distributed univariate $N(0, \sigma_{residual}^2)$.

Based on Figure 3B, model B included all variables retained in the final model A in addition to the fixed effects for potential confounders MN, MA, SCC, UC, and MC entered both individually and as a group to assess confounding using the method of change in estimates. Furthermore, separate lactation-specific models based on model A or model B were estimated (depending on evidence of confounding). For lactation 1 and 2 models, a 2-level MRM with cows nested within herd and with the random DIM slope coefficients at the cow level was specified. Lactation and herd-lactation interaction were removed from lactation 1 and 2 models due to collinearity. For lactations >2, a 3-level MRM similar to model A or B was specified.

For each herd, locally weighted scatterplot smoothing (**LOWESS**) was used to plot the 4% FCM predictions ($X\beta$) from the lactation-specific models to compare FCM produced by ELISA negative (S/P ratio = 0.05), ELISA weak positive (S/P ratio = 0.32), and ELISA strong positive (S/P ratio = 1); and FC negative, FC positive, and both FC positive and ELISA strong positive. The cut-offs for the different ELISA categories were based on prior recommended values for likelihood ratios (Collins, 2002). The lactation-specific plots were generated using the fixed effects of the final model (A or B; depending on presence of confounding), the mean lactation-specific DIM at peak reported in both herds' last monthly DHI test [dairy 1 (2) lactation 1 = 102 (94) DIM, lactation 2 = 74 (77) DIM, and lactation 3 = 71 (78) DIM], and the industry standard lactation length of 305 DIM.

Maximum likelihood was used to fit models (Jiang, 2007), and model fit was assessed by evaluating the normality of the empirical BLUP for herd, cow, lactation, and pre- and post-peak DIM. Model fit of the study's 2-piece linear function that used the observed peak and lactation length was compared with the similar function that assumed a fixed peak at 60 DIM and 305-d lactations but on an equivalent subset of the study data truncated at 305 DIM. Comparison of the two 2-piece functions was based on the AIC and the magnitude of variability attributable to each of the lactation splines as a percentage of total variability in the data. The model with the lower AIC and variability attributable to the pre-peak and post-peak lactation slopes as a percentage of the total variability was considered a better fit. All analyses were performed using Stata (Stata/IC 10.0, College Station, TX).

RESULTS

Descriptive Statistics

A total of 10,378 MAP ELISA and 5,580 MAP FC results were matched to 22,278 and 125,254 monthly DHI records from dairies 1 and 2, respectively. Overall, 2,598 and 14,499 lactations of 1,375 and 4,551 cows on dairies 1 and 2, respectively, were matched to the MAP test results. The study cows had a mean of 1.8 and 3.3 ELISA tests/cow, and 1.6 and 2.1 FC tests/cow on dairies 1 and 2, respectively. On both dairies, a total of 110 and 64 cows switched ELISA and FC status, respectively. The total number of cows that tested positive on ELISA or FC was 272 and 868 cows on dairies 1 and 2, respectively, and the total number of cows that tested positive by both ELISA and FC was 56 and 274 cows on dairies 1 and 2, respectively.

Two-Piece Mixed-Effects Regression Models

Model A based on Figure 3A showed a significant decrease of 2.47 and 2.16 kg of 4% FCM per day for every 1-unit increase in S/P ratio in serum ELISA and a change of status from negative to positive on FC, respectively. Addition of DIMprePK² and DIMpostPK² to model A resulted in a better fit compared with the same model without the 2 variables (AIC: 1,011,180 vs. 1,012,243). In contrast, there was no significant difference in FCM production for calving seasons April to May, June to August, and September to December (P -values = 0.14, 0.43, 0.48, respectively) compared with the January to March calving season; and no significant association between switch in ELISA status (P -value = 0.29) or FC status (P -value = 0.20) and FCM production. However, HerdLact was significant (P -value < 0.01) and hence, was retained in the model. Results of the likelihood ratio test comparing model A with and without the group of variables ELISAswitch, FCswitch, and CALVSEAS showed no significant contribution to the model (P -value > 0.99) and hence were excluded from further models.

