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Development of Species-Specific Enzyme-Linked Immunosorbent Assay for Diagnosis of Johne's Disease in Cattle

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The previously described (M. De Kesel, P. Gilot, M.-C. Misonne, M. Coene, and C. Cocito, J. Clin. Microbiol., 31:947–954, 1993) a362 recombinant polypeptide of *Mycobacterium paratuberculosis* was used as reagent for an enzyme-linked immunosorbent assay (ELISA). This ELISA, which is endowed with species specificity with respect to the other mycobacteria, was applied to the analysis of bovine paratuberculosis (Johne's disease), an endemic mycobacteriosis of cattle caused by *M. paratuberculosis*. The distribution of anti-a362 antibodies in the cattle population was analyzed by a computer program (mixture population model) to determine a cutoff value for the test. The prevalence of a362 seropositivity in the Belgian bovine population was estimated to be 12%. The sensitivity of the a362 assay was 70%, as determined with reference sera from the U.S. National Repository of Paratuberculosis Specimens. Some 40% of the animals in the herds with paratuberculosis analyzed were found to be positive by the a362 assay. The latter proved to be 95% specific with respect to both healthy and tuberculous cattle.

Paratuberculosis (Johne's disease), a chronic enteritis produced by *Mycobacterium paratuberculosis*, affects a large proportion of ruminants in all continents and is the cause of important economic loss. An initial asymptomatic, nonexcretory stage of the disease evolves to an asymptomatic, excretory stage and, finally, to a symptomatic stage in which the clinical signs of chronic diarrhea and the shedding of large quantities of bacteria are predominant (6, 7).

Early diagnosis of infected cattle is essential for avoiding the spread of infection, but the available diagnostic tests have serious limitations. In fact, microbiological identification is of limited use, because the replication of this mycobactin-dependent organism is slow (positive cultures are obtained after 2 to 4 months) (21). Specific nucleic acid probes allow the identification of *M. paratuberculosis* in the feces of cattle without preliminary culture of the microorganism, but are not able to diagnose nonexcretory infected animals (27, 32). The available immunological tests, although apparently adequate to detect infected animals at the initial stage of the disease, have serious limitations because of cross-reactions with related bacteria of the *Corynebacterium-Mycobacterium-Nocardia* group (11, 14, 16, 20, 25, 26). Cross-reactions with *Mycobacterium bovis* are particularly feared, because tuberculous cattle are highly contagious for humans and are subject to compulsory slaughtering. Cutaneous testing with extracts of either *M. paratuberculosis* (johnin) or *Mycobacterium avium* (avian tuberculin) lacks specificity and yields negative reactions in animals with advanced paratuberculosis (6, 15, 19). The complement fixation (CF) test with crude mycobacterial lysates has been widely used for years, despite its lack of specificity and its poor sensitivity (8, 14, 20, 25). The agarose gel immunodiffusion (AGID) test has similar limitations. The reagent of the commercial AGID test (Rapid Johne's test; Immucell Corp., Portland, Maine) is represented by the whole cytoplasm of *M. paratuberculosis* 18, which was recently identified as *M. avium* serotype 2 (29). Immunoenzymometric serological tests (enzyme-linked immunosorbent assays [ELISAs]), which monitor the level of antimycobacterial antibodies in infected cattle, are endowed with a high degree of sensitivity; however, ELISAs based on specific *M. paratuberculosis* antigens have not been described (11, 18, 34). To avoid the large number of false-positive results that spoil the performances of these assays, a step of preabsorption of sera on *Mycobacterium phlei* sonicates was introduced to remove part of the cross-reacting antibodies (4, 22, 23, 35). Two such tests are commercially available: Allied-ELISA (Allied Laboratories, Fayette, Mo.) and CSL ELISA (Johne's absorbed EIA; Commonwealth Serum Laboratories, Parkville, Australia; also distributed by IDEXX Laboratories, Westbrook, Maine). The former assay is based on a partly purified antigen preparation of *M. paratuberculosis* 18, whereas the latter uses whole cytoplasm of *M. paratuberculosis* VRI 316:102-2 (9).

