Evaluation of *Mycobacterium avium* subsp *paratuberculosis* infection of dairy cows attributable to infection status of the dam

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Objective—To estimate the extent to which infection with *Mycobacterium avium* subsp *paratuberculosis* (MAP) of cows in a large dairy was attributable to the infection status of their dams.

Design—Retrospective longitudinal study.

Animals—625 dam-daughter pairs of Holstein cows.

Procedure—Serologic test results were compared between cows and their dams. Logistic regression was used to assess whether a cow's serologic status was associated with its dam's serologic status. Infection with MAP attributable to being born to a seropositive dam was estimated for individual cows and for the herd.

Results—Cows with seropositive dams were 6.6 times as likely to be seropositive, compared with cows of seronegative dams. For seropositive cows born to seropositive dams, 84.6% of seropositivity was attributable to being born to a seropositive dam and 15.4% to other exposures, including exposure as calves to flush water that contained feces of adult cattle. For the herd as a whole, the seropositive status in 34% of seropositive cows was attributable to being born to a seropositive dam.

Conclusions and Clinical Relevance—For dairy herds that breed seropositive cows, subsequent transmission of MAP to their daughters, either congenitally or via exposure to feces and colostrum of the dam shortly after birth, can contribute substantially to maintaining prevalence of MAP in a herd. Removal of seropositive, clinically unaffected cows and their daughters would be necessary to reduce infection with MAP attributable to congenital or periparturient transmission from dam to daughter. (*J Am Vet Med Assoc* 2005;227:450–454)

Paratuberculosis, otherwise known as Johne's disease, is a chronic debilitating disease of cattle and other ruminants caused by the intracellular bacterium ***Mycobacterium avium*** **subsp *paratuberculosis*** **(MAP)**. The disease is found worldwide and primarily causes an untreatable chronic enteritis[1,2]; a study[3] in California dairy herds estimated a true prevalence of infection with MAP in California dairy cows of 9.4%.

Cattle < 2 years of age have higher risk of becoming infected than do older cattle. The incubation period may be several years, and subclinically infected cattle can seroconvert and shed the organism in their feces, colostrum, and milk.[4] Clinically affected animals may have intermittent diarrhea, especially after calving[5]; decreased milk production; diminished reproduction; and weight loss, despite a normal appetite. Terminally, cachexia, weakness, profuse watery diarrhea without tenesmus, and submandibular edema are seen.[6] Only a few infected cows in a herd may have clinical signs of the disease[6]; the other infected cattle may not be identifiable by use of serologic testing, or they may shed the agent and have no clinical signs.[7] Reviews[6,8,9] of the disease suggest infection can be transmitted congenitally; from milk or colostrum of infected animals; or from milk, colostrum, or feed contaminated by feces of infected animals. In a study[10] of cows that were slaughtered, culled, or suspected of being infected, 26.4% of recovered fetuses were infected with MAP. Congenital transmission also was suspected in another study[11] in which 7.7% of variation in antibodies against MAP (measured by log optical density of an ELISA) in the milk of a cow was attributable to variation in antibodies in the milk of its dam. A correlation between dam and female offspring (daughter) anti-MAP antibodies in milk could be associated with congenital transmission or transmission of the bacteria to offspring shortly after birth via consumption of colostrum, milk, or feces of the infected dam.

Control of paratuberculosis is difficult because of the long incubation period, inaccurate detection of subclinically infected animals when only a single test is used, expense of supplemental tests, and need for strict management. Management includes culling of infected cows on the basis of results of serologic testing, bacteriologic culture of feces, or both to prevent exposure of uninfected animals and contamination of the environment. In the absence of information attributing increases in herd prevalence of infection to the retention of subclinically infected cows, herd managers may be reluctant to cull every infected cow, especially if a cow maintains good milk production and does not have signs of disease. Although results of previous studies indicate that congenital transmission is possible[10] and that anti-MAP antibody status of the dam and daughter may be correlated in herds without a paratuberculosis control program,[11] the extent to which retention of clinically normal seropositive cows could contribute to herd infection in herds with long-standing management practices to control paratuberculosis is not known. One possible means by which retention of seropositive cows (assuming that seropositivity is a reasonably accurate estimate of infection) could contribute to sustained herd infection is transmission of MAP to their daughters, either via congenital transmis-

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sion or by exposure of calves shortly after birth to MAP in feces or colostrum.

