Evaluation of an Enzyme-Linked Immunosorbent Assay(ELISA) Using *Mycobacterium phlei* –Absorbed Serum for the Diagnosis of Bovine Paratuberculosis in a Field Study

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Introduction

Paratuberculosis is a chronic gastro-intestinal disease of domestic and wild ruminants caused by *Mycobacterium paratuberculosis*. The disease is transmitted primarily by the fecal-oral route via contaminated feed and water. Besides losses due to emaciation and occasional death of clinically ill animals, subclinically infected animals decrease productivity and increase susceptibility to other infections.[1,7]

In Japan, bovine paratuberculosis was first recorded in 1931. Since then, the incidence of the disease has been limited to a few reports of dairy cattle imported from the United States of America and Canada.[4,14] In 1980 the disease was newly disclosed in the Japanese native beef cattle herds.[17] More recent records of our government regulatory agencies indicated a considerable increase in reports of the disease over a five year period, mostly in the native beef cattle.

Paratuberculosis is designated as a reportable disease in Japan. Clinically ill animals confirmed by the microscopic examination of fecal smears for acid-fast organisms and animals which gave a positive reaction both in the complement-fixation(CF) test[18] and johnin skin test have been condemned with indemnity paid. In the proceeding with these control measures, we have been frequently hampered with a situation in which the reliability of the CF and johnin test may be queried. In epizootiologic investigations also, we encountered quite a few false-positive and false-negative test results.

An enzyme-linked immunosorbent assay (ELISA) for detecting antibodies to *M.paratuberculosis* has been reported,[2,5,11,15] and those results suggested that the technique would have potential to become a valuable diagnostic tool, because of its high sensitivity. Our previous report indicated that the specificity of the ELISA technique could be improved by the selective detection of the IgG1 subclass antibodies to protoplasmic antigen of *M. paratuberculosis*.[15] Furthermore, false-positive reactions could be controlled successfully by an absorption treatment of test bovine sera with heat-killed organisms of *Mycobacterium phlei*.[16]

The objectives of the present study were to test several antigens for ELISA and then to examine their potential for the diagnosis of naturally and experimentally occurring *M. paratuberculosis* infections in cattle. The study includes a comparison of the specificity and sensitivity of the ELISA with the CF test for the detection of paratuberculous cattle.

Materials and methods

1) Serum samples

Sera were obtained from 494 cattle in Japanese

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black beef herd which paratuberculosis was diagnosed in 1982. Blood samples were collected from all cattle 12 months of age or older. Additional sera for the study came from cattle infected experimentally as described below. The specificity of our serologic method was checked with sera obtained from Holstein-Friesian cattle in 3 commercial dairy herds with no history of paratuberculosis. Blood samples were collected from 2 years of age or older.

2) Fecal culture test

Approximately 1 g of fecal specimens was shaken with 25 m*l* of distilled water for 30 min and the straw and heavy particles were allowed to settle for 1 hr. Five m*l* of the supernatant was mixed with 20 m*l* of 0.6% hexadecylpyridinium chloride.[10] After 20 hr at 22°C, 0.2 m*l* portion of the sediment that was formed was transferred to each of 4 slants of Herrold's egg-yolk agar medium, three of which contained J-mycobactin.[9] The samples were incubated at 37°C and examined every 4 weeks for up to 16 weeks for the presence of *M. paratuberculosis*. Identification was based on colonial morphology and mycobactin dependence.

3) Experimental infection

Holstein-Friesian calves were used. All calves were purchased at 3 days of age from local herds. Each animal was given, via stomach tube, 100 mg (wet wt.) of live *M. paratuberculosis* suspended in 50 m*l* of commercial dairy milk at 5 days of age. Blood samples were collected before inoculation and at 4 weeks intervals thereafter. Fecal samples from each animal were collected twice a month for 15 months after the calves were inoculated.

