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## Performance of a Johne's disease enzyme-linked immunosorbent assay adapted for milk samples from goats

Miguel Salgado, Elizabeth J. B. Manning, Michael T. Collins<sup>1</sup>

**Abstract.** Antibody detection-based tests for paratuberculosis offer speed and economy, 2 diagnostic test attributes important to animal industries with narrow profit margins. Application of such tests to individual milk samples instead of serum samples can further improve testing efficiency and decrease testing cost. Accuracy of a commercial bovine paratuberculosis enzyme-linked immunosorbent assay (ELISA) adapted for use on goat serum and milk samples was determined. Fecal, blood, and milk samples were collected from 159 goats belonging to 2 Wisconsin goat herds with a prior history of paratuberculosis and 1 herd of 50 goats from a paratuberculosis-free Wisconsin herd. Fecal samples were cultured using the modified BACTEC 12B media. Sera were tested according to the manufacturer's instructions for bovine samples. Milk samples were centrifuged and mixed with the ELISA kit's *Mycobacterium phlei*-containing diluent at a ratio of 1:2. Using fecal culture as the "gold standard," the sensitivity of the ELISA on goat serum was 64% and the sensitivity of the ELISA on goat milk was 48%. The milk ELISA had higher agreement with fecal culture results ( $\kappa = 0.525$ ) than the serum ELISA ( $\kappa = 0.425$ ). ELISA specificity was 100% on both serum and milk. Regression analysis also showed good correlation between serum and milk S/P values ( $r^2 = 0.67$ ). Although less sensitive, the ELISA on goat milk samples appears to offer a useful, low-cost alternative for detection of goats with paratuberculosis that have progressed to the stage of shedding *M. paratuberculosis* in their feces.

**Key words:** Diagnosis; ELISA; goats; Johne's disease; milk; paratuberculosis; serum.

Paratuberculosis (Johne's disease) is a chronic infectious enteric disease of ruminants. It is seen pri-

marily in cattle, sheep, and goats and is caused by *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*). Clinical expression of the disease in goats is weight loss and, at times, diarrhea and hypoproteinemia. The infection has a prolonged incubation period and thus many infected animals in a typical herd are in a preclinical stage of the disease. At the clinical stage of infection, the ileum may be markedly

From the School of Veterinary Medicine, University of Wisconsin, Madison, WI. Current address (Salgado): Instituto de Microbiología, Facultad de Ciencias, Universidad Austral de Chile, Valdivia, Chile.

<sup>1</sup>Corresponding Author: Michael T. Collins, University of Wisconsin School of Veterinary Medicine 2015 Linden Drive Madison, WI 53706-1102.

thickened by diffuse granulomatous inflammation and serosal lymphatic vessels are often distended and prominent, although in sheep and goats with clinical paratuberculosis, enteric lesions may be less obvious than that seen in cattle.<sup>10</sup> Caseous tubercle-like foci or areas of calcification may also be noted in some cases.<sup>7</sup>

Fecal culture is widely accepted as a most reliable method for diagnosis of paratuberculosis in live animals. However, this diagnostic method is slow, expensive, and not well standardized across laboratories.<sup>4</sup> Serologic tests, in particular those using enzyme-linked immunosorbent assay (ELISA) technology, are popular because blood is a convenient sample, and the assays are fast, inexpensive, and easily automated. However, the utility of serology is compromised by antibody rarely being produced at detectable levels in the early stages of natural *M. paratuberculosis* infection.<sup>1,14,16,19</sup> Most studies agree that in whole-herd testing, the sensitivity of serologic assays for paratuberculosis is less than 50% when compared with fecal culture done at the same time.<sup>8,15,20,25</sup>

The dairy goat industry has not given Johne's disease the same degree of attention that it has received from the cattle industry in recent years. The cost of diagnostic testing is 1 obstacle to regular surveillance for this infection. Use of milk instead of serum samples could make paratuberculosis surveillance more accessible to the dairy goat industry because the industry already collects milk samples for quality testing. Monitoring of herd health data with electronic data management and reporting systems is in place. If laboratories that process milk samples for quality testing also performed paratuberculosis testing, the per sample cost could be reduced further and no additional sample collection or shipment costs would be incurred. The aim of this study was to compare the sensitivity and specificity of a commercial ELISA kit using both serum and individual milk samples collected from dairy goats.

