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C. H. J. Kalis, H. W. Barkema, J. W. Hesselink, C. van Maanen and M. T. Collins

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## Evaluation of two absorbed enzyme-linked immunosorbent assays and a complement fixation test as replacements for fecal culture in the detection of cows shedding *Mycobacterium avium* subspecies *paratuberculosis*

C. H. J. Kalis, H. W. Barkema, J. W. Hesselink, C. van Maanen, M. T. Collins

**Abstract.** Control of paratuberculosis in dairy herds is based on preventing the transmission of *Mycobacterium avium* subsp. *paratuberculosis* (*Mptb*) from cows to calves by management measures, supported by removal of cows excreting these bacteria by the fecal route (*Mptb* shedders). Fecal culture is the most accurate test for identifying *Mptb* shedders, but this technique is expensive and takes up to 16 weeks for results to be available. Serologic tests are inexpensive, rapid, and easy to perform. Of serologic tests, the complement fixation test (CFT) and absorbed enzyme-linked immunosorbent assay (ELISA) are the serologic tests used most frequently; the CFT is considered less accurate than the ELISA with respect to sensitivity and specificity. The commonly accepted absorbed ELISA is from the Australian Central Serum Laboratory. However, a European supplier has marketed a second ELISA that is supposed to be more sensitive in detecting *Mptb* shedders. These 2 absorbed ELISAs, designated ELISA-A and ELISA-B, and an in-house CFT were compared with data from 2 serum panels. The *Mptb* shedding panel consisted of sera from 198 culture-positive cows from 53 infected herds. The method used for culture of fecal samples was a modified Jørgensen method on individual samples. The *Mptb* shedder detection rate by the 3 serologic tests ranged from 29.8% to 39.4%. Detection rate for ELISA-A was lower than that for ELISA-B and CFT. For all 3 tests, detection rate was dependent on the level of *Mptb* shedding and the age of the animals. Detection rates increased as cattle age increased to 4 years. The specificity panel was initially composed of sera from 811 cows randomly selected from 41 herds without clinical paratuberculosis that were negative for *Mptb* based on whole-herd fecal culture. The modified Jørgensen method for culture was used on pooled fecal samples. Serologic test specificity ranged from 93.4% to 99.8%. The specificity of ELISA-A was higher than that of ELISA-B and CFT. Specificity of ELISA-B between herds was 75–100%. Specificity of CFT between herds was 62–100%. The low specificity of ELISA-B and CFT could not be explained by a higher sensitivity for *Mptb*-infected cows before onset of shedding, because in the 19 herds with 8 more subsequent negative whole-herd fecal cultures in the 4 years after sampling, specificity was not improved. The insufficient specificity of ELISA-B was not corrected sufficiently by heightening the cutoff value because *Mptb* shedder detection rate was lowered to 28.9%, equal to that of ELISA-A, and specificity only rose to 97%, much lower than that of ELISA-A. Taking into account the different test characteristics, serologic tests are a cost-effective alternative to fecal culture in high-prevalence herds. For certification programs, only ELISA-A is recommended because in a large number of nonsuspect herds specificity remained almost 100%.

Paratuberculosis, or Johne's disease, in cattle is a chronic, infectious enteric disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Mptb*). Clinical signs of the disease include decreased milk production, weight loss, and diarrhea. Cattle become infected in the first months of life, but the infection remains unrecognized until the onset of clinical signs.

These signs usually appear by >4 years of age, although they may not be observed for a majority of the infected cattle in a dairy herd because the cows are culled for economic reasons prior to the onset of clinical disease.<sup>2,21,24</sup>

Paratuberculosis control is based on hygienic measures and removal of cows shedding *Mptb* in their feces. The currently available tests for the diagnosis of paratuberculosis are not adequate for detecting all infected cows at a single point in time.<sup>3,21,25</sup> Identification of *Mptb* shedders is most effectively achieved by culture of fecal samples,<sup>25</sup> but this technique is expensive and requires at least 16 weeks for completion.<sup>3,9,12,13,25</sup> Serologic tests are less expensive, faster, and easier to perform, but in general results are considered less reliable because of false-positive and false-negative re-

