Evaluation of Four Serological Tests for Bovine Paratuberculosis

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The standard complement fixation (CF) test, a commercial agarose gel immunodiffusion (AGID) test (ImmuCell Corporation, Portland, Maine), and two commercial enzyme-linked immunoabsorbent assays (ELISAs; Allied Laboratories, Glenwood Springs, Colo. [Allied ELISA] and the CSL, Limited, [Parkville, Victoria, Australia] enzyme immunoassay [CSL ELISA]) were evaluated by using sera from the Repository for Paratuberculosis Specimens. The case definition of subclinical bovine paratuberculosis was isolation of Mycobacterium paratuberculosis from fecal samples or internal organs of dairy cattle without diarrhea or weight loss. Animals designated as free of the disease originated exclusively from certified paratuberculosis-free herds in Wisconsin. None of the cattle evaluated had been vaccinated for paratuberculosis. Among 177 M. paratuberculosis-infected cattle, the CF test, the AGID test, the Allied ELISA, and the CSL ELISA had test sensitivities of 38.4, 26.6, 58.8, and 43.4%, respectively, and specificities of 99.0, 100.0, 95.4, and 99.0%, respectively. Only 108 of the infected cattle were confirmed by culture or by a commercial DNA probe (IDEXX Corporation, Portland, Maine) to be shedding the organism in fecal samples at the time of serological testing. Among the 108 M. paratuberculosis fecal shedders, the CF test, the AGID test, the Allied ELISA, and the CSL ELISA were positive for 54.6, 40.7, 65.7, and 56.5% of the cows, respectively; and among the 69 cows that were nonshedders, the tests were positive for 14.5, 4.3, 47.8, and 24.6% of the cows, respectively. There was a significant difference (P < 0.05) in the rate of positive test results for all four tests between these two groups of animals. The CF test performed well when it was compared with the other serological tests if a titer of ≥1:8 was classified as a positive test result.

Clinical bovine paratuberculosis (John's disease) is characterized by chronic diarrhea and weight loss that is not responsive to treatment (3). However, once paratuberculosis has been confirmed in a herd, the diagnosis of clinical disease, while important, is not the key to controlling or eradicating the disease (3). Changes in herd management to decrease the rate of infection transmission are used with diagnostic tests to identify subclinically infected animals for removal from the herd (6). Several tests have been developed for the diagnosis of Mycobacterium paratuberculosis infections in cattle. Some detect the organism directly in fecal or tissue samples (5, 13, 42, 44), and others detect serum antibodies, with the most common tests being the complement fixation (CF) test (2, 10, 15, 20, 21, 32), the agarose gel immunodiffusion (AGID) test (31–33), and the enzyme-linked immunosorbent assay (ELISA) (8, 19, 39, 47–49). Reports of diagnostic test accuracy differ among publications, making comparison of test performance difficult. These discrepancies are likely due to differences in case definition or the use of imperfect "gold standards" to define the infection status of animals. In this report, we describe the evaluation of four serological tests for paratuberculosis by using samples obtained from the Repository for Paratuberculosis Specimens (35). The tests evaluated include the standard CF test used in the United States (20), a commercial AGID test (ImmuCell Corporation, Portland, Maine), and two commercial ELISAs (Allied Laboratories, Glenwood Springs, Colo. [Allied ELISA] and the CSL, Limited, [Parkville, Victoria, Australia] enzyme immunoassay [CSL ELISA]).

MATERIALS AND METHODS

Specimens. The specimen collection used for this study is described in detail elsewhere (34, 35). Briefly, serum and fecal specimens were collected concurrently from 828 dairy cattle in 13 Wisconsin herds, catalogued, cultured (fecal samples only), and stored at −70°C. None of the cattle tested had been vaccinated for paratuberculosis. Nine of the herds, containing a total of 632 dairy cattle, were infected with M. paratuberculosis and had infection prevalences that ranged from 7 to over 60%. Four of the herds, containing a total of 196 cattle, were certified to be free of paratuberculosis by the state of Wisconsin Department of Agriculture, Trade and Consumer Protection on the basis of a regular program of annual whole-herd testing for paratuberculosis by the conventional fecal culture method (6, 43). These specimens formed part of the Repository for Paratuberculosis Specimens housed at the University of Wisconsin-Madison (35).