4) Antigen used for ELISA

Crude protoplasmic antigen was prepared from live cells of a culture of *M. paratuberculosis* strain 18 as previously described.[8] The antigens examined included semipurified protoplasmic antigen, johnin purified protein derivative (PPD) and lipopolysaccharide(LPS) antigen[18] of *M. paratuberculosis*.

5) Chromatography of antigen

Crude protoplasmic antigen was applied to a column packed with DEAE-Sepharose 6B. Stepwise elution was performed with increasing molar concentrations of NaCl in 0.1 M tris buffer, pH 8.0. and gave rise 7 peaks (Fig 1). The fractions of each peak were pooled, concentrated and lyophilized.

6) Immunoabsorbent and absorption treatment

M. phlei strain ATCC 354 was grown in Roux bottle at 37°C as a pellicle on broth medium with 2% glycerin for 2 weeks. The pellicle was harvested by pouring the culture over filter paper to collect the organisms, and then was homogenized in high speed blender. The bacterial suspension was

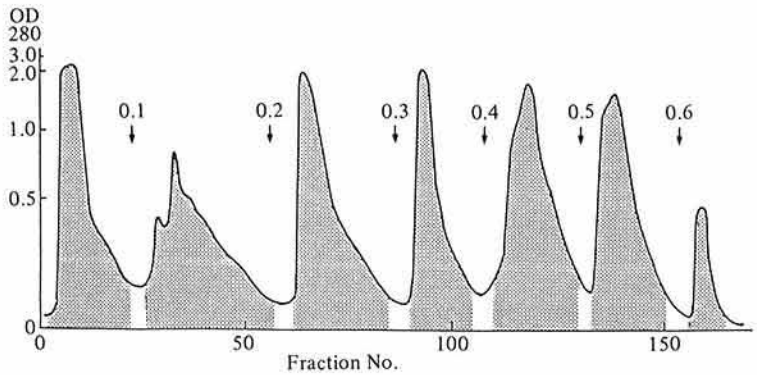

Fig. 1. DEAE-Sepharose ion-exchange column-chromatography of crude protoplasmic antigen of *M. paratuberculosis*
The column was equilibrated with 0.1 M tris buffer, pH 8.0: antigen was dissolved in the same buffer and eluted from the column with tris buffer following the step-wise gradient with 0.1~0.6 M NaCl in 0.1 M tris buffer.

allowed to stand for 3 hr and then the supernatant was decanted. The precipitates were mixed with 10 volume phosphate buffered saline (PBS). After repeating this washing process, the precipitate was suspended in 10 volume of PBS and sodium azide was added to 0.1%.

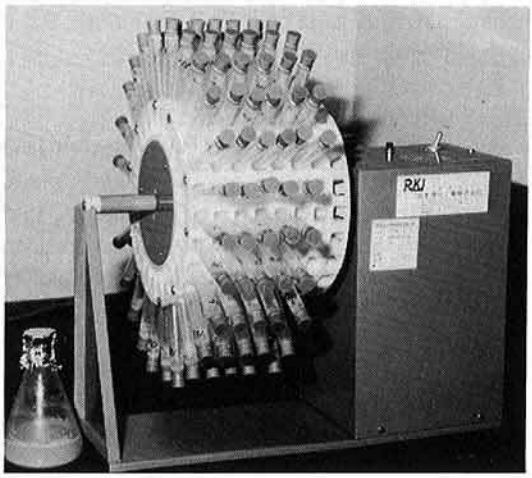

Plate 1. Absorption treatment of bovine serum with a suspension of *M. phlei* prior to ELISA test

A 50 μl sample of each test serum was mixed with 950 μl of the immunosorbent solution of *M. phlei*. Each reaction mixture was allowed to react for 1 hr (Plate 1), then it was centrifuged for 5 min at 1,000 rpm. A 200 μl sample of the supernatant was mixed with PBS containing 0.1% Tween 80, 1 M NaCl and 0.25% gelatin.