Three Wisconsin goat herds were selected for participation in this study. A confirmed history of paratuberculosis existed for 2 herds (A and B) that were just beginning to implement an *M. paratuberculosis* infection control program. The spectrum of disease in these herds was broad, that is, the herds contained goats in uninfected, subclinical, and clinical phases of infection. The apparent prevalence by fecal culture was 17.7% and 5.5% in herds A and B, respectively. The third population was a closed milking herd (C) considered free of the infection on the basis of lack of clinical cases and 2 years of negative whole-herd paratuberculosis serology and fecal culture results.

Fecal, blood, and milk samples were collected from all female goats >1 year old during October and November 2003 (18 goats from herd A, 141 goats from herd B, and 50 goats from herd C). Feces was col-

lected per rectum and transferred to plastic bags. Blood samples (5 ml) were obtained from the jugular vein using Vacutainers<sup>®</sup>,<sup>a</sup> and individual milk samples by manual milking into sterile 50-ml plastic tubes without preservative. All samples were immediately transported to the laboratory with a refrigerant. After clotting and centrifugation, serum was harvested from blood samples. Both sera and milk samples were frozen at -20 C until tested. Fecal samples were processed without freezing. Fecal samples were cultured using the modified BACTEC 12B medium as previously described.<sup>5</sup> All acid-fast bacteria recovered were tested using a multiplex polymerase chain reaction (PCR) for IS900, IS901, IS1311, IS1245, and the 16s ribosomal DNA (rDNA) specific for mycobacteria (Uppal M. et al. 2002. Ann. Meet. Am. Soc. Microbiol. abstract # Z-58, p. 518). Only bacteria testing positive for both mycobacterial 16s rDNA and IS900 were considered to be *M. paratuberculosis*.

Serum and milk samples both were tested in duplicate wells using a commercial *M. paratuberculosis* antibody test kit.<sup>b</sup> This kit is not licensed for use in goats but uses a protein-G conjugate capable of reacting with antibody from a wide range of animal species.<sup>9</sup> Sera were tested according to the manufacturer's instructions for bovine serum samples. Milk samples were centrifuged, and a portion of the skim milk fraction was pipetted from below the cream layer. This milk fraction was then treated like a serum sample with 1 protocol exception: the milk was mixed with ELISA kit diluent at a ratio of 1:2 instead of 1:20. The absorbance reading in all ELISA plate wells was measured at 620 nm by an ELISA reader.<sup>c</sup> ELISA optical density readings were transformed to S/P values as per manufacturer's directions. All assays were run in duplicate and any assay with a between-well coefficient of variation of 10% was repeated; the second result was used for data analysis.

The case definition for a *M. paratuberculosis*-infected goat was isolation of *M. paratuberculosis* from a fecal sample. The case definition for a noninfected goat was any goat tested from the paratuberculosis-free herd (found again to be 100% fecal culture-negative during this study as had been the case in previous years' testing).

Sensitivity was defined as the percentage of fecal culture-positive goats testing ELISA positive. Specificity was defined as the percentage of goats in the paratuberculosis-free herd testing ELISA-negative. Agreement between categorical (pos/neg) serum and milk ELISA results and fecal culture results was evaluated using McNemar's chi-square test and the kappa statistic. The relationship between serum and milk ELISA S/P values was evaluated by linear regression analysis. Statistical analyses were done using Instat<sup>®</sup>.<sup>d</sup>

**Table 1.** ELISA results on serum and milk samples from goats of 3 infection statuses: *Mycobacterium paratuberculosis* infected (fecal culture-positive), noninfected, and status uncertain (fecal culture negative but resident in infected herd).

