JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1981, p. 396-403
0095-1137/81/100396-08$02.00/0

Vol. 14, No. 4

BrucELISA: an Enzyme-Antibody Immunoassay for Detection of *Brucella abortus* Antibodies in Milk: Correlation with the *Brucella* Ring Test and with Shedding of Viable Organisms

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Received 9 December 1980/Accepted 20 May 1981

An indirect enzyme-antibody immunosorbent assay (BrucELISA) is described for the detection of antibody to *Brucella abortus* in cow's milk. Three series of milk samples were obtained from an adult-vaccinated dairy herd infected with *B. abortus*. The BrucELISA system was used as a screening test for individual milks diluted 1:200 (BE 200 test), for undiluted bulk milks, and to determine antibody titer (BrucELISA titration assay). The BrucELISA results correlated highly with positive *Brucella* ring test reactions and culture positivity, eliminated false-positive *Brucella* ring test reactions, detected antibody in some samples which were *Brucella* ring test negative, and distinguished between vaccinated and infected animals. BrucELISA titration assay titers of ≥1:800 were correlated with shedding, or were prognostic for animals which eventually became shedders. Binding of the enzyme-antibody conjugate to bovine immunoglobulin in the absence of rabbit anti-bovine immunoglobulin occurred with culture-positive or -negative milks showing titers of ≥1:1,600 (the beta effect); the effect was also of predictive value in identifying eventual shedders. The BrucELISA system is a sensitive, specific, and inexpensive method for screening large numbers of individual or bulk milk samples for the presence of antibody to *B. abortus*.

The *Brucella* ring test (BRT) and bacterial culture have been used for many years for the detection of dairy cows infected with *Brucella abortus* (1, 18–20). Unfortunately, the BRT does not show sufficient sensitivity in early infections or in bulk milk samples taken from large herds, so that its use as a screening test for the detection of antibody is limited. Furthermore, the BRT, when performed using undiluted whole milk, has been reported to yield a high percentage of false-positive reactions which do not correlate with concomitant serological analyses (28 as reported in reference 27). Culture too has serious limitations: *B. abortus* is a relatively slow-growing organism, is often missed since relatively few cultivable organisms may be present, and can be obscured by the overgrowth of other bacteria during culture. On the other hand, milk constitutes a highly desirable source of antibody for routine screening purposes, and for the identification of infected individuals, since sample collection is simple and noninvasive.

Several investigators recently reported the use of various enzyme-antibody immunoassays for bovine antibody to *B. abortus* in milk or serum. Thoen et al. (27) devised a direct enzyme-linked immunosorbent assay (ELISA) procedure for the detection of antibody to *B. abortus* in cow's milk, using an immunoglobulin fraction of rabbit anti-bovine immunoglobulin conjugated with peroxidase. Their results showed that the ELISA was of comparable sensitivity to the BRT. Carlsson et al. (6) reported the use of an ELISA for measuring serum antibody in rabbits immunized with antigen extracted from *B. abortus*. Stemshorn et al. (24) used a similar approach to study the onset of serum antibody production in cows immunized with S19 vaccine. All of these studies were carried out using enzyme-antibody conjugates directed to bovine immunoglobulin.

The indirect ELISA system described in this report (BrucELISA) detected antibody to *B. abortus* in cow's milk at levels far lower than those detected in the BRT, provided for rapid and inexpensive screening of both individual and bulk milk samples, and tested the possibility that the need for culture might be circumvented. The results indicate that the assay is not only sensitive and specific, but is able to distinguish between infected and vaccinated animals, and is of diagnostic value in predicting which animals

are shedding or will eventually shed cultivable *B. abortus*.

MATERIALS AND METHODS

Dairy herd. The Florida herd studied in this report first showed evidence of infection with *B. abortus* in March 1975. Test-and-slaughter procedures eventually eliminated reactor animals by November 1977. In February 1979, one reactor was found in the herd, which numbered 208 animals. Many more reactors were found with subsequent tests. On 1 August 1979, the herd of 175 adult animals was vaccinated with S19. From time to time since then, reactors have been found in the herd, including four in the fall of 1979 and those revealed by our studies. On 21 January 1980, the first series of milk samples for our study were obtained from the herd. Serological tests were performed at that time by the State of Florida laboratory. Their results, on 244 animals, were: card positive, 20 (8.2%); rivanol positive, 17 (7.0%); and complement fixation positive, 14 (5.7%).

Milk samples. Three separate sets of milk samples were shipped on ice to Vermont for analysis. The samples were acquired on the following dates: series 1, 21 January 1980; series 2, 1 March 1980; series 3, 2 April 1980. All shipments were received within 24 h of drawing the samples; no preservatives were used. All aliquots used for the BrucELISA testing were routinely pasteurized at 62°C for 60 min and then stored at −20°C.