7) ELISA procedure

The ELISA was conducted as described previously.[16] The serum was assayed for IgG1 antibody titer against *M. paratuberculosis* antigens. Plate 2 shows a reaction of ELISA in the microtitration plate.

8) CF test

Antibody titers against LPS antigen of *M. para-*

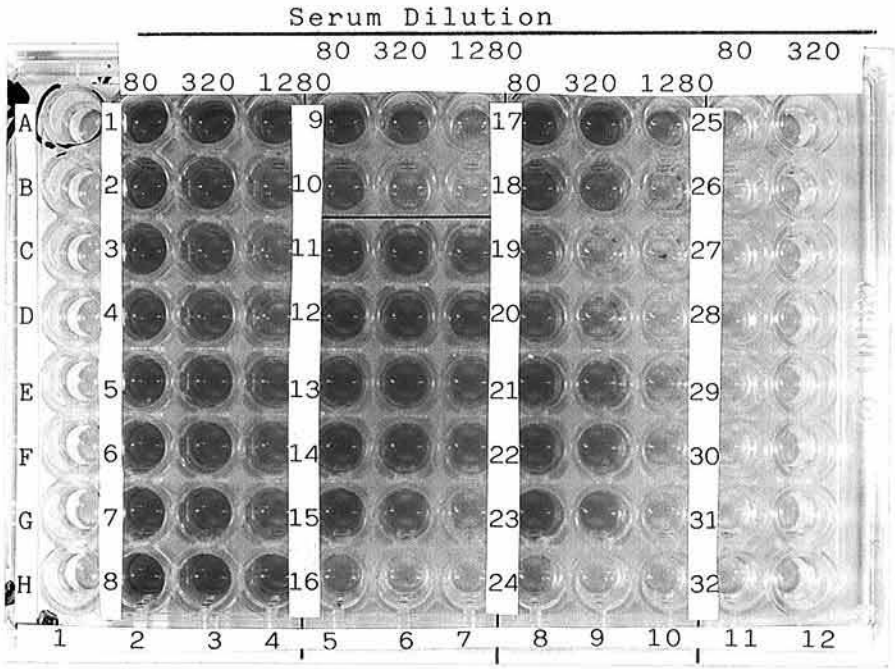

Plate 2. Microtitration plate showing reactions of the ELISA
Well A-1, blank. Seven wells (B~H) of column 1 show the reaction with negative reference serum. Columns 2 through 10 (serum No. 1~24) show the reactions with sera diluted at 1:80 to 1:1,280 of 24 paratuberculous cattle. Columns 11 through 12 (serum No. 25~32) show the reaction with sera diluted at 1:80 to 1:320 from 8 nonparatuberculous cattle.

tuberculosis were assayed by a method previously described.[18]

Results

Determination of optimal dilution of protoplasmic antigen and the fractionated antigens is shown in Fig 2. Results were recorded as optical density(OD) value at 492 nm. The OD value reached higher level with 0.3M fraction of the protoplasmic antigen than other fractions. The 0.3M fraction was used as the semipurified protoplasmic antigen and was named 0.3M-P antigen. Comparison of reliability of the ELISA with 4 different kinds of antigen was examined with sera of cattle collected from the fields. In the first situation, the ELISA antibody was tested with 1,226 sera obtained from 3 herds that had no history of paratuberculosis infection. Fourteen(1.1%) of those sera gave positive results in the test with 0.3M-P antigen. The ELISA with johnin PPD antigen and LPS antigen were positive in much higher percentages of those tested sera (Table 1).

