

## *Mycobacterium paratuberculosis* Cultured from Milk and Supramammary Lymph Nodes of Infected Asymptomatic Cows

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**Milk and supramammary lymph node samples were obtained from asymptomatic cows infected with *Mycobacterium paratuberculosis* at the time of slaughter. Of 81 supramammary lymph node samples, 22 (27%) were culture positive for *M. paratuberculosis*. Of 77 milk samples, 9 (11.6%) were culture positive. The prevalence of supramammary lymph node or milk infection was highest with heavy fecal shedding of *M. paratuberculosis* and lowest with light shedding. The serologic status of the cow was not useful for predicting the risk of supramammary lymph node or milk infection. Shedding of *M. paratuberculosis* occurs in the milk of asymptomatic infected cows but, apparently, less frequently than previously reported for symptomatic cows.**

Paratuberculosis is a chronic granulomatous intestinal disease of ruminants caused by *Mycobacterium paratuberculosis*. Paratuberculosis is generally regarded as an enteric infection, although dissemination of *M. paratuberculosis* to various organs (liver [2, 3, 10], spleen [3, 8], genital organs [8, 9], kidneys [4], and uterus [7, 13]) and semen (8) has been reported. Control programs are designed to prevent transmission, which is widely believed to occur when young calves ingest *M. paratuberculosis* organisms after the contamination of feed sources with feces from infected animals (11, 21). However, direct shedding of *M. paratuberculosis* into milk or colostrum could result in transmission, even when normal control measures are observed. The isolation of *M. paratuberculosis* from udder tissue (3, 19), supramammary lymph nodes (2, 3), and milk (2, 3, 15, 19) of cows with clinical signs of paratuberculosis has been reported. Similar studies of asymptomatic cattle have not been reported, although asymptomatic infected cows outnumber symptomatic cows in most herds with paratuberculosis (1, 11, 21).

The purpose of the study reported here was to determine the prevalence of isolation of *M. paratuberculosis* from milk of infected asymptomatic cows and to perform a systematic evaluation of both milk and supramammary lymph nodes from infected cows, which has not been reported previously.

### MATERIALS AND METHODS

**Animals.** Asymptomatic fecal-culture-positive cows were identified by reviewing results of fecal cultures for *M. paratuberculosis* from the Commonwealth of Pennsylvania Diagnostic Laboratory (Tunkhannock, Pa.) and the Mycobacterial Laboratory, New Bolton Center, University of Pennsylvania School of Veterinary Medicine. Culture-positive asymptomatic animals were monitored until slaughter. Samples were collected from 86 asymptomatic cows from 25 known-infected dairy herds participating in a control program which included annual or semiannual fecal cultures from all herd members. Herds were selected on the basis of geographic accessibility to the slaughterhouse employed. All asymptomatic infected cows available for study were included.

**Sample acquisition.** At the time of slaughter, whole blood

was collected for serologic testing and feces were collected from the rectums for mycobacterial culture. The skin of the teats was disinfected with a 4% solution of 10.5% *o*-phenylphenol and 0.5% *o*-benzyl-*p*-chlorophenol (Amphyl; National Laboratories, Montvale, N.J.), and a 50-ml milk sample (composite of all four quarters) was obtained. After the removal of the udder from the carcass, a supramammary lymph node was removed with sterile instruments. Milk samples and supramammary lymph nodes were obtained from 72 cows, supramammary lymph nodes but no milk samples were obtained from 9 cows, and milk samples but no supramammary lymph nodes were obtained from 5 cows. Samples were transported on ice to the laboratory.