The purpose of the study reported here was to estimate the extent to which infection with MAP, as estimated by results of serologic testing in a large dairy, was attributable to infection of the dam.

Materials and Methods

In April and May 2003, a retrospective longitudinal study of pairs of cows (cows and their female offspring [daughters]) was conducted on a commercial dairy in which cows had been tested serologically for several years as part of a paratuberculosis control program and in which management changes had been made to reduce transmission of MAP. The herd offered an opportunity to assess whether daughters born to seropositive cows were more likely to be seropositive than daughters born to seronegative cows. For each cow-daughter pair, results of serologic testing (ELISA) for MAP were obtained from herd laboratory records. The serologic status of the dam, as determined by use of all historical serologic test results, was considered to be a proxy for its infection status at the time its daughter was born, as was the serologic status of the daughter. The finding that seropositive dams tended to have seropositive daughters was interpreted to indicate evidence of transmission of MAP from dam to daughter, either congenitally or by exposure to MAP in feces or colostrum of the dam shortly after birth.

Study herd—Cows studied were on a dairy located in the San Joaquin Valley of California. The herd was composed of approximately 1,500 Holsteins being milked, 280 nonlactating cows, and 1,400 calves and replacement heifers. Cattle were managed under a closed-herd policy that precluded introduction of new animals, with the exception of adult replacement bulls that were not tested for MAP. Prior to the study, several management changes had been implemented to reduce transmission of MAP. Flushing of calf pen alleys with water from the milking parlor and adult cow pens was discontinued in May 1999. At the same time, a new policy was initiated to remove newborn calves from their dam immediately after birth to minimize exposure to the dam's feces and reduce the likelihood the calf would suckle the dam. However, some calves born at the start of the milking shift when personnel were unavailable were removed 1 to 2 hours after birth. After each calving in individual calving pens, bedding was removed, the calving area was disinfected with a commercial phenolic disinfectant,[a] and rice straw was used for new bedding. Prior to October 2001, newborn calves were fed pooled colostrum from dams of unknown serologic status regarding MAP. After October 2001, they were fed only colostrum from cows that consistently yielded negative results (**sample-to-positive [S:P]** ratio < 0.2) for antibodies against MAP, as measured by use of a commercial ELISA kit.[b] Since 1987, calves from 2 to 8 weeks of age were fed waste milk from sick or treated cows after the milk was pasteurized by heating at 72.2° to 79.4°C (162° to 175°F) for 60 seconds. Newborn calves were housed in individual calf hutches until 2 weeks of age, after which they were moved to elevated pens under a roof until weaning at 2 months of age. Calves were located away from adult cows until at least 14 months of age and had no access to flush water or feces from adult cow pens, except for sick calves that were temporarily located in pens adjacent to sick adult cows where they could have had cross-fence exposure to seropositive cows and their feces. After 14 months of age, replacement heifers were moved to pens adjacent to adult cattle where they were exposed to flush water from pens holding adult cattle.

Serologic testing—From May 1999 to April 2003, blood samples were collected from lactating cows and tested at the California Animal Health and Food Safety Laboratory for antibodies against MAP by use of a commercial ELISA kit.[b] Cows were tested every 6 months, except for those due to calve within 110 days; those cows were tested when their lactation ended. In October 2002, the testing protocol was revised to include only cows due to cease their lactation. Heifers, therefore, were tested at 35 months of age at the earliest, approximately 10 months after calving at 25 months of age. Seropositive cows were marked with a colored leg band and preferentially culled if they developed lameness or other health or production problems.

On the basis of the ELISA kit manufacturer's recommended cutoff ratio (S:P ratio, 0.25), a single test has an estimated sensitivity of 45% and a specificity of 99%.[12-14] Serologic status was determined by use of the manufacturer's recommended S:P ratio cutoff value of 0.25 and by use of the reported[15] 85% prediction intervals of S:P ratios for infected and uninfected animals. For the latter, an S:P ratio from 0.2 to 0.35 was designated as suspicious, a ratio > 0.35 was designated as positive, and a ratio < 0.2 was designated as negative.[15] Sensitivity was enhanced by use of results of multiple serologic tests for each cow, rather than only 1 test result. Thus, a cow was classified as seropositive if it had at least 1 positive test result among all test results recorded, and a cow was classified as seronegative if all the recorded serologic results were negative. Cows with a test result from 0.2 to 0.35 and that were still in the herd were retested and reclassified accordingly. Infection status of cows that continued to have S:P ratios from 0.2 to 0.35 was designated as suspicious.