BRT. The BRT was performed by the method of Alton et al. (1).

Antigen. The plate agglutination antigen used in these experiments was obtained from the United States Department of Agriculture Animal and Plant Health Inspection Service, Ames, Iowa, as a suspension of stained *B. abortus* 1119-3 in 0.85% NaCl containing 0.5% phenol. The suspension was centrifuged at 5,000 × *g* for 15 min. The supernatant was passed through a 0.45-μm filter (Gelman Sciences, Inc., Ann Arbor, Mich.) to remove residual particulate matter; the sedimented bacteria were discarded. The solution was stored at 4°C until used.

Microtiter plates. Cooke Micro-Elisa microtiter plates with flat-bottom wells (Dynatech Laboratories, Inc., Alexandria, Va., model 1-223-29) were used throughout the study.

Enzyme-antibody conjugates. Conjugates were constructed by coupling the immunoglobulin fraction of goat anti-rabbit immunoglobulin G (IgG; Cappel Laboratories, Downingtown, Pa.) to β-galactosidase (*Escherichia coli* 3.2.1.23, grade IV, Sigma Chemical Co., St. Louis, Mo.; abbreviated GZ) with glutaraldehyde (29). Five milligrams of crystalline GZ in 2.2 M $(NH_4)_2SO_4$ was centrifuged at room temperature for 10 min at 7,000 × *g*; the supernatant was discarded, and the pellet was dissolved in 0.9 ml of 0.05 M phosphate-buffered saline (PBS; pH 7.2). The enzyme solution and an aliquot of the immunoglobulin fraction of goat anti-rabbit IgG were separately dialyzed overnight at 4°C against 6 liters of PBS. The dialyzed goat immunoglobulin was then adjusted to 20 mg/ml using $E^{1\%}_{1cm} = 13.0$. Two milligrams of immunoglobulin in 0.1 ml of PBS was added to the dialyzed enzyme solution, and 2% electron-microscopy grade glutaraldehyde (Polysciences, Warrington, Pa.; made by diluting 8% glutaraldehyde 1:4 with distilled water) was added slowly with stirring to a final concentration of 0.2% (vol/vol). The preparation was allowed to stand without stirring at room temperature in a closed vial for 3 h, at which time 0.1 ml of 1 M lysine was added to stop the glutaraldehyde reaction. The straw-colored, slightly cloudy solution was then dialyzed against 4 liters of PBS containing 0.02% (wt/vol) NaN_3. The dialyzed conjugate was stored at 4°C in a closed container. Such preparations have shown no significant alteration in activity after storage for more than 3 years.

ELISA procedure. The ELISA procedure used is a modification of the method of Voller et al. (29). The supernatant from the *Brucella* antigen preparation was diluted 1:100 in pH 9.6 carbonate coating buffer. Appropriate microtiter plate wells were then incubated at room temperature with 200 μl of diluted antigen or coating buffer alone for 30 min. The plates were washed three times with PBS containing 0.05% Tween 20 (PBST). Two hundred microliters of each diluted milk sample was then dispensed to each well; the plates were covered and incubated for 2 h at room temperature. The plates were then washed three times with PBST, and 200 μl of a 1:1,000 dilution of normal rabbit serum or a 1:1,000 dilution of rabbit anti-bovine IgG antiserum (Miles Laboratories Inc., Elkhart, Ind., catalog no. 64-113-1) in PBST was added to appropriate wells. After 2 h at room temperature, the plates were again washed three times with PBST, and 200-μl volumes of a 1:5,000 dilution of GZ-goat anti-rabbit IgG conjugate in PBST were added to each well. After overnight incubation at 4°C, the plates were again washed three times with PBST and then with 200 μl of substrate solution (4 mg of *o*-nitrophenyl-β-D-galactopyranoside [Sigma Chemicals] per ml in 0.1 M phosphate buffer [pH 7.2] containing 5 mM Mg^{2+} and 0.1 M 2-mercaptoethanol). The plates were incubated at room temperature (21 to 24°C) for 30 min, at which time 50 μl of 1 M Na_2CO_3 was added to each well to stop the enzyme-substrate reaction and intensify the yellow chromophore. A 200-μl volume was withdrawn from each well and diluted 1:5 in distilled water, and the optical density was determined in a Beckman DB-G double-beam spectrophotometer at 405 nm using a quartz cuvette and 1-cm light path.

In some experiments, a through-the-well ELISA colorimeter ("Minireader," Dynatech) was used to determine chromophore concentrations from BrucELISA tests directly in ELISA plates, thus eliminating the need to dilute each sample.