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sults.<sup>1,2,9,16</sup> The serologic tests most frequently used are the *Mycobacterium phlei*-absorbed enzyme-linked immunosorbent assay (absorbed ELISA) and the complement fixation test (CFT). In Europe, the CFT is recommended to confirm a clinical diagnosis of paratuberculosis.<sup>2,9</sup> Reported sensitivity of the CFT ranges from 10% to 90%.<sup>1,2,5,9,17,20,22,26</sup> Specificity of the CFT is considered to be approximately 70% in dairy herds.<sup>1,2,9,16</sup> The specificity rate reported in those studies may be influenced by an underestimation of the infection rate. This underestimation may have occurred because infected animals were identified based on clinical investigation, macroscopic necropsy, or insensitive culture techniques on individuals, without reference to negative whole-herd cultures used as the “gold standard” for absence of infection. A higher CFT specificity (95–99%) has been reported for sera from uninfected dairy herds from Wisconsin and northwestern Australia.<sup>17,18,22</sup> The absorbed ELISA has been reported to surpass the CFT in accuracy because of a  $\geq 2$ -fold higher sensitivity<sup>17,18,22</sup> and a specificity of almost 100%.<sup>4,6,8,17,21–23,26</sup> Comparisons of commercially available absorbed ELISAs demonstrated little difference in test specificity, and lower specificity contributed to differences in cutoff values.<sup>8,26</sup> The company<sup>a,b</sup> that originally delivered the absorbed ELISA from the Australian central serum laboratory<sup>c</sup> (CSL) changed to another absorbed ELISA, made in Sweden from New Zealand components,<sup>d</sup> because of a promised better test sensitivity and high specificity.

The aim of the present study was to examine the effectiveness of serologic tests in detecting *Mptb* shedders to determine the optimal cost effectiveness of these tests in paratuberculosis control programs. Two different absorbed ELISA kits commercialized by the same company were compared to study the effect of test kit design. As a control, the in-house CFT<sup>e,f</sup> for the diagnosis of paratuberculosis in cattle in The Netherlands was used. As factors influencing test specificity, herd history and cutoff value of test interpretation were studied. In addition, the association of age and level of fecal *Mptb* shedding of the cows was examined.

## Materials and methods

**Animals.** The “positive” serum panel included samples from fecal culture–positive cows originating from 53 infected dairy herds. The “negative” serum panel included samples from randomly selected cows from 41 clinically nonsuspect herds in a herd certification program for which the first whole-herd fecal culture was negative for *Mptb*. These herds were followed further in the certification program, and 19 of the 41 herds were still negative after a total of 9 whole-herd fecal cultures 4 years later.

**Fecal sampling.** Fecal samples were collected from the rectum of each animal using disposable plastic rectal ex-

amination gloves without lubrication gel. After the fecal sample was collected, the gloves were tied up and identified with preprinted self-adhesive labels that showed the bar code for each cow, as recorded in the Dutch Identification and Registration system.<sup>15</sup> Samples were kept at 4 C during transport and were processed for isolation of *Mptb* within 24 hr after arrival at the laboratory. Pooling was performed by mixing fecal samples from 5 animals ordered by age.<sup>11</sup>

**Fecal culture procedure.** Fecal samples were cultured using a modified Jørgensen method.<sup>12</sup> A fecal sample of approximately 2 g (range, 1.8–2.2 g) was decontaminated for 30 min with 8 ml of 4% NaOH and centrifuged (1,000  $\times$  g), and after removal of the supernatant the remaining sediment was exposed for 30 min to 5 ml of a mixture of oxalic acid<sup>g</sup> (5 mg/ml) and malachite green (1 mg/ml). After another centrifugation step (1,000  $\times$  g), the sediment was re-suspended in 4 ml of a mixture of neomycin<sup>h</sup> (0.5 mg/ml) and amphotericin B<sup>g</sup> (50 mg/ml) and incubated overnight at room temperature. The separation layer between the particulate matter and the clear antibiotic solution was inoculated onto modified Löwenstein-Jensen agar slants. Four tubes containing mycobactin<sup>g</sup> were inoculated. The tubes were inspected at 8, 12, 16, and 26 wk of incubation for evidence of *Mptb* growth. A sample was considered to be culture positive when 1 or more colonies were recorded as *Mptb*, confirmed by polymerase chain reaction, in 1 or more culture tubes.

