

## Herd prevalence and geographic distribution of, and risk factors for, bovine paratuberculosis in Wisconsin

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**Summary:** A random sample of Wisconsin dairy herds, stratified by herd size, were tested for paratuberculosis by use of an absorbed ELISA procedure. The ELISA was optimized for overall accuracy by means of receiver operating characteristic curve analysis, and had a sensitivity and specificity of 50.9 and 94.9%, respectively. Herd prevalence was analyzed for correlation with responses to a management practices questionnaire completed by the herd owners. One hundred and fifty-eight herds and 4,990 cattle were tested. Of these, 50% of herds and 7.29% of cattle had positive test results. Calculation of true prevalence from the apparent prevalence indicated that 4.79% of cattle and 34% of the Wisconsin dairy herds tested had serologic evidence of paratuberculosis. Among the 54 herds classified as positive on the basis of true prevalence estimation, the mean number of test positive cattle was 20.3%. The geographic distribution of herds with positive results was not uniform. More infected herds were found in the southern and western districts of Wisconsin than in the eastern district. The west-central district had a larger number of infected herds than did other districts. By use of  $\chi^2$  analysis, the only management factor found to be significantly associated with herd prevalence was housing of calves after weaning ( $P = 0.03$ ). Specifically, in herds with higher prevalence, calves were separated after weaning into calf barns and hutches rather than into pens in the cow barn more often than in herds with lower prevalence. This factor was also considered significant by use of logistic regression analysis. Logistic regression analysis also revealed that herd size and location of farm by district of the state were significantly related to herd paratuberculosis prevalence.

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Funded in part by a grant from the Wisconsin Agricultural Experiment Station, project No. 3370.

The authors thank the State and Federal Veterinary Medical Officers in Wisconsin for collection of the blood samples used in this study, and the University of Wisconsin Cartographic Laboratory for preparation of the map shown in Figure 3.

In most surveys, prevalence of bovine paratuberculosis has been estimated on the basis of results of bacteriologic culture of fecal or tissue samples collected from culled cows at the time of slaughter. In these studies, depending on the region of the country in which the study was conducted, between 2.6 and 18% of cows were found to be infected with *Mycobacterium paratuberculosis*, the cause of bovine paratuberculosis.<sup>1-5</sup> In 2 such studies in Wisconsin, 10.8 and 7.8% of culled dairy cattle were found to be infected.<sup>2,6</sup> On the basis of results of a serologic test, apparent prevalence of paratuberculosis in Florida cows was reported to be 17.1%.<sup>7</sup> However, accuracy of the test was not reported, so true prevalence of paratuberculosis could not be determined, and samples were not collected from a random cross-section of cows in Florida dairy herds.

Previous paratuberculosis surveys have been cross-sectional in nature, and the individual animal was used as the unit of measurement. Such surveys are only appropriate for contiguous populations, that is, populations in which extensive movement or mingling of animals occurs. Cattle populations are not contiguous, but are separated, and a more appropriate unit of measure for prevalence surveys is, therefore, the herd.<sup>8</sup>

Kopecky<sup>9</sup> reported that there was a nonuniform geographic distribution of *M paratuberculosis*-infected dairy herds in Wisconsin and suggested that soil type, in particular soil pH, and location of infected herds were associated. Unfortunately, data were drawn only from records of the Wisconsin Animal Health Laboratory and consisted of results for samples submitted by veterinarians seeking confirmation of a clinical diagnosis of paratuberculosis. Because of the stigma associated with paratuberculosis and because of veterinarians' concerns about the effect of such a diagnosis on their clients' businesses, the data may have been biased.

The purpose of the study reported here was to conduct a random cross-sectional survey of Wisconsin dairy herds to determine the geographic distribution of herds with paratuberculosis and herd prevalence rates. Owners of herds included in

the study were asked to complete a questionnaire on herd management so that factors associated with a higher prevalence of paratuberculosis could be identified.

