JOURNAL OF CLINICAL MICROBIOLOGY, May 1980, p. 499-502
0095-1137/80/05-0499/04$02.00/0

Vol. 11, No. 5

Enzyme-Linked Immunosorbent Assay for Detecting Antibodies in Cattle in a Herd in Which Anaplasmosis Was Diagnosed

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An enzyme-linked immunosorbent assay (ELISA) was developed for detecting antibodies in anaplasmosis with an antigen prepared from infected bovine erythrocytes. Protein A labeled with horseradish peroxidase was used as conjugate. A comparison was made of results of ELISA, complement fixation test (CFT), and card test (CT) on sera from 97 cows in a herd in which anaplasmosis had been diagnosed. Positive ELISA reactions were observed in serum dilutions of 1:20 or greater in each of 26 cows positive by CFT and in 18 of 22 cows suspected by CFT. Of 49 cows negative by CFT, 31 were negative by ELISA. Positive ELISA reactions were observed in 45 cows positive by CT; 27 of 44 cows negative by CT were negative by ELISA. No ELISA reactions were detected in the sera of 23 cows found negative in the CFT and CT or in 8 cows negative in CFT and positive in CT. No positive CFT, CT, or ELISA reactions were observed in sera of cattle in a noninfected herd.

Serological procedures have been developed for use in the diagnosis of anaplasmosis in cattle (2, 5, 7, 9). The complement fixation test (CFT) and card test (CT) are the methods currently used most often in the United States. The CT is a rapid procedure which has been used successfully to detect infected herds for treatment. Since the CT is generally used for screening herds, it has been suggested that the results should be supported by supplemental tests (1). The CFT has been shown to provide suitable sensitivity and specificity; however, application of CFT has been limited by its complexity. Therefore, it would be desirable to develop a rapid, simple serological test with sensitivity and specificity comparable to CFT which could be readily automated.

Enzyme-linked immunosorbent assay (ELISA) has been used for detecting antibodies in bacterial, viral, and certain parasitic diseases of cattle and other animals (6, 10, 11). Recent reports indicate that ELISA is a reliable test for detecting brucella antibodies in the milk of cows naturally infected with *Brucella abortus* (14). The sensitivity and specificity of ELISA have been demonstrated by its application in characterizing antigenic determinants in certain gram-negative and acid-fast bacteria (12; C. O. Thoen, W. G. Eacret, and J. L. Jarnagin, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, C49, p. 318). The purpose of this study was (i) to develop an ELISA for detecting serum antibodies in cattle infected with *Anaplasma marginale* and (ii) to compare the ELISA reactions with results obtained on CFT and CT in 97 cows in a herd in which anaplasmosis had been diagnosed.

MATERIALS AND METHODS

Antigen. *A. marginale* antigen (Scientific Services Laboratory, National Veterinary Services Laboratories, Ames, Iowa) was prepared from infected bovine erythrocytes by a method previously described (3). Initial tests were made with varying concentrations of antigen (Table 1). For routine tests, a 1:40 dilution of antigen was prepared with a phosphate-buffered saline solution.

ELISA conjugate. The ELISA conjugate was prepared by modification of the method of Nakane and Kawaoi (8). A 5-mg amount of horseradish peroxidase type VI (Sigma Chemical Co., St. Louis, Mo.) was dissolved in 1.0 ml of fresh 0.3 M sodium bicarbonate (pH 8.1). To this was added 0.1 ml of 1% dinitrofluorobenzene (Sigma Chemical Co.) in absolute ethanol, and the solution was mixed gently for 1 h at room temperature. One ml of 0.01 M $NaIO_4$ (Sigma Chemical Co.) in distilled water was added and gently mixed for 30 min at 20°C. A 1-ml quantity of 0.16 M ethylene glycol was then added, and the solution was mixed for 1 h. This was dialyzed in 3 liters of 0.01 M sodium bicarbonate buffer, pH 9.5, at 4°C. A 5-mg amount of protein A (Pharmacia, Piscataway, N.J.) was added to 3 ml of horseradish peroxidase-aldehyde solution and

TABLE 1. *ELISA on reference sera from an animal experimentally infected with A. marginale and on sera from a negative noninfected control cow*

Antigen[a] dilution	Intensity of reaction at serum dilution:											
	Positive serum[b]						Negative serum					
	1:5	1:10	1:20	1:40	1:80	1:160	1:5	1:10	1:20	1:40	1:80	1:160
1:10	4	3	3	2	1	0	1	0	0	0	0	0
1:20	4	4	3	2	1	0	0	0	0	0	0	0
1:30	4	4	3	2	1	0	0	0	0	0	0	0
1:40	4	4	2	2	1	0	0	0	0	0	0	0
1:50	4	4	2	1	1	0	0	0	0	0	0	0
1:60	3	3	2	1	0	0	0	0	0	0	0	0
1:70	2	2	1	0	0	0	0	0	0	0	0	0
1:80	2	0	0	0	0	0	0	0	0	0	0	0

[a] Complement fixation antigen was produced by Scientific Services Laboratory, National Veterinary Services Laboratories, Ames, Iowa.

