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Enzyme Immunoassay for Detecting *Brucella* Antibodies in Cow's Milk

CHARLES O. THOEN,[1]* DONALD E. PIETZ,[2] AMY L. ARMBRUST,[1] AND RUBE HARRINGTON, JR.[2]

Department of Veterinary Microbiology and Preventive Medicine, College of Veterinary Medicine, Iowa State University, Ames, Iowa 50011[1] and National Veterinary Services Laboratories, U. S. Department of Agriculture, Ames, Iowa 50010[2]

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An enzyme immunoassay (EIA) was developed for detecting *Brucella* antibodies in milk of cows infected with *Brucella abortus*. The enzyme immunoassay using heat-killed cells of *B. abortus* strain 19 was of comparable sensitivity to the *Brucella* ring test in detecting antibodies in milk of a reference positive control cow experimentally infected with *B. abortus* strain 2308 and in milk of 16 naturally infected cows from which *B. abortus* was isolated. No detectable enzyme immunoassay reactions were present in milk of 11 noninfected controls. The enzyme immunoassay is a procedure which can be readily automated so that screening tests for brucellosis could be conducted at a reference laboratory where uniform conditions can be maintained.

The *Brucella* ring test (BRT) has been used widely for screening milk samples for the presence of *Brucella* antibodies. Available information indicates that only 25% of the samples positive by the BRT are traced to herds containing cows with positive serological reactions (12). Current procedures necessitate the tracing of all BRT-positive samples to the herd of origin, and blood samples are collected from cows in the herd. Considerable time is required for collecting these samples and conducting laboratory examinations. Since approximately 75% of the positive BRT herds do not contain serological reactor animals, considerable savings could be realized if a test with comparable sensitivity and improved specificity could be developed for detecting antibodies to *Brucella abortus* in milk.

An enzyme immunoassay (EIA) has been developed for detecting the presence of A and M antigens on *Brucella* cells (9); however, no information is available on the use of the EIA for detecting antibodies in secretions of cattle. The purpose of this investigation was to develop a modification of the EIA that was comparable in sensitivity to the BRT for detecting *Brucella* antibodies in the milk of cows from which *B. abortus* was isolated.

MATERIALS AND METHODS

Antigens. *B. abortus* strain 19 vaccine was reconstituted with diluent. A 0.2-ml amount was used to inoculate 5-ml quantities of tryptose broth which were incubated at 37°C for 72 h. A 2-ml amount of the 72-h broth culture was used to inoculate Roux flasks containing tryptose agar; the flasks were incubated at 37°C for 4 days. The cells were harvested by washing the surface with sterile phosphate-buffered saline. The cell suspension was autoclaved at 121°C for 15 min and washed two times with phosphate-buffered saline. The killed cell suspension was adjusted to a McFarland no. 4 standard.

Brucella soluble antigen (BSA) was prepared from a subculture of *B. abortus* strain 1119-3 grown in liquid medium as previously described (1). A 50-g amount of cells from a regular harvest was resuspended in 200 ml of sterile distilled water. The cell suspension was autoclaved at 121°C for 20 min, and the supernatant (BSA) was prepared and stored at −20°C (3). The BSA used in this study (lot 13) contained 6.01 mg of protein per ml.

Milk samples. A BRT-positive milk sample obtained from a cow experimentally infected with *B. abortus* strain 2308 was used as a reference standard. A BRT-negative milk sample obtained from a noninfected cow was included as a negative control. Serial dilutions of the milk samples for the EIA were made by using 0.5 M NaCl containing 1% Tween 80 adjusted to pH 7.5 with 1 M K_2HPO_4. The serial dilution ring test was conducted as previously described (1). The study was extended to include milk samples from 16 cows from which *B. abortus* was isolated and from 11 cows from which no *Brucella* was isolated.

Conjugate. Rabbit anti-bovine immunoglobulin labeled with horseradish peroxidase (type VI; Sigma Chemical Co., St. Louis, Mo.) was prepared by modification of the method of Nakane and Kawaoi (5). Column filtration was not used. A 5-mg amount of horseradish peroxidase was used to label 20 mg of immunoglobulin. The filtered conjugate was stored at 4°C. The conjugate was used at a dilution of 1:200 in 0.5 M NaCl containing 1% Tween 80 adjusted to pH 7.5 with 1 M K_2HPO_4.

Substrate. A working solution of substrate was

prepared by using hydrogen peroxide and 2,2'-azino(3-ethyl benthiozaline-6-sulfonate) (ABTS) in citric acid. The procedure for preparing ABTS was obtained from Mary Louise Bartlett, Los Alamos Scientific Laboratory, Los Alamos, N. M. (unpublished data).