**Mycobacterial cultures.** Fecal specimens (2 g) were suspended in 35 ml of sterile distilled water and mixed on a laboratory shaker for 30 min. After an additional 30 min to allow settling of the particulate matter, 5 ml of the supernatant was transferred to 35 ml of 0.9% hexadecylpyridinium chloride (HPC; final concentration, 0.75%; Sigma, St. Louis, Mo.) and allowed to decontaminate for 18 h. The sample was then centrifuged for 30 min at 900 × *g* to concentrate the mycobacterial organisms (6, 20). The supernatant was discarded, and the pellet was resuspended in 1 ml of water with amphotericin B (100 µg/ml). Five drops (0.15 ml) of inoculum were placed on each of four slants of Herrold's egg yolk medium (12) (HEYM) with mycobactin J (Allied Laboratories Inc., Fayette, Mo.) and incubated at 37°C for 16 weeks. The tubes were examined every 2 weeks for mycobacterial growth. Colonies of acid-fast bacteria were subcultured to test for mycobactin dependency. Slowly growing mycobactin-dependent organisms with appropriate colony morphology and acid-fast staining characteristics were identified as *M. paratuberculosis*. The number of colonies per culture tube was recorded for each culture-positive specimen. Cows with fecal culture colony counts of >70 per tube were reported as having organisms too numerous to count and classified as heavy shedders. Cows with colony counts of 10 to 70 and of <10 were classified as intermediate and light shedders, respectively. A colony count of one colony in each of four tubes corresponded to approximately 40 to 80 CFU per g of feces, on the basis of recovery experiments in our laboratory.

Tissue samples (1 g) were homogenized in 10 ml of HPC with Ten Broeck tissue grinders (25 cows) or a Stomacher

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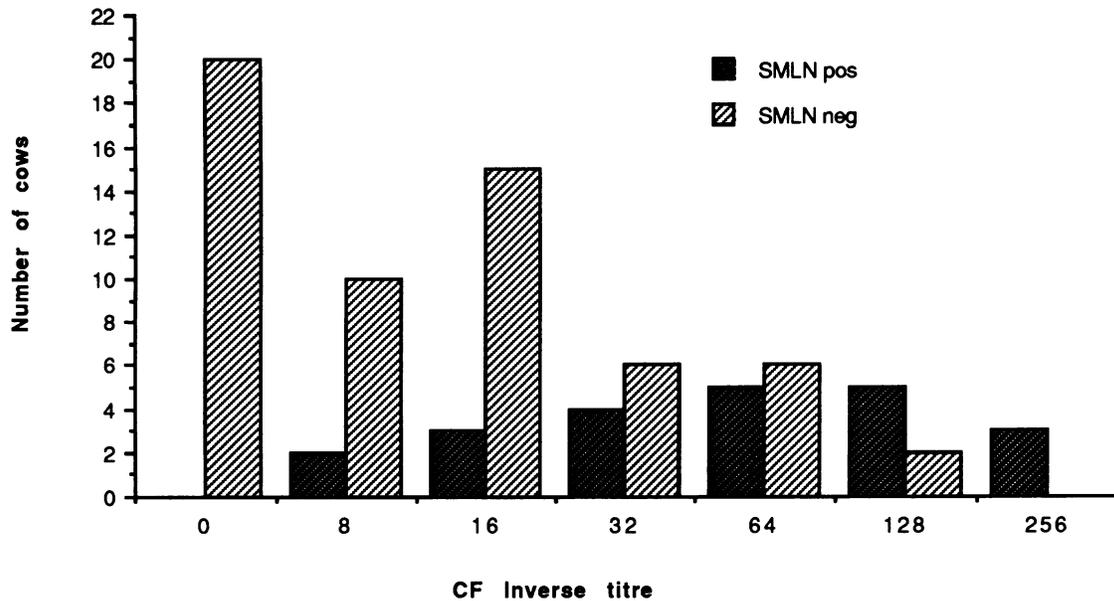


FIG. 1. Distribution of results of serum CF test for antibodies to *M. paratuberculosis* (CF inverse titer) for cows with supramammary lymph nodes that were culture positive (SMLN pos) and culture negative (SMLN neg). An inverse titer of  $\geq 32$  was considered positive.

(Tekmar, Cincinnati, Ohio) lab blends (56 cows). The homogenate was decontaminated for 4 h in 0.75% HPC and then centrifuged at  $900 \times g$  for 30 min. The pellet was resuspended in 1 ml of Middlebrook 7H9 broth (Difco Laboratories, Detroit, Mich.), and 5 drops were inoculated onto HEYM slants and incubated as described above.