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Statistical analyses—Data collected included date of birth, ELISA test results, and whether or not the daughter was exposed as a calf to flush water from adult pens. Daughters were paired with their dams, and no animal was considered twice in the analysis, either as a daughter or as a dam. For any cow that was tested from May 1999 to April 2003, only 1 daughter could be identified that also had been tested in that interval.

The **apparent period herd prevalence (AP)** was calculated as the number of seropositive cattle divided by the total number of cattle tested from May 1999 to April 2003. True prevalence was calculated by use of the estimated AP and the test sensitivity and specificity, as reported.[12-14] The Pearson χ^2 test and logistic regression were used to test the hypothesis that the serologic status of daughters was independent of that of their dams. The **odds ratio (OR)** was calculated to estimate the strength of any association between infection status of the dam and daughter. The **attributable fraction (AF)** and the **population AF (PAF)** were calculated as follows:

$$AF = (OR - 1)/OR \text{ and}$$

$$PAF = 1 - \frac{\text{No. of seropositive daughters born to seronegative dams} \times \text{No. of seronegative daughters}}{\text{No. of seronegative daughters born to seronegative dams} \times \text{No. of seropositive daughters}},$$

respectively.[16] The AF and PAF were calculated to obtain estimates of the proportion of the seropositive daughters born to seropositive dams that was attributable to being born to a seropositive dam and the proportion of seropositive animals in the herd that was attributable to being born to a seropositive dam, respectively.

A second analysis that used logistic regression of cow serologic status determined by use of the 85% prediction intervals was used to assess the relationship between dam and daughter status and to estimate an adjusted OR. Dam serologic status, age of the dam at the birth of its daughter, and exposure of the daughter as a calf to flush water (0 = no and 1 = yes) were included initially as covariates in the logistic regression model. Dam age at the birth of its daughter was dichotomized as ≤ 36

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months and > 36 months. Confounding of the association between daughter and dam status by dam age at calving was evaluated by comparing estimated ORs for dam status with and without the variables in the model. Variation in ORs by > 15% was interpreted to indicate significant confounding. A 5% level of significance was used to identify significant effects of covariates. The OR was calculated by use of coefficients (β) estimated by logistic regression for variables with a value of $P \leq 0.05$. The OR of daughters of seropositive dams being seropositive, compared with daughters of seronegative dams being seropositive, was calculated as follows:

$$OR = \frac{e^{(\beta_0 + \beta_{1\text{Dam positive}})}}{e^{(\beta_0 + \beta_{1\text{Dam negative}})}},$$

where β_0 is the intercept and β_1 is the coefficient for the effect of dam serologic status on daughter serologic status.

Results

All of the 2,585 animals serologically tested from May 1999 to April 2003 were born before October 2001, when the herd's colostrum program was changed. Of the 2,585 animals, 329 (12.7%) were classified as seropositive by use of the manufacturer's recommended cutoff S:P ratio of 0.25 and 283 (10.9%) were classified as seropositive by use of the 85% prediction intervals.

Daughters were tested a mean of 2.45 times, and dams were tested 3.1 times; the minimum number of tests performed for a single animal was 1, and the maximum number of tests was 10. No records existed for tested dams that had more than 1 daughter tested.