Estimates of ELISA and FC from model A with and without MN decreased by <0.1% and <3.7%, respectively; hence, MN did not substantially confound the association between MAP and milk production in this study and was removed from the final model A. In model B, introducing MN, MA, SCC, UC, and MC individually or as a group of potential confounders resulted in a <10% change in estimates of the ELISA and FC coefficients. Table 1 compares the final models

Table 1. Estimates of model parameters for the association between *Mycobacterium avium* ssp. *paratuberculosis* (MAP) and milk production in 2 California dairies based on 2-piece mixed effects regression models¹

Item	Model A ²			Model B ²		
	β^3	SE	P-value	β	SE	P-value
Fixed effects ⁴						
Intercept	36.09	0.26	<0.01	35.85	0.27	<0.01
Herd 1 (Holstein)	Reference			Reference		
Herd 2 (Jersey)	−1.26	0.27	<0.01	−1.20	0.27	<0.01
Lactation	3.20	0.15	<0.01	3.17	0.15	<0.01
HerdLact	−1.14	0.15	<0.01	−1.13	0.15	<0.01
DIMprePK	19.34	0.26	<0.01	19.49	0.26	<0.01
DIMpostPK	−21.56	0.24	<0.01	−21.63	0.23	<0.01
DIMprePK squared	7.92	0.29	<0.01	8.22	0.29	<0.01
DIMpostPK squared	5.66	0.24	<0.01	5.81	0.24	<0.01
ELISA	−2.47	0.20	<0.01	−2.40	0.20	<0.01
FC	−2.16	0.17	<0.01	−2.09	0.17	<0.01
Monensin (MN)				0.47	0.09	<0.01
Mastitis (MA)				−0.91	0.12	<0.01
SCC				<−0.01	<0.01	<0.01
Uterine condition (UC)				−1.34	0.61	0.03
Metabolic condition (MC)				−3.57	0.50	<0.01
Random effects ⁴	Variance	SE		Variance	SE	
Herd	<0.01	<0.01		<0.01	<0.01	
Cow	6.27	0.29		5.92	0.29	
Lactation	34.77	0.65		34.34	0.65	
DIMprePK	25.40	1.14		26.14	1.15	
DIMpostPK	58.65	1.44		57.61	1.42	

¹Cow MAP status was determined using a commercial serum ELISA and a liquid fecal culture followed by 2 confirmatory PCR assays (FC).

²Model A assumed that MA, SCC, UC, and MC are not confounders of the association between MAP and milk production. No evidence of confounding by MN; hence, removed from model A. Model B assumed MN, MA, SCC, UC, and MC are potential confounders of the association between MAP and milk production.

³Kilograms of 4% FCM.

⁴HerdLact = herd-lactation interaction; DIMprePK and DIMpostPK = 2-piece linear function for DIM pre- and post-peak, respectively.

A and B and shows a <0.1% and <3.2% decrease in estimates of ELISA and FC coefficients, respectively, on milk production, which can be interpreted as no evidence of confounding by MN, MA, SCC, UC, or MC in this study. Furthermore, the difference in estimates between model A and B (with and without confounders) for effects of ELISA and FC was not practically important (78 and 70 mL, respectively).

Table 2 presents results of the lactation-specific models based on model A in Table 1 as there was no evidence of confounding. There was no significant association between MAP and FCM in first-lactation cows in the study herds. However, the association between MAP on FCM was significant in second-lactation cows and its magnitude (decrease in milk production) continued to increase as cows progressed to higher lactations. Figure 4 presents the LOWESS plots of the lactation-specific models' predicted 4% FCM production by herd and lactation for the different ELISA and FC results. The 2-piece linear function that used the observed peak and lactation length compared with the similar function that assumed a fixed peak at 60 DIM and 305 d lactations resulted in a lower AIC (928,814 vs. 952,956,

respectively), lower variability attributable to the pre-peak slopes (16.5% vs. 21.3%), and lower variability attributable to the post-peak slopes (32.5% vs. 35.6%), both as a percentage of total variability.

DISCUSSION

To our knowledge, this is the first longitudinal study to estimate the magnitude of the association between MAP and FCM production considering possible causal relationships among variables in large dairies using bST. This study found that cows that tested positive for MAP by ELISA or FC produced significantly less FCM per day than their respective MAP test-negative herd mates. Cows that tested positive by ELISA produced 2.5 kg less of 4% FCM per day than cows that tested negative, holding all other variables constant. In contrast, cows that tested positive on FC produced 2.2 kg of 4% FCM less per day than cows that tested negative, holding all other variables constant. In addition, cows that tested positive by both ELISA and FC produced 4.7 kg of 4% FCM less per day than cows that were negative on both tests (Table 1). The decrease in

Table 2. Estimates of model parameters for the association between *Mycobacterium avium* ssp. *paratuberculosis* (MAP) and milk production in 2 California dairies based on lactation-specific 2-piece mixed effects regression models¹