BE 200 test. The BrucELISA 200 screening test (BE 200) was devised as a screening method for detecting antibody to *Brucella* antigen in milk samples from individual cows. Each milk sample was diluted 1:200 in PBST and then assayed in the fashion described above. After termination of the substrate incubation step, the plates were inspected visually to identify reactive milk samples. Those samples which showed no reactivity above the control wells were considered to be free of antibody to *Brucella* antigen. Positive

samples were subjected to the BrucELISA titration assay (BET) described below.

BET. Positive milk samples, detected in the BE 200 screen, were diluted from 1:200 to 1:6,400 (and occasionally further) in PBST, using twofold dilutions. Each dilution was then assayed as above. The titer of each sample was determined by comparing the optical density of the sample treated with normal rabbit serum to that of the rabbit anti-bovine IgG-treated sample. The titration endpoint was considered to be that milk dilution which yielded an optical density value in the 1:5 dilutions $\geq$ the optical density value above the activity of the normal rabbit serum control.

Culture. Each milk sample was cultured by streaking 0.1 ml on tryptose serum agar containing bacitracin, cycloheximide, and polymixin in a concentration specified by the WHO Laboratory Manual (1), or recommended by the National Veterinary Service Laboratories at Ames, Iowa. If a milk sample was BRT positive, five 0.1-ml volumes from that animal were cultured. Plates were incubated in 5% CO_2 at 37°C and examined as early as 36 h and as late as 2 weeks after inoculation. Suspicious colonies were tested with phage 3. Such procedures enabled us to tentatively identify *B. abortus* by bacteriophage-specific action within 60 to 72 h after planting of the original milk specimen. Isolated colonies were tested for growth in the absence of CO_2 with 5 U of penicillin per ml, in 1:500,000 thionine blue, and tested for enhancement or inhibition of growth at 1 mg of erythritol per ml in order to prove the organism was S19. All phage-specific cultures were tested for urease activity, H_2S production, sensitivity to thionine, and basic fuschin. In addition, API (Analytical Products, Plainview, N.Y.) diagnostic microplates were inoculated with the isolates to determine sugar metabolic abilities. Using oxidation fermentation medium (Difco Laboratories, Detroit, Mich.), both aerobic and anaerobic tests using glucose were also done. Isolates tested conformed to the pattern on *B. abortus* biotype 1 with one exception, which was biotype 2. One strain of S19 was isolated from an infected cow. National Animal Disease Laboratory, Ames, Iowa, confirmed these identifications.

Artifically constructed bulk milk samples. To determine the efficacy of detecting antibody to *B. abortus* in bulk milk samples, artificially constructed bulk samples were tested with a modification of the BE 200. A panel of BET-positive Florida milk samples was diluted 1:100, 1:200, 1:400, and 1:800 with BET-negative, BRT-negative, culture-negative fresh non-pasteurized bulk milk obtained from a Vermont herd, or with PBST. Fifty microliters of PBS containing 10% (vol/vol) Tween 20 were then added to 1-ml volumes of each of the dilutions made with milk. The final concentration of Tween 20 in these dilutions (0.5%) was therefore 10 times the concentration of detergent used in the BET assay to compensate for the potential loss of detergent through emulsification of lipid. (Previous experiments had shown that, if the BET is performed in the absence of Tween 20, bovine immunoglobulin from all milk samples binds nonspecifically to the microtiter plates, thus increasing the background optical activity and obscuring the detection of specific antibody.) These artificially constructed "bulk milk" samples were then assayed for antibody activity, together with dilutions of the same BET-positive samples diluted in PBST. After development of the chromophore, and addition of stopping solution, the optical activity of each well was determined at 405 nm using a through-the-well ELISA colorimeter.

RESULTS

The BrucELISA, BRT, and culture results are given in Table 1. Of 132 BRT-positive samples analyzed in series 1, 2, and 3, 72 (54.5%) of these samples were positive using only undiluted milk, showed no demonstrable antibody in the BET, and were culture negative. Thus, more than half of these BRT-positive reactions were not associated with detectable shedding of *B. abortus* nor with the detection of antibody by a sensitive independent test.

Elevated BET titers were obtained in animals which were actively shedding *B. abortus* (Tables 1 and 2). In all cases, the BET titer for culture-positive samples was $\geq$1:800. In addition, several samples yielded titers $\geq$1:800, but no organisms were isolated; we interpreted such results in the BET to indicate a high degree of suspicion with regard to cryptic infection. Indeed, in those cows for which samples in two or three series were available, 7 of 8 cows which yielded a BET titer of $\geq$1:800 were either culture positive or became positive in a subsequent sample (Table 2). No sample in any of the three series which gave a BET titer of $\leq$1:400 was culture positive (Table 1) or was derived from a cow which eventually became culture positive (Table 2). Cow no. 140 (Table 2) was the single exception, since the BET titer steadily declined from 1:800 (series 1) to 1:200 (series 3), with no evidence of detectable shedding. Of particular importance is the fact that cows 150, 10, 210, 240, and 57 gave BET titers of $\geq$1:1,600 in series 1, but were culture negative. Yet these cows were shown to be active shedders in series 2 (or, in the case of cow 150, not until series 3 samples were acquired). Thus the BrucELISA provided evidence of antibody to *Brucella* at a time when our culture methodology was unable to detect viable organisms.