Six hundred twenty-five dam-daughter pairs were identified for which a serologic diagnosis obtained by use of the 85% prediction intervals for seropositivity existed for both dam and daughter (**Table 1**). Of these, information on the dam's age when its daughter was born was available for 610, and information on dam age at birth of the daughter and whether the daughter was born before or after calf alley flushing was discontinued was available for 510. Median ages at the last test for daughters and dams were 33.4 and 57.2 months, respectively. Median ages at the end of follow-up for daughters exposed and not exposed to flush water from adult cows were 46.7 and 32.8 months, respectively. Both univariate logistic regression analyses, one with dam serologic status as a categoric variable (seropositive, suspicious, or seronegative; n = 625; model 1) and another with dam serologic status as a binary variable (seropositive or seronegative; 599; model 2), yielded an estimated OR of 3.6 (95% **confidence interval [CI]**, 1.5 to 8.3) for a daughter being seropositive given that it was born to a seropositive dam ($P = 0.003$). The OR for the effect of dam serologic status, estimated from multivariate logistic regression analysis that included dam serologic status (categoric), exposure to flush water, and dam age at birth of the daughter (n = 510; model 3), was 6.6 (95% CI, 2.2 to 19.5; $P = 0.001$). The OR for the effect of exposure to flush water was 28.5 (95% CI, 8.6 to 94.1; $P < 0.001$; **Table 2**). No evidence was found for an interaction between dam age at the birth of the daughter and dam serologic status ($P = 0.173$). No evidence for confounding by dam age or by exposure to flush water was detected (change in OR < 15%).

The percentage of seropositive daughters for which seropositive status was attributable to being born to a seropositive dam (AF) was 84.8% on the basis of the OR estimated by use of logistic regression (OR, 6.6; Table 2) and 71.9% on the basis of the OR calculated from frequency data (OR, 3.6; 95% CI, 1.4 to 8.9; $P = 0.002$; Table 1). The percentage of herd seroprevalence that was attributable to an animal being born to a seropositive dam (PAF) was 34.4%.

Considering results obtained by use of the manufacturer's S:P ratio cutoff value (0.25), 11 of 23 seropositive daughters were from seropositive dams and 139 of 602 seronegative daughters were from seropositive dams (OR, 3.1; 95% CI, 1.2 to 7.6; $P = 0.006$; AF, 66.6%; PAF, 32.3%).

Table 1—Distribution of results of an ELISA for serum antibodies against *Mycobacterium avium* subsp *paratuberculosis* (MAP) in dairy cows (dams) and their female offspring (daughters) as determined by use of 85% prediction intervals.

	Daughters	
Dams	**Seropositive**	**Seronegative**
Seropositive	11	118
Seronegative	12	458
Subtotal	23	576
Suspicious	0	26
Total	**23**	**602**

Seropositive = Sample-to-positive (S:P) ratio > 0.35. Seronegative = S:P ratio < 0.2. Suspicious = S:P ratio 0.2 to 0.35.

Discussion

The strong and significant positive association between a daughter's serologic status and its dam's serologic status, although not direct evidence of causal-

Table 2—Results of multivariable logistic regression analysis of the association between dam and daughter serologic status as classified by use of 85% prediction intervals for results of an ELISA for MAP.

					95% Confidence interval of OR		
Variable	**No. of pairs**	**β coefficient**	**SE of β**	**OR***	**Lower**	**Upper**	***P* value**
Dam serologic status (seropositive)	510	1.880	0.557	6.55	2.20	19.53	0.001
Exposure to flush water	510	3.350	0.609	28.49	8.63	94.06	< 0.001

*OR is the ratio of the odds of disease in exposed daughters to the odds in unexposed daughters.

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ity, does suggest that in the herd studied, there was considerable transmission of MAP from infected cows to their daughters, either from in utero exposure or from exposure to the dam's feces or colostrum shortly after birth. Daughters born to seropositive dams were 3.6 to 6.6 times as likely to be seropositive as those born to seronegative dams. The estimate of 6.6 was derived from a model that adjusted the effect of dam serologic status on daughter serologic status for dam age at calving and for exposure of the calf to flush water containing feces from adult animals. The AF estimated here suggested that 71.9% to 84.8% (OR, 3.6 to 6.6) of infection (ie, seropositivity) in seropositive cows born to seropositive dams was attributable to being born to the seropositive dam; the remaining 15.2% of infection was attributable to other exposures unrelated to the dam's serologic status. By use of a conservative estimate for the OR of 3.6, the estimated PAF of 0.34 suggested that 34% of seropositivity in the herd was attributable to being born to a seropositive dam. These estimates were interpreted to mean that had there been no transmission from seropositive cows to their daughters, the prevalence of infection in the herd would be diminished by 34%. Thus, in the herd studied, 66% of herd prevalence was attributable to infection acquired by means other than transmission from an infected dam to its daughter.