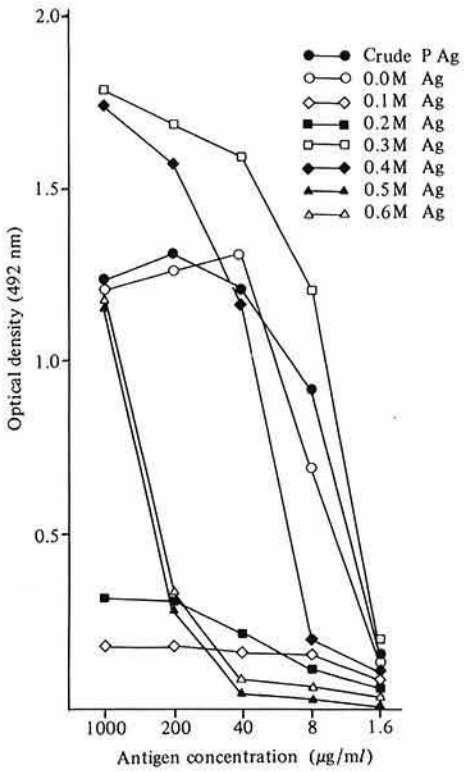

Fig. 2. ELISA titration curves for DEAE-Sepharose-fractionated antigen of crude protoplasmic antigen of *M. paratuberculosis* as obtained with positive standard serum of paratuberculosis cattle

In the second situation, ELISA results were compared with fecal culture tests in the infected herds. Fifty-eight of the 494 tested animals yielded positive results in fecal culture tests. Among these culture positive animals, 38 (66%) were positive in the ELISA with 0.3M-P antigen, 31 (53%) were positive with crude protoplasmic antigen, 25 (43%) were positive with johnin PPD antigen, and 18 (31%) were positive with LPS antigen (Table 2). The results of Tables 1 and 2 indicate that the 0.3M-P antigen is the most promising antigen among the tested antigen preparations. Tests of sera from 20 animals that were fecal culture positive, but ELISA negative showed that 12 animals (60%) seroconverted by 5 months after the first sampling (Table 3). Eventually, the ELISA test identified 50 (86%) animals among 58 fecal culture positive animals at the point when all fecal culture results were available. At necropsy examination, 21 of 31 animals which were positive in the ELISA test using 0.3M-P antigen but negative in fecal culture tests were confirmed as paratuberculosis.

Table 1. Comparison of specificity among ELISA using 0.3M protoplasmic antigen(Ag), crude protoplasmic Ag, johnin PPD Ag, and LPS Ag in 3 paratuberculosis-free herds

Herd	No. of cattle	No. of animals with positive reactions at 1:80 dilution of serum			
		0.3M protoplasmic Ag	Crude protoplasmic Ag	Johnin PPD Ag	LPS Ag
A	429	6 (1.4)*	6 (1.4)	17 (4.0)	13 (3.0)
B	502	5 (1.0)	6 (1.2)	22 (4.4)	8 (1.6)
C	295	3 (1.0)	5 (1.7)	9 (3.0)	18 (6.1)

* Percentage in parenthesis

Table 2. Comparison of sensitivity among ELISA using 0.3M protoplasmic antigen(Ag), crude protoplasmic Ag, johnin PPD Ag, and LPS Ag for detection of *M. paratuberculosis* -infected cattle in a paratuberculosis affected herd

Fecal culture	No. of cattle	0.3M protoplasmic Ag					Crude protoplasmic Ag					Johnin PPD Ag					LPS Ag				
		<80	80	320	1280	Total	<80	80	320	1280	Total	<80	80	320	1280	Total	<80	80	320	1280	Total
+++*	8	0	1	1	6	8(100)**	0	1	1	6	8(100)	1	1	0	6	7(87.5)	1	1	5	1	7(87.5)
++	12	4	1	1	6	8(66.7)	4	1	2	5	8(66.7)	5	1	1	5	7(58.3)	6	1	5	0	6(50.0)
+	38	16	12	5	5	22(57.9)	23	7	5	3	15(39.5)	27	5	4	2	11(28.9)	33	3	2	0	5(13.2)
Total	58	20	13	7	18	38(65.5)	27	9	8	14	31(53.4)	33	7	5	13	25(43.1)	40	5	12	1	18(31.0)
−	436	405	17	10	4	31(7.1)	423	3	10	0	13(3.0)	424	9	3	0	12(2.8)	422	6	8	0	14(3.2)