[b] Complement fixation titer positive at 1:5.

mixed for 3 h at 20°C. The conjugate was extensively dialyzed against phosphate-buffered saline and then stored at −20°C. The conjugate was used at a 1:5,000 dilution in 0.5 M NaCl containing 1% Tween 80 adjusted to pH 7.5 using 1 M K_2HPO_4.

Sera. Serum was collected at day 180 postinfection from a cow experimentally inoculated with *A. marginale*. Serum was also collected from a noninfected (control) cow which was found negative for anaplasmosis by CFT and CT. Sera from 97 cattle in a herd in which anaplasmosis had been diagnosed previously and sera from 35 cattle in a noninfected herd were examined by ELISA. The sera were tested by CFT and CT as previously described (2, 7). Dilutions of test serum were made with 0.5 M NaCl containing 1% Tween 80 adjusted to pH 7.5 with 1 M K_2HPO_4. For routine testing, serial dilutions of 1:5, 1:10, 1:20, 1:40, 1:80 and 1:160 were made on each of the sera.

Substrate. A working solution of substrate was prepared using 3% hydrogen peroxide and 2,2′-azino (3-ethyl benthiozaline 6-sulfonate) (ABTS) in 0.05 M citric acid. The procedure for preparing ABTS was obtained from Mary Louise Bartlett, Los Almos Scientific Laboratory, Los Almos, N.M. (unpublished data).

ELISA test protocol. The ELISA test was conducted by modification of a procedure described previously (14). Tests with *A. marginale* antigen were made by adding a 0.05-ml sample of a 1:40 dilution of antigen to each test well of a flat-bottomed microtiter tissue culture tray (type IS-FB-96T; Linbro Scientific, Hamden, Conn.). The plates were allowed to air dry at 37°C for 16 h. Each plate was washed once with a wash solution of 0.5 M NaCl containing 0.5% Tween 80 adjusted to pH 7.5 with 1 M K_2HPO_4. Sera from an animal experimentally infected with *A. marginale* and a noninfected control were used in the antigen evaluation (Table 1). A volume of 0.05 ml of diluted serum was added to each test well and incubated for 8 min at 22°C on a horizontal shaker (Arthur H. Thomas Co., Philadelphia, Pa.). The wells were washed eight times with the wash solution. The conjugate, at a dilution of 1:5,000, was added in 0.05-ml volumes to each well. After 8 min of incubation on the shaker at 22°C, the wells were washed as before. Then 0.05 ml of ABTS substrate solution was added to each well and incubated for 10 min at 22°C. The green color developed by the reaction was observed, and the intensity was graded from 1 to 4. No apparent color change (0) was considered negative. The ELISA test reactions were stopped by adding 0.1 ml of 0.1 M hydrofluoric acid to each test well.

RESULTS

The results of ELISA on reference serum from an animal experimentally inoculated with *A. marginale* and on serum from negative control are shown in Table 1. Positive ELISA reactions (2+) were observed at 1:40 serum dilutions in the animal experimentally inoculated with 1:10, 1:20, 1:30, and 1:40 dilutions of CFT antigen. When the antigen dilutions of 1:50 and 1:60 were used, positive ELISA reactions were present in serum dilutions of 1:20; only suspicious reactions (+) were observed at the 1:40 serum dilutions. Higher dilutions of antigen (1:70 and 1:80) failed to produce positive ELISA reactions at serum dilutions of 1:20 or 1:40. No positive ELISA reactions (2+) were observed in the serum of a noninfected control cow. The 1:40 dilution of CFT antigen was selected for use in ELISA tests conducted on sera from animals in a herd in which anaplasmosis had been diagnosed.