In attempting to control paratuberculosis, it may not always be economically feasible or practical to cull all seropositive, clinically normal cows or all daughters born to seropositive cows, especially if the cows are maintaining acceptable milk production and are otherwise healthy. Thus, over the long-term course of a paratuberculosis control program, herd seroprevalence may decline initially as animals with clinical disease are removed and young stock are segregated from older animals. As observed for the herd studied here, the seroprevalence may then reach a plateau that is maintained in part by retention of seropositive cows and their daughters. Prior to this study, several management changes had been made to reduce transmission of MAP, including culling of seropositive cows with clinical signs of paratuberculosis or with other production problems, use of individual calving pens, rapid removal of calves from the dam at calving, reduced exposure to feces of adult animals for cattle < 14 months of age, feeding of heat-treated waste milk to unweaned calves, and, since October 2001, feeding individual cow colostrum from seronegative cows to newborn calves. These practices likely contributed to the initial decline in seroprevalence from 1999 to 2003, as indicated by the lower prevalence in May 2003 of 5.4%, compared with the previous 5-year period prevalence of 22.6%. As estimated here, a 34% further reduction in the 5.4% prevalence would be expected if seropositive cows had not been bred back or if their daughters had not been retained. Further reductions in prevalence in this herd may yet be realized by segregating sick calves away from adult animals and by the policy initiated in October 2001, in which calves would be fed colostrum only from cows with a negative ELISA test result. It also should be noted that no appreciable improvement in the overall ability to detect infected animals would necessarily be expected if bacteriologic culture of feces had also been implemented, especially given the low herd prevalence toward the end of the study.[17,18]

A possible explanation for some of the results we obtained could be a genetic predisposition to postnatal infection with MAP, in which the higher risk of infection for daughters of seropositive cows would be related to an inherited susceptibility of infection. In another study,[10] however, infection with MAP was detected in 26.4% of fetuses collected at slaughter from cows with clinical signs of paratuberculosis and from culled cows, suggesting that the findings here were not attributable to genetic factors per se. If cows in the herd studied here had a similar rate of congenital transmission as in the study of slaughtered cows, the AF of 84.6% found here would suggest that perhaps 58.2% (84.6% minus 26.4%) of the infection attributable to being born to a seropositive dam was acquired after the calf was born, presumably by ingestion of feces or colostrum from the dam. In a study[11] of MAP-infected Danish herds with no paratuberculosis control program, only 7.7% of the variation in milk anti-MAP antibody status of daughters could be explained by variation in the milk anti-MAP antibody status of their dams. The low correlation could be a reflection of the considerable postnatal transmission expected in herds with no control program and the comparative diminished overall effect of congenital transmission. As postnatal transmission declines following control efforts aimed at eliminating animals that are shedding bacteria in the environment, the relative contribution of congenital transmission to overall herd prevalence increases. That is, congenital infection will have a greater proportional effect on prevalence as postnatal transmission is reduced.

An anticipated problem with this study was the low sensitivity of the ELISA test and the likelihood of false-negative test results for infected animals that were tested only once. The study addressed the issue of low sensitivity by use of multiple test results for each animal, which was expected to increase the probability of eventually detecting an infected animal. In addition, comparison of the OR designated by use of the single cutoff value recommended by the ELISA test manufacturer (OR, 3.1) with that designated by use of the 85% prediction intervals described by others[15] (OR, 3.6) indicated that any reduction in sensitivity that resulted from use of the 85% prediction intervals had little, if any, effect on the estimated ORs.

An interesting finding was that daughters exposed as calves to flush water from adult pens were 28.5 times as likely to yield positive test results as unexposed calves. This association suggested that exposure to flush water, which is common on many large dairies, could constitute a substantial risk for exposure to MAP. It is possible, however, that this estimate was somewhat inflated by the age difference in exposed and unexposed animals. Because the likelihood of seroconversion in MAP-infected animals increases with age[19] and because daughters exposed as calves to flush water containing feces from adult cattle were older at their last test than those not exposed, those exposed to flush water were more likely to be seropositive than those not exposed.

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This study found that for a herd in which paratuberculosis control efforts had already considerably reduced the risk of transmission of MAP from a contaminated environment, continued transmission of MAP from retained seropositive cows to their daughters, either in utero or postnatally, can contribute substantially to maintaining infection with MAP in the herd.

a. LpH, Steris Corp, Mentor, Ohio.
b. HerdChek Mpt, IDEXX Inc, Portland, Me.

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