Fecal samples were collected from the rectum of each cow with a disposable plastic obstetrical glove, and approximately 50 g of the fecal sample was placed in a plastic bag (Whirl-pack; Nasco Laboratories, Fort Atkinson, Wis.). Fecal samples were then placed in a styrofoam cooler, chilled with ice packs, taken to the laboratory, and processed for the isolation of M. paratuberculosis by conventional and radiometric methods, after which they were frozen at −70°C. Later, these samples were tested for M. paratuberculosis by using a commercial DNA probe (42). Blood samples were collected simultaneously with fecal samples via the middle coccygeal vein by using a 20-gauge, 1-in. (2.54-cm) needle and a 10-ml Vacutainer tube (Corning Glass Works, Corning, N.Y.). After clotting and within 12 h, the sample was centrifuged and serum was harvested and stored at −70°C in a sterile cryogenic vial (Tekmar Co., Cincinnati, Ohio).

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M. paratuberculosis detection methods. Conventional fecal culture on Herrold’s egg yolk agar (HEY) was performed by the Wisconsin Central Animal Health Laboratory. In Wisconsin at the time of the study, the conventional method used 0.25% hexadecylpyridinium chloride as the decontaminant and two tubes of HEY with mycobactin and one tube without mycobactin. Cultures on HEY were incubated and observed for 3 months. In addition, fecal samples from the nine infected herds were cultured four more times by conventional methods at the Wisconsin Central Animal Health Laboratory during the next 2 years. Isolation of a slow-growing, acid-fast organism with a colony morphology typical of that of M. paratuberculosis on HEY with mycobactin but not on HEY without mycobactin was considered a positive culture.

For radiometric fecal culture, we used a recently described adaptation of the BACTEC system (5). Briefly, it uses BACTEC 12B medium supplemented with 0.1 ml of a 40-μg/ml mycobactin J solution (Allied Laboratories); 1.0 ml of egg yolk suspension (Difco, Detroit, Mich.); and 0.1 ml of an antibiotic cocktail containing vancomycin, amphotericin B, and nalidixic acid (final concentrations of the antibiotics in the radiometric broth, 10, 20, and 30 μg/ml, respectively). Specimen samples or tissue homogenates were decontaminated in 1.0% hexadecylpyridinium chloride at a ratio of 3 g/30 ml. After 24 h, when large fecal or tissue debris had settled to the bottom of the tube, the top 10 ml was filter concentrated by using a 10-ml syringe fitted with a 3-μm pore-size filter in a Swinex filter holder (Millipore Corp., Bedford, Mass.). The entire filter was then placed into the vial containing the radiometric culture medium, and the vial was resealed. Vials of radiometric culture medium were read weekly on a BACTEC 460 ionization detector (Beckton Dickinson Microbiologic Systems, Towson, Md.). All positive vials (growth index, >30) were subcultured on plate media, and the identity of M. paratuberculosis was confirmed by mycobactin dependency.

The DNA probe assays were performed with the commercial kits (U.S. Veterinary License no. 313) according to the directions of the manufacturer (IDEXX Corp.). With this probe kit, the polymerase chain reaction is used to improve the specificity of the test (42). Oligonucleotide primers derived from the insertion element IS900 are used to amplify a 1.0-kb fragment that is detected by a hybridization probe internal to the polymerase chain reaction primers. Fecal samples were frozen prior to probe analyses but had undergone a maximum of two previous thawings. A 1.0-g sample was used for each test. Positive and negative control fecal samples and positive and negative (amplified product) control samples were run with each assay. Previous studies have shown this test to be 100% specific for detecting M. paratuberculosis from bovine fecal samples (34, 42).