We have recently shown that a 34-kDa protein of the *M. paratuberculosis* A36 complex is immunodominant in animals with Johne's disease (11, 16) and contains B-cell epitopes specific toward all of the tested mycobacteria, including *M. bovis* and *M. avium* (11). The gene coding for the 34-kDa protein was isolated by molecular cloning and sequenced. The 897-bp open reading frame coded for a novel protein containing well-defined hydrophobic and hydrophilic portions (17). A DNA fragment coding for the hydrophilic carboxyl end of the 34-kDa protein has been isolated from a λgt11 genomic library of *M. paratuberculosis* (clone a362). This DNA fragment coded for a polypeptide containing B-cell epitopes present in all *M. paratuberculosis* strains tested but not in *M. bovis* or related bacteria, including many strains of the *M. avium-Mycobacterium intracellulare-Mycobacterium scrofulaceum* group (12).

In the present work, the recombinant a362 polypeptide, which represents the first specific recombinant antigen of *M. paratuberculosis*, was used as the reagent for an ELISA for paratuberculosis.

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MATERIALS AND METHODS

Serum samples. We tested some 750 serum samples from cattle, which were divided into five groups. The first group included 300 serum samples provided by the Centers for Animal Diseases from the Belgian provinces of Liège, Hainaut, and Namur. They were serum samples from cows submitted for brucellosis serology. These 300 serum samples (100 by the center in each province), which were collected by systematic random sampling, were obtained from cattle that are representative of the bovine population of a large section of the country (33). The second group was a reference set of 30 serum samples from cattle with paratuberculosis obtained from the U.S. National Repository for Paratuberculosis Specimens (30) (M. Collins, University of Wisconsin). The third group included sera from 38 animals from a herd (herd T) containing tuberculous cattle; these animals yielded positive reactions to bovine tuberculin or cultures of biopsy specimens from these animals were positive for *M. bovis*. They were also positive in an ELISA based on the cross-reactive mycobacterial antigen complex A36 (11). The fourth group was composed of an additional 175 control serum samples from two Belgian herds (herds H1 and H2) with no history of the disease. Finally, 208 serum samples from two Belgian herds (herds P1 and P2) included animals with clinical symptoms of Johne's disease and positive fecal culture (fifth group).

ELISA. The recombinant polypeptide a362 was synthesized as a fusion protein with the first 25 amino acids of mouse tumor necrosis factor and was purified on a metal chelate adsorbent as described previously (17). Multiwell microtiter plates (high binding capacity; Microwell module F16, Nunc, Roskilde, Denmark) were coated with the purified a362 recombinant polypeptide (0.5 μg in 100 μl of 0.05 M sodium carbonate buffer [pH 9.6] per well), air dried overnight, and saturated (300 μl of 0.1% [wt/vol] serum albumin–0.15 M NaCl solution per well for 2 h at 37°C). After incubation (100 μl per well for 1 h at 37°C) of bovine sera diluted 1/75 in PBST (0.15 M NaCl, 0.005% Tween 80, 0.02 M phosphate buffer [pH 7.2]) containing 0.1% bovine serum albumin, washing with PBST, and the addition of peroxidase-labeled rabbit anti-cow immunoglobulin (Ig) (Dako, Copenhagen, Denmark) (100 μl of a 1/5,000 dilution in PBST containing 0.1% bovine serum albumin per well for 1 h at 37°C), excess reagent was removed by washing with PBST and peroxidase substrate was added (100 μl [per well] of a 17 mM sodium citrate buffer pH 6.3 containing 0.2% [wt/vol] *o*-phenylenediamine and 0.015% [wt/vol] hydrogen peroxide for 30 min at 37°C in the dark). The reaction was stopped by the addition of 100 μl of 2 M H_2SO_4 per well, and the A_{492} was measured in a colorimetric plate reader (SLT 210; Kontron Analytical, Watford, United Kingdom). Samples were tested in duplicate. Those with more than 20% discrepancy were retested. The IDEXX ELISA was used as a control, and we followed the instructions of the manufacturer.

Mixture population modeling. Upon logarithmic transformation, ELISA data were divided in frequency classes of 0.1 logarithmic optical density units. By using the likelihood function (24), an iterative computer program (based on the algorithm of Agha and Ibrahim [1]) was used to calculate the maximum likelihood estimates of a set of parameters (proportion of the total number of serum samples, mean value, and variance for each hypothetical population present in the entire population analyzed). ELISA data were sequentially made to fit models of increasing population numbers until an additional population did not significantly improve the likelihood value. Once the best set of parameters was found, a Gaussian distribution was then designed for each population by using the estimated mean and variance values.

