Evaluation of Enzyme-Linked Immunosorbent Assay in Comparison with Complement Fixation Test for the Diagnosis of Subclinical Paratuberculosis in Cattle

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(Received 12 October 1990/Accepted 2 April 1991)

ABSTRACT. An enzyme-linked immunosorbent assay (ELISA) was evaluated and compared in parallel with the standard complement fixation test (CFT) for the diagnosis of bovine subclinical paratuberculosis. Bovine sera preabsorbed with the mixture of *Mycobacterium phlei* and kaolin suspension were assayed for antibody activities to the crude protoplasmic antigen of *Mycobacterium paratuberculosis* in the ELISA. ELISA antibody titer was expressed as ELISA antibody index (EAI) value: EAI = (At - An) / (Ap - An), where At, Ap and An are the absorbance values of a 1:200 dilution of unknown test sera, a 1:400 dilution of positive control serum, and a 1:200 dilution of negative control serum. An EAI of 0.6 or greater was established as a reasonable cutoff point for a positive antibody titer by ELISA. Of the 156 sera from cattle with subclinical *M. paratuberculosis*-infection, 106 (67.9%) were positive by ELISA and 41 (26.3%) by CFT. Of the 3,880 sera from cattle in the herds which had no history or evidence of paratuberculosis, 3,875 (99.9%) were negative by ELISA, and 3,787 (97.6%) by CFT. Positive ELISA titers were detectable 1 to 5 months earlier than positive CFT titers in experimentally infected cattle, and 7 to 10 months earlier in naturally infected cattle. These results indicate that the ELISA should replace the CFT as the routine test of choice for the diagnosis of bovine paratuberculosis.—KEY WORDS: cattle, diagnosis, paratuberculosis.

J. Vet. Med. Sci. 53(4): 577–584, 1991

Paratuberculosis is an insidious infectious disease of ruminants caused by *Mycobacterium paratuberculosis*. Cattle with clinical paratuberculosis are easily recognized by the occurrence of chronic, intermittent diarrhea accompanied by severe weight loss and debilitation. In most herds affected with the disease, the incidence of subclinically infected animals usually exceeds that of clinical cases. Identification of subclinically infected animals will be important for successful control, because animals which are shedding the organisms in their feces, but are not clinically ill, may serve as dangerous reservoirs of new infection in the herd.

Complement fixation test (CFT) has been applied widely as the standard test for serological diagnosis of bovine paratuberculosis [10, 13, 15] and it is known as a valuable aid for identifying clinical cases [7, 15]. However, this test proved to be unsatisfactory for detecting subclinically infected cattle, due to the high proportion of false-positive and false-negative results [1, 4, 14, 16]. Isolation of *M. paratuberculosis* from fecal samples is a reliable method for antemortem diagnosis of paratuberculosis, but this is extremely time consuming, usually requiring 8 to 12 weeks for the identification of the organism by its cultural properties [6, 11] .

Therefore, alternative methods for the rapid and specific diagnosis of bovine paratuberculosis are obviously needed. A variety of serological methods have been evaluated in comparison with the conventional CFT. However, most of them lacked sufficient specificity and sensitivity to render them clinically useful as a diagnostic tool of subclinical paratuberculosis in cattle [6].

Using an enzyme-linked immunosorbent assay (ELISA), we previously reported a significant increase in IgG1 antibodies to crude protoplasmic antigen of *M. paratuberculosis* in sera from paratuberculous cattle [18]. Furthermore, the specificity of the ELISA could be improved significantly by absorption of test bovine sera with heat-killed organisms of *Mycobacterium phlei* [20]. This finding has been supported by other researchers [3, 9, 11, 12, 17].

Thus, the ELISA might serve as a promising alternative to the CFT, if the procedure was standardized and the diagnostic reliability was evaluated on the proper scale in field studies. The object of the present investigation was to standardize the procedure of the ELISA and to evaluate the relative usefulness of the ELISA and CFT for identifying subclinically infected cattle.