Correlation between BRT and the BE 200 screening test. Of the 414 milk samples tested, 341 were BRT negative using undiluted milk (Table 3). However, 16 of these BRT-negative samples were judged positive in the BE 200 screening test by visual inspection and subsequent colorimetry; when assayed in the BET, two samples revealed a titer of 1:400, and a subsequent sample from one of these animals was shown to be BRT positive and culture positive in series 3. A third sample, from series 1,

TABLE 1. *Correlation between BRT titer and BrucELISA titer of all samples from series 1, 2, and 3*[a]

BET titer	BRT titer									
	Negative	Undiluted	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
<1:200	473	75	15	2	0	0	0	0	0	0
1:200	14	5	1	0	0	0	0	0	0	0
1:400	2	5	2	1	0	1	0	0	0	0
1:800	0	1	1[b]	0	1[b]	2(1 + 1[b])	2(1 + 1[b])	0	0	0
1:1,600	0	0	1	0	1	1[b, c]	1[b, c]	1[b, c]	0	0
1:3,200	1[b, c]	0	0	2(1 + 1[b, c])	0	2(1 + 1[c])	1[b, c]	0	0	0
1:6,400	0	0	0	0	0	5(3[b, c] + 2[c])	3(2[b, c] + 1[c])	2[b, c]	0	1[b, c, d]

[a] The number of individual milk samples which were beta positive or culture positive or both are given in parentheses.
[b] Culture positive.
[c] Beta positive.
[d] Yielded a BET titer of 1:51,200 on further study.

TABLE 2. *BET titers, beta effect, and culture results for all individual milk samples from series 1, 2, and 3 which showed BET titers of ≥1:800 in one or more of the three series*

Cow no.	Series 1			Series 2			Series 3		
	BET titer^{-1}	Beta	Culture	BET titer^{-1}	Beta	Culture	BET titer^{-1}	Beta	Culture
410	200	−	−	400	−	−	800	−	+
140	800	−	−	400	−	−	200	−	−
208	800	−	+	NS[a]			NS		
451	NS			800	−	−[b]	NS		
150	1,600	−	−	3,200	−	−[b]	800	−	+
113	1,600	+	+	NS			NS		
223	1,600	−	OG[b, c]	NS			NS		
242	3,200	+	+	3,200	+	+	800	−	+
10	3,200	−	−	6,400	+	+	1,600	+	+
210	3,200	+	−	6,400	+	+	1,600	+	+
240	6,400	+	−	6,400	+	+	3,200	+	+
8A	6,400	+	+	NS			NS		
22	6,400	+	+	NS			NS		
57	6,400	+	−	6,400	+	+	6,400	+	+
124	6,400	+	+	NS			NS		
162	6,400	+	OG[b]	NS			NS		

[a] NS, No sample; cow dry, eliminated from herd, new to herd, or otherwise not available for testing
[b] Subsequent samples cultured by P. Nicoletti were found to contain cultivable field strain of *B. abortus*.
[c] OG, Overgrown; presence of contaminating bacteria obscured culture results.

TABLE 3. *BE 200 and BET analyses of BRT-negative milk samples from series 1 and 2*

Series	Total no. of samples	No. samples BRT negative[a]	No. samples BE 200 positive	No. samples culture positive	No. samples in BET giving titer of:				
					1:200	1:400	1:800	1: 1,600	1: 3,200
1	209	173	11	1	9	1	0	0	1[b]
2	205	168	5	0	4	1[c]	0	0	0

[a] No visible ring using undiluted milk.
[b] Culture positive, beta positive; became BRT positive in series 2.
[c] Became BRT positive, culture positive in series 3.

showed a titer of 1:3,200, and a subsequent sample from this animal was shown to be BRT positive in series 2.

Beta effect: nonspecific binding of enzyme-antibody conjugate to bovine antibody correlated with shedding. All milk samples were tested with normal rabbit serum in addition to rabbit anti-bovine IgG, as a control for the specificity of binding of the goat anti-rabbit IgG-galactosidase conjugate. When the

data from the series 1 BE 200 and BET experiments were analyzed, it became apparent that the conjugate adsorbed to many antigen-coated wells treated with 1:200 dilutions of milk in the absence of rabbit anti-bovine IgG or with normal rabbit serum. The effect seemed to be correlated with high-titer BET results and, more importantly, with the presence of cultivable *B. abortus* in the sample (Tables 1 and 2). We elected to call this phenomenon the beta effect.