	Infected	Noninfected	Status uncertain	Total
ELISA positive* on serum	16 (64%)	0 (0%)	22 (16.4%)	38
ELISA positive on milk	12 (48%)	0 (0%)	5 (3.7%)	17
Total tested	25	50	134	209

\* Positive defined as S/P  $\geq 0.25$

Among the 159 goats in the 2 *M. paratuberculosis*-infected herds, 25 (15.7%) were fecal culture-positive. None of the 50 goats tested in the paratuberculosis-free herd had positive fecal cultures (Table 1). Because of the small sample size for non-*M. paratuberculosis*-infected goats and because all S/P values from goats in the paratuberculosis-free herd for both serum and milk samples were 0.05, ROC analysis was not possible, i.e., the assay was 100% specific at every cutoff above 0.05. Therefore, the manufacturer's recommended cutoff for interpretation of serum ELISA results for cattle, S/P 0.25, was used for further data analysis.

The sensitivity of the ELISA on goat serum was 64% (95% CI; 54–74%) and the sensitivity of the ELISA on goat milk was 48% (95% CI; 38–58%). ELISA specificity was 100% when applied to serum and milk samples at the 0.25 S/P cutoff. In comparison with fecal culture, the serum ELISA had lower agreement of 85.2% ( $\chi^2 = 5.25$ ,  $P < 0.05$ ; kappa = 0.425) than did the milk ELISA (91.4% agreement,  $\chi^2 = 3.56$ ,  $P > 0.05$ , kappa = 0.525). The correlation between serum and milk S/P values by linear regression analysis was fair (Fig. 1,  $r^2 = 0.67$ ).

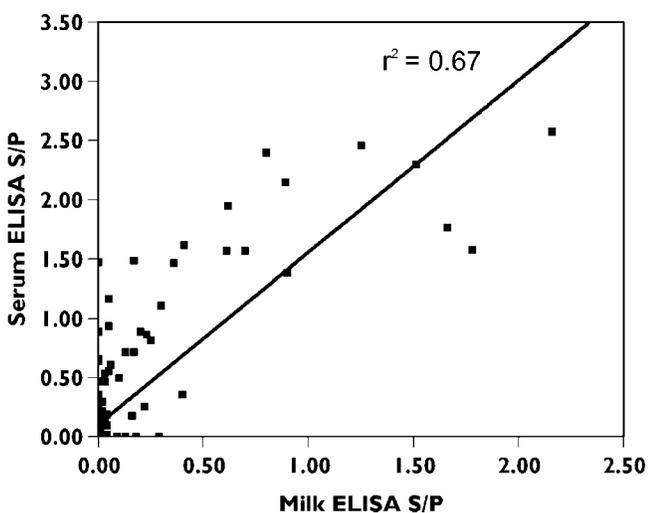
Published reports on natural *M. paratuberculosis* infection in goats are few.<sup>3,11,13</sup> Little is known about the prevalence and economic impact of this disease on the

goat industry in the United States or elsewhere. However, on the basis of discussions with senior officials of the American Dairy Goat Association and on the Johne's Testing Center's caprine diagnostic testing history, it is apparent that paratuberculosis is not uncommon and that its effects on production can be economically significant. More precise determination of caprine paratuberculosis prevalence at the animal and herd levels and an estimation of the economic impact of paratuberculosis on goat producers require use of affordable diagnostic tests of known sensitivity and specificity.

