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Detection of Serum Antibody to *Brucella abortus* in Cattle by Use of a Quantitative Fluorometric Immunoassay†

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A quantitative fluorometric immunoassay (FIAX) was adapted for the detection of serum antibodies to *Brucella abortus* in cattle. Results are expressed in nanograms of immunoglobulin binding the antigen carrier. The FIAX was compared with the standard tube agglutination, Rivanol precipitation, and complement fixation tests, using 285 serum samples from vaccinated, challenged, or control cattle. Linear regression analysis indicated a significant correlation among all four serological tests; the FIAX test correlated best with the Rivanol test. Ninety sera were from vaccinated and nonvaccinated cattle that were challenged with virulent *B. abortus* 2308. The sensitivity and specificity of each serological test were determined based on culture results from these cattle. The FIAX was the most sensitive of the four serological tests, detecting 79.2% of the culture-positive animals. The FIAX was the least specific, with 15.4% of the culture-negative animals being classified as positive. Eighty-eight sera were from cattle vaccinated with strain 19 but not challenged. All four serological tests had a statistically significant ability to distinguish sera from control and vaccinates on the basis of mean titers. The mean titer of vaccinates was also significantly different from that of challenged animals. Advantages and disadvantages of the FIAX test for bovine brucellosis are discussed.

Bovine brucellosis caused by *Brucella abortus* is an economically important disease associated with abortions and infertility. Despite an active vaccination program with the live bacteria *B. abortus* 19, infections and disease are still prevalent in the United States (8, 12).

Several serological methods are currently used to indicate infection with *B. abortus* in live cattle (1, 11, 14). These include complement fixation (CF), Rivanol precipitation (RIV), and standard tube agglutination (ST) tests. These serological tests rely on secondary reactions, including the ability of antibody to bind complement or to cause agglutination. And, because they require subjective determinations, they are prone to variation among laboratories. There are several factors related to the host-parasite interaction that cause these serological tests to be less than optimal. First, animals in the early stages of infection may not have a detectable serum antibody titer (12). Second, cattle that are chronic carriers of the organism may not have detectable antibody titers to the organism. Other chronic carriers frequently will have a decline in antibody titer before abortion, and this titer subsequently may remain diminished (4, 12). Third, cattle that have received strain 19 vaccine may have titers that are indistinguishable from those of cattle with virulent field strain infection (11, 12, 14).

Recently, a semiautomated quantitative fluorometric immunoassay (FIAX; International Diagnostic Technology, Inc., Santa Clara, Calif.) has been described as a means of detecting serum antibody to viruses, bacteria, fungi, and parasites in humans and animals (3, 6, 7, 16). In cattle, the FIAX system has been reported to quantitate the antibody response to *Pasteurella haemolytica*, *Anaplasma marginale* (3, 7), and *Pasteurella multocida* (R. J. Panciera, R. E. Corstvet, A. W. Confer, and J. A. Rummage, Am. J. Vet. Res., in press). Because the FIAX system is a primary binding assay and is rapid, simple, and inexpensive, adaptation of it as a diagnostic test for bovine brucellosis should be considered.

The purpose of this manuscript is to describe the adaptation of the FIAX test for the detection of antibodies to *B. abortus* in cattle. Results obtained by the FIAX test are compared with those obtained by three conventional serological tests.

MATERIALS AND METHODS

FIAX test. The basic protocol for detecting antibody with the FIAX system has been previously described (3, 6, 7). In general, FIAX is an indirect immunofluorescence test, in which specific fluorescence due to binding of a fluorescein isothiocyanate-conjugated antiglobulin is quantitated as a fluorescence signal unit (FSU) by a fluorometer. Preliminary studies showed optimal conditions for this particular test to be a 1:51 working dilution of unknown serum and a 1:800 dilution of fluorescein isothiocyanate-conjugated rabbit anti-bovine immunoglobulin G (IgG) (heavy and light chain specific) (Cappell Laboratories, Cockranville, Pa.).