Tables 1 and 2 show that the beta effect was associated with the presence of cultivable *B. abortus* in samples yielding a BET titer ≥1:1,600. In addition, Table 2 shows that cows 210 and 240 were positive for the beta effect before the detection of *Brucella* in subsequent samples. An additional beta-positive sample was detected in series 1 among the BRT-negative samples (Table 1); this sample was also culture positive and showed a BET titer of 1:3,200. Finally, the beta effect was not detected in culture-positive samples which yielded a BET titer ≤1:800 (Tables 1 and 2).

All BET titers reported in this study have been corrected for the beta effect, and therefore reflect specific reactions between *B. abortus* antigen and bovine antibody to *B. abortus*.

Antibody detection in artificially constructed bulk milk samples. The results of the BrucELISA tests on artificially constructed bulk milk preparations are given in Table 4. The optical density readings of the 1:200 dilutions are given for comparison, along with the titers. Of the 17 BET-positive samples showing titers of ≥1:800 in PBST, 14 were found to have the same titer when diluted in milk. Two other samples in this category were reduced to 1:400, and a third was reduced to 1:200. The four remaining BET-positive samples with titers <1:800 in PBST also showed diminution of titer when diluted in milk; the sample from cow 150 (series 1) showed a titer of 1:100 in PBST, but was negative when diluted with milk.

Some loss of antibody activity in artificially constructed bulk milk preparations is seen in Table 4 by comparing the optical density readings of the same sample diluted either in PBST or milk-Tween. With the exception of the series 1 and 3 samples of cow 150 (which showed low optical density readings close to background), all samples showed lower optical density readings when milk was used as a diluent as compared with those achieved with PBST.

DISCUSSION

Several recent reports have applied ELISA technology to the detection of bovine serum antibody to *B. abortus*. These investigators have found their particular ELISA adaptation to be as sensitive (4, 9, 21, 24) or more sensitive (6, 14, 22) than other commonly used serum antibody tests (e.g., complement fixation, rivanol, tube agglutination) for the detection of serum antibody. The ability to construct enzyme-antibody conjugates directed to specific isotype classes of immunoglobulins may result in greater understanding of infectious processes involved in brucellosis (13). Additional attractive aspects of ELISA assays for bovine brucellosis include the potential for greater sensitivity, the possibility of visual evaluation in screening tests, ease of performance and automation, and the possibility of identifying suspect herds by analyzing bulk milk samples.

Serological analyses using ELISA technology have already begun to produce new information regarding the biology of bovine brucellosis and the nature of the immune response to the complex antigens of *B. abortus* (3, 13). However, such approaches require the acquisition of serum samples, an invasive procedure which complicates the need for routine surveys of individual animals or herds. In addition, serum antibody produced in response to S19 vaccination may confound interpretation of ELISA test results (3). Diaz et al. (8) have shown that S19 vaccinates could be distinguished from infected animals in a radial immunodiffusion test using serum. Although these observations are encouraging with respect to development of a discriminating serological assay, the technique has so far been limited to serum samples from individual animals. On the other hand, bulk milk samples from dairy cows are readily available and represent natural secretions which circumvent invasive procedures. If such samples could be used to survey large numbers of herds for the presence of antibody to *B. abortus*, and if the assay procedure were sensitive enough to detect a single infected cow in bulk milk samples from herds of 100 or more animals, then such an assay would provide a significant contribution to the early detection of brucellosis and effective management of this disease.

The BrucELISA assay reported here was devised to determine whether we could improve upon the sensitivity and specificity of the BRT. Thoen et al. (27) recently reported the use of a direct ELISA system for detecting lacteal antibody to *B. abortus*. Their system employed visual inspection of the chromophore generated with a peroxidase-labeled rabbit anti-bovine immunoglobulin conjugate. The BRT and ELISA titers achieved with milk samples from culture-positive cows were quite similar in both tests. Our results, on the other hand, showed a consid-

TABLE 4. *BrucELISA analysis of artificially constructed bulk milk samples prepared by diluting selected BET-positive samples with negative milk or with PBST*