Isolation of *M. paratuberculosis* from fecal samples has been the primary method used to diagnose paratuberculosis in goats.<sup>21</sup> High cost and lack of speed are 2 disadvantages of culture-based diagnostic methods. Molecular diagnostics overcome the second but not the first of these disadvantages. Serologic tests are rapid, low cost, and able to detect the *M. paratuberculosis*-infected animals in the latter stages of infection that are most infectious (excreting highest number of the pathogen) and less productive.<sup>6,24</sup>

The commercial ELISA used for this study uses a protein-G conjugate capable of binding antibodies from a broad range of mammals including goats.<sup>9,11</sup> Using the same protocol and cutoff as for bovine sera, the ELISA on goat sera (tested at a 1:20 dilution) had a sensitivity and specificity (relative to fecal culture) of 64% and 100%, respectively. This is comparable with the findings of a comparable study that reported a sensitivity (relative to fecal IS900 PCR on both clinically normal goats and goats with signs of Johne's disease) of 54% (95% CI; 36–71%) and specificity of 100% for the same commercial absorbed ELISA and a serum dilution of 1:249.<sup>3</sup> Other studies evaluating ELISAs for paratuberculosis in goats using various ELISA techniques and differing reference tests reported sensitivity and specificity values of 88% and 94%,<sup>12</sup> 77.3% and 96.8%,<sup>18</sup> and 27% and 84% using a ELISA based on the lipoarabinomannan antigen.<sup>13</sup> Collectively, these reports indicate that detection of antibody to *M. paratuberculosis* in goat serum has potential utility for *M. paratuberculosis* infection diagnosis.

The goat milk sample ELISA results agreed with those obtained by fecal culture (kappa = 0.525) and correlated with those of the ELISA on serum (Fig. 1,



**Figure 1.** Regression analysis of ELISA S/P values on serum and milk ( $n = 209$  pairs of results).

$r^2 = 0.67$ ). Specifically, 48% of fecal culture–positive goats were milk ELISA positive and 76% of goats with positive ELISA results on serum also were ELISA positive on milk samples. This is in agreement with reports on *M. paratuberculosis* antibody detection in bovine milk and serum.<sup>6,23</sup> Antibodies to *M. paratuberculosis* in milk likely represent passive spillover of immunoglobulin-G (IgG)<sub>1</sub> and IgG<sub>2</sub> from the serum immunoglobulin pool rather than active secretion specifically into milk.<sup>2</sup> Consequently, it is to be expected that antibody concentrations in milk will generally be lower than in serum, accounting at least in part for a lower ELISA sensitivity on milk samples. Stage of lactation also affects ELISA sensitivity with milk samples as was shown for cattle.<sup>14</sup> The effect of lactation stage was not evaluated in this study. Because of the lower sensitivity, ELISA on serum would be preferred over the ELISA on milk until the cost differential is sufficiently large for the milk ELISA to provide a better benefit-to-cost ratio.

Reports on the efficacy of antibody detection for diagnosis of bovine paratuberculosis indicate that serum antibody level is directly related to the likelihood of the animal being a heavy fecal shedder of *M. paratuberculosis* as detected by fecal culture.<sup>6</sup> Although diagnostic sensitivity is less for antibody detection–based assays done on individual milk samples as compared with serum, these so-called milk ELISAs are effective at detecting heavy fecal shedders. Goat producers wanting to initiate a paratuberculosis control program would benefit from the availability of this low-cost testing technology as 1 option for herd surveillance and infection management. Low-cost tests are particularly important to agricultural businesses with low profit margins, such as the dairy goat industry.

Paratuberculosis control programs require careful attention to herd management. Diagnostic testing can be used to supplement control programs and to document progress in *M. paratuberculosis* infection reduction with time. Economic factors often bar the use of diagnostic tests. A milk ELISA for individual goat milk samples appears to offer a useful, potentially low-cost alternative for detection of goats with paratuberculosis that have progressed to the stage of shedding *M. paratuberculosis* in their feces. A milk ELISA could encourage producers to begin a paratuberculosis control program by easily and inexpensively identifying the most infectious goats in a herd. As herd prevalence decreases, more sensitive tests would be recommended. Availability of low-cost tests with easy sample collection can promote more investigations of possible paratuberculosis problems in goat herds. Antibody detection–based tests will have greater positive predictive value in herds that have been confirmed to be infected by isolation of *M. paratuberculosis* from an-

imals that are true members of the herd or flock (i.e., not recently purchased). There have been reports that infections by other members of the mycolata, a super-generic taxon that includes *Rhodococcus*, *Corynebacterium*, *Mycobacterium*, and *Nocardia*, may cause false-positive serologic tests for paratuberculosis in small ruminants.<sup>17,22</sup> Thus, thorough knowledge of the herd's health status and careful interpretation is needed when using antibody detection assays in herds of unknown *M. paratuberculosis* infection status.