Serologic tests evaluated. The four diagnostic tests evaluated were the standard CF test that is used in the United States (20), a commercial AGID test (ImmuCell), and two commercial ELISAs, the Allied Laboratories ELISA (Allied ELISA) and the John’s Absorbed Enzyme Immunooassay (CSL, Limited [CSL ELISA]). The CF test was performed by the staff of the Wisconsin Central Animal Health Laboratory by using the standard procedure with antigen and positive control sera obtained from the National Veterinary Services Laboratory (20). The CF test antigen is derived from heat-killed Mycobacterium tuberculosis cells and dried bovine intestinal mucosa (41). The AGID test was performed by one of the authors (D.C.S.) according to the directions of the manufacturer, with the interpretation of test results provided in the instructions packaged with the kit. This test uses M. paratuberculosis 18 protoplastic antigen, as does the Allied ELISA (33, 48, 49). The CSL ELISA uses M. paratuberculosis VRI 316/102-2 protoplastic antigen (9, 19). Both ELISAs preabsorb the test sera with Mycobacterium phlei to reduce nonspecific reactions (9, 19, 49). The Allied ELISA was performed blindly in duplicate wells by Allied Laboratories. Samples were considered positive if the mean optical density of the test serum was more than twice the optical density of the negative control serum. The CSL ELISA was performed with commercial kits according to the directions of the manufacturer (8). Negative and high-positive control sera were included in the kit, and each has a specified range of optical density values (450-nm filter) for an assay to be valid. The cutoff value for valid assay was the mean of duplicate negative controls plus 0.100. Sera with discrepant results between wells or with borderline values were retested. Results were reported as (optical density of test serum − cutoff value) × 100. All values greater than zero were considered positive. Results of evaluation of the CSL ELISA have been reported in part previously (8).

Resolution of discrepant results. The infection status of animals that were serologically positive but fecal culture or probe negative were resolved by surgical biopsy of the ileum and regional lymph nodes or collection of these tissues at a local abattoir. The biopsy procedure collected a full-thickness (1- by 2-cm) piece of terminal ileum taken 10 to 20 cm proximal to the ileocecal junction and an ileocecal lymph node through a right-flank laparotomy incision. Histopathology with both hematoxylin-eosin and acid-fast stains was done on half of each tissue sample, and the remainder of the tissue sample was homogenized and cultured for M. paratuberculosis by the radiometric technique (5). Tissues collected at slaughter were of the same size and from the same anatomical locations and were processed and cultured in the same way that the surgically obtained tissues were.

Gold standard for test specificity analysis. Isolation of M. paratuberculosis from fecal samples is generally considered definitive for the diagnosis of paratuberculosis; therefore, by definition, the culture-based procedures were considered 100% specific. For serological test specificity analysis, only animals from herds certified to be free of paratuberculosis were used as the negative control population. Herds in which the animals were certified to be free of paratuberculosis were those in which all animals of ≥20 months of age have been tested and found to be negative for paratuberculosis by the conventional fecal culture method performed on at least three consecutive samples taken not less than 10 months but not more than 14 months apart (6). Herd certification is renewed annually, and all four herds examined in this study passed at least one annual recertification. One hundred ninety-six animals from the paratuberculosis-free herds were thus used for serologic test specificity analysis.

Gold standard for test sensitivity analysis. The diagnostic difficulty in paratuberculosis is detection of M. paratuberculosis infections in clinically normal animals. Most animals become infected soon after birth, and the disease progresses through three distinct stages. Stage one animals are infected but do not shed the organism in their feces, stage two animals shed the organism in their feces but are clinically normal, and stage three animals shed the organism in their feces and have clinical disease. In this report, only stage one and two animals (subclinical infections) were evaluated. The case definition for subclinical paratuberculosis was isolation of the causative agent, M. paratuberculosis, from any fecal or tissue sample collected. Thus, by using this microbiolog-
ical case definition, all M. paratuberculosis-infected but clinically normal animals were used for diagnostic test sensitivity analysis. Of the 632 animals in the nine M. paratuberculosis-infected herds that were tested, 177 were confirmed to be infected. Herd prevalence for M. paratuberculosis infection ranged from as low as 7% in herd A to greater than 60% in herd I (Table 1).

Statistical methods. Test sensitivity and specificity estimates with 95% confidence limits were calculated by standard methods (29). Diagnostic test sensitivity determinations for the four tests were compared by using McNemar’s test (23). In addition, animals that shed the organism (stage two animals) were placed in one group, and animals that did not shed the organism (stage one animals) were placed in another group to determine whether the stage of disease affected test performance. Test results were evaluated by binomial distribution (24).