Cow no.	Optical density values of 1:200 dilutions at 405 nm[a] with:		Titer^{-1}, determined by dilution with:[b]	
	PBST[b]	Milk[b, c]	PBST	Milk
Series 1				
8A	0.55	0.30	≥800	≥800
10	0.38	0.19	≥800	≥800
22	0.99	0.63	≥800	≥800
57	0.88	0.29	≥800	≥800
124	0.64	0.31	≥800	≥800
150	0.09	0.07	100	<100
210	0.51	0.23	≥800	≥800
240	0.53	0.23	≥800	≥800
242	0.22	0.08	≥800	≥800
Series 2				
10	0.60	0.20	≥800	≥800
57	0.41	0.32	≥800	≥800
150	0.20	0.11	400	100
210	0.67	0.37	≥800	≥800
240	0.75	0.39	≥800	≥800
242	0.15	0.11	400	100
Series 3				
10	0.60	0.32	≥800	≥800
57	0.66	0.29	≥800	≥800
150	0.13	0.44	≥800	400
210	0.46	0.16	≥800	200
240	0.55	0.22	≥800	400
242	0.22	0.09	200	100
Control milk[a]	0.00	0.00	Negative	Negative

[a] Samples read using a Dynatech Minireader microtiter plane colorimeter; specimens read directly from plate after zeroing on control milk wells. Light path = 0.79 cm.

[b] Artificially constructed bulk milk samples were diluted 1:100, 1:200, 1:400, and 1:800 with PBS-Tween 20 (0.05%) or with milk to which Tween 20 was then added to a final concentration of 0.5%.

[c] Milk: fresh nonhomogenized, nonpasteurized bulk milk acquired from a Vermont herd, determined to be BET negative, BRT negative, and culture negative.

erable increase in sensitivity with the BrucELISA as compared to the BRT.

Our BrucELISA results were highly correlated with BRT titer, but positive reactions in the BrucELISA often showed titers 100-fold greater than comparable BRT results. False-positive, low-level BRT reactions commonly reported using undiluted milk (27) were virtually eliminated. Furthermore, these was a correlation between high-titer *Brucella*-positive milk samples in the BrucELISA and the presence of cultivable *B. abortus* in the same samples. High titers in the BrucELISA often preceded the detection of organisms by our culture methods, and were therefore of prognostic value.

In spite of the report by Cameron and Kendrick (5) that lacteal antibody wanes within 3 months of adult vaccination, as measured by the whey agglutination test, we have anticipated the possibility of finding some antibody in this herd with the sensitive BrucELISA, even though adult vaccination with S19 had been carried out 7 months before the beginning of the study. This was not the case, since 90% of the samples were negative in the BET assay. It seems likely, therefore, that the BrucELISA system offers the additional benefit of being able to discriminate between vaccinated and infected dairy cows. However, additional studies of herds with different vaccination histories, and controlled experiments with vaccinated and intentionally infected animals, should be done to bolster this conclusion.

One additional advantage emerging from this study derives from the apparent prognostic value of the BET in detecting animals which did not shed sufficient numbers of viable brucellae to be diagnosed by the culture technique used in the present study, but which were culture positive on subsequent testing. Since the animals

used for this study were from a commercial dairy herd, no data are available regarding time of onset of udder infection, the source of the infection, or the mode of transmission. The commonly employed method of culturing the centrifugate and cream from 20 ml from each quarter was not followed in this study for logistical reasons, and it is possible that culturing larger volumes of milk might have detected shedders which went undetected in our study. Nonetheless, the data suggest that lacteal antibody is shed during the early incubation stage of infection with *B. abortus,* and that such antibody can often be detected before the time when the organisms can be readily cultivated from milk samples. Early detection of such animals, using the BE 200, could contribute to removal of infected animals before significant shedding, thus breaking the chain of infection.

Antibody to *B. abortus* was detected in artificially constructed bulk milk samples diluted 1:100 or greater with BE 200-negative bulk milk. Although the antibody in BET-positive milks was detectable when diluted with negative milk, the titers were often lower than those achieved with PBST as a diluent. In view of the association between bovine immunoglobulin and the cream layer (the basis for the BRT), it is conceivable that the antibody became partitioned between the aqueous and cream components of the homogenized milk used as a diluent, and that much of the antibody was therefore unavailable for adsorption to solid-phase antigen, thus lowering the titer. However, the experiment shows that the BrucELISA test is capable of detecting *Brucella* antibody in constructed bulk milk preparations which mimic the dilutions one might expect in bulk milk taken from the vast majority of dairy herds in the United States. Roepke and Stiles (20) analyzed similar artificially constructed bulk milk samples with the BRT. Statistical analysis of their data led to the conclusion that the BRT had a 50% likelihood of detecting 1 reactor cow in herds of less than 100 animals, and a 65% chance of detecting 1 reactor in more than 95% of the herds in most dairy states (using census figures published in 1966). Although the number of BrucELISA-positive animals in this study is not sufficient to warrant extensive statistical analysis, the data suggest that, even with some loss of antibody activity when positive samples were mixed with milk as compared to PBST, the assay was capable of detecting one animal in 100 with a probability approaching 100%. In view of the estimated probabilities of Roepke and Stiles (20), cited above, and the fact that up to 75% of BRT-positive herds do not contain animals which are positive by serological tests (27, 28; 54.5% in our study), we conclude that the BrucELISA system shows both greater sensitivity and greater specificity than the BRT when bulk milk samples are analyzed for positive reactors.