## Methods

**Serologic test**—The absorbed ELISA test for serum antibodies to *M paratuberculosis*, as originally described by Yokomizo et al,<sup>10,11</sup> was used. Briefly, *M paratuberculosis* strain 18 protoplasmic antigen<sup>a</sup> was coated onto flat-bottom wells in 96-well microtitration plates by placing 100  $\mu$ l of a solution containing 10  $\mu$ g of antigen/ml in each well, and incubating plates for 16 hours at 4 C. Undiluted test sera were preabsorbed with *M phlei* cells to remove cross-reacting mycobacterial antibodies. A suspension of 20 mg of lyophilized whole *M phlei* cells per ml of saline (0.9% NaCl) solution containing 1.0% Tween 20 was used. Preabsorption was performed on U-bottom 96-well microtitration plates. Equal volumes (25  $\mu$ l) of test sera and absorbing antigen suspension were placed in wells on the plate, and plates were agitated on an orbital shaker twice for 1 minute. Plates were then allowed to stand for 30 minutes at approximately 20 C to allow the *M phlei* cells to settle. Sera were removed, diluted 1:1,000 in buffer (phosphate buffered saline solution and Tween), and transferred, in quadruplicate, to microtitration plates with *M paratuberculosis*-coated wells. Plates were incubated for 30 minutes at 37 C and washed 4 times with buffer. After washing, 100  $\mu$ l of biotin-SP-goat anti-bovine IgG (heavy and light chain),<sup>b</sup> diluted 1:2,000 in buffer, was added to each well. Plates were again incubated for 30 minutes at 37 C and washed 4 times with buffer; 100  $\mu$ l of hydrogen peroxide-2,2'-azino-bis(3-ethylbenzthiazolin-6-sulfonic acid; ABTS) substrate was then added to each well (3  $\mu$ l 3% H<sub>2</sub>O<sub>2</sub>/10 ml ABTS substrate in citric acid buffer). The plates were incubated for 3 minutes at approximately 20 C, and the reaction was stopped by adding of 33  $\mu$ l stopping reagent (hydrofluoric acid + EDTA) to each well. Optical density (OD) was read at 410 nm, using an ELISA reader.<sup>c</sup>

Positive and negative control sera were included on each plate. Mean OD was calculated for each sample, and results for test sera were expressed as a percentage of the OD of the positive control sample (EV%: ELISA value percentage) using the following equation: EV% =

$$\frac{\text{Mean OD test serum} - \text{Mean OD negative control serum}}{\text{Mean OD positive control serum} - \text{Mean OD negative control serum}}$$

**Accuracy determination**—Sera from 177 cattle proven on the basis of results of bacteriologic culture to have paratuberculosis and from 196 cattle in herds certified to be free from paratuberculosis

<sup>a</sup>Allied Laboratories Inc, Glenwood Springs, Colo (now known as Allied Monitor Inc, Fayette, Mo).

<sup>b</sup>Jackson Immunoresearch Laboratories, West Grove, Pa.  
<sup>c</sup>Dynatech 360, Dynatech Corp, Torrance, Calif.

were obtained from a repository for paratuberculosis specimens<sup>12</sup> and tested. The resulting EV% data were analyzed by use of receiver operating characteristic (ROC) curve to determine the EV% that when used as a cutoff between positive and negative test results would yield the highest test accuracy.<sup>13</sup>

**Survey design**—The Wisconsin Agricultural Statistics Service generated a random list of 475 herds stratified by herd size (herds classified as having between 1 and 25 cattle, 26 and 50 cattle, 51 and 100 cattle, or > 100 cattle) from a list of all livestock producers in Wisconsin.

A cover letter, explaining the study and inviting participation, and a questionnaire were mailed to each herd owner. Herd owners were assured that the study was confidential in an attempt to allay their fears that if paratuberculosis were found in their herd, this would become public knowledge. A code number was assigned to each herd, and the identity of the herd was never made known to the investigators. The questionnaire (Appendix) was designed to elicit information from herd owners about herd management practices thought to affect the prevalence of *M paratuberculosis* infection in dairy cattle herds.<sup>14</sup>

Blood samples were collected between October 1989 and June 1990 from a random sample of adult milking cattle in herds whose owners agreed to participate in the study. In each herd, the number of cattle from which blood was collected was determined on the basis of herd size. Standard sampling tables were used to determine the number of samples needed to be 95% confident that at least 1 sample would yield a positive result if  $\geq 1$  cow in the herd was truly infected.<sup>15</sup> Blood samples were collected by state and federal veterinarians into 10 ml evacuated tubes, and shipped immediately with refrigerant to the testing laboratory. Sera were harvested and frozen at -20 C until tested.