A comparison of the results of ELISA and CFT on sera from 97 cattle is shown in Table 2. These results revealed that 26 animals positive by CFT were positive by ELISA (2+ reaction at serum dilution of 1:20). Suspicious CFT reactions were observed in sera of 22 animals; 18 were positive by ELISA and 4 were negative (82% agreement). Of the 18 animals positive on the ELISA, 13 were positive by CT. Four animals negative by ELISA were negative by CT. Thirty-one of forty-nine animals negative by CFT were negative by ELISA (63% agreement);

TABLE 2. *Comparison of results of ELISA and CFT on sera from 97 cattle in a herd in which anaplasmosis had been diagnosed*

CFT results	ELISA results		% Agreement
	Positive	Negative	
Positive	26	0	100
Suspect	18[a]	4	82
Negative	18	31[b]	63

[a] Sera from 13 cattle were positive by CT.
[b] Sera from eight cattle were positive by CT.

eight of these were sera from cattle positive by CT. Eighteen animals negative by CFT were positive by ELISA; 13 of these animals had positive CT reactions.

A comparison of CT and ELISA reactions on sera from 97 cattle is shown in Table 3. Sera from 45 animals were positive in both tests; 13 of these animals were negative by CFT. Eight animals positive by CT were negative by ELISA and CFT. Seventeen animals negative by CT were positive by ELISA; eight of these were positive by CFT, four were suspect, and five were negative. Twenty-seven animals were negative by the ELISA and CT; 23 were negative by CFT and four were suspect by CFT.

Results of CT and CFT are presented in Table 4. Of the 26 animals positive by CFT, 18 were positive by CT; 8 were negative by CT. All 26 were positive by ELISA. Of 22 animals suspect by CFT, 14 were positive by CT and ELISA. Eight sera suspect by CFT were negative by CT: four were positive by ELISA and four were negative. Of 49 sera negative by CFT, 21 were positive by CT; 13 of these sera were positive by ELISA. Twenty-three of twenty-eight sera negative by CFT and CT were negative by ELISA.

The results of CFT, CT, and ELISA on sera from 35 cattle in a noninfected control herd are shown in Table 5. Negative reactions were observed on CFT, CT, and ELISA conducted on sera from 34 cows. One serum was unsuitable for CFT because of anticomplementary activity; this serum was negative by CT and ELISA. Serum from one animal was suspect by CT, but negative by CFT and ELISA.

DISCUSSION

Protein A, a major cell wall component of certain strains of *Staphylococcus aureus*, binds immunoglobulin molecules with high affinity (4). This unique property of protein A has been utilized in the modified ELISA system described herein for detecting antibodies to *A. marginale* in the sera of cattle. Anti-species conjugates of bovine immunoglobulins prepared in goats failed to provide suitable specificity. The use of protein A-labeled horseradish peroxidase conjugates eliminates the time-consuming, laborious, and expensive procedures related to inoculating animals for the production of anti-species immunoglobulin. Moreover, the protein A enzyme-labeled conjugate was found to be stable for at least 2 months.

The major problem with the CFT for use in diagnosis of anaplasmosis is its complexity. The standardization of CFT system components such as complement and erythrocytes requires considerable time. A major advantage of ELISA is that it is a simple procedure that can be readily automated for screening large numbers of serum samples at a reference laboratory where uniform test conditions can be maintained.

The CFT has been shown to be a reliable serological procedure for identifying diseased animals; however, some animals infected with *A. marginale* are not detected. The information reported herein shows that the results of ELISA are in 100% agreement for sera positive by CFT and CT. The findings also revealed that a high

TABLE 3. *Comparison of results of ELISA and CT on sera from 97 cattle in a herd in which anaplasmosis had been diagnosed*

CT results	ELISA results		% Agreement
	Positive	Negative	
Positive	45	8[a]	85
Negative	17[b]	27	61

[a] Sera from all eight were negative by CFT.
[b] Sera from five cattle were negative, four were suspect, and eight cattle were positive on CFT.

TABLE 4. *Comparison of results of CFT and CT on sera from 97 cattle in a herd in which anaplasmosis had been diagnosed*

CFT results	CT results		% Agreement
	Positive	Negative	
Positive	18[a]	8[a]	69
Suspect	14[a]	8	64
Negative	21[b]	28[c]	57

[a] Sera from all cattle were positive by ELISA.
[b] Sera from 13 cattle were positive by ELISA.
[c] Sera from 23 cattle were negative by ELISA.

TABLE 5. *Results of CFT, CT, and ELISA on sera from 35 cattle in a noninfected control herd*

Test	No. positive	No. suspect	No. negative
CFT	0	0	34[a]
CT	0	1	34
ELISA	0	0	35

[a] One serum of 35 was not suitable for CFT because of anticomplementary activity.

percentage of the animals identified as suspect by CFT (18/22) were positive by ELISA. Moreover, it was found that eight animals positive by the CT were negative by CFT and ELISA. These findings suggest that ELISA may provide an improved test procedure for detecting infected animals in herds in which *A. marginale* infection persists.

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