The beta effect emerged from controls performed on series 1 milk samples as "nonspecific" interaction between bovine immunoglobulin (in the form of antibody adsorbed to solid-phase *B. abortus* antigen) and the GZ-goat anti-bovine conjugate. Rabbit immunoglobulin, or other rabbit serum constituents, played no role in the beta effect, and galactosidase alone did not bind to bovine immunoglobulin or to *Brucella* antigen. Furthermore, the beta effect only occurred when titers were ≥1:1,600 in the BET, and was almost always detectable when milk samples were culture positive with a BET titer ≥1:1,600. Of equal importance was the finding that four animals with BET titers ≥1:1,600, but with no demonstrable shedding of viable brucellae, were nonetheless beta positive. Two of these animals were found to be culture positive when series 2 samples were tested; the other two animals had been sent to slaughter. This observation implies that the beta effect, like BET titers ≥1:1,600, may be of prognostic value in predicting which animals will eventually become shedders of *B. abortus.*

The mechanism of the beta effect is not known. However, we have considered the possibility that the effect is due either to conglutinin or to immunoconglutinin (10–12). Recently, we found (D. K. Boraker, W. R. Stinebring, and J. R. Kunkel, unpublished observations) that the beta effect is not affected by ethylenediaminetetraacetic acid under conditions known to dissociate the conglutinin-C3b complex (12). The beta effect may be due to the presence of immunoconglutinin in the hyperimmune goat immunoglobulin fraction used to construct the conjugate used in this study; the presence of immunoconglutinin-GZ copolymeric complexes in the conjugate might allow for specific binding of such complexes to aggregates of solid-phase *Brucella* antigen, bovine antibody to *Brucella,* and bound C3b (the target for immunoconglutinin). Immunoconglutinin (but not conglutinin) is known to exist in "normal" goat serum (14) and to increase in titer upon intensive immunization (10, 11) such as that which might be experienced by repeatedly immunizing goats with bovine immunoglobulin to produce high-titer commercial antisera.

ACKNOWLEDGMENTS

We thank Leslie Lerea and Vickie Shaddock for able technical assistance; John Aleong for review of the manuscript; W. Bolton for the ELISA plate reader; and Jan Huber for assist-

ance in obtaining milk samples. Paul Nicoletti isolated *Brucella abortus* from samples indicated in the manuscript, and we wish to thank him for informing us of the results. David Walker aided us in many ways, for which we are most grateful.

This study was supported by grant 519-8-776 from the U.S. Department of Agriculture to W.R.S., and U.S. Department of Agriculture-S.E.A. Grant 909 to the Vermont Agricultural Experiment Station.