MATERIALS AND METHODS

Serum specimens: Sera were obtained from 156

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cattle over 15 months of age which were identified as infected with *M. paratuberculosis* by bacteriological culture of feces in 2 herds of Japanese black beef cattle, 1 herd of Japanese brown beef cattle and 2 herds of Holstein-Friesian cows. All the infected cattle had no overt clinical signs of paratuberculosis at the time of serum sampling. Two hundred other sera of fecal culture-negative cattle were also collected from 2 Japanese black beef herds which had no history or evidence of paratuberculosis infection. In addition, sera were obtained from several other sources, as follows: (1) 3,805 apparently healthy cattle over 13 months of age in 4 beef and 15 dairy herds which had no history or evidence of paratuberculosis infection. All the cattle were negative for tuberculin skin test within the previous year. (2) 75 dairy cows over 25 months of age, which were slaughtered due to positive tuberculin skin test results, but were negative for subsequent bacteriological culture and had no demonstrable lesions consistent with tuberculosis. All the animals were from herds which had no history or evidence of paratuberculosis infection. (3) 4 calves infected experimentally as described below. (4) 3 Japanese black beef cattle approximately 2 years of age purchased as naturally infected animals which were identified by isolation of *M. paratuberculosis* from feces.

Experimental infection: Four Japanese black calves in this experiment were natural offspring of cows from herds without a history of paratuberculosis. One- to three-day-old male calves were inoculated by gastric tube with 50 m*l* of dairy milk containing 200 mg (wet wt.) of live *M. paratuberculosis* strain J-3. Serum and fecal samples were collected at one-month intervals for 11 to 13 months after the inoculation. These animals were positive for bacterial isolation of *M. paratuberculosis* from feces at 2 to 3 months after the inoculation and remained so throughout the study, though without clinical signs.

Bacterial culture of feces: Fecal samples were collected directly from rectum at the time of blood sample collection. Each sample was treated with 0.75% hexadecylpyridinium chloride solution at 25°C for 16 hr, and the deposit sown on to three slopes of Herrold's egg yolk medium containing mycobactin. The samples were incubated at 37°C and examined for the presence of *M. paratuberculosis* every four weeks for up to 12 weeks. Identification was based on acid fast staining, colonial morphology and mycobactin dependence.

Antigen used for ELISA: The antigen used for ELISA was prepared from live cells of *M. paratuberculosis* strain P-18 as previously described [18]. Briefly, the bacterial pellets were suspended in distilled water and ruptured in a refrigerated French press (Aminco, Urbana, Ill, U.S.A.) at 35,000 $1b/in^2$. The disrupted cell suspension was centrifuged 50,000 × g for 2 hr to remove any unbroken cells. The pooled supernatant was added with ammonium sulfate to 40% saturation. The resulting precipitate was harvested, dialyzed against distilled water for 2 days and then lyophilized. The resulting powder was designated as crude protoplasmic (CP) antigen. Sixty milligram of the lyophilized preparations were reconstituted with 150 m*l* of distilled water containing 0.1% NaN_3 and each lot was passed through 0.22 μm membrane filter. The antigen solution was dispensed in 1 m*l* quantities into 5 m*l* glass ampules and lyophilized.

M. phlei-absorbent: Strain ATCC 354 of *M. phlei* was grown in nutrient broth (Eiken Chemical Co., Ltd., Tokyo, Japan) with 2% glycerin in a Roux bottle at 37°C for 3 weeks. The culture was heated at 90°C for 30 min, harvested by pouring the culture over filter paper, and washed 5 times with sterilized distilled water. Bacterial pellets of 700 g (wet wt.) were suspended in 2,500 m*l* of distilled water, dispensed in 15 m*l* quantities into 100 m*l* glass vials and lyophilized.

