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Use of age and milk production data to improve the ability of enzyme-linked immunosorbent assay test results to predict *Mycobacterium avium* ssp. *paratuberculosis* fecal culture status

Roy D. Berghaus,¹ Thomas B. Farver, Randall J. Anderson, John M. Adaska, Ian A. Gardner

Abstract. Cows from 2 California dairies were tested for paratuberculosis at the end of lactation by using fecal culture and a commercially available serum enzyme-linked immunosorbent assay (ELISA) test kit. Individual cow characteristics and production variables were evaluated along with ELISA testing results as predictors of fecal culture status. In multivariable logistic regression analysis, age and a herd-standardized version of 305-day mature equivalent (305 ME) milk production were significant predictors of fecal culture status after adjusting for herd, quarter of the study year, and ELISA sample-to-positive (S/P) ratio. The area under a nonparametric receiver operating characteristic curve was significantly greater for a multivariable model that included age and the level of milk production when compared with a model without these covariates. In conclusion, consideration of cow-level covariates was useful as an aid in predicting *Mycobacterium avium* ssp. *paratuberculosis* (MAP) fecal culture status. For a given ELISA S/P ratio, older cows and those with lower 305 ME milk production relative to other cows in the herd were significantly more likely to be shedding MAP in their feces at the end of lactation.

Key words: Dairy cattle; fecal culture; Johne's disease; *Mycobacterium paratuberculosis*.

Introduction

Paratuberculosis, or Johne's disease, is a chronic granulomatous enteric disease of ruminants that is caused by *Mycobacterium avium* ssp. *paratuberculosis* (MAP).¹⁵ Cattle are most susceptible to infection with MAP shortly after birth, though the disease has a long latent period, and infected animals do not typically develop clinical signs of illness before adulthood. Diagnosis of infection is accomplished by detecting the causative organism, as with bacterial culture, or by detecting a pathogen-specific immune response. The absorbed enzyme-linked immunosorbent assay (ELISA), in which serum is pretreated with an extract of *Mycobacterium phlei* to reduce the potential for nonspecific binding, is currently the most commonly used serologic method for detecting antibodies directed against MAP in cattle.^{3,26}

Bacterial culture of tissues or feces is generally regarded as the most accurate diagnostic test for identifying animals infected with MAP, with a sensitivity estimate of 38–55% and specificity of nearly 100%.^{16,20,25} Culture of MAP requires long incubation periods, with the newer automated liquid-culture systems taking 4–6 weeks and conventional growth on solid media taking as long as 12–16 weeks. Consequently, the long wait for results from the laboratory, and the higher costs compared with serology, have made culture a less desirable test from the viewpoint of many producers and veterinarians.

Several absorbed serum ELISA test kits are available commercially, with recent estimates of sensitivity and specificity in the ranges of 28–29% and 95–100%, respectively.⁷ Although ELISA testing has lower sensitivity and specificity when compared with fecal culture, it offers the advantages of a 2–3-day turnaround time and a substantially lower cost. As a result, ELISA testing is often used to make decisions about individual animals that may be infected with MAP. Previous studies have demonstrated that considering quantitative information, such as sample-to-positive (S/P) ratios, can dramatically improve the predictive ability of these tests as compared with an approach that simply classifies animals as positive or negative based on an arbitrary cutoff value.^{5,6} The goal of the present study was to determine whether cow-level demographic and pro-

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duction information could be used in conjunction with quantitative ELISA results to further improve the prediction of fecal culture status in dairy cows tested for MAP infection at the end of lactation.

Materials and methods

Study herds

Two dairies, located in California's Central Valley, were enrolled in the current study as part of a USDA-sponsored Johne's disease demonstration herd project. One was composed of approximately 2,500 adult Jersey cows, and the other was a herd of approximately 1,370 adult Holsteins. These herds were selected for participation in the demonstration herd project because previous testing had identified >5% seroprevalence for MAP infection, the owners expressed a strong interest in participating, both herds used Dairy Herd Improvement Association (DHIA) testing services, and both herds used a computerized record system to maintain information on individual animals.

Sample collection

Beginning in March 2004, milking cows from both herds were routinely tested for paratuberculosis by fecal culture and serum ELISA when they reached the end of lactation (i.e., dry-off). A veterinary medical officer (RJA) from the California Department of Food and Agriculture visited the farms on a weekly basis to collect samples. Blood samples were collected from the coccygeal vein directly into a sterile 10-ml tube without anticoagulant, and fecal samples were collected from the rectum. Approximately 25 g of feces was collected from each animal with a new disposable obstetric sleeve and placed in a 50-ml flip-top plastic container. Blood and fecal samples were kept cool after collection and were transported via courier service to the California Animal Health and Food Safety (CAHFS) Laboratories in Fresno and Tulare, California, respectively.