EIA test protocol. The EIA test was conducted by using a modification of procedures described previously (8–10; C. O. Thoen, A. L. Armbrust, and M. P. Hopkins, Am. J. Vet. Res., in press); 0.05 ml of whole cell *B. abortus* antigen (WCA) was added to each well of a microtiter tissue culture tray (type IS-FB-96T; Linbro Scientific, Hamden, Conn.), and 0.05 ml of BSA was added to each well of a separate tray. The plates were allowed to dry at 22°C for 16 h and then washed with 0.5 M NaCl containing 0.05% Tween 80 adjusted to a pH of 7.5 with 1 M K_2HPO_4. A comparison of WCA and BSA was made by using serial dilutions of the reference standard positive BRT milk sample (see Table 1). A 0.05-ml volume of diluted milk was added to each well and incubated for 10 min at 22°C on a horizontal shaker (Arthur H. Thomas Co., Philadelphia, Pa.). The wells were washed eight times with 0.5 M NaCl containing 0.5% Tween 80 adjusted to pH 7.5 with 1 M K_2HPO_4. Then 0.05 ml of a 1:200 dilution of conjugate was added to each well. After 5 min of incubation at 22°C, the tray was washed as before. Then 0.05 ml of ABTS substrate solution was added to each well and incubated for 12 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 was considered negative.

The development of EIA reactions in the serial dilutions of milk of 16 cows from which *B. abortus* was isolated and of milk from 11 noninfected cows was made by using WCA. The results were compared with the results of the serial dilution BRT.

RESULTS

The serial dilution BRT titer of the reference standard positive milk sample was 1:2,048 (Table 1). Positive EIA reactions (2+ or greater) with WCA and BSA were observed at dilutions of 1:4,096 and 1:128, respectively. Triplicate tests failed to reveal important variations in EIA reactions when either WCA or BSA was used. The EIA reactions were visible at 10 min; however, the optimal time for reading reactions was determined to be 12 min. Nonspecific reactions were apparent in lower dilutions of milk after 16 min of incubation and after 25 min of incubation in the higher dilutions of milk. No EIA reactions were observed using WCA or BSA for the BRT-negative milk sample.

TABLE 1. *Comparison of an EIA using heat-killed cells of B. abortus and a BSA with the BRT for detecting antibodies in a reference standard positive milk sample from a cow experimentally infected with B. abortus strain 2308*

Milk dilution	EIA test reaction		BRT reaction
	B. abortus cells	BSA	
1:2	4+	4+	4+
1:4	4+	4+	4+
1:8	4+	4+	4+
1:16	4+	4+	4+
1:32	4+	4+	4+
1:64	4+	4+	4+
1:128	4+	2+	4+
1:256	4+	+	4+
1:512	4+	−	4+
1:1,024	4+	−	4+
1:2,048	4+	−	4+
1:4,096	2+	−	+
1:8,192	±	−	−
1:16,384	−	−	−

Positive serial dilution EIA and BRT titers were observed at milk dilutions of 1:32 in all 16 cows from which *B. abortus* was isolated (Tables 2 and 3). Positive EIA reactions were observed at milk dilutions of 1:64 in 15 of 16 cows, whereas positive BRT titers were observed in only 13 of 16 cows. Positive EIA and BRT reactions were observed in milk dilutions at 1:128 in 8 of the 16 cows. Two cows (cows 549 and 332) were positive by EIA and negative by BRT; one cow (190) was positive by BRT and negative by EIA. Positive EIA and BRT reactions were observed at milk dilutions of 1:256 in 5 of the 16 cows; two cows (549 and 596) were negative by BRT and positive by EIA. One cow (917) was positive by BRT and negative by EIA. Three of 16 cows (549, 596, and 732) had positive EIA reactions at milk dilutions of 1:512 or greater, whereas only one cow (732) was positive by BRT. No positive EIA or BRT reactions were observed in serial dilutions of milk from 11 noninfected control cows.

DISCUSSION

The BRT which detects the presence of *Brucella* antibodies in milk is used in the Cooperative State-Federal Brucellosis Eradication Program as a survey procedure for monitoring milk marketing herds for brucellosis. Although the BRT is a relatively simple procedure, there are several factors that may contribute to false-negative or false-positive results. The cream obtained from milk of individual cows can provide extremely variable BRT results (7). The variation was reduced when pooled cream samples were used; however, fourfold differences in replicate tests were observed.

The type of preservative and concentration (i.e., bichloride of mercury) used in the milk sample can affect the sensitivity of the BRT reaction (6). Other factors, such as time and temperature of storage and the time and temperature of heating samples, influenced the sensitivity of the BRT on Babcock test samples.