Absorption of bovine serum: In our preliminary tests, several sera of adult cattle obtained from paratuberculosis-free herds were recognized to yield false-positive results in the ELISA test performed by the method described previously [20]. Subsequently, those false-positive reactions were found to be reduced to the level of negative range by dual absorption of the sera with 10% kaolin (18) and *M. phlei* suspension. For this reason, in the present experiments, preabsorption of all test sera was carried out with the following modification using the mixed suspension of 10% kaolin and *M. phlei* instead of *M. phlei* suspension. The lyophilized *M. phlei* in a vial was reconstituted with 60 m*l* PBS containing 0.1% NaN_3. The *M. phlei* suspension (70 mg wet wt./m*l*) was mixed with an equal volume of 10% (w/v) kaolin suspension in PBS, and was used as absorbent. A 25 μl of each test bovine serum or of control sera was mixed with 975 μl of the absorbent, and shaken for 30 min at room temperature. The mixture was centrifuged for 3 min at 1,000 rpm or

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allowed to stand at 4°C overnight. This absorption step had no effect on the sensitivity of the ELISA test.

Preparation of ELISA reagents and storage: Bovine IgG1, IgG2 and IgM were prepared as previously described [19]. Antisera against bovine IgG1 were prepared in outbred rabbits (New Zealand White). The antisera were raised by three intramuscular injections of the bovine IgG1 (1 mg/m*l*) emulsified in Freund's incomplete adjuvant containing 0.01% (w/v) muramyl dipeptide at 3 weeks intervals. Rabbits were exsanguinated on days 60, and the sera were pooled for rabbit hyperimmune sera to bovine IgG1. The serum was absorbed with glutaraldehyde insolubilized IgG2 (10 mg wet wt.) to render subisotype-specificity as previously described [19]. After centrifugation at 10,000 × g for 1 hr, the supernatant was diluted 1:10 in PBS containing 3% bovine serum albumin, fraction V (Sigma Chemical Co., St. Louis, Mo., U.S.A.) (PBS-BSA) containing 0.1% NaN_3. The specificity of antiserum to IgG1 was determined in the gel diffusion and immunoelectrophoresis by a procedure described by Duncan *et al.* [8]. The preparations of the rabbit anti-bovine IgG1 serum did not react with the IgG2 and IgM, but reacted with IgG1 in the tests.

Affinity purified goat anti-rabbit IgG peroxidase conjugate (Kirkegaard & Perry Laboratories Inc., Gaithersburg, Md., U.S.A.) was used. One milligram of lyophilized conjugate was reconstituted with 100 m*l* PBS-BSA containing 0.01% thimerosal, and dispensed in 0.5 m*l* quantities into 5 m*l* plastic tubes. The stock preparations of rabbit anti-bovine IgG1 antiserum and conjugate were stored at −70°C and diluted just before use.

Control sera for the ELISA test: A positive control serum was obtained from an experimentally infected cow. The cow had been inoculated intravenously with 100 mg (wet wt.) of live *M. paratuberculosis* ATCC 19698 at 30 days of age and exsanguinated at 18 months post-infection when the CF antibody titer of serum reached 1:80. The cow was shedding large number of *M. paratuberculosis* bacilli in the feces over extended periods of time. A negative control serum consisted of pooled serum from 500 cattle obtained from dairy herds which had no history or evidence of paratuberculosis. The preparations of sera were dispensed in 0.5 m*l* quantities into 2 m*l* glass ampules and lyophilized. An EAI value (see below) of the positive control serum diluted 1:1,600 was calculated as 0.6. The absorbance value (see below) of the negative control serum was calculated as 0.08. Those values of the control sera did not show a significant variation during a period of at least 15 months storage at 4°C.