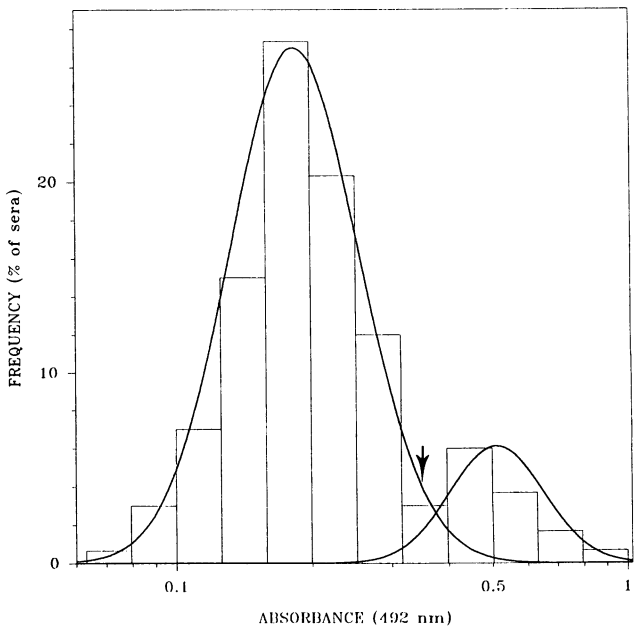

FIG. 1. Distribution of anti-a362 Ig titers in randomly chosen samples from a large bovine population. Serum samples from 300 cattle representative of cattle from a wide region of Belgium were analyzed for the presence of anti-*M. paratuberculosis* Ig by the a362 ELISA. Sera were distributed in frequency classes of 0.10 logarithmic unit of optical density (A_{492}) (rectangles), and the Gaussian curves that best fit the experimental data were computed (continuous lines). The arrow separating the two populations indicates the chosen cutoff value.

Estimation of cutoff value. The optimum cutoff value (3), estimated by the method of Anderson (2), was the value c minimizing the relationship

$$(1 - p) \int_c^{\infty} d_1(x)\,dx + rp \int_{-\infty}^{c} d_2(x)\,dx,$$

where p is the prevalence of the tested parameter in the global population, d_1 and d_2 are the density distributions of test values from populations 1 and 2, respectively (determined by the mixture population modeling), and r is the ratio of the cost of a misclassified result in population 1 over the cost of a misclassified result in population 2.

RESULTS

Determination of a cutoff value for the a362 ELISA using a mixture population model. The establishment of the cutoff value is of paramount importance for a serological test of diagnostic significance. Such a reference value for the immunoassay used in the present work was determined as follows. The titers of anti-a362 immunoglobulins were measured by applying the a362 ELISA to the sera from 300 randomly chosen cattle within a large bovine population. ELISA values were divided in frequency classes of 0.1 logarithmic units of optical density (A_{492}) (Fig. 1). The distribution of the frequency classes was then analyzed according to the likelihood function. Models involving an increasing number of populations were considered, and the model best-fitting experimental data was chosen. A bimodal model was found to be the most

appropriate for accommodating our results; it included 87.7% low-titer sera (mean A_{492}, 0.184) and 12.3% high-titer sera (mean A_{492}, 0.513). The Gaussian distribution of each population, computed from the estimated mean values and variance, is shown in Fig. 1.

The optimum cutoff value separating these two populations (population 1, characterized by a low titer of Ig; population 2, characterized by a high titer of Ig) was estimated by the method of Anderson (2). For this purpose, we assumed that the prevalence of antibodies to peptide a362 is equal to the proportion of cattle with a high Ig titer ($P = 0.12$) and that the cost of a misclassified result in population 1 is greater than the cost of a misclassified result in population 2 ($r = 2$). Accordingly, five serum samples with ELISA absorbance values corresponding to the determined cutoff value (Fig. 1, arrow) were chosen and included as standards in each ELISA. The absorbance values for the sera below the mean value for these five serum samples were considered a negative result in the a362 ELISA, while those with higher absorbances were regarded as positive.