RESULTS

Test specificity. When applied to the 196 paratuberculosis-free animals, the Allied ELISA, the CSL ELISA, the AGID test, and the CF test (for the CF test, a titer of \( \geq 1:8 \) was considered positive) had test specificities of 95.4% \( \pm 2.9% \), 99.0% \( \pm 1.4% \), 100.0%, and 99.0% \( \pm 1.4% \), respectively (\( \pm \) values are 95% confidence intervals). For CF test titers of \( \geq 1:16 \) and \( \geq 1:32 \), the specificities were both 100.0%. There was no difference in specificity for the CF test, the AGID test, and the CSL ELISA; but these three tests were more specific (\( \chi^2 = 3.27; P < 0.07 \)) than the Allied ELISA was. Test specificity estimations were almost identical for the four herds examined that were certified to be free of paratuberculosis.

Test sensitivity. Test results for each of the nine M. paratuberculosis-infected herds are summarized in Table 1. When applied to all 177 M. paratuberculosis-infected animals, the Allied ELISA, the CSL ELISA, the AGID test, and the CF test (a titer of \( \geq 1:8 \) was considered positive) had test sensitivities of 58.8% \( \pm 7.3% \), 43.4% \( \pm 7.3% \), 26.6% \( \pm 6.5% \), and 38.4% \( \pm 7.2% \), respectively (Table 2). The Allied ELISA was significantly more sensitive (\( P < 0.001 \)) than the other three serological tests evaluated. The CSL ELISA and the CF test had similar sensitivities but were significantly more sensitive (\( P < 0.001 \)) than the commercial AGID test (Table 3).

Stage of disease effect. One hundred eight cows were found to shed the organism in their feces at the time that the serum sample was collected. Sixty-nine cows were culture or DNA probe negative at the time of serum collection and were thus classified as nonshedders. Of these, 34 cows were confirmed to be M. paratuberculosis infected by radiometric culture of surgical biopsy specimens or internal organs collected at slaughter, and 35 were later diagnosed by conventional fecal culture (35).

When animals were grouped according to their shedding status, a significantly lower test sensitivity was found for all four tests in the nonshedder group (Allied ELISA, \( z = 2.37, P < 0.05 \); CSL ELISA, \( z = 4.52, P < 0.0001 \); AGID test, \( z = 6.8, P < 0.0001 \); CF, \( z = 6.8, P < 0.0001 \)). Sensitivity estimates for the four tests in the two groups of animals are listed in Table 2. The Allied ELISA was more sensitive (\( P < 0.04 \)) than the other three tests for both groups (shedders and nonshedders) of animals (Table 3). The CSL ELISA was more sensitive than the AGID test (\( P < 0.001 \)) and was similar in performance to the CF test for both shedders and nonshedders. The CF test was more sensitive than the AGID test only in the shedding group of animals (\( P < 0.003 \)) (Table 3).

DISCUSSION

All four diagnostic tests evaluated were relatively specific, with the AGID test, the CF test, and the CSL ELISA being more specific (\( \geq 99.0% \)) than the Allied ELISA (95.4%). The diagnostic specificities found in this study were comparable to those found in other recent studies (9, 19, 26, 31, 32, 47).

| Test | Sensitivity (%) for:
<table>
<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td>Nonshedders (n = 69)</td>
</tr>
<tr>
<td>Allied ELISA</td>
<td>47.8 ± 11.9</td>
</tr>
<tr>
<td>CSL ELISA</td>
<td>24.6 ± 10.2</td>
</tr>
<tr>
<td>AGID</td>
<td>4.3 ± 4.8</td>
</tr>
<tr>
<td>CF (( \geq 1:8 ))</td>
<td>14.5 ± 8.4</td>
</tr>
<tr>
<td>CF (( \geq 1:16 ))</td>
<td>5.8 ± 5.6</td>
</tr>
<tr>
<td>CF (( \geq 1:32 ))</td>
<td>1.4 ± 2.8</td>
</tr>
</tbody>
</table>