ELISA procedure: Ninety-six-well flat bottomed microtitration polystyrene plates (Immunoplate I: Nunc, Oskitde, Denmark) were used. Each well was coated with 200 μl of appropriate concentration of CP antigen (see below) in 0.05 M carbonate buffer (pH 9.6) containing 0.001% Tween 80 and 0.05% NaN_3 (coating buffer) and stored overnight at 4°C. The plates were then washed three times with PBS containing 0.05% Tween 80 (washing buffer). Thereafter, 300 μl of PBS containing 0.2% gelatin and 0.1% NaN_3 were added to each well. After an incubation of 1 hr at 25°C, the plates were washed twice for immediate use. For assays of IgG1 antibody activity, the 1:40 dilution of the supernatant of the absorbed bovine sera, as mentioned above, was diluted 1:5 (at a final dilution of 1:200) with phosphate buffer (pH 7.2) containing 1 M NaCl, 0.2% gelatin and 0.1% Tween 80 (ELISA-PBS). Each serum was diluted in triplicate wells of microtitration plate which was not coated with antigen. Volumes of 200 μl of the diluted serum were transferred to antigen coated plates with an eight-channel dispenser. After 2 hr-incubation at 25°C, the plates were washed three times and 200 μl of rabbit anti-bovine IgG1 antiserum diluted appropriately (see below) in ELISA-PBS were added to each well. After 2 hr-incubation at 25°C, the plates were washed as indicated above and 200 μl of conjugate diluted appropriately (see below) in ELISA-PBS was added. After 2 hr-incubation at 25°C, the plates were washed four times, and 100 μl of substrate solution containing 2 mM hydrogenperoxide and 0.4 mM 2,2,-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in 0.05 M citrate buffer (pH 4.0) was added. After 30 min-incubation at 25°C, 50 μl of 5% sodium dodecyle sulfate solution were added to stop the color reactions. The absorbance value was read at 415 nm and 492 nm on a dual wavelength with a model MTP-22 microplate reader (Corona Electric Co., Ltd., Ibaraki, Japan). Each test was accompanied by the following controls: a 1:200 dilution of negative control serum, a 1:400 and 1:1,600 dilution of positive control serum.

Computer interface and calculations: The absorb-

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ance value-reading was transmitted directly to a model PC-9801 microcomputer (NEC, Tokyo, Japan) for data storage and rapid data processing, where the mean absorbance reading from a sample tested in triplicate was calculated and processed by the program selected. If the coefficients of variation of the absorbance values exceeded 15%, the value was flagged to alert the operator that the samples should be retested. The computer was programmed to calculate ELISA antibody index (EAI) values: EAI =(At - An) / (Ap - An), where At, Ap and An are the absorbance values of a 1:200 dilution of unknown test sera, a 1:400 dilution of a positive control serum and a 1:200 dilution of a negatiave control serum, respectively. To minimize the influence of day-to-day fluctuation of EAI values on the results, a positive control serum diluted 1:1,600 was tested alongside unknown test sera on the same plate and the EAI value was corrected as follows. The expected EAI of 0.60 (mean value of EAI of a 1:1,600 dilution of the positive control serum obtained in 10 experiments over a period of 10 days) was divided by the observed EAI (on test day) of the same dilution of the positive control serum to obtain a correction factor (COF). After determining the COF, all EAI values for all sera tested on that same day were corrected by multiplying with the COF. These calculations were also performed using the computer program. A value of EAI was rounded off in numbers to the first decimal place.

Determination of optimum assay conditions: Optimum concentration of CP antigen and optimum dilutions of anti-bovine IgG1 and conjugate were determined by checkerboard titration. The procedure was virtually identical to that described for ELISA procedure, with the exception of the dilution of positive control serum used in the test for the determination of antigen concentration. Each well of the microplates for ELISA test was filled with 200 μl of CP antigen solution (1.25, 2.5, 5, 10, 20, 40, 80 μg of coating buffer per ml) and was tested against a 1:3,200 dilution of the positive control serum and a 1:200 dilution of the negative control serum. Rabbit anti-bovine IgG1 antiserum and conjugate were used at a 1:200 (1 unit/ml; see below) dilution of stock preparations. An antigen concentration which gave the best separation between the absorbance value of positive and negative control sera was defined as one unit per ml (data not shown). Two units per ml of CP antigen was used in ELISA tests. The optimum concentration of the antigen preparation did not show a significant variation between lots during a period of at least 15 months storage in lyophilized condition.