**Data analysis**—Standard methods were used to estimate true herd prevalence from apparent herd prevalence.<sup>16,17</sup> The distribution of herd prevalence of paratuberculosis was found to be skewed, and many herds had a prevalence less than the overall mean prevalence for the state. Use of residual plots and the Shapiro-Wilk test for normality<sup>18</sup> demonstrated that data were not normally distributed, and none of several transformations of the prevalence data produced a normal distribution. Therefore, because ANOVA could not be used, the  $\chi^2$  test of association was used to identify associations between management variables and herd prevalence. For these analyses, herds were grouped according to apparent prevalence of paratuberculosis in the herd (0%, > 0% but < 15%, and  $\geq 15\%$ ). Management variables were categorized according to survey responses. Stepwise logistic regression, with herd paratuberculosis prevalence as the dependent variable, was performed. Survey variables

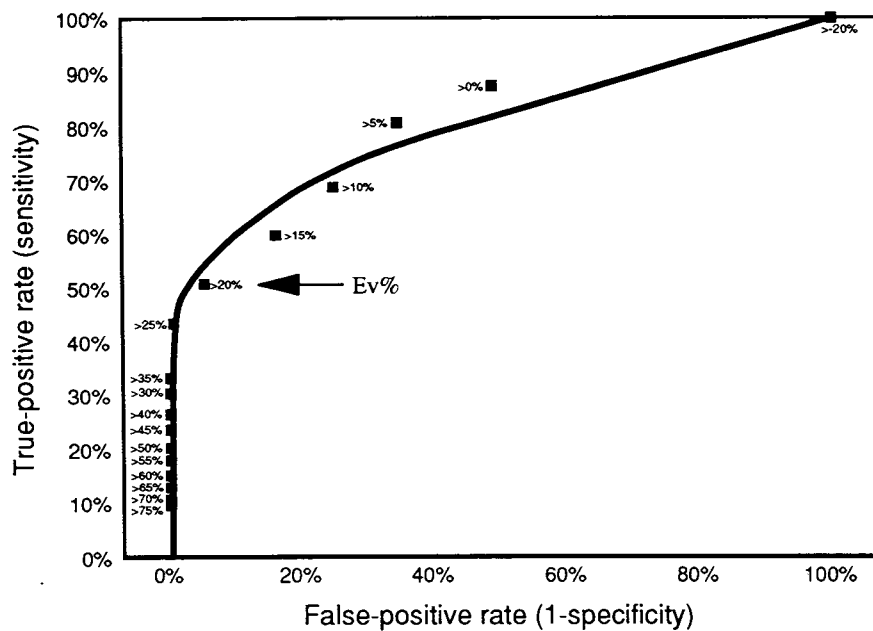


Figure 1—Receiver operating characteristic curve for results of an ELISA to determine paratuberculosis in cattle. Data represents results of testing blood samples from 177 cattle known to have, and 196 cattle known not to have, paratuberculosis. EV% = ELISA value percentage of the optical density of a positive control sample.

from the  $\chi^2$  analysis with  $P$  values  $< 0.25$  and variables of known biological importance were used as independent variables. Variables with  $P$  values as high as 0.25 were used, because use of only those variables with  $P$  values  $< 0.05$  will often fail to identify variables known to be important.<sup>19,20</sup>

## Results

**Assay accuracy**—From the ROC curve, the optimal cutoff for EV% was determined to be 20 (Fig 1). Thus, EV%  $> 20$  were considered positive. At this cutoff, the ELISA had sensitivity of 50.9% and specificity of 94.9% for individual cow samples. The 95% confidence interval for sensitivity was (43.6, 58.2) and for specificity was (92.0, 97.8).