**Shedders panel.** To compare relative sensitivity of fecal culture and serologic tests, serum samples were collected from 198 *Mptb* culture-positive cows originating from 53 dairy herds. None of these animals showed clinical symptoms of paratuberculosis. Samples were taken from the coccygeal vein using Clot Activator Vacutainer<sup>®i</sup> serum tubes. Tubes were identified with self-adhesive labels preprinted that showed the bar code for each cow, as recorded in the Dutch Identification and Registration system.<sup>15</sup>

**Specificity panel.** To compare specificity of the serologic tests, blood samples were collected from approximately 20 randomly chosen cows per herd from 41 nonsuspect dairy herds. Sampling technique was identical to collection of samples of the shedders panel. Selected herds were managed under a closed-herd system and were free of clinical signs of paratuberculosis. Closed herd management for >3 yr and absence of clinical signs for >5 yr were verified by the Animal Health Service. Fecal pools of all adult cows (>2 yr) were cultured for *Mptb*, and only negative results were obtained, and blood samples were collected as soon as culture results were available.

**Serologic test procedures.** Blood samples were tested using 3 serologic tests. The exact details of the antigen preparation methods for the *Mycobacterium paratuberculosis* Antibody Test Kit<sup>a</sup> (ELISA-A) and the *Mycobacterium paratuberculosis* Antibody ELISA<sup>b</sup> (ELISA-B) were not available, but bound antibodies are detected by an anti-bovine IgG1 conjugate for ELISA-A and by a protein G conjugate for ELISA-B. A CFT was also performed,<sup>c</sup> using commercial reagents. All samples were tested in the serologic tests according to the manufacturers’ instructions supplied with the kits. Negative and positive control samples were provided with the kits and were processed in duplicate on each 96-

**Table 1.** Detection rate of *Mycobacterium avium* subsp. *paratuberculosis* and specificity of 2 absorbed ELISAs and a complement fixation test (CFT) for the diagnosis of paratuberculosis in culture-positive cows ( $n = 198$ ) from 53 infected herds, cows ( $n = 811$ ) from 41 nonsuspect herds with  $\geq 1$  negative herd cultures, and cows ( $n = 346$ ) from 19 nonsuspect herds with 9 negative herd cultures.

Test	Infected herds		Nonsuspect herds			
	No. positive cows	Detection rate (%)	$\geq 1$ negative culture		9 negative cultures	
			No. positive cows	Specificity (%)	No. positive cows	Specificity (%)
ELISA-A	59	29.8*†	809	99.8*‡§	345	99.7*‡§
ELISA-B	78	39.4‡	754	93.4‡	322	93.1‡
ELISA-B, double cutoff value	59	29.8†	796	97.6§	335	96.8§
CFT	70	35.4	771	95.1	323	93.3

\* Different ( $P < 0.005$ ) from results of ELISA-B.

† Tendency for difference ( $0.05 < P < 0.10$ ) from results of CFT.

‡ Different ( $P < 0.05$ ) from results of ELISA-B, double cutoff value.

§ Different ( $P < 0.05$ ) from results of CFT.

well plate, together with 4 control sera with different antibody concentrations. Results of the ELISA-A were classified as positive when the optical density (OD), i.e., the absorbance reading of the sample at 450 nm (OD450) minus the OD450 of the negative control, was  $>0.1$  units. Results of the ELISA-B were classified as positive when the ratio between the corrected sample OD value and the corrected positive control OD value (S/P ratio) was  $>0.15$ . In addition, because results indicated an unsatisfactory specificity, a higher cutoff value (S/P  $> 0.30$ ) was used to study the effect of a higher cutoff value on the specificity of the test. CFT results were interpreted as negative at values of 0 or 10 and as positive at values of 20, 50, and  $>50$ , corresponding to titers of 1/8, 1/16, and  $\leq 1/32$ .