To determine a suitable working dilution for rabbit anti-bovine IgG1 antiserum and conjugate, various dilutions of them and a constant amount of antigen (2 units/ml), a 1:400 and 1:1,600 dilution of the positive control serum, a 1:200 dilution of the negative control serum were used in the test. Five dilutions were run (1:100, 1:200, 1:300, 1:400, 1:500), and the highest dilution yielding an EAI of an approximately 0.6 for a 1:1,600 dilution of the positive control serum was defined as one unit per ml. One unit per ml was adopted as a working concentration for the stock preparations of rabbit anti-bovine IgG1 antiserum and the conjugate in ELISA tests. The optimum dilution of both reagents did not show a significant variation between lots during a period of at least 15 months storage at -70°C.

Complement fixation test (CFT): Phenol water extracted polysaccharide antigen of *M. paratuberculosis* strain TEPS [22] was used as the antigen for the CFT. Antibody titers were assayed by a method previously described [22], and sera giving complete fixation of complement at a dilution of 1:10 or more were regarded as positive.

RESULTS

Assay reproducibility: Reproducibility of the ELISA was determined by using the positive control serum. Interassay variability of the EAI value was assessed by using 6 assays performed over a 15 month period and was found to have a coefficient of variation ranging from 5.6% to 6.7%.

Determination of cutoff for positive ELISA test: The distribution of the values of EAI by the ELISA in 156 fecal culture-positive and 200 culture-negative cattle is shown in Table 1. For this analysis, cattle with clinical signs of paratuberculosis were excluded. The EAI values of all the fecal culture-negative animals were of $\leqq 0.2$. On the basis of these results, a tentative assumption was made that an EAI of $\geqq 0.3$ should be defined as positive and an EAI of $\leqq 0.2$ as negative. This positive/negative threshold could classify correctly 126 (80.8%) of the 156 culture positive animals.

The validity of this assumption on this baseline was examined by analysis of specificity of the ELISA using the other sets of sera. Results similar to those

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Table 1. Sensitivity of ELISA compared with CF test (CFT) in sera of 156 cattle with positive fecal culture and 200 cattle with negative fecal culture of *M. paratuberculosis*

Fecal culture	No. of cattle	No. of positive cattle (%)						
		Cutoff value in ELISA						CFT
		0.1[a]	0.2	0.3	0.4	0.5	0.6	
+++[b]	78	78 (100)	78 (100)	75 (96.2)	75 (96.2)	73 (93.6)	66 (84.6)	32 (41.0)
++	45	42 (93.3)	39 (86.7)	32 (71.1)	28 (62.2)	28 (62.2)	26 (57.8)	8 (17.8)
+	33	29 (87.9)	24 (72.7)	19 (57.6)	17 (51.5)	15 (45.5)	14 (42.4)	1 (3.0)
+++~+	156	149 (95.5)	141 (90.4)	126 (80.8)	120 (76.9)	116 (74.4)	106 (67.9)	41 (26.3)
−	200	25 (12.5)	1 (0.5)	0	0	0	0	0

a) ELISA antibody index = At − An/Ap − An where At, Ap and An are the absorbance values of a 1:200 dilution of unknown test sera, a 1:400 dilution of a positive control serum and a 1:200 dilution of negative control serum, respectively.
b) + = 1~4 colonies of *M. paratuberculosis* per agar slant, ++ = 5~49 colonies, +++ = ≧50 colonies.

Table 2. Specificity of ELISA compared with CF test (CFT) in 2 groups of cattle from paratuberculosis noninfected herds

Cattle group	Tuberculin test	No. of cattle	No. of negative cattle (%)				
			Cutoff value in ELISA				CFT
			0.3[a]	0.4	0.5	0.6	
1	Negative	3,805	3,770 (99.1)	3,789 (99.6)	3,794 (99.7)	3,803 (99.9)	3,749 (98.5)
2	Positive	75	43 (57.3)	63 (84.0)	68 (90.7)	72 (96.0)	38 (50.7)
Total		3,880	3,813 (98.3)	3,852 (99.3)	3,862 (99.5)	3,875 (99.9)	3,787 (97.6)

a) ELISA antibody index: Refer to the footnote in Table 1.