Cow-level covariate information

A single veterinary medical officer recorded body condition scores (BCS) (scale 1–5) for cows at the time of sample collection. Information on BCS was not recorded for 272 (9.9%) of the 2,756 cows included in the analysis. Other cow-level demographic and production data were extracted from the computerized record systems of each herd. Because the 2 farms used different software packages, the analysis was limited to variables that were comparable between the 2 systems. Specific variables that were evaluated as predictors of MAP fecal culture status included age (in years) at the time of dry-off, lactation number, BCS, length of the lactating period (i.e., days in milking), calving-to-conception interval (i.e., days open), number of times bred during the lactation, 305-day mature equivalent (305 ME) milk production, relative value (%), lactation total milk fat (%), and lactation total milk protein (%).

With respect to the production variables, 305 ME is a measure of milk production calculated by DHIA that has been adjusted for age and season of calving. This is a projected value before 305 days in lactation, but afterward it is the actual value of milk that had been

produced, taking the adjustment factors into account. Relative value compares an individual cow's 305 ME with the herd average 305 ME, such that a cow with an adjusted milk production value equivalent to the herd mean would have a relative value of 100%. The total milk fat (%) and the total milk protein (%) were calculated by dividing the total weights of milk fat and protein produced during the lactation, respectively, by the total weight of milk produced and then multiplying by 100. Measures of milk fat and protein percentages that had been adjusted for age and the length of lactation were not comparable between the different software packages, and consequently, only the crude measures for these variables were evaluated in the analysis.

Laboratory methods

ELISA testing. ELISA testing was performed by the CAHFS Laboratory in Fresno, California, with a commercially available serum ELISA test kit.^a Results of the ELISA were recorded as an optical density (OD) and reported as an S/P ratio, where $S/P = (\text{OD of unknown sample} - \text{OD of negative-control sample}) / (\text{OD of positive-control sample} - \text{OD of negative control sample})$. Samples were initially assayed in a single well on the ELISA plate. Because the variability in ELISA results increases with the mean OD of the sample,^{2,8} samples with an S/P ratio ≥ 0.2 were reassayed in duplicate, and the mean of the 2 readings was used as the final result to increase precision of the assay.

Fecal culture testing. MAP fecal cultures were performed by the CAHFS Laboratory in Tulare, California, with an automated liquid-culture system^b as previously described.²³ Briefly, 2 g of feces was mixed with 35 ml sterile water and allowed to stand at room temperature for 30 min. Then, a 5-ml aliquot of the supernatant was mixed with 25 ml of brain heart infusion (BHI) broth containing 0.9% cetylpyridinium chloride, and the mixture was incubated at 37°C for 18–24 hr. After centrifuging at $900 \times g$ for 30 min, the pellet was resuspended in 1 ml of BHI broth containing an antibiotic solution, and the sample was incubated again at 37°C overnight. The sample was then combined with an equal volume of egg yolk supplement, and 1 ml was transferred to a bottle containing a proprietary mix of broth, growth supplements, antibiotic, and buffer solutions. Bottles were incubated at 37°C for 6 wk. The liquid-culture instrument periodically measured the headspace pressure of incubation bottles and gave a positive signal when the partial pressure of oxygen was sufficiently decreased to indicate mycobacterial growth.

At the end of the 6-wk incubation period, all samples were stained to screen for the presence of acid-fast bacilli and were also tested by the polymerase chain reaction (PCR) to confirm the presence of MAP by amplifying a segment of the IS900 sequence. For a culture to be considered positive, a positive PCR result was required.

PCR testing of cultures. Conventional PCR was used to confirm the presence of MAP.¹⁴ Briefly, DNA was extracted by boiling 500 μl of the culture broth solution in a sterile screw-cap microcentrifuge tube for 20 min.

Tubes were centrifuged at $18,300 \times g$ for 2 min, and 100 μ l of the supernatant was transferred to a 1.5-ml tube. Polymerase chain reaction was performed by adding 5 μ l of the extracted sample to a reagent mixture containing 3 mM $MgCl_2$, 2.5 μ l $10\times$ PCR buffer II, 200 μ M dNTP, 0.5 μ l DNA polymerase, 5 μ l DNA template, 0.4 μ M IS900 primer (5'-CCGCTAATTGAGAGATGCGATTGG-3'), and 0.4 μ M IS900 primer (5'-AATCAACTCCAGCAGC-GCGGCCTCG-3'). Reaction conditions were 95°C for 5 min (1 cycle); 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min (50 cycles); and 72°C for 7 min (1 cycle). Products were analyzed by electrophoresis on a 1% agarose gel, with visualization of a 229 base-pair product being considered a positive finding.

Statistical analysis

Cows that completed their lactations between March 15, 2004, and March 14, 2005, were eligible for inclusion in the analysis. Some cows completed more than a single lactation during the study year, but information corresponding to only the first completed lactation was used, so that each cow contributed only 1 observation to the final data set. Laboratory and production data were screened by range and logic checks, and unexpected values were checked against the original records. Only cows that had been tested within 14 days of their recorded dry-date and those that had completed a lactation of at least 240 days were included in the analysis.