A linear regression curve was used to convert serum sample FSU obtained from the FIAX fluorometer into nanograms of immunoglobulin binding per StiQ sampler. This curve was calculated by using four known concentrations of purified bovine IgG (Cappell Laboratories), as determined by the Bio-Rad method (Bio-Rad Laboratories, Richmond, Calif.) with bovine albumin as a standard. For each FIAX test, 25 μl of four concentrations (5, 10, 20, and 40 μg/ml) of immunoglobulin, five replicates per concentration, were applied directly to the StiQ sampler and allowed to dry overnight at 37°C. These StiQs were washed in phosphate-buffered saline containing 0.15% Tween 20 for 10 min, followed by incubation in the conjugated antibovine IgG for 20 min, and again washed in phosphate-buffered saline–0.15% Tween 20 for 10 min. An FSU was obtained on all replicates, and the arithmetic mean was calculated for each of the four concentrations of immunoglobulin G. The

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natural logarithm of the protein concentration (in micrograms) was then plotted against the logarithm of the FSU to obtain a linear regression curve. The Pearson product-moment correlation was calculated for the regression (r = 0.993), and a significant correlation was found ($P < 0.01$). A mean FSU was calculated from duplicate or triplicate samples for each test serum and plotted on the regression curve to determine micrograms of immunoglobulin binding for each serum sample. These values were multiplied by 1,000 (to convert micrograms to nanograms) and designated as FIAX titers. For the purpose of evaluation, a FIAX titer was defined as positive when more than 51.5 ng of immunoglobulin was bound per StiQ (highest value obtained for the negative controls).

Antigen. The antigen used for the FIAX test was derived from a soluble *B. abortus* 1119 antigen (BASA) (2) obtained from the National Veterinary Services Laboratories, U.S. Dept. of Agriculture, Ames, Iowa. The original preparation was obtained from autoclaved cells suspended in distilled water. BASA was modified to BASA-protein (BASA-P) by extensive dialysis, centrifugation, and ammonium sulfate precipitation. (L. B. Tabatabai, and B. L. Deyoe, Dev. Biol. Stand., in press). BASA-P contained 0.289 mg of carbohydrate and 2.42 μg of 2-keto-3-deoxyoctulosonic acid per mg of protein (5, 15). BASA-P was suspended in phosphate-buffered saline (0.01 M, pH 7.4) at a concentration of 25 μg of protein per ml as determined by the Lowry method (9) with bovine serum albumin as a standard. Twenty-five microliters of this suspension (0.625 μg) was applied to one side of the StiQ samplers (International Diagnostic Technology, Inc.) and allowed to dry overnight at 37°C before use in the FIAX test.

Conventional tests. The standard serological tests (CF, RIV, and ST) were performed on all serum samples, using standard protocols (1, 11). A positive CF test was defined by a 3+ or greater reaction at a serum dilution of 1:10. A positive RIV test was defined as precipitation at a serum dilution of 1:25 or greater. A positive ST test reaction was defined as agglutination at a serum dilution of 1:100 or greater.

Sera. A total of 285 serum samples were used in this study. Of these sera, 90 were taken from 90 cattle 10 to 12 weeks after challenge with ca. 10^7 CFU of virulent *B. abortus* 2308. Of these 90 challenged cattle, 63 had received strain 19 vaccine 8 months before challenge. Eighty-eight sera were from cattle receiving strain 19 vaccine alone: 24 received 10^9 CFU, and 22 received 10^{10} CFU. Sera were collected from these cattle at 1 and 6 months postvaccination only (22 samples from the low dose and 20 samples from the high dose were available for the 1-month sampling). One hundred and seven sera represented negative controls. These were from cattle that had received neither strain 2308 nor strain 19 and were from certified brucellosis-free herds.

Culture techniques. Standard culture techniques were used to detect *B. abortus* in the 90 challenged animals, beginning at 14 weeks after challenge (1). The following tissues were collected and cultured for *B. abortus*: spleen, uterine washings, each quarter of the udder, and parotid, mandibular, retropharyngeal, bronchial, hepatic, prescapular, prefemoral, popliteal, internal iliac, and supramammary lymph nodes. All *B. abortus* isolated were examined for characteristics of *B. abortus* 19 and 2308. Animals were considered negative when cultures were negative for isolation of strain 2308.

Statistical analyses. Mean antibody titers were compared by multiple t tests. A t test for equal and unequal variances was calculated for the mean titers for each of the comparisons. An F statistic was calculated to determine whether unequal variances were present. If the probability of F was less than 0.05, unequal variances were used in calculating t test values. Cross-tabulation comparisons of positive and negative classifications of sera by the four tests were performed by chi-square analysis. A correlation of serological tests was determined by the Pearson product-moment correlation. Correlations of serological and culture results were compared by chi-square analysis also. All analyses were calculated by using the statistical analysis system (13).