Milk samples were centrifuged at  $900 \times g$  for 30 min, and the supernatant was discarded. The pellet was resuspended in 0.75% HPC, allowed to decontaminate for 4 h, and then processed as described above for tissue samples.

**Serologic tests.** Serum was harvested by centrifugation and frozen ( $-70^\circ\text{C}$ ) for batch testing. Samples were submitted to the Commonwealth of Pennsylvania Diagnostic Laboratory (Summerdale, Pa.) for complement fixation (CF) testing; a titer of  $\geq 1:32$  was considered positive. The agar gel immunodiffusion (AGID) test was conducted by using agar plates with 3-mm wells. Samples of test or control serum (15  $\mu\text{l}$ ) were placed in the peripheral wells, and 10  $\mu\text{l}$  of protoplasmic antigen (10 mg/ml; Allied Laboratories Inc.) was placed in the central well. Plates were read for precipitation bands of identity at 48 h (14).

Enzyme-linked immunosorbent assay (ELISA) testing was done with the L-arabinomannan antigen (17). Flat-bottomed 96-well microtitration plates (Nunc, Roskilde, Denmark) were coated with 0.2 ml of L-arabinomannan antigen per well (0.5  $\mu\text{g}/\text{ml}$  in 0.1 M carbonate-bicarbonate buffer, pH 9.6; provided by E. A. Sugden, Agriculture Canada, Animal Disease Research Institute, Nepean, Ontario, Canada). Plates were sealed, left at  $24^\circ\text{C}$  for 18 h, and then frozen ( $-20^\circ\text{C}$ ) until needed ( $<2$  weeks). After being thawed at  $24^\circ\text{C}$  for 1 h, plates were washed four times with 0.05% Tween 20 (Sigma) in phosphate-buffered saline (PBS-T20) by using an automated plate washer (Titertech; Flow Laboratories, Inc., McLean, Va.). Test serum samples were diluted 1:200 in PBS-T20 containing 0.01% merthiolate (Sigma). Dilute test serum samples (0.2 ml each) were added to test wells in triplicate, and 11 control serum samples were added to each plate. Plates were incubated at  $37^\circ\text{C}$  for 1 h in a sealed humid container and washed four times as before.

After the plates were washed, 0.2 ml of a 1:3,000 dilution (in PBS-T20 containing 0.01% merthiolate) of mouse monoclonal anti-bovine immunoglobulin G1 conjugated to horseradish peroxidase (K. H. Nielsen, Agriculture Canada, Animal Disease Research Institute) was added to each well, incubated at  $24^\circ\text{C}$  for 30 min, and washed as described above. The substrate [2,2'-azino-bis(3-ethylbenzthiazoline sulfonic acid)] (Sigma) was diluted in citrate buffer (pH 4) to a final concentration of 210  $\mu\text{g}/\text{ml}$ , with 0.1 ml of 0.5 M  $\text{H}_2\text{O}_2$  added to each 25 ml of substrate solution. The  $A_{405}$  was read three times at 2-min intervals with an ELISA reader (Titertech Multiscan Mark II; Flow Laboratories). A kinetics-based enzyme-linked immunoassay (KELA) software package (5) was used to normalize results between runs (on the basis of the 11 control serum samples) and provide a KELA score, which was proportional to the substrate reaction rate (slope of the optical density versus time) and, thus, the amount of antibody in the test sample. A KELA score of  $\geq 40$  was considered positive, on the basis of prior experience with positive and negative control serum samples, and provided a test sensitivity of 80% and a specificity of 80% relative to fecal culture (18).