Univariate descriptive statistics were calculated separately for each herd, and 2-sample *t*-tests or Pearson chi-square statistics were used to compare herd means and proportions, respectively. The bivariate associations between categories of cow-level covariates and fecal culture status were evaluated by a Pearson chi-square statistic, and multivariable analysis was conducted by binary logistic regression with fecal culture status as a dichotomous outcome variable.

The multivariable analysis was performed by a split-sample approach, with two thirds of the observations being randomly assigned to a training sample used for model fitting and the remaining one third of observations being held in reserve as a validation sample. Model selection was performed by a backward selection procedure, with variables having an association ($P < 0.2$) with fecal culture status in the bivariate analysis being included as part of a maximum potential model. Terms were sequentially removed from the maximum model on the basis of likelihood ratio testing until a preliminary main-effects model was reached. The initial model selection procedure was performed with variables included as continuous predictors. Milk production variables including 305 ME, lactation total milk fat (%), and lactation total protein (%), were centered and standardized for animals within each herd by subtracting the mean and dividing by the standard deviation (SD).

After a preliminary main-effects model was attained, categorizations of the predictor variables based on quartiles were evaluated one-by-one to determine whether the relationships between predictors and the outcome variable were approximately linear in the log odds. Additional

categorical and quadratic forms were evaluated based on these results. Categorical or quadratic variable forms were preferred over the simple continuous variables only if they significantly improved the fit of the multivariable model as determined by likelihood ratio testing. Next, an attempt was made to add-back each of the variables that had been dropped during the initial model selection procedure, and finally all possible 2-way interactions between variables in the preliminary main-effects model were evaluated. During the model selection procedure, significance of main-effect terms was assessed with $\alpha = 0.05$, and significance of interaction terms was assessed with $\alpha = 0.01$.

The Hosmer-Lemeshow (H-L) test was used to assess goodness-of-fit for the multivariable model, and predictive ability was evaluated by cross-classifying animals according to their observed and predicted fecal culture status (with a predicted probability of 0.5 as the cutoff value). An overall measure of discriminatory accuracy for the model was estimated by calculating the area under a nonparametric receiver operating characteristic (ROC) curve, which is a plot of sensitivity versus (1 – specificity) over the range of all possible cutoff values.¹⁰ The area under the curve (AUC) for the final (full) model was compared with that of a reduced model that did not include cow-level covariate information to quantitatively compare the discrimination ability of the 2 models.⁹

Additional model checking was performed by plotting the predicted probabilities of the observations versus the change in the Pearson chi-square statistic ($\Delta\chi^2$), the change in deviance (ΔD), and the change in estimated coefficients (Pregibon's $\Delta\beta$) associated with deleting individual observations from the final model.¹¹ Influential observations were investigated and their records were checked for accuracy. All analyses were performed by using commercially available statistical software.^c

Results

Univariate analysis and herd comparisons

Descriptive statistics for the 2 herds are shown in Table 1. The herds were similar with respect to age and BCS of cows at the time of dry-off, but they differed significantly with respect to the remaining demographic and production parameters. As expected, the Holstein herd had a significantly higher mean milk production (305 ME) and significantly lower mean percentages of milk fat and protein. Surrogate measures of base reproductive performance, including the length of the lactating period, the calving-to-conception interval, and the number of times bred during the lactation, were all significantly higher (i.e., less favorable) for the Holstein herd. The mean lactation number of cows in the Holstein herd was also significantly lower than for the Jersey herd, despite the similar age distributions.

The estimates of MAP infection prevalences based on serologic and fecal culture were not significantly different between the 2 herds during either the first or

Table 1. Summary of demographic and production variables for cows at the time of dry-off between March 15, 2004, and March 14, 2005, in 2 California dairy herds.

| Variable | Holstein herd | | Jersey herd | |
|--|-----------------------|-----------------|-----------------------|----------------|
| | <i>n</i> [†] | Mean (SD) | <i>n</i> [†] | Mean (SD) |
| Age at dry-off (yr) | 836 | 4.4 (1.6) | 1920 | 4.4 (1.7) |
| Lactation number | 836 | 2.1 (1.4)** | 1920 | 2.5 (1.6)** |
| Body condition score (scale 1–5) | 780 | 2.8 (0.4) | 1704 | 2.8 (0.4) |
| Length of the lactating period (days in milking) | 836 | 358.4 (92.2)** | 1920 | 329.6 (74.1)** |
| Calving-to-conception interval (days open) | 836 | 141.4 (97.7)** | 1920 | 113.6 (78.5)** |
| Number of times bred during the lactation | 836 | 2.9 (2.0)** | 1920 | 2.5 (2.2)** |
| 305-day mature equivalent milk production (kg) | 836 | 12,470 (1400)** | 1920 | 9,060 (1290)** |
| Relative value (%) | 836 | 103.8 (11.5)* | 1920 | 102.1 (14.8)* |
| Lactation total fat (%) | 836 | 3.6 (0.3)** | 1920 | 4.6 (0.4)** |
| Lactation total protein (%) | 836 | 3.0 (0.2)** | 1920 | 3.6 (0.2)** |

[†] Number of cows tested at dry-off after a lactation of ≥ 240 days in length.