**Serologic survey**—Of the 475 herd owners invited to participate in the survey, 199 (42%) returned a completed questionnaire and agreed to have their herd tested for paratuberculosis. Because of scheduling difficulties, blood samples could not be collected from all herds; however, 158 herds (33%) and 4,990 dairy cattle were tested. Of the herds tested, 31 had between 1 and 25 cattle, 56 had between 26 and 50 cattle, 67 had between 51 and 100 cattle, and 4 had  $> 100$  cattle. Responders did not differ from nonresponders, and tested herds did not differ from untested herds, with regard to herd size, geographic location, or participation in Dairy Herd Improvement Association programs. Overall, 7.29% of cattle had positive test results, and 50.0% of herds had 1 or more cows with positive results. Of the 158 herds, 79 (50.0%) had an apparent prevalence of 0%, 54 (34.2%) had an apparent prevalence between 0 and 15%, and 25 (15.8%) had an apparent prevalence of  $\geq 15\%$ . The distribution of apparent prevalence among tested herds was skewed (Fig 2). By inspection it

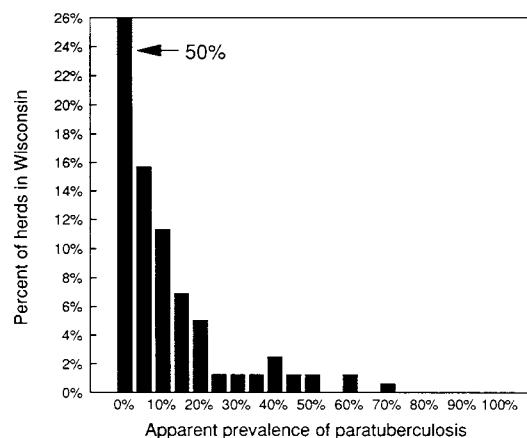


Figure 2—Histogram of the distribution of apparent herd prevalence rates for paratuberculosis among 158 dairy herds in Wisconsin. The left-most bar (0% prevalence) represents 50% of the herds tested.

appeared that the geographic distribution of apparent herd prevalence was not uniform (Fig 3). Calculation of true herd prevalence from apparent prevalence indicated that 4.79% of cattle and 34% of Wisconsin dairy herds tested had serologic evidence of paratuberculosis.

**Management factors**—By  $\chi^2$  analysis, only housing of calves after weaning was significantly ( $P = 0.03$ ) associated with true herd prevalence of paratuberculosis (Table 1). The number of herds tested was too small to allow analysis for an association between true herd prevalence and county; therefore, herds were grouped by state district for analysis. State districts were based on those regions of Wisconsin (divided on county lines) for which state and federal district veterinarians were assigned animal health control responsibilities. We