**Statistical analysis.** The chi-square test or Fisher's exact

TABLE 1. Results of culture of milk and supramammary lymph node samples for *M. paratuberculosis* from asymptomatic infected cows

Fecal culture status <sup>a</sup>	No. of culture-positive samples/no. of samples tested (%)	
	Supramammary lymph node	Milk
Heavy	18/39 (46) <sup>b</sup>	7/37 (19) <sup>b</sup>
Intermediate	2/9 (22)	1/9 (11)
Light	2/33 (6)	1/31 (3)

<sup>a</sup> Status based on colony counts from fecal cultures for *M. paratuberculosis*. See Materials and Methods for explanation.

<sup>b</sup> Differences between fecal shedder groups were significant (chi-square test for linear trend,  $P < 0.05$ ).

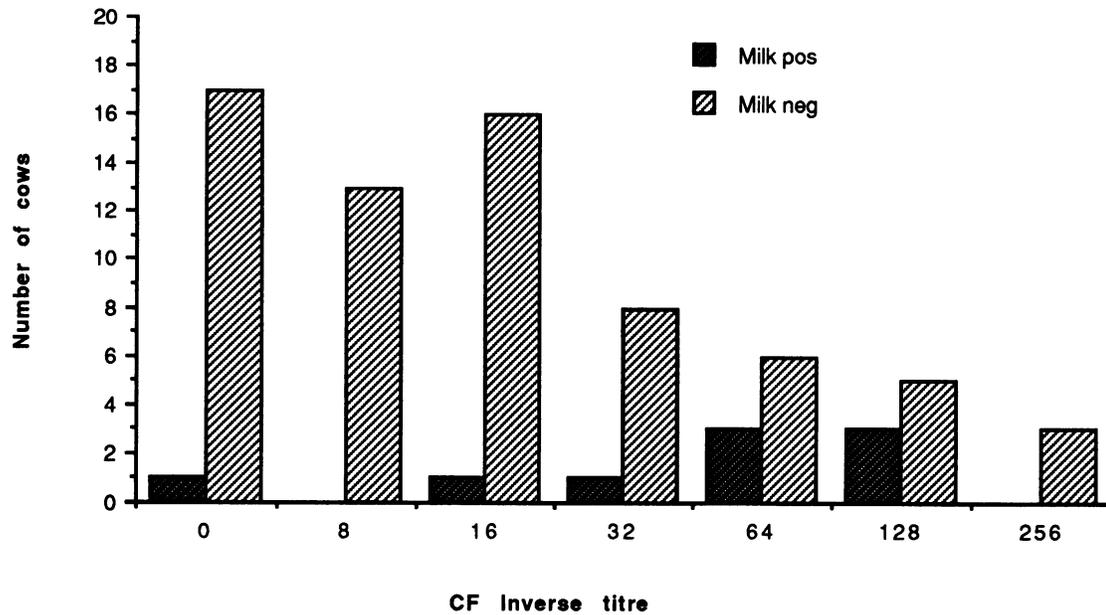


FIG. 2. Distribution of results of serum CF test for antibodies to *M. paratuberculosis* (CF inverse titer) for cows with culture-positive (Milk pos) and -negative (Milk neg) milk. An inverse titer of  $\geq 32$  was considered positive.

test was used to determine whether there was a significant difference in the proportion of cows that were seropositive by AGID of those with culture-positive milk or supramammary lymph nodes versus those that were culture negative. The Wilcoxon rank sums test was used to compare ELISA and CF scores for dams with culture-positive versus culture-negative milk and supramammary lymph nodes. A significance level of 0.05 was used. The chi-square test for linear trend (16) was used to determine whether the proportion of cows with culture-positive milk or supramammary lymph

nodes was significantly different as fecal shedding rate (i.e., low, intermediate, or high) increased.