* Comparison of herd means (*t*-test): $P < 0.01$.

** Comparison of herd means (*t*-test): $P < 0.001$.

second halves of the 1-year study period, though both herds had fewer positive test results during the second half of the study (Table 2). Compared with the first 6-months, both herds had a significantly lower proportion of fecal culture positive results during the second 6-months ($P < 0.001$), whereas the decreases in seroprevalence were not significant ($P > 0.2$).

Bivariate and multivariable analyses

Of the variables that were evaluated, only BCS and the percentages of total milk fat and protein were not significantly associated with fecal culture status in the bivariate analysis (Table 3). Older cows and those with a higher lactation number were significantly more likely to be fecal culture positive, as were cows with longer lactation periods, longer calving-to-conception intervals, and an increased number of times bred during the lactation. Cows with lower values of milk production and percent relative values were also at significantly increased risk for a positive fecal culture result, as were cows with higher ELISA S/P ratios.

Coefficients and odds ratios for the final multivariable logistic regression model are shown in Table 4. Compared with cows that were younger than 3 years of age at the time of dry-off, the odds of having a positive fecal culture result were twice as high for 3–4-year-old cows and 3 times higher for cows that were 5 years of age or older. Cows with a 305 ME milk production value that was 1 SD below the herd mean had a 20% higher odds of having a positive fecal culture result than did cows with a 305 ME that was equivalent to the herd mean. Higher ELISA S/P ratios were also associated with an increased risk of having positive fecal culture results, though the relationship was not linear and was best approximated by adding a quadratic term to the model. There was no significant difference in fecal culture prevalence between herds; however, as suggested from the univariate analysis, the odds of a cow being fecal culture positive were about 50% lower during the second half of the study. Herd and quarter of the study year were specifically included in the model so that the other coefficients would be adjusted for these

Table 2. Summary of paratuberculosis testing results for cows tested at the time of dry-off during the first and second halves of the study year in 2 California dairy herds.

| Test | Holstein herd | | Jersey herd | |
|-----------------------------------|---------------|-----------------|-------------|-----------------|
| | <i>n</i> * | % Positive (SE) | <i>n</i> * | % Positive (SE) |
| Serum ELISA [†] | | | | |
| March 15, 2004–September 14, 2004 | 427 | 8.0 (1.3) | 944 | 5.6 (0.7) |
| September 15, 2004–March 14, 2005 | 409 | 5.9 (1.2) | 976 | 4.6 (0.7) |
| Fecal culture | | | | |
| March 15, 2004–September 14, 2004 | 427 | 18.0 (1.9) | 944 | 17.4 (1.2) |
| September 15, 2004–March 14, 2005 | 409 | 9.8 (1.5) | 976 | 9.4 (0.9) |

* Number of cows tested after a lactation of ≥ 240 days in length.

[†] ELISA = enzyme-linked immunosorbent assay. Using the test-kit manufacturer's recommended cutoff of sample-to-positive ratio ≥ 0.25 .

Table 3. Bivariate analysis of fecal culture status by categories of covariate values at the time of dry-off for cows from 2 California dairies.

| Variable | Fecal culture positive (%) | <i>n</i> † | <i>P</i> -value* |
|--|----------------------------|------------|------------------|
| Age at dry-off (yr) | | | <0.001 |
| <3 | 4.8 | 705 | |
| 3–4 | 13.8 | 1,274 | |
| 5+ | 21.0 | 777 | |
| Lactation number | | | <0.001 |
| 1 | 7.0 | 1,064 | |
| 2 | 15.6 | 652 | |
| 3 | 14.2 | 486 | |
| 4+ | 23.1 | 554 | |
| Body condition score (scale 1–5) | | | 0.871 |
| ≤2.5 | 13.2 | 660 | |
| 2.75+ | 13.4 | 1,824 | |
| Length of the lactating period (days in milking) | | | 0.004 |
| ≤304 | 11.6 | 1,254 | |
| 305–364 | 13.5 | 801 | |
| 365+ | 17.0 | 701 | |
| Calving-to-conception interval (days) | | | <0.001 |
| ≤149 | 12.0 | 2,059 | |
| 150+ | 18.1 | 697 | |
| Number of times bred during the lactation | | | 0.001 |
| ≤2 | 11.9 | 1,767 | |
| 3+ | 16.5 | 989 | |
| 305-day mature equivalent milk production | | | 0.030 |
| ≤herd mean | 15.0 | 1,363 | |
| >herd mean | 12.1 | 1,393 | |
| Relative value (%) | | | 0.008 |
| ≤100 | 15.5 | 1,172 | |
| 101+ | 12.1 | 1,584 | |
| Lactation total fat (%) | | | 0.134 |
| ≤herd mean | 14.5 | 1,393 | |
| >herd mean | 12.5 | 1,363 | |
| Lactation total protein (%) | | | 0.176 |
| ≤herd mean for dry cows | 12.6 | 1,368 | |
| >herd mean for dry cows | 14.4 | 1,388 | |
| ELISA S/P ratio‡ | | | <0.001 |
| <0.10 | 10.3 | 2,409 | |
| 0.10–0.24 | 19.4 | 191 | |
| 0.25–0.39 | 27.0 | 37 | |
| 0.40–0.99 | 47.8 | 46 | |
| 1.0+ | 76.7 | 73 | |

factors, though there was little change (<10%) in the estimates for the effects of age, milk production, and ELISA S/P ratio if these 2 variables were removed (model not shown).