**Data analysis.** *Mptb* shedder detection rate was defined as the percentage of *Mptb* shedders correctly classified by the serologic tests. Detection rate and specificity were calculated using Statistix 2.0 for Windows. Detection rates and specificities of the 3 tests were compared using McNemar's test for paired samples. Differences were considered significant at  $P = 0.05$ . Three levels of *Mptb* shedding were recognized. Chi-square analysis and contingency tables<sup>19</sup> were used to compare relative sensitivity for the 3 *Mptb* shedding levels, detection rate and specificity between age groups, level of *Mptb* shedding in age groups, and within-herd prevalence for the different tests. The correlation between test results in the specificity panel was studied using correlation analysis.<sup>19</sup>

## Results

The *Mptb* shedder detection rate was 29.8% for ELISA-A, 39.4% for ELISA-B, and 35.4% for CFT (Table 1). Increasing the cutoff value of ELISA-B from 0.15 to 0.3 decreased the detection rate to that of ELISA-A (29.8%). Results of all 3 tests were positively associated with the level of *Mptb* shedding in the feces (Table 2). Detection rates of ELISA-A, ELISA-B (manufacturer's cutoff), and CFT increased from 12%, 24%, and 19%, respectively, for cows having 1–10 colony-forming units (CFU) of *Mptb* per 2 g feces to 68%, 79%, and 75%, respectively, for cows having  $>100$  CFU per 2 g feces. In the culture-positive cattle used to make up the shedders serum panel, there was an age-dependent increase of the level of *Mptb* shedding up to 4 years of age, and a decrease for cattle  $>4$  years of age. At 2, 3, 4, and  $>4$  years of age, the culture positive samples with  $>10$  CFU were 21%, 48%, 57%, and 43%, respectively ( $P = 0.01$ ). *Mptb*

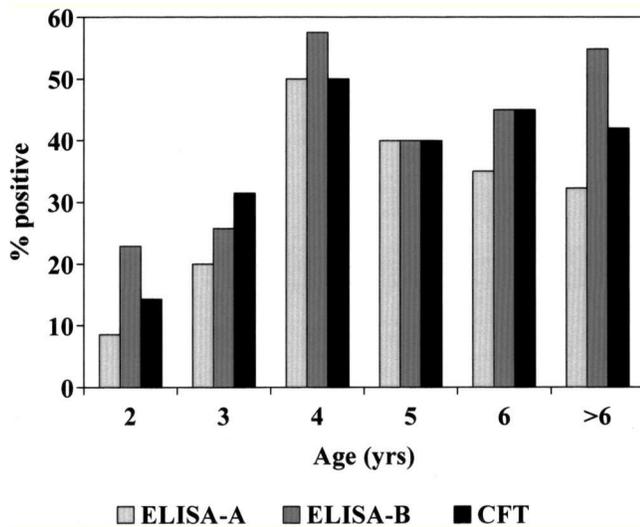
**Table 2.** Sensitivity of 2 absorbed ELISAs and a complement fixation test for detection of antibodies against *Mycobacterium avium* subsp. *paratuberculosis* related to the level of bacterial shedding.\*

Test	1–10 CFU		10–100 CFU		$>100$ CFU	
	No. positive cows	Sensitivity (%)	No. positive cows	Sensitivity (%)	No. positive cows	Sensitivity (%)
ELISA-A	11	12††	18	40‡	19	68
ELISA-B	22	24††	22	50‡	22	79
ELISA-B, double cutoff value	15	17††	16	36‡	20	71
CFT	16	19††	21	52‡	21	75
All	7	8††	13	30‡	18	64
None	62	69††	16	46	5	18

\* CFU was not determined for all samples.