## RESULTS

Milk samples and supramammary lymph nodes were obtained from 72 cows, supramammary lymph nodes but no milk samples were obtained from 9 cows, and milk samples but no supramammary lymph nodes were obtained from 5 cows. Of 81 supramammary lymph node samples, 22 (27%)

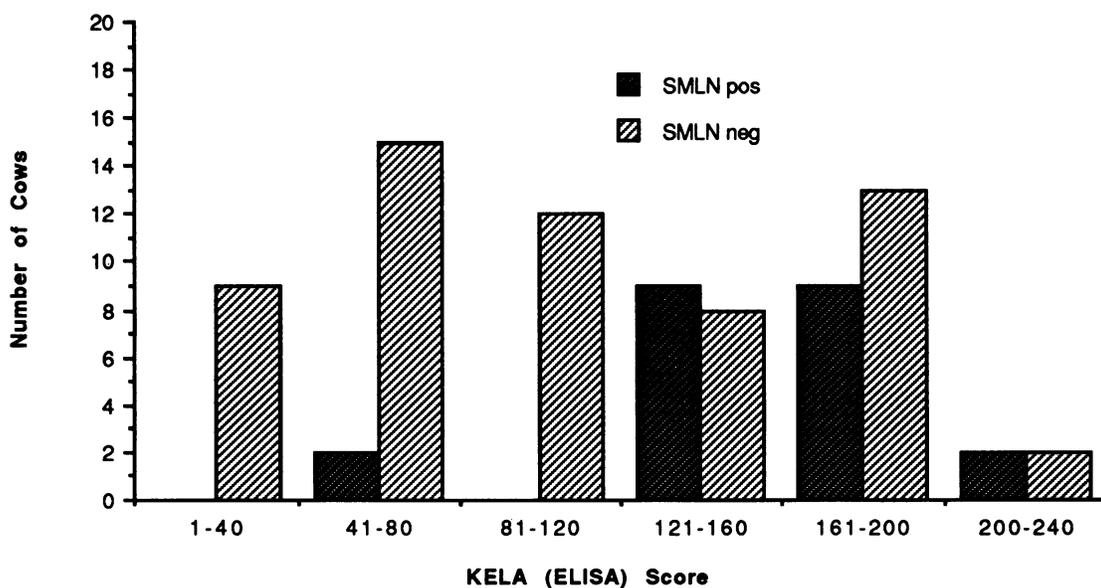


FIG. 3. Distribution of results of serum ELISA test for antibodies to *M. paratuberculosis* (KELA ELISA score) for cows with supramammary lymph nodes that were culture positive (SMLN pos) and culture negative (SMLN neg). A KELA score of  $\geq 40$  was considered positive.

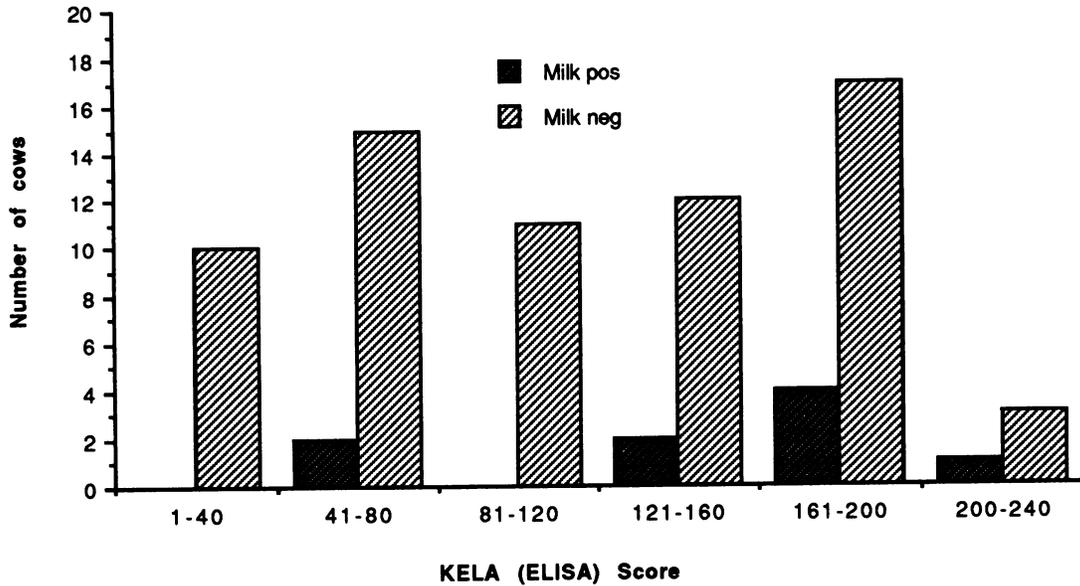


FIG. 4. Distribution of results of serum ELISA test for antibodies to *M. paratuberculosis* (KELA ELISA score) for cows with culture-positive (Milk pos) and -negative (Milk neg) milk. A KELA score of  $\geq 40$  was considered positive.