The sensitivity and specificity of each serological test was calculated: sensitivity = ([number of true positives]/[number of true positives + number of false negatives]) × 100%; specificity = ([number of true negatives]/[number of true negatives + number of false positives]) × 100% (10). For the purpose of comparison, culture results were considered absolute in that true positives referred to all cattle that were culture positive and true negatives referred to all cattle that were culture negative. False-negative referred to cattle that were serologically negative but culture positive. False-positive were cattle that were serologically positive but culture negative.

RESULTS

Comparison of mean titers. The mean antibody titers as determined by all four serological tests were significantly different between challenged cattle and negative controls ($P < 0.001$) (Table 1). For all four serological tests, a significant difference ($P < 0.001$) also was observed when the mean antibody titers for challenged cattle were compared with the mean titer for strain 19 vaccinates. There was a significant difference ($P < 0.05$) between mean antibody titers determined by the four tests for strain 19 vaccinates and control cattle.

In the cattle receiving strain 19 only, at 1 month after vaccination there was no significant difference ($P > 0.10$), by any of the four tests, between mean antibody titers for cattle vaccinated with 10^9 CFU and those for cattle vaccinated with 10^{10} CFU. In these vaccinated cattle, antibody titers were significantly higher ($P < 0.001$) at 1 month than at 6 months after vaccination.

For analysis, challenged cattle (Table 2) were subgrouped according to culture status and whether they were vaccinated or nonvaccinated. Analysis of data from all four serological tests for these subgroups of challenged cattle revealed that mean antibody titers for culture-positive cattle were significantly higher ($P < 0.001$) than mean antibody titers for culture-negative cattle. For all tests except FIAX, there was a significant difference ($P < 0.05$) between mean antibody titers of vaccinated and challenged versus nonvaccinated and challenged cattle. All four serological tests showed significantly higher ($P < 0.05$) mean antibody titers in animals that were vaccinated and culture positive than in those that were vaccinated and culture negative. All four serological tests also showed a significantly higher ($P < 0.05$) mean antibody titer in animals that were nonvaccinated and culture positive than in those that were nonvaccinated and culture negative. No significant difference ($P > 0.05$) was observed in any of the four tests when vaccinate mean titers and nonvaccinate mean titers of culture-negative animals were examined. Of animals that were culture positive, mean titers were significantly higher ($P < 0.05$) in the nonvaccinates as compared with the vaccinates by all tests except FIAX.

Comparison of serological and culture results. Results of

culture status versus serological classification for challenged cattle are presented (Table 3). There was a significant association ($P < 0.01$) for contingency comparisons of the FIAX, ST, RIV, and CF tests with culture results. There was a significant association ($P < 0.001$) between the percentage of positive and negative sera detected by each of the four tests in a contingency table analysis. Results of linear regression analysis indicated that there was a significant linear association ($P < 0.0001$) among the four serological tests (Table 4).

Sensitivity and specificity. The FIAX test had the highest sensitivity and the lowest specificity of the four tests examined (Table 5). Specificity was best for the CF test.

DISCUSSION

The results of these studies indicate that the FIAX test is readily adaptable for the detection of antibodies to *B. abortus* in cattle. The FIAX test readily detected titers due to vaccination or challenge. The FIAX test demonstrated low nonspecific binding of antibody as demonstrated by animals without detectable levels of immunoglobulin binding to the antigen. This may be inherent in the antigen used (BASA-P) and not due to the FIAX test itself. Preliminary results, in this laboratory with heat-killed, phenol-preserved