† Different ( $P < 0.01$ ) from 10–100 CFU shedders.

‡ Different ( $P < 0.05$ ) from  $>100$  CFU shedders.



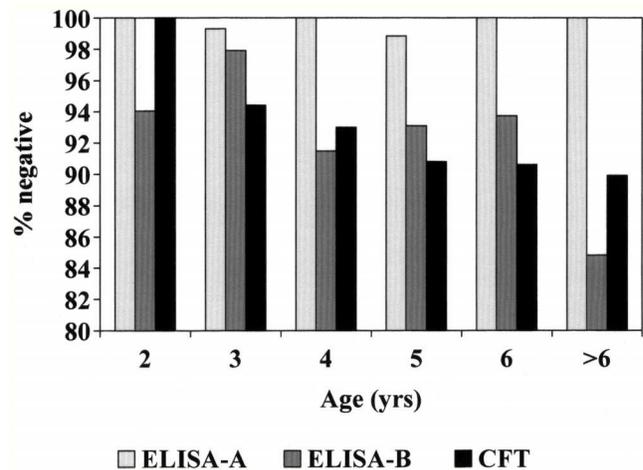
**Figure 1.** Distribution of the sensitivity of 2 absorbed ELISAs and a complement fixation test for detection of antibodies against *Mycobacterium avium* subsp. *paratuberculosis* in culture-positive cattle per year of age.

shedder detection rates for the 3 serologic tests increased up to 4 years of age (highest  $P = 0.002$ ; Fig. 1). The shedder detection rate of ELISA-B was higher than that of ELISA-A in animals 2 and >4 years of age ( $P = 0.02$  and  $0.03$ , respectively). Detection rates of ELISA-B and CFT did not differ among any of the age groups (lowest  $P = 0.11$ ).

Specificity of ELISA-A was 99.8% (Table 1); specificities of ELISA-B (93.4%) and CFT (95.1%) were lower. When the cutoff value of ELISA-B was changed from the recommended S/P ratio of 0.15 to a ratio of 0.30, specificity (97%) was still lower than that of ELISA-A. Positive results from the ELISA-B and from the CFT for the serum panel of the 41 nonsuspect herds were correlated (Pearson's correlation coefficient = 0.19,  $P < 0.0001$ ). Of the ELISA-B-positive samples, 28.3% were also CFT positive, and 6.9% of the ELISA-B-negative samples were CFT positive. Prevalence of positive results from the ELISA-B and CFT for the specificity panel was different among herds ( $P = 0.05$ ,  $P < 0.0001$ , respectively). Specificity of ELISA-B between herds ranged from 75% to 100%, and specificity of CFT between herds ranged from 62% to 100%. Specificity of ELISA-B decreased among cattle >6 years old ( $P = 0.03$ ; Fig. 2), and specificity of CFT decreased with increasing cattle age ( $P < 0.0001$ ; Fig. 2). Specificities of ELISA-B and CFT did not increase when herds not reaching the highest level in the certification program were excluded from the analysis (Table 1).

### Discussion

The detection rate by the absorbed ELISA-A of around 30% of *Mptb* shedding cows was much lower



**Figure 2.** Distribution of the specificity of 2 absorbed ELISAs and a complement fixation test for detection of antibodies against *Mycobacterium avium* subsp. *paratuberculosis* in cattle from certified paratuberculosis-free herds per year of age.