Other variations in the BRT may be attributed to the formation of the cream layer, which

TABLE 2. *Results of EIA on milk from 16 cows from which B. abortus was isolated and on milk from 11 noninfected control cows*

Cow no.	EIA test reactions with the following milk dilutions:											*B. abortus* isolated
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1,024	1:2,048	
124	4+	4+	4+	4+	4+	3+	3+	2+	+	–	–	+
190	4+	4+	3+	3+	NT[a]	2+	+	±	–	–	–	+
192	4+	4+	4+	4+	4+	3+	2+	+	±	–	–	+
243	4+	4+	4+	4+	3+	2+	+	+	±	–	–	+
332	4+	4+	4+	4+	2+	2+	2+	±	–	–	–	+
358	4+	4+	4+	3+	2+	2+	+	±	–	–	–	+
375	4+	4+	4+	4+	4+	4+	3+	2+	+	–	–	+
531	4+	4+	3+	3+	2+	2+	+	–	–	–	–	+
549	4+	4+	4+	4+	4+	4+	4+	4+	3+	2+	–	+
596	4+	4+	4+	4+	4+	4+	4+	4+	3+	2+	+	+
732	4+	4+	4+	4+	4+	4+	4+	3+	2+	+	–	+
915	4+	4+	4+	4+	4+	4+	3+	2+	+	±	–	+
917	2+	4+	4+	4+	4+	3+	3+	+	±	–	–	+
973	4+	4+	4+	3+	2+	+	+	–	–	–	–	+
985	4+	4+	4+	4+	4+	4+	3+	2+	+	±	–	+
37	4+	4+	4+	4+	3+	2+	+	±	–	–	–	+
7135	–	–	–	–	–	–	–	–	–	–	–	–
7156	–	–	–	–	–	–	–	–	–	–	–	–
7275	–	–	–	–	–	–	–	–	–	–	–	–
7389	–	–	–	–	–	–	–	–	–	–	–	–
7391	–	–	–	–	–	–	–	–	–	–	–	–
7392	–	–	–	–	–	–	–	–	–	–	–	–
7396	–	–	–	–	–	–	–	–	–	–	–	–
7397	–	–	–	–	–	–	–	–	–	–	–	–
7505	–	–	–	–	–	–	–	–	–	–	–	–
7560	–	–	–	–	–	–	–	–	–	–	–	–
7572	–	–	–	–	–	–	–	–	–	–	–	–

[a] NT, Not tested.

TABLE 3. *Results of BRT on serial dilutions of milk from 16 cows from which B. abortus was isolated and on milk from 11 noninfected control cows*

Cow no.	BRT reactions with the following milk dilutions:										
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1,024	1:2,048
124	4+	4+	4+	4+	4+	4+	4+	2+	–	–	–
190	4+	4+	4+	4+	4+	4+	2+	–	–	–	–
192	4+	4+	4+	4+	4+	4+	2+	–	–	–	–
243	4+	4+	4+	4+	2+	–	–	–	–	–	–
332	4+	4+	4+	4+	3+	–	–	–	–	–	–
358	4+	4+	4+	4+	4+	2+	–	–	–	–	–
375	4+	4+	4+	4+	4+	4+	4+	3+	–	–	–
531	4+	4+	4+	4+	4+	3+	–	–	–	–	–
549	4+	4+	4+	4+	4+	2+	–	–	–	–	–
596	4+	4+	4+	4+	4+	4+	3+	–	–	–	–
732	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	2+
915	4+	4+	4+	4+	4+	4+	4+	4+	2+	–	–
917	4+	4+	4+	4+	4+	4+	4+	3+	–	–	–
973	4+	4+	4+	4+	4+	3+	–	–	–	–	–
985	4+	4+	4+	4+	4+	4+	4+	2+	–	–	–
37	4+	4+	4+	4+	3+	–	–	–	–	–	–
7135	–	–	–	–	–	–	–	–	–	–	–
7156	–	–	–	–	–	–	–	–	–	–	–
7275	–	–	–	–	–	–	–	–	–	–	–
7389	–	–	–	–	–	–	–	–	–	–	–
7391	–	–	–	–	–	–	–	–	–	–	–
7392	–	–	–	–	–	–	–	–	–	–	–
7396	–	–	–	–	–	–	–	–	–	–	–
7397	–	–	–	–	–	–	–	–	–	–	–
7505	–	–	–	–	–	–	–	–	–	–	–
7560	–	–	–	–	–	–	–	–	–	–	–
7572	–	–	–	–	–	–	–	–	–	–	–

is dependent on a fat globule agglutinin necessary to aggregate fat globules in clusters (2, 4; A. J. Kenyon, Ph.D. thesis, University of Minnesota, St. Paul, 1961). The agglutinin, which is reported to be a lipoprotein, is labile to heat and agitation.

The EIA has been used previously for screening sera for antibodies in viral and bacterial diseases (8, 10; Thoen et al., in press). Recently a modification of EIA was developed for detecting the presence of A and M antigens of brucella cells (9). The EIA reported herein is comparable in sensitivity to the BRT for detecting antibodies in milk of cows from which *B. abortus* has been isolated. However, further studies are needed to obtain information on the specificity of the EIA in comparison to the BRT.

In herds where cows were vaccinated as adults with a reduced dosage of strain 19, it has been found that BRT-positive reactions frequently remained positive for long periods of time after the removal of *Brucella*-infected cows (11). Additional investigations are needed to evaluate the EIA in these herds.

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