Table 3. Response of ELISA and CF antibodies in cattle experimentally infected with *M. paratuberculosis*

Animal No.	Test	Months after inoculation											
		1	2	3	4	5	6	7	8	9	10	11	12
1	ELISA	0.1[a]	0.1	0.1	0.1	0.1	0.1	1.2	0.9	1.1	1.1	1.1	1.1
	CF	—	—	—	—	—	—	—	—	—	—	—	10[b]
2	ELISA	0.1	0.1	0.1	0.1	0.1	0.1	0.3	0.8	0.9	1.1	1.1	0.8
	CF	—	—	—	—	—	—	—	—	20	20	20	10
3	ELISA	0.1	0.1	0.1	0.1	0.1	0.1	0.7	1.1	1.1	1.0	1.1	0.8
	CF	—	—	—	—	—	—	—	10	10	5	10	—
4	ELISA	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	1.1	1.1	1.2	1.1
	CF	—	—	—	—	—	—	—	—	—	20	20	10

a) ELISA antibody index: Refer to the footnote in Table 1. The index of ≧0.6 was regarded as positive.
b) CF antibody titer; The titer of ≧10 was regarded as positive.

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Table 4. Response of ELISA and CF antibodies in cattle naturally infected with *M. paratuberculosis*

Animal No.	Test	Months for observation													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
501	ELISA	0.2[a)]	0.3	0.4	0.6	0.7	0.7	0.7	0.7	0.9	1.0	0.9	1.0	1.0	1.0
	CF	—	—	—	—	—	—	—	—	—	—	—	—	—	10[b)]
502	ELISA	0.5	0.6	0.6	0.7	0.8	0.8	0.8	0.9	1.0	1.0	1.1	1.0	1.0	1.0
	CF	—	—	—	—	—	—	—	—	10	5	5	—	10	10
503	ELISA	0.3	0.2	0.5	0.5	0.5	0.6	0.8	0.9	0.9	0.8	0.9	0.9	0.7	0.7
	CF	—	—	—	—	—	5	—	—	5	—	—	5	10	5

a) ELISA antibody index: Refer to the footnote in Table 1. The index of ≧0.6 was regarded as positive.
b) CF antibody titer: Refer to the footnote in Table 3.

described above were obtained with the tuberculin-negative 3,805 healthy cattle from noninfected herds as shown in Table 2. However, greater variation in ELISA antibody levels was encountered in the sera from the group of tuberculin-positive cattle: among the 75 sera, 29 (39%) had EAI values ranging from 0.3 to 0.5 and 3 (4%) had an EAI value of ≧0.6. From the practical point of view, the EAI value of 0.6 was chosen as a more stringent cutoff point. An ideally high specificity of 99.9% was recorded for the cutoff point in a total of 3,880 cattle from both groups 1 and 2. By moving the cutoff point from 0.3 up to 0.6, the sensitivity of the ELISA was reduced from 80.8% to 67.9% as shown in Table 1. The sensitivity of ELISA was influenced by the intensity of fecal bacterial shedding and was higher in animals with high level of fecal shedding. The CFT showed a sensitivity of 26.3% and a specificity of 97.6% as shown in Tables 1 and 2.

Seroconversion in experimentally and naturally infected cattle: Positive ELISA titers were detected as positive in the sera from 4 experimentally infected cattle at 7 to 9 months postinfection. Seroconversion of the infected animals was detectable with the ELISA 1 to 5 months earlier than with CFT as shown in Table 3. Similar pattern of seroconversions was found in naturally infected cattle. ELISA antibodies were detected as positive 7 to 10 months earlier than CF antibodies (Table 4). ELISA antibody levels tended to remain at positive level more consistently than CF antibodies in both experimentally and naturally infected cattle.