than the 57–65% reported previously.<sup>4,6,14</sup> The high correlation between detection rate and level of fecal *Mptb* shedding found in this study, in agreement with other reports,<sup>18,23,25</sup> could explain these differences if in earlier reports the serum panel included animals with a higher level of fecal shedding. The low detection rate of ELISA was in that case a reflection of more sensitive fecal culture techniques, in agreement with recent reports.<sup>17,25</sup> The logical misunderstanding that fecal culture and ELISA are equally effective in paratuberculosis control programs because both are reported to have a sensitivity of 40–50% is also caused by use of different “gold standards” based on different diagnostic techniques.<sup>25</sup> The findings in this study support those of a recent report in which regular fecal culture was recommended as 3-fold more effective in eliminating *Mptb* shedding cows as regular testing by ELISA.<sup>25</sup> The high detection rate for cows shedding high numbers of *Mptb* in feces, however, makes serology more effective in test and cull programs, because the most infectious cows are removed from a herd quickly and at low cost. The difference in detection rates between the ELISAs was in agreement with earlier reports,<sup>17,22</sup> but the high detection rate of CFT was unexpected. In earlier comparative studies,<sup>4,5,17,24</sup> CFT was reported as having a much lower sensitivity than the absorbed ELISA. This contradiction may be explained by the lack of standardization of the CFT among laboratories, whereas ELISAs are provided as diagnostic kits with set protocols. The positive association between age and detection rate of the 3 described tests for serum antibodies in infected cows was in agreement with other reports.<sup>1,20</sup> The CFT tended to outperform the ELISA-A when applied to young infected cows (Figs. 1, 2).

The frequently described higher specificity of tests based on the absorbed ELISA compared with the CFT<sup>3,16–18,20,22,26</sup> was not found in this study. Specificity of CFT was lower than that of ELISA-A but higher than that of ELISA-B. The high specificity of ELISA-A found in this study is in accordance with other observations with the basically identical CSL absorbed ELISA.<sup>4,6,8,22,26</sup> The low specificity of ELISA-B was not found in an earlier comparison of the CSL ELISA and an ELISA based on New Zealand components.<sup>17</sup> However, experiences in Norway with the equivalent of ELISA-B support these findings; only 8% of the ELISA-positive cows could be confirmed by histopathology or bacteriology.<sup>7</sup> The correlation of positive results between ELISA-B and CFT for sera from non-suspect herds also indicates that factors involved in low specificity of CFT may also account for the specificity problems of ELISA-B. These cross-reactions may be induced by bacterial infections with *Corynebacterium* or *Nocardia* species.<sup>13</sup> Alternatively, detection of total immunoglobulin antibodies by the ELISA-B kit (using a protein G conjugate) instead of only IgG1 antibodies as in ELISA-A could be responsible. In an earlier comparison of ELISAs produced with either Australian or New Zealand components, the specificity of the ELISA produced with New Zealand components (similar to the ELISA-B) was, in contradiction with the results of the present study, higher (99.7%) instead of lower than that of ELISA-A (97.3%).<sup>17</sup> This contradiction emphasizes the importance of using serum panels originating from local cattle to validate serologic tests. The high herd influence on specificity of CFT and ELISA-B emphasizes the need for a large number of herds to be included in the panel. Lack of a sufficient number of herds in serum panels used by the manufacturer for evaluation of newly developed tests may explain the disappointing results obtained with these panels. Changing the cutoff level from 0.15 to 0.30 for ELISA-B still resulted in an inappropriately low specificity of 97%. The most logical explanation for low specificity should have been detection of cows from the nonsuspect herds included in the panel that had *Mptb* infections but were not (yet) shedding the bacteria in their feces. In a recent study, a high percentage of herds in which clinical symptoms of paratuberculosis were never observed were unexpectedly infected with *Mptb*.<sup>10</sup> However, the assumption of the presence of infected nonshedders was not correct because in those 19 herds that were still culture negative after 5 years and 9 whole-herd fecal cultures, specificity of the serologic tests was not improved. Specificity of CFT was 95%, a value higher than the 70% reported earlier in European and US dairy herds<sup>2,9,16</sup> but in accordance with comparative studies using sera of cer-

tified paratuberculosis-free herds from Wisconsin and uninfected herds from northwestern Australia.<sup>17,18,22</sup>