were culture positive for *M. paratuberculosis*. Of 77 milk samples, 9 (11.6%) were culture positive. The proportion of culture-positive milk samples and supramammary lymph nodes was higher in the heavy shedders than in the intermediate and light shedders (Table 1). The trend for an increased proportion of culture-positive milk or supramammary lymph node samples with an increased fecal shedding rate is significant ( $P < 0.05$ ). Of 72 cows for which both milk and supramammary lymph node samples were available, 48 had specimens that were both culture negative, 5 had samples that were both culture positive, 3 had culture-positive milk

and culture-negative lymph node samples, and 16 had culture-positive lymph node samples and culture-negative milk samples. From the culture-positive specimens, colony counts ranged from 2 to 8 CFU per 50 ml of milk sample and 2 to too-numerous-to-count CFU per g of lymph node tissue.

Of the 86 cows tested, 78, 31, and 25 were seropositive by the ELISA, the CF testing, and AGID, respectively. There is a significant difference ( $P < 0.001$ ) in CF scores for cows with culture-positive versus -negative supramammary lymph node tissues (Fig. 1) and for culture-positive versus -negative milk samples ( $P < 0.05$ ; Fig. 2). There is a significant

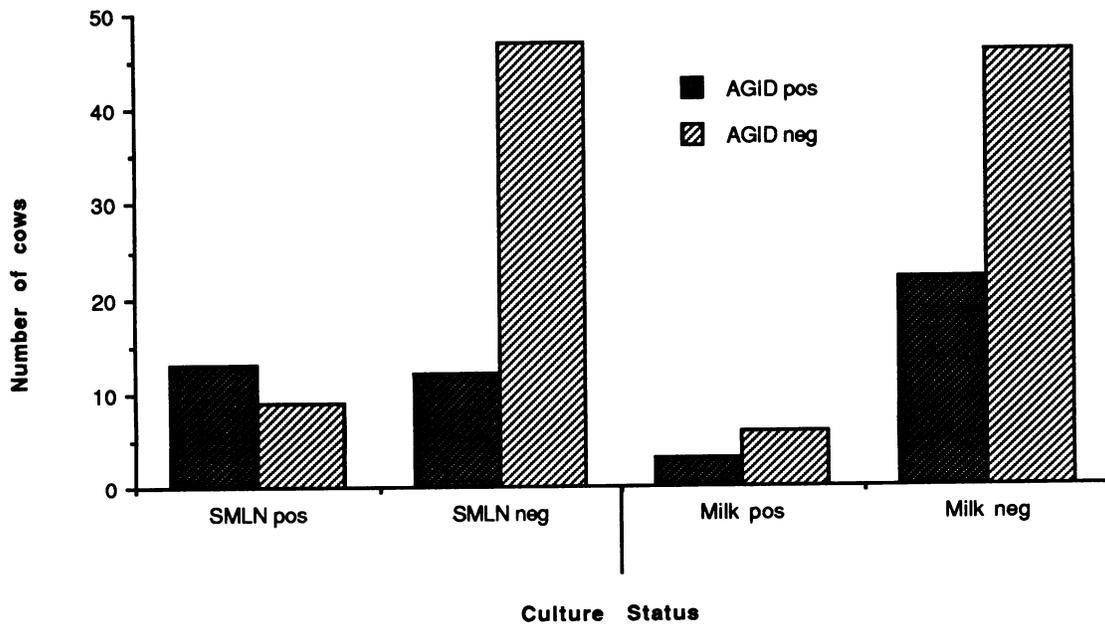


FIG. 5. Results of serum AGID test for antibodies to *M. paratuberculosis* for cows with supramammary lymph node culture-positive (SMLN pos) and -negative (SMLN neg) results and for cows with culture-positive (Milk pos) and -negative (Milk neg) milk.