DISCUSSION

The study presents the data on the development of the ELISA test as a practical diagnostic tool for subclinical bovine paratuberculosis. A very important part of developing a practical serodiagnostic test is setting the criteria for determining an upper limit of negative value and a lower limit of positive value. In our preliminary report [20], positive was defined as an absorbance value equal to or greater than twice the absorbance value obtained with the negative control serum. However, with this criteria, day-to-day variation occurred with the low levels of antibody, aging of the tracer, change in room temperature and other alterations in experimental conditions. The assay used in the present study was standardized by comparing the absorbance value of the test sera to that of negative and positive control sera and by expressing the antibody titer as EAI. The standardized ELISA test allowed greater reproducibility and solved the problem of intertest variation.

To ascertain the success of the test, sensitivity and specificity values were calculated. Sensitivity considers the number of false-negatives and specificity considers the number of false-positives. Ideally, both sensitivity and specificity of the ELISA should be 90% or above. When the lower limit of positive was set at the EAI of 0.3, sensitivity of the ELISA was 80.8% and specificity was 98.3%. Although the ELISA seems to be more specific than the CFT, 39% of tuberculin-positive cattle gave false-positive results with an EAI values ranging from 0.3 to 0.5 and 4% with an EAI value of ≧0.6. Therefore, we decided that the EAI value of 0.6 should be considered as a lower limit for the positive in our ELISA test. Ideal specificity approaching almost 100% was recorded for this selected cutoff point of 0.6 under the conditions of this experiment, but it reduced sensitivity to 67.9%. Even with this stringent criteria, the ELISA was two fold more sensitive than the CFT. Such increased sensitivity of the ELISA was also confirmed by monitoring seroconversion in experimentally or naturally infected cattle. Although the ELISA had poor sensitivity in

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infected cattle shedding only low numbers of *M. paratuberculosis* (Table 1), it may be possible to detect those animals by repeatedly applying the ELISA. The ELISA test may offer some practical advantages over fecal culture test for routine testing. The most outstanding advantage is the rapidity of obtaining test results. Results by ELISA were available within 36 hours, while the results of culture test required 7 to 15 weeks. The ELISA may therefore be useful to eliminate profuse excretors, and thus help to reduce the weight of environmental contamination.

IgM specific ELISA was found to be very useful in the early and specific diagnosis of human leprosy as demonstrated by Young *et al.* [21]. The ELISA for detecting IgM antibodies to the CP antigen did not seem to improve the sensitivity of the ELISA for paratuberculosis, because the paratuberculous cattle did not show significantly elevated levels of IgM antibody to CP antigen [20]. We used crude antigen in the present assay, because of the favorable results that we and others have previously reported [12, 20]. In contrast to detecting the antibodies to specific antigenic components of *M. paratuberculosis*, we expected that the multiple antigens present in CP antigen would be more likely to detect the presence of antibody. Abbas *et al.* [2] achieved sensitivity of over 80% for paratuberculous cattle without clinical signs, by the ELISA using affinity-purified antigen, but the specificity was 85%. Recently, Camphausen *et al.* [5] reported the isolation of glicopeptide lipid antigen specific to *M. paratuberculosis*. This antigen was highly reactive against hyperimmune bovine serum, but was reactive in only 1 of 9 clinically infected cattle. Therefore, it is likely that the ELISA using these purified antigens is not more effective than our ELISA method using CP antigen.

The results of the present study demonstrate that the ELISA for detecting IgG1 antibody to the CP antigen in bovine sera preabsorbed with the mixture of *M. phlei* and kaolin suspension is more effective than the conventional CFT in the detection of subclinical bovine paratuberculosis. We conclude that the ELISA should replace the CFT as the serological test of choice for diagnosis of bovine paratuberculosis.

ACKNOWLEDGEMENTS. This work was supported in part by a Grant-in-Aid (Bio Media Program) from the Ministry of Agriculture, Forestry and Fisheries in Japan (BMP 91 IV-1-3).

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