LITERATURE CITED

1. **Alton, G. G., L. M. Jones, and D. E. Pietz.** 1975. Laboratory techniques in brucellosis. World Health Organization, Geneva.
2. **Barta, O., V. Barta, O. P. Miniats, and D. G. Ingram.** 1970. Complement and conglutinin in the serum of germ-free and conventional piglets. J. Immunol. **105:** 350–354.
3. **Berman, D. T., B. L. Wilson, E. Moreno, R. D. Angus, and L. M. Jones.** 1980. Characterization of *Brucella abortus* soluble antigen employed in immunoassay. J. Clin. Microbiol. **11:**355–362.
4. **Byrd, J. W., F. C. Heck, and R. J. Hidalgo.** 1979. Evaluation of the enzyme-linked immunosorbent assay for detecting *Brucella abortus* antibodies. Am. J. Vet. Res. **40:**896–898.
5. **Cameron, H. S., and J. W. Kendrick.** 1957. Differentiating postvaccination reactions in brucellosis from virulent infection. J. Am. Vet. Med. Assoc. **130:**90–91.
6. **Carlsson, H. E., B. Hurvell, and A. A. Lindberg.** 1976. Enzyme-linked immunosorbent assay (ELISA) for titration of antibodies against *Brucella abortus* and *Yersinia enterocolitica*. Acta Pathol. Microbiol. Scand. Sect. C. **84:**168–176.
7. **DeCueninck, B. J.** 1974. C142 complement activity and conglutinogen in bovine milk. Int. Arch. Allergy Appl. Immunol. **59:**323–327.
8. **Diaz, R., P. Garatea, L. M. Jones, and I. Moriyon.** 1979. Radial immunodiffusion test with a *Brucella* polysaccharide antigen for differentiating infected from vaccinated cattle. J. Clin. Microbiol. **10:**37–41.
9. **Heck, F. C., J. D. Williams, and J. Pruett.** 1980. Interpretation of spectrophotometric absorbance values to define results of enzyme-linked immunosorbent assays. J. Clin. Microbiol. **11:**398–401.
10. **Ingram, D. G., and D. A. Barnum.** 1965. Fluctuations in the level of conglutinin in bovine serum. Can. Vet. J. **6:**162–169.
11. **Lachman, P. J.** 1967. Conglutinin and immunoconglutinins. Adv. Immunol. **6:**479–527.
12. **Lachman, P. J., D. E. Elias, and A. Moffett.** 1972. Conglutinin and immunoconglutinins, p. 202–214. *In* D. G. Ingram (ed.), Biological activities of complement. S. Karger, Basel.
13. **Lamb, V. L., L. M. Jones, G. G. Schurig, and D. T. Berman.** 1979. Enzyme-linked immunosorbent assay for bovine immunoglobulin subclass-specific response to *Brucella abortus* lipopolysaccharide. Infect. Immun. **26:**240–247.
14. **Magee, J. T.** 1980. An enzyme-labelled immunosorbent assay for *Brucella abortus* antibodies. J. Med. Microbiol. **13:**167–172.
15. **Mittal, K. R., and T. N. Jaiswal.** 1973. Conglutinating activity in apparently healthy human and animal sera. Immunology **25:**237–240.
16. **O'Beirne, A. J., and H. R. Cooper.** 1979. Heterogeneous enzyme immunoassay. J. Histochem. Cytochem. **27:** 1148–1162.
17. **O'Sullivan, M. J., J. W. Bridges, and V. Marks.** 1979. Enzyme immunoassay: a review. Ann. Clin. Biochem. **16:**221–240.
18. **Roepke, M. H., K. G. Paterson, F. C. Driver, L. B. Clausen, L. Olson, and J. E. Wentworth.** 1950. The *Brucella abortus* ring test. Am. J. Vet. Res. **11:**199–205.
19. **Roepke, M. H., J. M. Patterson, and B. L. Deyoe.** 1974. Brucella ring test sensitivity of individual and pooled bovine milks with various preservatives. Am. J. Vet. Res. **35:**115–118.
20. **Roepke, M. H., and F. C. Stiles.** 1970. Potential efficiency of milk ring test for detection of brucellosis. Am. J. Vet. Res. **31:**2145–2149.
21. **Ruppanner, R., M. E. Meyer, P. Willeberg, and D. E. Behymer.** 1980. Comparison of the enzyme-linked immunosorbent assay with other tests for brucellosis, using sera from experimentally infected heifers. Am. J. Vet. Res. **41:**1329–1332.
22. **Saunders, G. C., E. H. Clinard, M. L. Bartlett, and W. M. Sanders.** 1977. Application of the indirect enzyme-labelled antibody microtest to the detection and surveillance of animal diseases. J. Infect. Dis. **136** (suppl.): S258–S266.
23. **Schuurs, A. H. W. M., and B. K. vanWeemen.** 1977. Enzyme-immunoassay. Clin. Chem. Acta **81:**1–40.
24. **Stemshorn, B., K. Nielsen, and D. G. Ingram.** 1979. Evaluation of an enzyme-labelled antiglobulin test for anti-*Brucella* IgG in cattle sera. J. Am. Vet. Med. **175:** 613.
25. **Stemshorn, B. W., K. H. Nielsen, B. S. Samagh, L. B. Forbes, and D. G. Ingram.**1980. Evaluation of an enzyme-labelled antiglobulin test for anti-*Brucella* immunoglobulin G among 3 cattle populations. Am. J. Vet. Res. **41:**1779–1784.
26. **Thoen, C. O., A. L. Armbrust, W. G. Eacret, R. Harrington, and G. M. Brown.** 1979. Use of an enzyme immunoassay test for characterizing the A and M antigens of *Brucella*. J. Clin. Microbiol. **9:**485–487.
27. **Thoen, C. O., D. E. Pietz, A. L. Armbrust, and R. Harrington.** 1979. Enzyme immunoassay for detecting *Brucella* antibodies in cow's milk. J. Clin. Microbiol. **10:**222–225.
28. **United States Department of Agriculture.** 1968–1977. Progress Reports, Cooperative State-Federal Brucellosis Eradication Program. Animal Plant Health Inspection Service, U. S. Department of Agriculture, Hyattsville, Md.
29. **Voller, A., D. Bidwell, and A. Bartlett.** 1976. Microplate enzyme immunoassays for the immunodiagnosis of virus infections, p. 506–512. *In* N. R. Rose and H. Friedman (ed.), Manual of clinical microbiology. American Society for Microbiology, Washington, D. C.