* The \( \pm \) values represent 95% confidence intervals. n, number of animals tested.
TABLE 3. Comparison of test sensitivities by using McNemar's test

<table>
<thead>
<tr>
<th>Tests</th>
<th>Nonsheddors (n = 699)</th>
<th>Shedders (n = 108)</th>
<th>All infected animals (n = 177)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\chi^2$</td>
<td>P</td>
<td>$\chi^2$</td>
</tr>
<tr>
<td>Allied ELISA versus CSL</td>
<td>12.5</td>
<td>&lt;0.001</td>
<td>4.5</td>
</tr>
<tr>
<td>Allied ELISA versus AGID</td>
<td>28.0</td>
<td>&lt;0.001</td>
<td>19.3</td>
</tr>
<tr>
<td>Allied ELISA versus CF*</td>
<td>21.0</td>
<td>&lt;0.001</td>
<td>4.7</td>
</tr>
<tr>
<td>CSL ELISA versus AGID</td>
<td>12.1</td>
<td>&lt;0.001</td>
<td>11.1</td>
</tr>
<tr>
<td>CSL ELISA versus CF*</td>
<td>2.1</td>
<td>0.150</td>
<td>0.5</td>
</tr>
<tr>
<td>AGID versus CF*</td>
<td>0.4</td>
<td>0.546</td>
<td>8.5</td>
</tr>
</tbody>
</table>

* n, number of animals tested.
** $\chi^2$, chi-square distribution (1 degree of freedom).
*** For the CF test, a titer of ≥1:8 was considered positive.

The high ELISA specificity was surprising, considering the low antibody detection limit of ELISA technology, the extensive cross-reactivity among mycobacteria (37), and the high prevalence of antibodies to mycobacteria in cattle (1). The diagnostic specificities of the two ELISAs studied were apparently achieved by the absorption of antibodies common to mycobacteria by using M. phlei, as demonstrated by Western blot (immunoblot) analysis by Milner et al. (18). The AGID and CF tests do not use heterologous antigens to absorb nonspecific antibodies, however, and the specificities of these tests were probably due to their high antibody detection limits, e.g., their low sensitivities.

When applied to the total population of subclinically M. paratuberculosis-infected cows, the Allied ELISA was the most sensitive test; this was followed by the CSL ELISA and the CF test (a titer of ≥1:8 was considered positive), which had equivalent test accuracies. The commercial AGID test was the least sensitive of the four serological tests evaluated. The reason for the lower test sensitivity of the CSL ELISA compared with that of the Allied ELISA was possibly due to differences in how optical density data were interpreted, i.e., the cutoff for classification of test results as positive or the degree of absorption of test sera with M. phlei before running the assay. Raising the cutoff for the Allied ELISA from ≥2.1 to ≥2.6 resulted in sensitivity and specificity estimates that were almost identical to those of the CSL ELISA. The two ELISAs were more sensitive than the AGID test mainly because ELISA techniques are able to detect lower concentrations of antibody than gel diffusion assays are (40).

When evaluating diagnostic accuracy, it is important to include patients with the entire spectrum of disease or infection severity as it is normally found in the population being tested (see Ranshoff and Feinstein [22] for a comprehensive discussion of spectrum). For subclinically bovine paratuberculosis, this should include unequivocally disease-free populations of animals and M. paratuberculosis-infected animals, both those that shed the organism in their feces and nonshedders. The distribution of animal specimens found in the Repository for Paratuberculosis Specimens met this criterion and represented the proportions normally found in infected herds prior to implementation of paratuberculosis control procedures (34, 35). Inclusion of only fecal culture-positive animals in test sensitivity estimation and use of fecal culture-negative animals that reside in M. paratuberculosis-infected herds for specificity estimation does not give a proper determination of diagnostic test accuracy, particularly for those tests that are not based on detection of the organism (35). This is most important for specificity analysis, since one cannot be certain that a fecal culture-negative animal is disease-free if it resides in an infected herd because of the long prepatent period of the disease. To illustrate this point, if sensitivity analysis was done by using only conventional HEY fecal culture-positive cows (n = 126) and specificity analysis was done on HEY fecal culture-negative animals, even after a minimum of three HEY fecal cultures taken over a 2-year period (n = 505), the sensitivity and specificity of the Allied ELISA would have been estimated at 60% (76 of 126) and 75% (378 of 505), respectively, while, in fact, the Allied ELISA had a sensitivity and specificity of 58.8 and 95.4%, respectively (data not shown). This bias in paratuberculosis diagnostic test evaluations has occurred repeatedly, giving a false impression that serological tests lack specificity (2-4, 11, 12, 14-17, 21, 25, 27, 32, 36, 38, 46). This has been particularly true for the CF test, the oldest serological test for paratuberculosis. In this report, the CF test (a titer of ≥1:8 was considered positive) performed well when it was compared with other serological tests. Ridge et al. (26) reported similar findings with the CF test used in Australia.