Comparison of the a362 ELISA with other serological tests for paratuberculosis. The a362 ELISA was compared with other serological tests for paratuberculosis. Some 30 reference serum samples from cattle certified to have paratuberculosis (from the U.S. National Repository for Paratuberculosis Specimens) were analyzed by the a362 ELISA and the commercial IDEXX ELISA. The cutoff value was established as described above for the a362 ELISA and according to the manufacturer's recommendations for the IDEXX ELISA. In Table 1, our results are compared with CF test, AGID test, and Allied, UW, and CSL ELISA data from the U.S. National Repository. The UW ELISA was a test performed at the University of Wisconsin by using an antigen complex sold by Allied (8a). Some 57% of the 30 reference serum samples were positive by the CF test, 53% were positive by the AGID test, 87% were positive by the Allied ELISA, 80% were positive by the IDEXX ELISA, 80% were positive by the UW ELISA, 77% were positive by the CSL ELISA, and 70% were positive by the a362 ELISA.

Application of the a362 ELISA to herds with healthy, tuberculous, and paratuberculous animals. The serological test based on the a362 recombinant polypeptide was applied to sera from five herds of cattle: two herds of healthy animals (herds H1 and H2), animals from a herd with tuberculosis (herd T), and animals from two herds with paratuberculosis (herds P1 and P2). The five bovine serum samples chosen as standards in the first part of the work were added in each test to establish the cutoff value. Herds with paratuberculous animals yielded a much higher percentage of positive results (mean of positive results in herds P1 and P2, 40.8%) than those without paratuberculous animals (mean of positive results in herds H1, H2, and T, 5.1%) (Table 2). From the percentage of animals in herds H1, H2, and T that tested false positive, the specificity of the a362 ELISA with respect to healthy animals was found to be 95.0%, and the species specificity with respect to tuberculous animals was 94.7%.

The Gaussian distributions of the ELISA absorbance values of sera from herds H1, P2, and T were clearly different (Fig. 2). In the case of the healthy (Fig. 2A) and tuberculous (Fig. 2C) populations, the distribution of anti-a362 Ig followed a monomodal model, whereas a bimodal distribution was found for the herd with paratuberculous animals (Fig. 2B); 58% of the serum samples displayed a low mean absorbance value and 42% displayed a higher value (Table 2). Similar distributions with respect to disease status were found for the other herds that were studied (data not shown).

TABLE 1. Comparative serological analysis of paratuberculous cattle

Animal[a]	Serological test result[b]						
	CF test	AGID test	Allied ELISA	UW ELISA	CSL ELISA	IDEXX ELISA	a362 ELISA
103D9	+	+	+	+	+	+	+
107D9	+	+	+	+	+	+	+
109D9	+	−	+	−	+	+	−
120D9	+	+	+	+	+	+	+
201D9	−	−	+	+	−	−	−
205D9	+	−	+	+	+	+	+
207D9	−	−	+	+	−	−	−
208D9	+	+	+	+	+	+	+
211D9	−	+	−	+	+	+	+
217D9	−	−	+	+	−	+	−
225D9	−	−	−	−	−	−	−
227D9	+	+	+	+	+	+	+
233D9	−	−	−	−	−	−	−
236D9	−	−	−	−	−	−	−
301D9	+	+	+	+	+	+	+
349D9	−	−	+	−	+	+	−
122F9	+	+	+	+	+	+	+
125F9	+	+	+	+	+	+	+
126F9	+	+	+	+	+	+	+
129F9	+	+	+	+	+	+	+
132F9	+	+	+	+	+	+	+
136F9	+	+	+	+	+	+	+
138F9	+	+	+	+	+	+	+
159F9	−	−	+	+	+	+	+
164F9	+	+	+	+	+	+	+
177F9	−	−	+	−	−	−	−
190F9	−	−	+	+	+	+	+
191F9	−	−	+	+	+	+	+
199F9	−	−	+	+	+	+	+
200F9	+	+	+	+	+	+	+

[a] Accession number of the U.S. National Repository for Paratuberculosis Specimens.

[b] CF test, AGID test, Allied ELISA, UW ELISA, and CSL ELISA data are from the U.S. National Repository for Paratuberculosis specimens (8a). IDEXX ELISA and a362 ELISA data are from the present study. Results are considered positive or negative, as described in the text for the a362 ELISA and according to the manufacturer's instructions for the other tests.