In a competing multivariable model (not shown), ELISA S/P ratio was evaluated as a categorical variable with the following categories: <0.10, 0.10–0.24, 0.25–0.39, 0.40–0.99, and ≥1.0. Odds ratios (95% confidence interval) for these categories of S/P ratio compared with the reference category of <0.10 were 1.4 (0.9, 2.3), 1.8 (0.6, 4.9), 10.4 (5.0, 21.8), and 20.1 (9.9, 40.7), respectively. The continuous quadratic form of S/P ratio was ultimately preferred for the final model because it maximized the use of available data while requiring the estimation of fewer coefficients.

Graphs of predicted probabilities derived from the final model for different covariate values are shown in Fig. 1. The likelihood that a cow would be fecal culture positive increased dramatically with increasing ELISA S/P ratios. Probability curves were shifted upward for older as compared with younger cows, corresponding to the increased probability of culturing MAP in the feces of older cows with a given S/P ratio. Separate curves for milk production relative to the herd average demonstrate the extent to which cows with a low (–2 SD) versus high (+2 SD) 305 ME were more likely to be shedding MAP. The overall decrease in fecal culture prevalence during the second half of the study year is reflected by a shift downward and to the right for curves corresponding to the fall months as compared with the earlier spring.

The H-L statistic indicated that the final multivariable model fit reasonably well for both the observations ($n = 1,849$) that were included in the estimation sample (H-L = 8.14, 8 df, $P = 0.420$) and the observations ($n = 907$) that were used for model validation (H-L = 6.31, 10 df, $P = 0.788$). With a predicted probability of 0.5 as the cutoff value, the overall correct classification for observations in the estimation sample was 88.4%, with 17.8% of culture-positive cows and 99.1% of culture-negative cows being correctly classified. Cows that were classified as positive by the model were fecal culture positive 74.1% of the time (positive predictive value), and cows that were classified as negative were fecal culture negative 88.9% of the time (negative predictive value). With respect to the validation sample, overall correct

Table 3. Continued.

† Number of animals tested at dry-off in both herds after a lactation of ≥240 days.

‡ ELISA = enzyme-linked immunosorbent assay; S/P = sample-to-positive.

* *P*-value based on the Pearson chi-square statistic.

Table 4. Results of multivariable logistic regression modeling for the prediction of fecal culture status in cows ($n = 1849$) tested at dry-off from 2 California dairies.†

| Variable | Coefficient | Standard error | OR (95% CI) | <i>P</i> -value* |
|--------------------------------|-------------|----------------|----------------|------------------|
| Herd | | | | 0.770 |
| Holstein | Referent | Referent | Referent | |
| Jersey | 0.0482 | 0.1645 | 1.0 (0.8, 1.5) | |
| Quarter of the study year | | | | <0.001 |
| March 15–June 14, 2004 | Referent | Referent | Referent | |
| June 15–September 14, 2004 | 0.0306 | 0.1938 | 1.0 (0.7, 1.5) | |
| September 15–December 14, 2004 | −0.6875 | 0.2099 | 0.5 (0.3, 0.8) | |
| December 15–March 14, 2005 | −0.6259 | 0.2410 | 0.5 (0.3, 0.9) | |
| Age at dry-off (yr) | | | | <0.001 |
| <3 | Referent | Referent | Referent | |
| 3–4 | 0.8022 | 0.2426 | 2.2 (1.4, 3.6) | |
| 5+ | 1.1196 | 0.2474 | 3.1 (1.9, 5.0) | |
| Herd-standardized 305 ME‡ | −0.2326 | 0.0734 | 0.8 (0.7, 0.9) | 0.002 |
| ELISA S/P ratio | 3.6655 | 0.4747 | ND§ | <0.001 |
| (ELISA S/P ratio) ² | −0.9295 | 0.2065 | ND§ | <0.001 |
| Constant | −2.7494 | 0.2735 | NA | <0.001 |

* Based on Wald statistics.

† OR = odds ratio; CI = confidence interval; 305 ME = 305-day mature equivalent; ELISA = enzyme-linked immunosorbent assay; S/P = sample-to-positive; ND = not determined; NA = not applicable.

‡ Estimates correspond to a change of 1 standard deviation in 305 ME milk production relative to other cows in the same herd.

§ Not determined because S/P ratio is included as a quadratic variable.

classification was 87.2%, with 18.3% of culture-positive cows and 98.8% of culture-negative cows being correctly classified. The positive and negative predictive values for the validation sample were 72.7% and 87.8%.