* +=1~10 colonies of *M. paratuberculosis* per medium slant, ++=11~50 colonies, +++=50~100 colonies.
** Percentage of ELISA positive animals in parenthesis

Table 3. Seroconversion of ELISA antibodies in the cattle showing fecal culture positive but ELISA negative results at the first sampling

Fecal culture	No. of cattle	Cumulated No. of animals with seroconversion in the time of month after first sampling			Positive No.
		3	4	5	
++	4	0	2	2	2 (50)*
+	16	6	9	10	10 (63)
Total	20	6	11	12	12 (60)

* Percentage in parenthesis

Finally, the ELISA test was examined on the sequential sera from 3 experimentally infected calves. The antibody for *M. paratuberculosis* was not detected in any of the animals at the initial period of fecal shedding observed 1 to 4 months after infection (Fig 3). Seroconversion was observed at the later stages when they begun to show the second phase of shedding. The ELISA antibodies preceded the detection of antibodies with the CF test by 1 or 2 months.

Discussion

Program for the control or eradication of *M. paratuberculosis* in an infected herd should be oriented toward the early diagnosis of infected animals, thereby allowing for removal of carriers from the infected herds. The clinical diagnosis of the disease is difficult to establish because of the presence of asymptomatic carriers, which excrete variable numbers of *M. paratuberculosis* organisms.[6] Although the fecal culture test is the most reliable diagnostic test for the detection of subclinical shedders, the prolonged incubation period required for positive culture results allows the infected cattle to remain in the herd, thereby causing contamination of the environment.

Until now, the CF test has been used widely as a routine diagnostic test of bovine paratuberculosis. However, it has been pointed that the test lacks the sensitivity and specificity for the detection of infection in individual cattle.[3,12,13] Our previous report also indicated that quite a few cattle culled on the basis of the CF test were found at necropsy not being infected.[16] Such false-positive reactions are considered to be caused by infection with corynebacteria, nocardia, rhodococcus or other species of mycobacteria sharing antigens common with *M. paratuberculosis*. In our recent investigation, the cross-reacting antibodies found in the sera from *Mycobacterium bovis, Mycobacterium kansasii* or *Nocardia asteroides* infected cattle could be eliminated by absorbing the test serum with a suspension of *M. phlei*. The results of the field survey described herein confirmed that the ELISA has a