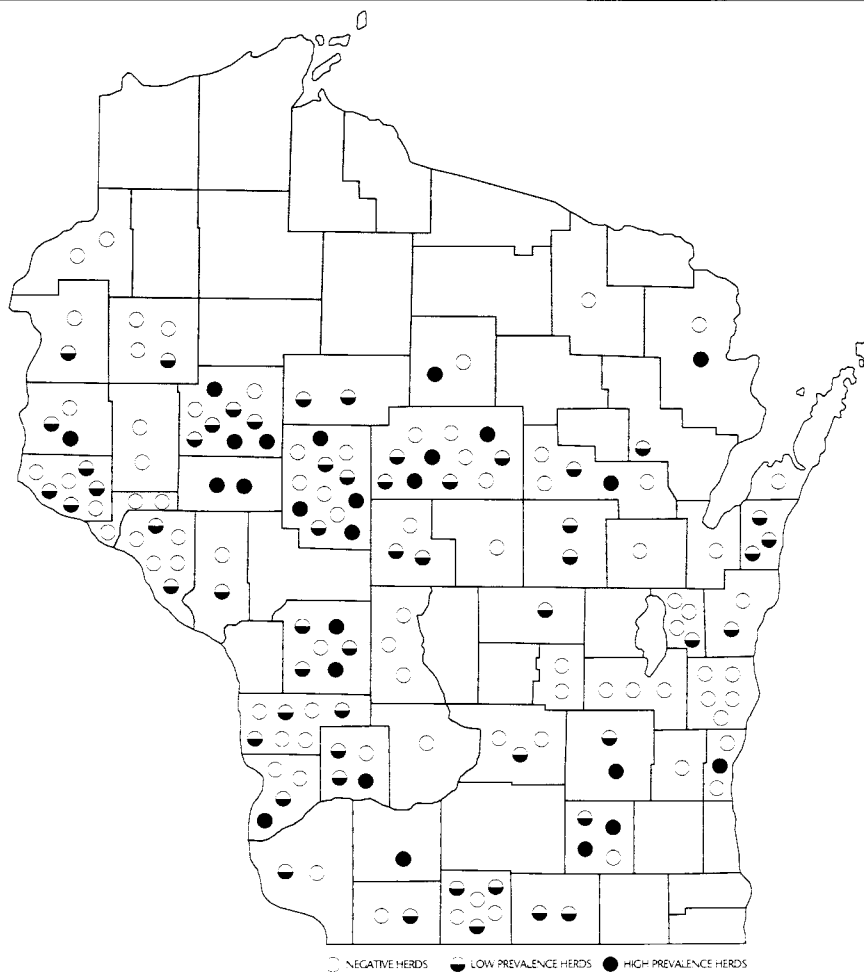


Figure 3—Geographic distribution of 158 Wisconsin dairy herds tested for paratuberculosis. Open circles, herds in which paratuberculosis was not detected; partially filled circles, herds with apparent herd prevalence between 0 and 15%; filled circles, herds with apparent herd prevalence  $\geq 15\%$ .

Table 1—Results of  $\chi^2$  analysis for associations between apparent herd paratuberculosis prevalence (0%, 0 to 15%,  $\geq 15\%$ ) and various management factors

Management factor	$\chi^2$ value (df)	P value
Herd size*	3.34 (2)	0.19
Herd location by state district	7.57 (4)	0.11
Herd tested under DHIA program	2.82 (4)	0.59
Open vs closed herdt	2.94 (2)	0.23
Location of calving (summer)	12.99 (8)	0.11
Location of calving (winter)	8.97 (8)	0.35
Calf/dam separation time	5.32 (4)	0.25
Calf housing before weaning	4.60 (4)	0.33
Calf housing after weaning	14.26 (6)	0.03
Cows with nonresponsive diarrhea	1.01 (2)	0.60
Cows tested for paratuberculosis	0.97 (2)	0.62
Herd veterinarian ever suspected paratuberculosis	4.14 (2)	0.13
Herd owner has read articles on paratuberculosis	0.30 (2)	0.86

\*Herds classified as having between 1 and 25 cattle, between 26 and 50 cattle, between 51 and 100 cattle, or  $>100$  cattle. †Herds classified according to possible responses on a questionnaire completed by herd owner (Appendix). df = degrees of freedom; DHIA = Dairy Herd Improvement Association. Data are results of testing, by use of ELISA, blood samples from 4,990 cattle in 158 herds.

did not detect, by use of  $\chi^2$  analysis, association between geographic location of herds grouped by district and true herd prevalence of paratuberculosis; however, geographic location was significant in the logistic regression model. Logistic regression revealed significant interactions between herd size,

geographic location of the herd, and calf housing after weaning. These 3 variables were retained in the final logistic regression model and their P values were; for herd size,  $P = 0.07$ , for district of the state,  $P = 0.08$ , and for housing after weaning,  $P = 0.05$ .

### Discussion

The absorbed ELISA was a simple, inexpensive, and reasonably accurate test for paratuberculosis.<sup>21</sup> Its sensitivity and specificity, 50.9 and 94.9%, respectively, were the same as those reported for the absorbed ELISA performed by others.<sup>22,b</sup> If a higher cut-off value was used to define positive tests (EV%  $\geq 25\%$ ), its accuracy would be the same as that of an absorbed ELISA test kit.<sup>21-24,d</sup> Although test sensitivity was low relative to the sensitivity of many other diagnostic tests, this sensitivity was calculated on the basis of results of testing individual animals. With the herd sampling strategy used, the ability of the test to detect infected herds was much higher. Specifically, the sampling fraction was determined to yield 95% confidence that if a perfect test (100% sensitive and 100% specific) were used, at least 1 positive test result would be obtained if

<sup>d</sup>Johne's Absorbed EIA, CSL Ltd, Melbourne, Australia.

the herd was truly infected. The 50.9% sensitivity of the ELISA used means that we were approximately 90% confident that we could detect infection among herds with at least 1 *M paratuberculosis*-infected cow.