In ROC curve analysis, predicted probabilities derived from the final model (Table 4) were used to estimate sensitivities and specificities over the entire range of possible cutoff values for the combined estimation and validation samples ($n = 2,756$). A plot of the sensitivity versus ($1 -$ specificity) over the range of cutoffs is shown in Fig. 2 for both the final model and a reduced model that did not include the effects of age and milk production. The AUC for the final (full) model was significantly greater than the AUC for the reduced model (0.736 vs. 0.705; $\chi^2 = 8.90$, 1 df, $P = 0.003$). If herd effects and quarter of the study year were excluded (unadjusted models not shown), the AUC for a model containing ELISA S/P ratio, age, and milk production as predictors was significantly greater than the AUC when ELISA S/P ratio was used as the sole predictor of fecal culture status (0.708 vs. 0.665; $\chi^2 = 5.65$, 1 df, $P = 0.017$).

Discussion

In the current study of dairy cattle that were tested for paratuberculosis at the end of their lactation, age and the level of milk production were significant predictors of whether MAP would be identified via fecal culture after adjusting for ELISA test results,

herd effects, and quarter of the study year. Compared with a reduced model that did not include the effects of age and milk production, a model that did include these covariates had a significantly greater AUC in ROC analysis, indicating that the overall discriminating ability of the model was improved by including this additional information. This improvement was not dependent on whether adjustment was made for herd effects and quarter of the study year.

Several variables related to reproduction were associated with fecal culture status during the bivariate analysis, but after adjustment for age and the level of milk production, these reproductive indicators were no longer significant predictors of fecal culture status. An association between calving-to-conception interval and ELISA test results has been reported in a previous study,¹³ with cows that had a positive ELISA result having significantly longer intervals. One surprising finding was the lack of association between BCS and the results of fecal culture. As the determination of BCS is subjective, it is possible that the variability inherent in the scoring process was a limiting factor, though the same individual conducted the scoring independent of knowledge of test results for all cows in the study. Rather, this finding may provide some support to the producers' contention that they were not observing clinical cases of paratuberculosis, despite the relatively high apparent prevalences.

Both age and lactation number were evaluated as predictors during the model selection process, and