difference ( $P < 0.001$ ) in the ELISA scores between cows with culture-positive and -negative lymph nodes (Fig. 3), but no difference was demonstrated for milk samples (Fig. 4). The proportion of AGID-seropositive cows with culture-positive supramammary lymph nodes is significantly different ( $P < 0.01$ ) from that for the cows with culture-negative lymph nodes, but the difference is not significant for milk samples ( $P = 0.054$ , Fig. 5).

## DISCUSSION

Previous authors have reported shedding of *M. paratuberculosis* organisms in milk in 3 of 4 cases (2), 1 of 20 cases (15), and, most recently, 9 of 26 cases (19). In all previous studies, only cows with clinical signs of paratuberculosis were tested. In general, the clinically affected animals represent a small proportion of the infected animals in the herd, with clinical signs detected months to years after fecal shedding begins, when the animal is in an advanced stage of infection (1, 11, 21). The results of the current study reveal that supramammary lymph node infection and direct shedding of *M. paratuberculosis* into the milk of asymptomatic infected cows occur. The 11.6% prevalence of milk shedding in the current study is lower than that most recently reported for symptomatic cows (19). This would suggest that the likelihood of shedding of the organism into the milk is greater with more advanced infection. This is further supported by the finding in the current study that the prevalence of *M. paratuberculosis* in milk was higher for cows with high fecal culture colony counts (19%) compared with those for cows with intermediate (11%) and low (3%) counts. A similar trend was seen for the prevalence of supramammary lymph node infection. The infection rate for the supramammary lymph nodes in the present study (46% of heavy fecal shedders, 27% of all cows) is difficult to compare with that reported previously (one of four cows) for symptomatic cows because of the small sample size in the previous report (19). Comparison of the prevalence of shedding into milk with the prevalence reported in older studies (2, 15) is difficult as culturing techniques varied significantly and current methods may be more sensitive.

The mechanism of the shedding of organisms into milk could not be determined from this study, although presumably this occurs by hematogenous or lymphatic spread. Previous work has shown that udder tissue may be infected with *M. paratuberculosis* (3, 19), and dissemination of *M. paratuberculosis* from enteric sites to other organs has been reported (2, 4, 7–10, 13).

Although the differences in the CF and ELISA scores between cows with culture-positive and -negative supramammary lymph nodes are significant, Fig. 1 and 3 demonstrate the considerable overlap of serologic results for these two groups. Similar overlap exists for the ELISA and CF scores of cows with culture-positive and -negative milk (Fig. 2 and 4), although the CF scores for the two groups differ significantly. Thus, the serologic status of the cow appears to be of little use in predicting the risk of milk or supramammary lymph node infection.

The results of this study demonstrate that asymptomatic cows infected with *M. paratuberculosis* could potentially transmit paratuberculosis via the milk. Although the concentration of organisms in the samples tested was low (2 to 8 CFU per 50 ml of sample), the risk of infection would be multiplied due to the large quantities of milk consumed by a calf (4 liters per day). While the feeding of powdered milk replacer to calves would eliminate this risk, many farms

continue to feed whole milk rather than milk replacer. While colostrum samples were not studied, presumably the organism may also be shed in colostrum, which must be fed to every calf and could serve as a source of infection in calves despite subsequent feeding of milk replacer. Although, on the basis of this study, the risk of paratuberculosis transmission from the feeding of milk from asymptomatic cows is less than that from the feeding of milk from symptomatic cows, milk from infected asymptomatic animals should not be fed to calves. Since asymptomatic animals may remain undetected in the herd, feeding of milk replacer or pasteurized milk to calves should be recommended for herds known to have paratuberculosis.

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