Item	Lactation 1			Lactation 2			Lactation >2		
	β^2	SE	<i>P</i> -value	β	SE	<i>P</i> -value	β	SE	<i>P</i> -value
Fixed effects ³									
Intercept	36.44	0.13	<0.01	43.97	0.20	<0.01	47.69	2.29	<0.01
Herd 1 (Holstein)	Reference			Reference			Reference		
Herd 2 (Jersey)	−4.25	0.13	<0.01	−2.28	0.19	<0.01	−2.86	2.31	0.215
Lactation	DC ⁴			DC			−0.39	0.74	0.59
HerdLact	DC			DC			0.20	0.74	0.79
DIMprePK	16.69	0.32	<0.01	18.65	0.49	<0.01	19.80	0.51	<0.01
DIMpostPK	−14.53	0.32	<0.01	−23.51	0.43	<0.01	−26.3	0.44	<0.01
DIMprePK squared	7.03	0.35	<0.01	7.34	0.56	<0.01	6.83	0.58	<0.01
DIMpostPK squared	4.02	0.33	<0.01	5.07	0.44	<0.01	8.03	0.45	<0.01
ELISA	0.28	0.34	0.42	−1.16	0.49	0.02	−2.83	0.25	<0.01
FC	−0.09	0.24	0.72	−1.91	0.31	<0.01	−2.78	0.25	<0.01
Random effects ³	Variance	SE		Variance	SE		Variance	SE	
Herd	<0.01	<0.01		<0.01	<0.01		<0.01	<0.01	
Cow	18.15	0.56		29.67	0.99		10.14	0.68	
Lactation	NI ⁵			NI			25.93	1.14	
DIMprePK	12.91	1.01		28.02	2.26		32.36	2.66	
DIMpostPK	19.86	1.16		49.19	2.48		47.16	2.60	

¹Cow MAP status was determined using a commercial serum ELISA and a liquid fecal culture followed by 2 confirmatory PCR assays (FC).

²Kilograms of 4% FCM.

³HerdLact = herd-lactation interaction; DIMprePK and DIMpostPK = 2-piece linear function for DIM pre- and post-peak, respectively.

⁴DC = dropped because of collinearity.

⁵NI = random effect not included in model.

milk production in MAP test-positive cows compared with test-negative cows commenced in their second lactation (Table 2).

Causal diagrams were used to explore the role of MA, SCC, UC, and MC as intermediate factors of the association between MAP and milk production (model A) and as potential confounders (model B). In the model with confounders, model B, the estimates of the magnitude of association between MAP and FCM decreased by <0.1% and 3.2% for ELISA and FC, respectively. This change was considered trivial and thus, indicated no evidence of confounding. Although the lack of evidence for confounding by MN, MA, SCC, UC, or MC may not apply to other herds, it may support the theory that one or more of these factors may be intermediate factors, in which case adjusting for them would be unnecessary and yield imprecise or even biased estimates.

Another finding of the present study was the lack of a significant association between a switch in ELISA or FC status and milk production. This finding may be explained by the small number of cows that switched either status, which is consistent with a previous report by Nielsen et al. (2009). Milk production did not differ significantly by calving season, which may be explained by the year-round calving and consistent rations fed year round.

Previous studies of the association between MAP and TD milk (Kudahl et al., 2004; Beaudeau et al.,

2007) noted a significant decrease in FCM in the first lactation. In contrast, this study found no significant association between MAP and FCM production in the first lactation, which may be because of the study cows' young age at first calving [mean (SD) = 2.0 (0.3) yr] and because MAP-infected cows are less likely to test positive at a young age. Another possible explanation may be the high level of milk production found in the study cows, which may have reduced any association between MAP and milk production in the first lactation. In the study by Kudahl et al. (2004), Danish Holsteins and Jerseys in their first lactation produced a peak of 27 and 23 kg of 4% FCM compared with California Holsteins and Jerseys in this study, which produced 36 and 32 kg of 4% FCM, respectively.

Comparison of estimates of the magnitude of the association between MAP and milk from prior studies of ME 305 with results of the present study is difficult because of differences in outcome measures, herds, diagnostic assays, and statistical models. However, prior studies of ME 305 may have underestimated the magnitude of the association between MAP and milk production compared with our findings. This may be attributable to less information inherent in ME 305 compared with TD milk yields. In addition, the use of both MAP antibody and antigen detection tests in this study and others (such as that reported by Beaudeau et al., 2007) provided more diagnostic information on a