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### Sources and manufacturers

- a. BD Diagnostics, Loveton, MD.
- b. IDEXX Laboratories Inc., Westbrook, ME.
- c. BioTek Quant Microplate Universal Spectrophotometer, BioTek Instruments Inc., Winooski, VT.
- d. InStat® version 3.00 for Windows, GraphPad Software, San Diego, CA.

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## Comparison of blood polymerase chain reaction and enzyme-linked immunosorbent assay for detection of *Mycobacterium avium* subsp. *paratuberculosis* infection in cattle and sheep

Ramon A. Juste,<sup>1</sup> Joseba M. Garrido, Marivi Geijo, Natalia Elguezabal, Gorka Aduriz, Raquel Atxaerandio, Iker Sevilla

**Abstract.** A study was carried out to compare the performance of enzyme-linked immunosorbent assay (ELISA) and blood polymerase chain reaction (PCR) for diagnosis of paratuberculosis in cattle and sheep. For cattle, a set of 278 samples from 1 paratuberculosis-affected Friesian farm was used; it included 80 ELISA-positive samples and 198 ELISA-negative samples from an age-matched group. Ninety-four samples were from heifers and 184 were from 2–5-year-old cows. The overall analysis showed a clear association (Fisher exact test [FET]  $P = 0.0049$ ) but a weak negative agreement (45.3%, kappa =  $-0.1665 \pm 0.0994$ ) between the 2 tests. It reflected a moderate agreement among heifers (87.7%, kappa =  $0.4471 \pm 0.2435$ ) and a moderate disagreement among cows (62.7%, kappa =  $-0.3670 \pm 0.1057$ ). For sheep, 496 blood samples from 53 Latxa dairy flocks were used; 180 of the blood samples were from dam/offspring pairs. The overall association between the 2 tests on ovine samples was strong (FET,  $P = 0.0005$ ), whereas the agreement was low (kappa =  $0.1622 \pm 0.1188$ ). There was slightly better agreement for ewes (kappa =  $0.2135 \pm 0.1992$ ) than for lambs (kappa =  $0.1193 \pm 0.1301$ ). There was also a highly unlikely proportion of dam/offspring positive results (FET,  $P < 0.0001$ , kappa =  $0.6269 \pm 0.1854$ ). Four of 6 lambs that were necropsied 1 year after testing had paratuberculosis microscopic lesions in the ileocecal valve (3 lambs) or a PCR-positive result (4 lambs). These results suggest that blood PCR testing might be a potentially useful new approach in paratuberculosis diagnosis, especially in young animals.

**Key words:** Cattle; ELISA; *Mycobacterium avium* subsp. *paratuberculosis*; PCR; sheep.

It is widely accepted that the most reliable method for diagnosis of paratuberculosis in live animals is fe-

cal culture.<sup>3</sup> However, this method has the drawbacks that it takes several weeks to yield a result and there is no good interlaboratory standardization (RH Whitlock, personal communication). Although the use of new growth detection methods can significantly reduce this time,<sup>16</sup> fecal culture is still an expensive and labor-intensive technique. The alternative use of serological

From the Instituto Vasco de Investigación y Desarrollo Agrario (NEIKER), Berreaga, 1, Derio 48160, Bizkaia, Spain.

<sup>1</sup>Corresponding Author: Ramon A. Juste, Instituto Vasco de Investigación y Desarrollo Agrario (NEIKER), Berreaga, 1, Derio 48160, Bizkaia, Spain. rjuste@neiker.net