The combination of low detection rate of *Mptb* shedders and comparatively low specificity of ELISA-B and CFT implies that the prevalence of *Mptb* shedders within herds determines the test characteristics and therefore usefulness in individual herds. In high prevalence herds (>30% *Mptb* shedders), the predictive positive value of positive test results with ELISA-B and CFT will be around 75%. The costs associated with serologic tests are lower than those associated with fecal culture, and serologically positive infected cows are probably shedding the highest number of bacteria; these advantages might compensate for the costs of culling some false-positive cows. However, because in low prevalence herds (<10% *Mptb* shedders) the predictive positive value of positive results of ELISA-B and CFT will be <50%, there remains the risk of losing support for the control program because healthy cows could be culled and infected cows could be missed. Because of severe economic consequences to farmers from culling >10% of a herd yearly, administration of ELISA-A will be the most cost-effective way to lower infection rate in high-prevalence herds, and individual fecal culture will be more cost effective in low-prevalence herds. The use of ELISA-B or CFT can only be advised for confirmation of clinical diagnosis because the high prevalence of infected cows among suspect cows will give a positive predictive value of nearly 100%. In certification programs, only noninfected herds or herds with a low infection rate are involved. The high specificity of ELISA-A is more critical than the low sensitivity for detection of infected individual cows, provided that adequate herd sampling strategies are used to improve herd-level sensitivity. In this way, herds can be certified as low risk so that animals can be sold. To improve the reliability of certification, a higher sensitivity is needed and fecal culture is recommended.<sup>10,25</sup>

Based on the results of this study, absorbed ELISA based on Australian components (ELISA-A) would be the preferred serologic test for herd certification and paratuberculosis control programs. The estimation of detection rate of *Mptb* shedders and occurrence of unnecessary culling based on results of serologic tests is influenced by a number of factors, including the design of the test and the corresponding test characteristics. In addition to the age of the animals tested, the level of *Mptb* shedding among infected animals, the sensitivity of fecal culture, and the inclusion of a sufficient number of herds in the serum panels influence test validity.

### Acknowledgement

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### Sources and manufacturers

- a. Idexx Laboratories, Westbrook, ME.
- b. Idexx Scandinavia AB, Österbybrook, Sweden.
- c. Commonwealth Serum Laboratories, Parkville, Victoria, Australia.
- d. Ministry of Agriculture and Fisheries, Upper Hutt, New Zealand.
- e. Animal Health Service, Deventer, The Netherlands.
- f. ID-Lelystad, Lelystad, The Netherlands.
- g. Allied Monitor, Fayette, MO.
- h. Sigma Chemical Co., St. Louis, MO.
- i. Becton Dickinson and Company, Plymouth, UK.