The response rate of 42% was lower than expected, and this could have been a result of the stigma associated with a diagnosis of paratuberculosis in a herd. However, we did not detect any bias in the survey sample.

Although half of Wisconsin dairy herds had seropositive cattle, nearly two-thirds of the herds in this survey would be considered seronegative for paratuberculosis on the basis of true prevalence estimation. For the 79 herds with seropositive cattle, 25 (31.6%) had > 15% of the cattle test positive for paratuberculosis. This high herd prevalence of paratuberculosis indicates that the disease is a more serious threat to Wisconsin's dairy industry than previously suspected on the basis of data from surveys of infection status among culled dairy cows. Evidence from historical data on farms, as well as from epidemiologic modeling, indicates that this disease will continue to spread within herds until effective control measures are instituted.

By logistic regression, herd paratuberculosis prevalence was found to be related to geographic district and to herd size, even though neither was found to be associated with herd prevalence by use of  $\chi^2$  analysis. This would suggest that large herds in certain districts were likely to have a higher prevalence of *M paratuberculosis* infection than smaller herds in those districts or small or large herds in other districts.

Responses to the question whether the owner or herd veterinarian had ever suspected paratuberculosis in the herd were not significantly associated with herd paratuberculosis prevalence ( $P = 0.13$ ). This reflects the long incubation period for paratuberculosis and the low ratio of clinically ill to subclinically infected cattle in a herd. More than 90% of *M paratuberculosis*-infected cattle in a herd may be subclinically infected.<sup>25</sup> In practical terms, this means that by the time clinical disease is detected, infection may have already spread through a large percentage of the herd. Moreover, it illustrates that clinical observation is not sufficient for early detection of *M paratuberculosis* infections in dairy herds.

By use of  $\chi^2$  analysis, the only management factor significantly associated with herd paratuberculosis prevalence was calf housing after weaning. In higher prevalence herds, calves tended to be moved to calf barns and hutches rather than to pens in the cow barn. This observation is not consistent with most recommendations on paratuberculosis control, and this result could either be spurious, be related to the proximity of calf barns to adult cattle, or could indicate that *M paratuberculosis* can be transmitted between calves. We do not believe that the association is spurious.

Surprisingly, herd paratuberculosis prevalence was not associated with whether the herd was closed or open. Spread of the disease between herds should be a direct function of the frequency with which replacement cattle are bought from other herds and the extent to which the cattle buyers try to insure that source herds are free of *M paratuberculosis* infection.<sup>26</sup> We did find that 70% of respondents thought that their herds were closed, even though this is not consistent with our experience with Wisconsin dairy herds. The difference between responses to this question and our observations may have been a result of misinterpretation of the definition of a closed herd by farmers or failure on our part to ask the question precisely.

Our questionnaire was constructed to be brief and to try to measure only those general management practices thought to affect *M paratuberculosis* transmission. A more quantitative and detailed assessment of farm management practices would be necessary to determine whether other factors are associated with spread of paratuberculosis.

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## Appendix

Questionnaire to determine management factors possibly associated with herd prevalence of paratuberculosis