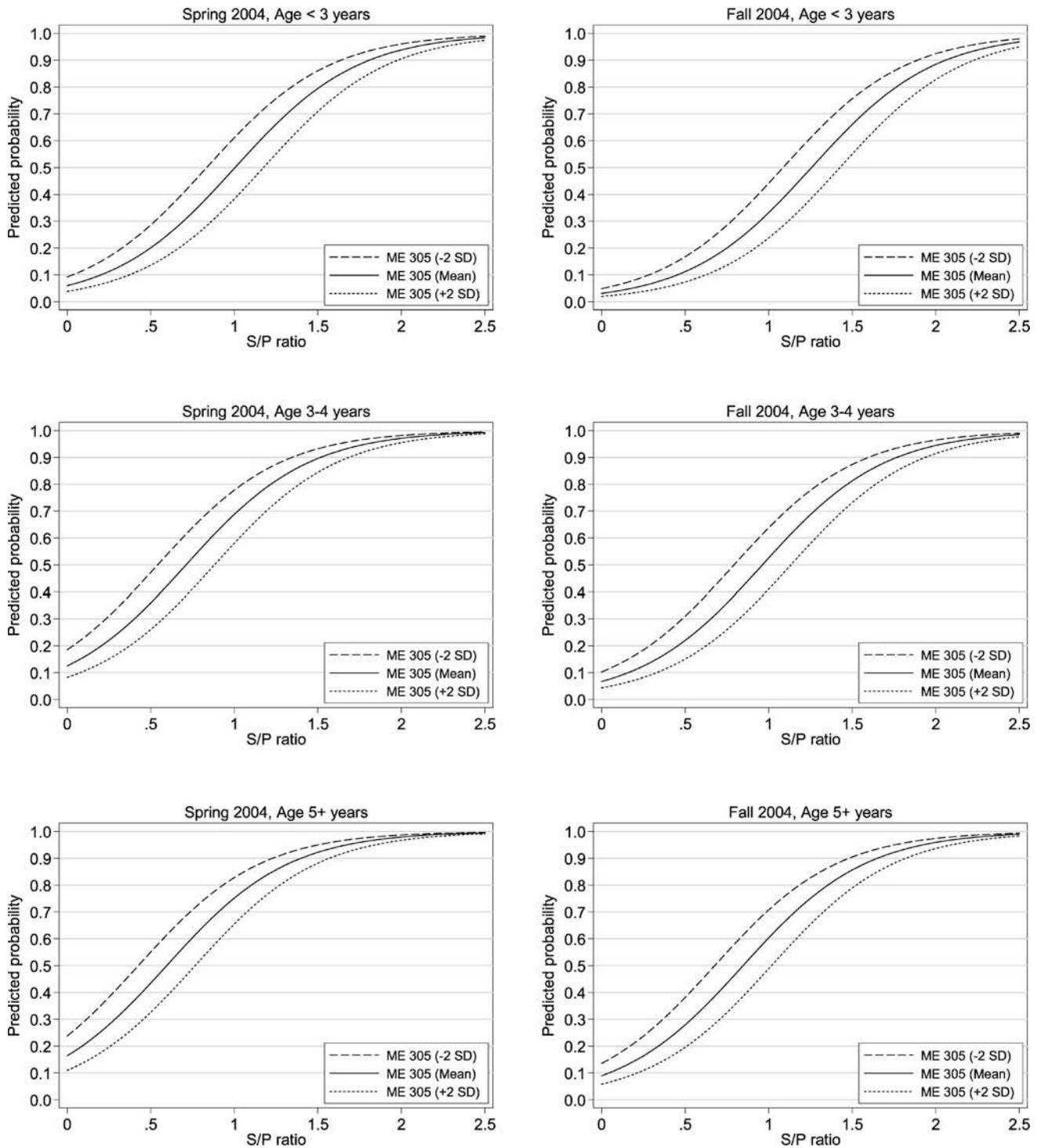


Figure 1. Predicted probabilities of isolating MAP on fecal culture by ELISA S/P ratio for Holstein dairy cows tested at the time of dry-off. Curves within each panel represent 305-day ME milk production (mean \pm 2 SD) relative to other cows in the herd. From top to bottom, panels represent increasing cow ages; from left to right, panels represent differences between the first and third quarters of the study year when overall fecal culture prevalences were 16.6% and 9.8%, respectively.

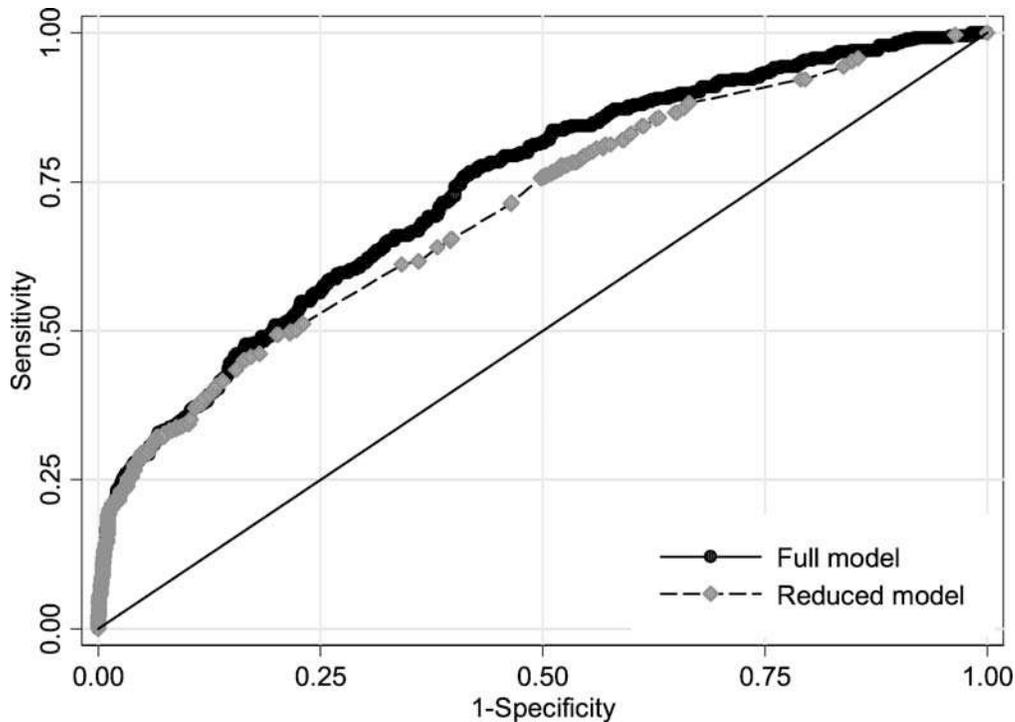


Figure 2. ROC curves based on predicted probabilities from the final (full) multivariable logistic regression model (with herd, quarter of the study year, age, herd-standardized 305-day ME milk production, and ELISA S/P ratio as predictors of fecal culture status) and a reduced model that did not include age and milk production as predictors.

though they are obviously correlated, age was consistently a better predictor of fecal culture status as measured by the model deviance. This may be because lactation number is confounded by factors associated with infertility and success of the herd's reproductive program. That is, cows that are consistently slow to conceive may have extended lactation periods relative to other cows in the herd and may be substantially older than other cows of the same parity. Previous studies have reported that cows in the second lactation and later have significantly higher ELISA values²⁴ and are 2–3 times more likely to have a positive ELISA result¹⁷ compared with cows in the first lactation.

Milk production was included in the logistic regression analysis as a herd-standardized transformation of 305 ME, such that the distribution of milk production values within each herd had a mean of 0 and an SD of 1. This approach was used because the herds were composed of different breeds of cattle and had markedly different values of milk production. Consequently, the transformation allowed a 1-unit change in the variable to be proportionally equivalent for both herds. For similar reasons, the percentages of total milk fat and protein were also evaluated as standardized variables in the multivariable analysis.