Most investigators believe that cattle primarily become infected with M. paratuberculosis by ingesting the organism as neonates, although in utero transmission of the bacterium has been shown to occur (39). Animals that actively shed the organism in their feces (stages two and three of disease) are most infectious. Therefore, it is desirable to know the percentage of fecal shedders that are also serologically positive. In this report, the AGID test detected the lowest number of animals that shed M. paratuberculosis in their feces (40.7%), and the Allied ELISA detected the largest percentage (65.7%). The CSL ELISA and the CF test were similar in their fecal shedder detection abilities (Table 2). We hasten to add that this shedder detection rate is not synonymous with test sensitivity, as the term is normally defined (22, 29).

No matter which serological test was used, many animals that shed M. paratuberculosis in their feces, and that were thus infectious, would not have been detected. This observation suggests that serological tests alone, particularly in herds with a high prevalence of paratuberculosis, may not be sufficient to control the disease by using a test-and-cull control program. Serological tests may be most useful as rapid, inexpensive screening tests to measure the prevalence of paratuberculosis in herds or to monitor herds once the disease has been eradicated or brought under control. This experience has already been reported for the CF test (15, 45). Once the prevalence of paratuberculosis has been estimated in herds with moderate to high prevalences of the disease, an organism detection-based test such as fecal culture or a gene probe should be used to identify M.
paratuberculosis fecal shedders in the cattle missed by serological testing. For commercial dairy herds, the cost of diagnostics plays a major role in the selection of tests for test-and-cull paratuberculosis control programs (7).

There were significantly lower sensitivities (P < 0.05) for all four tests when they were evaluated on stage one animals (nonshedders) compared with the sensitivities of the four tests when they were evaluated on stage two animals (fecal shedders) (Table 2). This was not surprising, since animals that shed the organism are thought to have more advanced disease and, thus, are more likely to be serologically positive (3, 10, 28); this observation was previously reported for the AGID and CF tests for animals with both clinical and subclinical forms of the disease (10, 31–33). This also has important implications for paratuberculosis diagnosis and control. As paratuberculosis becomes more severe in dairy herds, a higher proportion of adult infected animals also become fecal shedders (3, 34, 35). Thus, as the M. paratuberculosis infection prevalence increases in a herd, the sensitivity of the serological tests will probably increase, and conversely, as the prevalence in a herd declines, the sensitivity of a test will probably drop. This finding has already been reported for radiometric and conventional fecal culture and a commercial DNA probe (34). It is important to remember that diagnostic tests for paratuberculosis are used to select animals for culling from herds and that this is an integral part of any control program. If the sensitivity of diagnostic tests decreases as the severity of the disease in the herd decreases, it might be very difficult to eradicate paratuberculosis from infected herds by only test-and-cull methods. This is consistent with the experience of many veterinarians and livestock producers involved in paratuberculosis control or eradication programs (3).

The results presented in this report illustrate that when diagnostic test sensitivity estimations are reported for paratuberculosis, the severity of the disease in the herds and individual animals tested must be thoroughly described so comparison with previous studies can be made. Standards for the definition of M. paratuberculosis infection and the paratuberculosis-free status of animals or herds used for test sensitivity and specificity estimations should be rigorous. No animal resident in an M. paratuberculosis-infected herd should be assumed to be infection-free for diagnostic test specificity estimation purposes.

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