DISCUSSION

The available serological tests for Johne's disease are based on crude or partly purified cell components. They invariably lack species specificity because of strong cross-reactions among the corresponding antigens of related mycobacterial species. This is the case for the commercially available diagnostic tests used in the present work for comparison with the a362 ELISA and for the A36 ELISA that we have described previously (11). Recombinant proteins carrying species-specific B-cell epitopes would be the appropriate reagents for species-restricted ELISA. However, at present, only one *M. paratuberculosis*

TABLE 2. Characteristics of the bovine herds analyzed

Herd	Disease status	No. of animals	% a362 ELISA positivity[a]
P1	Paratuberculosis	139	39.6
P2	Paratuberculosis	69	42.0
H1	Healthy	110	5.5
H2	Healthy	65	4.6
T	Tuberculosis	38	5.3

[a] Percentage of positive results with respect to the reference sera, as described in the text.

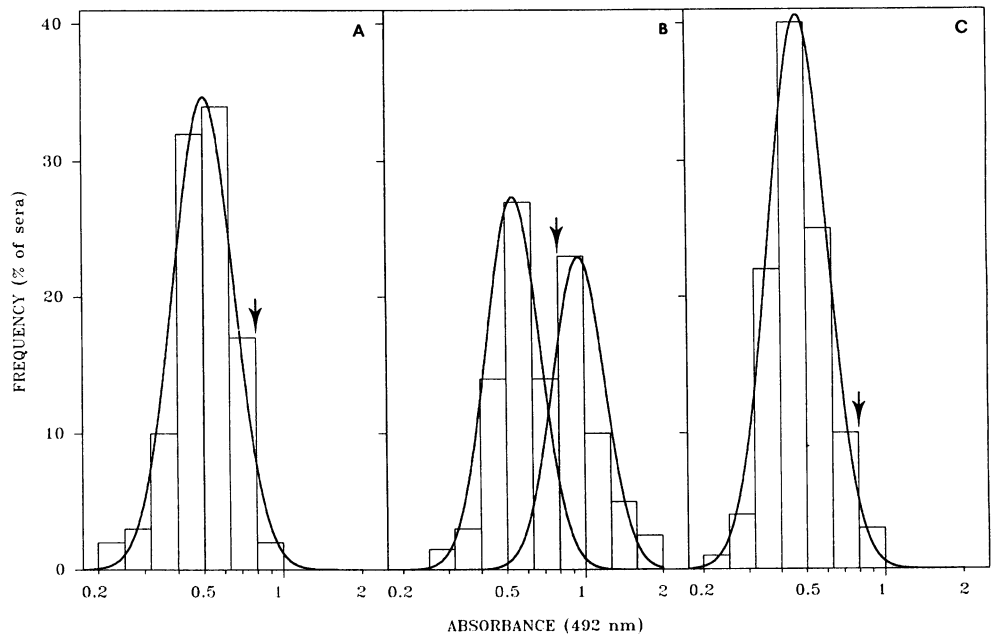

FIG. 2. Comparative distribution of anti-a362 Ig titers in healthy, paratuberculous, and tuberculous herds. Sera from healthy cattle (herd H1; $n = 110$) (A), a herd with paratuberculous animals (herd P2; $n = 69$) (B), and a herd with tuberculous animals (herd T; $n = 38$) (C) were submitted to the a362 ELISA. Sera were distributed in frequency classes of 0.10 logarithmic unit of optical density (A_{492}) (rectangles), and the Gaussian curves that best fit the experimental data were computed (continuous lines). The arrows indicate the mean absorbance ELISA value of the five serum samples selected as standard samples (corresponding to the arrow in Fig. 1), which were added in each experiment to determine the cutoff value of the test.

polypeptide (a362) containing specific B-cell epitopes has been obtained by genetic engineering (12, 17). This a362 polypeptide, produced in a recombinant *Escherichia coli* strain and devoid of cross-reacting mycobacterial components, was the reagent of the ELISA used in the present work to analyze paratuberculous cattle.