### References

1. Aalund O: 1984, Sensitivity, specificity and predictive value of diagnostic tests applied to animals with subclinical infections of paratuberculosis. *In: Paratuberculosis, diagnostic methods, their practical application and experience with vaccination*, ed. Jørgensen JB, Aalund O, pp. 9–39. Commission of the European Communities, Copenhagen, Denmark.
2. Benedictus G: 1984, Evaluation of organized control of bovine paratuberculosis in Friesland province, The Netherlands. *Tijdschr Diergeneeskd* 109:905–916.
3. Collins MT: 1996, Diagnosis of paratuberculosis. *Vet Clin North Am Food Anim Pract* 12:357–372.
4. Collins MT, Sockett DC, Ridge S, Cox JC: 1991, Evaluation of a commercial enzyme-linked immunosorbent assay for Johne's disease. *J Clin Microbiol* 29:272–276.
5. Colgrove GS, Thoen CO, Blackburn BO, Murphy CD: 1989, Paratuberculosis in cattle: a comparison of three serologic tests with results of fecal culture. *Vet Microbiol* 19:183–187.
6. Cox JP, Drane DP, Jones SL, et al.: 1991, Development and evaluation of a rapid absorbed enzyme immunoassay test for the diagnosis of Johne's disease in cattle. *Aust Vet J* 68:157–160.
7. Djønne B, Fredriksen B, Nyberg O, et al.: 2001, National bovine paratuberculosis program in Norway. *Bull IDF* 364:75–80.
8. Ellis TM, Carson BA, Kalkhoven MJ, Martin PA: 1998, Specificity of two absorbed ELISA's for surveys of *Mycobacterium paratuberculosis* in cattle. *Aust Vet J* 76:497–499.
9. Jørgensen JB: 1984, The diagnosis of clinical paratuberculosis in bovines. *In: Paratuberculosis, diagnostic methods, their practical application and experience with vaccination*, ed. Jørgensen JB, Aalund O, pp. 1–7. Commission of the European Communities, Copenhagen, Denmark.
10. Kalis CHJ, Barkema HW, Hesselink JW: 1999, Certification of dairy herds as free of paratuberculosis using cultures of strategically pooled fecal samples. *Proc Int Colloq Paratuberculosis* 6:55–58.
11. Kalis CHJ, Hesselink JW, Barkema HW, Collins MT: 2000, Culture of strategically pooled bovine fecal samples as a method to screen herds for paratuberculosis. *J Vet Diagn Invest* 12:547–551.
12. Kalis CHJ, Hesselink JW, Russchen EW, et al.: 1999, Factors influencing the isolation of *Mycobacterium avium* subsp. *paratuberculosis* from bovine fecal samples. *J Vet Diagn Invest* 11:345–351.
13. Merkal RS: 1984, Paratuberculosis: advances in cultural, serologic, and vaccination methods. *J Am Vet Med Assoc* 184:939–943.
14. Milner AR, Mack WN, Coates KJ, et al.: 1990, The sensitivity and specificity of a modified ELISA for the diagnosis of Johne's disease from a field trial in cattle. *Vet Microbiol* 25:193–198.
15. Nielen M, Jansen FCM, van Wuijckhuise LA, Dijkhuizen AA: 1996, Dutch cattle identification and registration (I&R) system: analysis of its use for controlling an outbreak of foot and mouth disease. *Tijdschr Diergeneeskd* 121:576–581.
16. Rankin JD: 1961, The non-specificity of a complement-fixation test used in the diagnosis of Johne's disease in cattle. *Res Vet Sci* 2:89–95.
17. Reichel MP, Kittelberger R, Penrose ME, et al.: 1999, Comparison of serological tests and faecal culture for the detection of *Mycobacterium avium* subsp. *paratuberculosis* infection in cattle and analysis of antigens involved. *Vet Microbiol* 66:135–150.
18. Ridge SE, Morgan IR, Sockett DC, et al.: 1991, Comparison of the Johne's absorbed EIA and the complement-fixation test for the diagnosis of Johne's disease in cattle. *Aust Vet J* 68:253–257.
19. Rothman KJ: 1986, *Modern epidemiology*. Little, Brown, and Co., Boston, MA.
20. Sherman DM, Gay JM, Bouley DS, Nelson GH: 1990, Comparison of the complement-fixation and agar gel immunodiffusion tests for diagnosis of subclinical bovine paratuberculosis. *Am J Vet Res* 51:461–465.
21. Sockett DC: 1994, Update on control and management of Johne's disease. *Bovine Pract* 28:136–138.
22. Sockett DC, Conrad TAA, Thomas CB, Collins MT: 1992, Evaluation of four serological tests for bovine paratuberculosis. *J Clin Microbiol* 30:1134–1139.
23. Sweeney RW, Whitlock RH, Buckley CL, Spencer PA: 1995, Evaluation of a commercial enzyme-linked immunosorbent assay for the diagnosis of paratuberculosis in dairy cattle. *J Vet Diagn Invest* 7:488–493.
24. Whitlock RH, Buergelt C: 1996, Preclinical and clinical manifestations of paratuberculosis (including pathology). *Vet Clin North Am Food Anim Pract* 12:345–356.
25. Whitlock RH, Wells SJ, Sweeney RW, van Tiem J: 1999, ELISA and fecal culture: sensitivity and specificity of each method. *Proc Int Colloq Paratuberculosis* 6:353–362.
26. Yokomoto Y, Kishima M, Mori Y, Nishimori K: 1991, Evaluation of enzyme-linked immunosorbent assay in comparison with complement fixation test for the diagnosis of subclinical paratuberculosis in cattle. *J Vet Med Sci* 53:577–584.