Figure 4. Locally weighted scatterplot smoothing of the magnitude of association between *Mycobacterium avium* ssp. *paratuberculosis* and 4% FCM as predicted by models in Table 2 and presented by dairy, lactation, ELISA status (A, B, C), fecal culture (FC) status (D, E, F), and both FC positive and ELISA strong positive (D, E, F). ELISA sample-to-positive ratios of 0.05, 0.32, and 1.0 were used to plot curves for ELISA negative, ELISA weak positive, and ELISA strong positive cows, respectively. Dairies 1 and 2 housed Holsteins and Jerseys, respectively.

cow's antibody response and fecal shedding compared with a single diagnostic assay. For example, when FC results were excluded from model A (results not shown), the estimate of the effect of MAP on milk production was a decrease of 2.9 kg of 4% FCM per day, which ignores the fecal shedding status of infected cows. In contrast, in model A with ELISA and FC results, the estimate of the effect of MAP on FCM in seropositive cows shedding MAP in their feces was a total decrease of 4.4 kg of 4% FCM.

Smith et al. (2009) reported a significant decrease in raw milk yield of MAP-infected cows based on TD milk weights in 3 dairies that milked primarily Holsteins. The analysis of crude TD milk yield may be suitable in cow cohorts of the same breed; however, FCM may be more suitable with cohorts of different breeds especially those varying widely in butter fat content such as Holsteins and Jerseys (Kudahl et al., 2004). To our knowledge, Smith et al. (2009) was the first report of the association between MAP and TD milk yield in US dairies but the study did not utilize causal diagrams or investigate the role of metabolic and uterine cow conditions as potential confounders.

Only milk production records from cows that gave birth to their first calf in the study herds were included in the data analysis. On dairy 1, milking cows were purchased before implementing a Johne's disease control program and their exclusion resulted in fewer cows and lactations in this herd than on dairy 2, which was also a larger herd. In addition, most of the results of the whole herd test for dairy 1 were excluded because most of the herd was made up of cows that did not meet the inclusion criteria of their first calving being in the study herd. In contrast, all 3 whole-herd test results for dairy 2 were included, which contributed to the greater number of test records for dairy 2 compared with dairy 1.

Prior studies do not describe how diagnostic test results were linked to more numerous TD milk records. In this study, a cow's first-lactation MAP test result before 305 DIM was linked to prior TD milk records because such a serum or fecal sample was representative of that lactation. The following MAP test result was then linked to the subsequent milk records prospectively. Results of MAP tests based on samples collected from first-lactation cows after 305 DIM were deemed too far in a lactation to be linked to previous milk records. Cows are commonly targeted for MAP testing at older ages (Collins et al., 2006) and hence, MAP test results for cows upon entry into their first lactation are rare. Hence, the issue of linking first-lactation TD milk records to MAP test results is common to such longitudinal studies and not necessarily this study.

The use of splines in a mixed model approach permitted us to model the shape of the lactation curve with inclusion of incomplete lactations. The modification of the piece-wise functions allowed for modeling a cow's lactation curve based on TD milk yield. To our knowledge, this is the first report of a milk model with cow-specific splines. A comparison between estimates of this study's model that uses a 2-piece linear function for complete lactations to a model that uses a function for 305-d lactations would not be valid because of their different underlying data sets. Alternatively, a comparison was made based on a data set with lactations truncated at 305 d, which showed that this study's 2-piece linear function resulted in better model fit. Random effects from MRM allowed for interpretation of the study results at the cow level rather than at the population level. Inclusion of the entire cohort of cows avoided the biases inherent in selecting cows with complete lactations because the cause of culling a cow or termination of its lactation may have been related to MAP status, the exposure of interest, which would result in biased conclusions.

A limitation of this study was the use of diagnostic assays with imperfect accuracies. Diagnostics assays used in the present study have higher specificities than sensitivities, which, given the moderate MAP prevalence in the study herds, led to fewer false positives and more false negatives, respectively. Such a difference may result in an underestimation of the true difference in milk production between test-positive and test-negative cows.

CONCLUSIONS

Cows that tested positive for MAP on serum ELISA or fecal culture produced less milk than cows that tested negative. The association between MAP and milk production was not significant in the first lactation but increased as the cows progressed to higher lactations. Mastitis, elevated SCC, and uterine or metabolic cow conditions did not confound the association between MAP and milk production in this study.

ACKNOWLEDGMENTS

The study was funded by Johne's disease Demonstration Herd Project, USDA, Animal and Plant Health Inspection Service, Veterinary Services; Austin Eugene Lyon's Fellowship and the Graduate Group in Epidemiology block grant funds, University of California, Davis; the Graduate Student Support Program and the Center for Food Animal Health, School of Veterinary Medicine, University of California, Davis.

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