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|---|---|
| <ol style="list-style-type: none"> <li>1. How many milking and non-milking cows in your present herd? ___</li> <li>2. How many of your milking and non-milking cows are registered? ___</li> <li>3. Is your herd tested under a DHIA program? (Circle one)             <ol style="list-style-type: none"> <li>1 Yes: Official test by DHIA personnel</li> <li>2 Yes: Owner collected samples</li> <li>3 No: Not tested</li> </ol> </li> <li>4. The dairy industry refers to a herd as OPEN if replacement animals are purchased or rented from outside the herd. Do you operate an OPEN herd or a CLOSED herd? (Circle one)             <ol style="list-style-type: none"> <li>1 Closed (Please go to next question)</li> <li>2 Open                 <ol style="list-style-type: none"> <li>a. Do you usually buy cows, springing heifers, or calves? (Circle one)                     <ol style="list-style-type: none"> <li>1 Cows</li> <li>2 Spinging heifers</li> <li>3 Calves</li> </ol> </li> <li>b. Where do you usually get replacement animals? (Circle one)                     <ol style="list-style-type: none"> <li>1 Local farmers</li> <li>2 Cattle dealers</li> <li>3 Sale barns</li> <li>4 Auctions</li> <li>5 Other (Please describe)</li> </ol> </li> <li>c. How many replacements do you usually buy in a year? ___</li> </ol> </li> </ol> </li> <li>5. Where do you house your milking cows? (Circle one)             <ol style="list-style-type: none"> <li>1 Stanchions</li> <li>2 Free stalls</li> <li>3 Loose housing</li> <li>4 Other (Please describe)</li> </ol> </li> <li>6. Where does calving usually occur on your dairy? (Circle one)             <ol style="list-style-type: none"> <li>1 Stanchions</li> <li>2 Free stalls</li> <li>3 Maternity pens</li> <li>4 Outdoor dry lot</li> <li>5 Pasture</li> </ol> </li> <li>7. Following calving, when do you separate calves from cows? (Circle one)             <ol style="list-style-type: none"> <li>1 Less than one hour after birth</li> <li>2 One to eight hours after birth</li> <li>3 More than eight hours after birth</li> </ol> </li> <li>8. Where do you house calves BEFORE weaning? (Circle one)             <ol style="list-style-type: none"> <li>1 Calf barn</li> <li>2 Calf hutches</li> <li>3 Pens in cow barn</li> <li>4 Other (Please describe)</li> </ol> </li> </ol> | <ol style="list-style-type: none"> <li>9. Where do you house calves AFTER weaning? (Circle one)             <ol style="list-style-type: none"> <li>1 Calf barn</li> <li>2 Calf hutches</li> <li>3 Pens in cow barn</li> <li>4 Other (Please describe)</li> </ol> </li> <li>10. Thinking now about the last 12 months, have any of your cows developed diarrhea that would not respond to treatment over several weeks or months? (Circle one)             <ol style="list-style-type: none"> <li>1 No</li> <li>2 Yes</li> </ol> </li> <li>11. What did you do with cows that developed the diarrhea? (Circle one)             <ol style="list-style-type: none"> <li>1 Culled the animal(s)</li> <li>2 Separated from other animals on farm</li> <li>3 Changed animal(s) diet</li> <li>4 Sought help from my veterinarian</li> <li>5 Other (Please describe)</li> </ol> </li> <li>12. Have you ever had any cows tested for paratuberculosis* by a laboratory? (Circle one)             <ol style="list-style-type: none"> <li>1 No (Please go to question 13)</li> <li>2 Yes                 <ol style="list-style-type: none"> <li>a. Did any cows test positive?                     <ol style="list-style-type: none"> <li>1 No</li> <li>2 Yes</li> </ol> </li> <li>b. If you had positive cows, how long ago was the first positive cow identified in your herd? (Circle one)                     <ol style="list-style-type: none"> <li>1 Within the past 12 months</li> <li>2 1 to 3 years ago</li> <li>3 3 to 5 years ago</li> <li>4 5 to 10 years ago</li> <li>5 More than 10 years ago</li> </ol> </li> <li>c. Has your WHOLE HERD (milking cows) ever been tested for paratuberculosis? (Circle one)                     <ol style="list-style-type: none"> <li>1 No</li> <li>2 Yes</li> </ol> </li> <li>d. How many cows tested positive? ___</li> </ol> </li> </ol> </li> <li>13. Have you or your veterinarian ever suspected that cows in your herd might have paratuberculosis? (Circle one)             <ol style="list-style-type: none"> <li>1 No</li> <li>2 Yes</li> </ol> </li> <li>14. Have you read any articles about paratuberculosis in magazines, trade journals, newspapers or other publications? (Circle one)             <ol style="list-style-type: none"> <li>1 No</li> <li>2 Yes</li> </ol> </li> </ol> |
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\*Copies of the questionnaire mailed to herd owners contained the term "Johne's disease," instead of paratuberculosis.