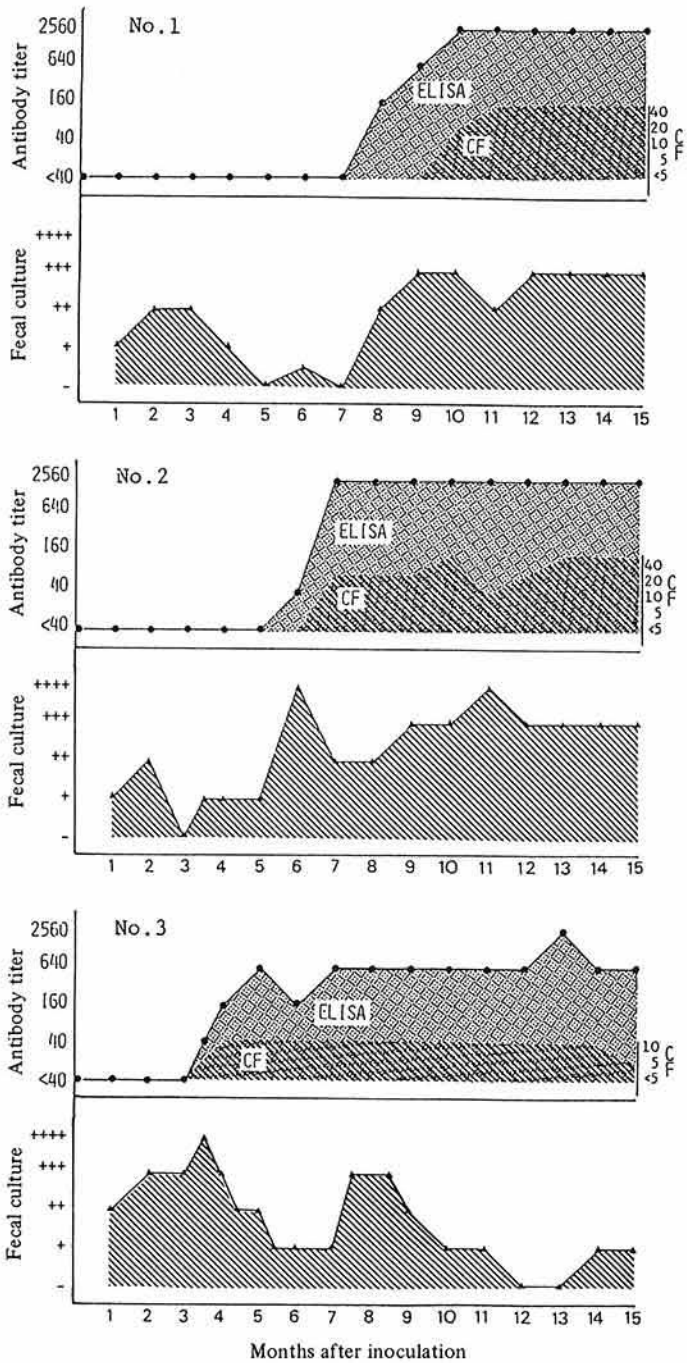

Fig. 3. Serological responses and fecal culture results of 3 calves following intraoral inoculation with live organisms(Wet wt. 100 mg) of *M. paratuberculosis*

* +=1~10 colonies of *M. paratuberculosis* per medium slant, ++=11~50 colonies, +++=50~100 colonies, ++++=more than 100 colonies.

superior specificity compared with the CF test.

Although the ELISA test used in the present study was not sufficiently sensitive for detecting all culture positive cattle tested, it has the advantages of reducing the time necessary to identify subclinically infected cattle. The ELISA was possible to detect more positive reactions in an infected herd than with the CF test. Moreover, it was shown that the seroconversion could be detected by the ELISA 1 to 2 months ealier than by the CF test. Thus, the application of the ELISA may significantly reduce the period of time for the identification of *M. paratuberculosis* infected cattle. The ELISA is a potentially useful aid for large scale epidemiological surveys, as it is less laborious, more economical and unaffected by contamination.

Animals should not be condemned on the basis of the ELISA results alone, as some animals with positive antibody titers may recover. We recommend the concurrent applications of the ELISA with the fecal culture test in the implementation of the control program of the paratuberculosis affected herds. ELISA positive animals should be segregated from the negative animal group until they gave a negative result in the fecal culture test. Preferably the ELISA positive animals even with negative fecal culture results should not be used as breeding animals. The ELISA using 0.3M-P antigen is a powerful adjunct to the isolation of *M. paratuberculosis* from feces for the diagnosis of bovine paratuberculosis.

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Summary

The specificity of an enzyme-linked immunosorbent assay(ELISA) for the diagnosis of paratuberculous cattle was improved by absorbing the bovine sera with a suspension of *Mycobacterium phlei*-killed organisms. The newly developed ELISA technique was evaluated and compared with the complement fixation (CF) test for the detection of antibodies to *Mycobacterium paratuberculosis*. Thirty-eight (66%) of 58 fecal culture positive cattle in a *M. paratuberculosis* infected herd were positive in the ELISA test, whereas the CF test yielded only 15 (26%) positive results. Calves experimentally infected with *M. paratuberculosis* seroconverted in the ELISA test at the earlier stage of infection than in the CF test. The ELISA was proposed as an alternative to the CF test for the detection of antibodies against *M. paratuberculosis* in bovine serum. Application of the ELISA as a diagnostic test for subclinical infection of cattle with *M. paratuberculosis* was proved to be very useful.

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