The percent relative value is directly related to 305 ME milk production within each herd but was not as

good a predictor as the herd-standardized 305 ME variable. The mean relative value of cows included in the analysis was significantly higher for the Holstein herd than for the Jersey herd (103.8% vs. 102.1%; $P < 0.01$), though the difference of 1.7% was not large enough to be practically important. In practice, relative values should equal 100% for cows with milk production equivalent to the herd mean, but the average relative value was slightly higher for cows included in the analysis because low-producing cows that were culled during midlactation were excluded. Hence, the standardized measure of 305 ME that was calculated may have been shifted 2–4% above the overall herd means, though this would be unlikely to have a meaningful impact on the interpretation of the model.

Previous researchers have reported that cows with clinical paratuberculosis have decreased milk production relative to their noninfected herd mates,⁴ though the relationship between subclinical infection and milk production is less clear. Some studies have found that milk production is significantly decreased in subclinically infected animals as identified by fecal culture or serum ELISA testing,^{1,18} whereas others have failed to find a significant difference between test-positive and -negative cattle with either type of test.¹² In the current study, cows that had a low 305 ME relative to other cows in the same herd had

a significantly greater probability of having a positive fecal culture after adjusting for age, ELISA test result, herd effects, and quarter of the study year.

Although it is tempting to interpret odds ratios in a manner analogous to that of likelihood ratios, it would be inappropriate to do so. Odds ratios are a measure of association that compare the odds of an outcome (i.e., disease) in individuals with different values of a predictor variable, whereas likelihood ratios compare the probabilities of observing a given predictor value (i.e., test result) in individuals with and without disease. Calculation of likelihood ratios is appropriate when the true disease status is known,⁵ which was not the case for the present study where test-negative cows may have been in an early stage of infection that could not yet be detected. Consequently, likelihood ratios were not derived as part of the present analysis, though it is possible to calculate covariate-adjusted likelihood ratios by using logistic regression estimates when true disease status is known.¹⁹

As part of a routine program, testing cows at the time of dry-off allowed producers to have information on ELISA results before parturition so that they could be considered in colostrum management, and fecal culture results were available before rebreeding during the subsequent lactation. Testing cows at the end of lactation was convenient for producers, and because it allowed testing in the herds to be spread out over time it was logistically beneficial for laboratory throughput. The quality and comparability of production data was also improved by using information from completed lactations.

Although producers and veterinarians generally regard culture as the definitive test for making decisions about paratuberculosis, it is not a perfect indicator of infection with MAP. With sensitivity estimates in the range of 38–55%, fecal culture may detect fewer than half of the infected individuals across the spectrum of disease. Although the specificity of culture is often cited as 100%, in practice this is likely an overestimate because of the potential for laboratory and clerical errors. Passive shedding of MAP has also been documented in noninfected animals within a few hours after experimental inoculation, and this phenomenon may occur naturally on farms where there is a high level of environmental contamination, complicating the interpretation of positive fecal culture results.²² Nevertheless, the prediction of whether animals are shedding MAP in their feces has practical application with respect to identifying those animals that are most likely to be contributing to environmental contamination. Identifying animals that are likely to be shedding a high concentration of organisms would also be important,

but inconsistent recording of MAP concentrations in feces (as measured by the number of days to a change in bottle pressure by the automated liquid culture system) prevented the evaluation of organism concentration in the present study.

For both herds, the fecal culture prevalence was significantly lower during the second half of the study year as compared with the first half. Although neither of the producers implemented substantial management changes during this study, they did have access to the results of ELISA and fecal culture testing for consideration in their culling decisions. Results from preliminary ELISA testing performed during the year before the study were also reported to producers. Having these results available may have increased the culling pressure on those cows that were most likely to be shedding MAP. The decreased prevalence during the second half of the study also corresponded with the cooler and wetter months of the year in central California (mid-September to mid-March), but without a longer study period it is not possible to infer a seasonal effect. A recent study of 539 Texas dairy cattle that were resampled at 3-month intervals over a 1-year period failed to identify any seasonal effects with respect to either ELISA S/P ratios or fecal culture positivity.²¹

In conclusion, information on cow-level covariates, including age and the level of milk production, was useful as an aid in predicting MAP fecal culture status. For cows with a given ELISA S/P ratio, older cows and those with a lower 305 ME relative to other cows in the herd were significantly more likely to be shedding MAP in their feces. Although the size and management style of these herds may differ from dairies in other regions, it is reasonable to expect that the relationships between paratuberculosis test status, age, and milk production would be similar in other areas and under different styles of management.

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Sources and manufacturers

- a. HerdCheck, IDEXX Laboratories Inc., Westbrook, ME.
- b. ESP para-JEM culture system II, TREK Diagnostic Systems Inc., Sun Prairie, WI.
- c. Stata Statistical Software: Release 8.2, StataCorp, College Station, TX.

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