The cutoff value, which is an essential parameter for interpretation of serological data, is usually determined by testing a large number of samples from healthy individuals. However, this uninfected population must be identified by independent criteria, such as established laboratory tests and clinical symptoms (which are lacking at the initial stage of the disease). Parker and coworkers (24) have recently developed a mathematical model to determine cutoff values from serological data for individuals of uncertain immunological status. By this method, we found two distinct populations in the 300 randomly sampled cattle of unknown immunological status and representative of a large geographical region in Belgium. One population with a low level of anti-a362 antibodies corresponded to 88% of the tested animals, whereas a second population with a higher level of antibodies corresponded to 12% of the animals tested (Fig. 1). A similar two-population pattern was also found in the herd with paratuberculous cattle (Fig. 2B), but not in the herds with healthy (Fig. 2A) or tuberculous (Fig. 2C) cattle. We infer, therefore, that the population with a high level of anti-a362 antibodies (Fig. 1) contained paratuberculous cattle displaying a specific immunological response to the a362 polypeptide. From the data in Fig. 1, the seroprevalence of paratuberculosis in Belgium was found to be 12%, a value close to those estimated by other investigators for different countries (5, 6). The cutoff value for the a362 ELISA was computed from our results, according to the formula reported in Materials and Methods, which also takes into account the effect of disease prevalence on the frequency of false-positive and false-negative results and the economic consequences of a misdiagnosis (28). In fact, if the ELISA is used as a screening test in an eradication plan for paratuberculosis, the cost of a false-negative result will be greater than the cost of a false-positive result.

In Table 1, the performance of the a362 ELISA was compared with those of several commercial immunoassays. This was done by submitting some 30 serum samples from the U.S. National Repository for Paratuberculosis Specimens to the a362 ELISA. These samples have been analyzed previously in a reference center (M. Collins, University of Wisconsin) by several assays including the CSL ELISA (corresponding to the IDEXX ELISA). With the exception of serum sample 217D9, similar results were obtained by the CSL ELISA (U.S. National Repository data) and the IDEXX ELISA (used in the present study as a control). From the data in Table 1, the sensitivity of the a362 ELISA was estimated to be 70%, a value likely to be much too high. The sensitivity levels that we computed for the commercial immunoassays were also higher than those estimated by other investigators in larger surveys. In fact, the sensitivities of the CF test, the AGID test, the Allied ELISA, and the CSL ELISA were claimed to be 38.4, 26.6, 58.8, and 43.4%, respectively, whereas the corresponding specificities were 99.0, 100.0, 95.4, and 99.0% (10, 31). The higher values obtained in our study were due to the fact that they are restricted to a few samples within a large collection of positive reference sera. However, the aim of this part of our work was merely to compare the performances of different diagnostic assays on a given number of serum samples from *M. paratuberculosis*-infected cattle. Since the same samples were analyzed by all of the assays, the relative value of each test can be established.

On the other hand, the prevalence of antibodies to peptide a362 in the herds with paratuberculous animals was 40.8%. This value is unlikely to correspond to the true sensitivity of the a362 ELISA, because not all animals from these herds were confirmed to be infected with *M. paratuberculosis*. The sensitivity of the a362 ELISA, based on a recombinant product, must thus be in the range of 40.8 to 70%, which was comparable to those of the other ELISAs, which are based on natural antigens or mixtures of mycobacterial components (31). This conclusion agrees with the previously observed immunodominance of the a362 polypeptide in Johne's disease (11, 12).

The serological tests previously developed for paratuberculosis were based on crude or partly purified antigens from *M. paratuberculosis*. To avoid large numbers of false-positive results, a step of preabsorption of sera on *M. phlei* sonicates was introduced to remove the cross-reacting antibodies. However, we have shown (11) that 96% of B-cell epitopes of the antigen complex A36 from *M. paratuberculosis* are common with those of *M. avium* and *M. bovis*, whereas only 45% of epitopes of A36 are common with those of *M. phlei*. After absorption with *M. phlei*, serum samples might still contain antibodies directed against *M. avium* or *M. bovis*. These unabsorbed antibodies might yield false-positive results in serological tests for paratuberculosis. The advantage of the a362 polypeptide is its specificity with respect to the other mycobacterial species (especially *M. bovis*, as indicated in Fig. 2C). A preabsorption step with *M. phlei* sonicates is thus unnecessary for the a362 ELISA.

According to the data in Table 2, the specificity of the a362 ELISA, with respect to both healthy and tuberculous animals, is 95%. It is possible that the small number of healthy or tuberculous cattle detected by the a362 ELISA were infected with *M. paratuberculosis* (animals that had recovered or that were at an early asymptomatic stage of the disease). Note that cattle with mixed *M. paratuberculosis* and *M. bovis* infections have been described previously (13). Because of its high species specificity, the a362 ELISA can thus be used in regions with endemic tuberculosis.

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