TABLE 1. Means and ranges of antibody titers to *B. abortus*

Status of animal	No. of sera	Test	Antibody titers[a]		SEM
			Range	Mean	
Controls	107	FIAX	0–51.5	3.5	0.8
		RIV	0	0.0	0.0
		CF	0–20.0	0.7	0.4
		ST	0–100.0	3.3	1.2
Challenged (strain 2308)	90	FIAX	0–891.0	163.5	19.4
		RIV	0–400	87.2	13.2
		CF	0–640	97.8	22.4
		ST	0–3,200	474.2	88.2
Vaccinated (strain 19)[b]	88	FIAX	0–318.4	34.7	6.5
		RIV	0–200.0	21.3	5.0
		CF	0–80.0	6.1	1.6
		ST	0–400.0	54.8	8.9
10^9 CFU (1-month postvaccinate)	22	FIAX	0–318.4	56.7	17.4
		RIV	0–200.0	33.0	12.7
		CF	0–80.0	10.0	3.9
		ST	0–400.0	62.5	19.9
10^9 CFU (6-month postvaccinate)	24	FIAX	0–33.1	6.9	2.5
		RIV	0	0.0	0.0
		CF	0	0.0	0.0
		ST	0–50.0	11.5	3.4
10^{10} CFU (1-month postvaccinate)	20	FIAX	0–236.6	76.7	2.8
		RIV	0–200.0	57.5	13.6
		CF	0–80.0	16.0	4.7
		ST	0–400.0	113.8	25.7
10^{10} CFU (6-month postvaccinate)	22	FIAX	0–56.4	4.7	2.8
		RIV	0	0.0	0.0
		CF	0	0.0	0.0
		ST	0–200.0	41.0	9.4

[a] FIAX titers are expressed as nanograms of immunoglobulin binding per StiQ.
[b] No subsequent challenge.

TABLE 2. Antibody titers to *B. abortus* in challenged cattle

Status of challenged animals	No. of sera	Test	Range	Antibody Titers[a]	
				Mean	SEM
Challenged (strain 2308)	90	FIAX	0–891.0	163.5	19.4
		RIV	0–400	87.2	13.2
		CF	0–640	97.8	22.4
		ST	0–3,200	474.2	88.2
Culture (positive)	57	FIAX	0–891.0	235.1	25.5
		RIV	0–400	136.8	17.9
		CF	0–640	154.4	33.2
		ST	0–3,200	736.4	127.2
Vaccinated[b]	36	FIAX	0–891.0	227.7	36.6
		RIV	0–400.0	93.1	20.2
		CF	0–640.0	71.7	30.3
		ST	0–1,600.0	313.2	77.7
Nonvaccinated	21	FIAX	5.3–444.5	247.8	30.5
		RIV	0–400.0	211.9	27.6
		CF	0–640.0	296.2	63.3
		ST	0–3,200	1,461.9	251.3
Culture (negative)	33	FIAX	0–307.8	39.7	11.6
		RIV	0–50	1.5	1.5
		CF	0	0.0	0.0
		ST	0–100	21.2	3.8
Vaccinated	27	FIAX	0–307.8	40.7	14.1
		RIV	0–50.0	1.9	1.9
		CF	0	0.0	0.0
		ST	0–100.0	20.4	4.4
Nonvaccinated	6	FIAX	23.5–55.5	35.5	5.5
		RIV	0	0.0	0.0
		CF	0	0.0	0.0
		ST	0–50	25.0	6.5
Cumulatively vaccinated[b]	63	FIAX	0–891.0	147.6	24.6
		RIV	0–400	54.0	12.9
		CF	0–640	41.9	17.8
		ST	0–1,600	187.7	47.8
Cumulatively nonvaccinated	27	FIAX	5.3–444.5	200.5	29.3
		RIV	0–400	164.8	27.5
		CF	0–640	230.4	54.6
		ST	0–3,200	1,142.6	227.0

[a] FIAX titers expressed as nanograms of immunoglobulin binding per StiQ.
[b] Vaccinated with strain 19 before challenge.

B. abortus (standard tube agglutination test antigen) as an antigen source, indicated a higher nonspecific binding of immunoglobulin than was observed with BASA-P. BASA-P has been shown to be adaptable to the enzyme-linked immunosorbant assay as well (L. B. Tabatabai and B. L. Deyoe, personal communication). Therefore, the choice of antigen may be critical.

The sensitivity of the FIAX test was greater than the sensitivity of the ST, RIV, or CF test. The specificity, however, of the FIAX test appeared less than the specificity of the other tests. A FIAX titer of more than 51.5 was defined as positive. This value represented the highest titer obtained for negative controls. Allowing a margin between the highest negative control value and the lowest designated positive response would increase the specificity but decrease the sensitivity of the test. The apparent lower specificity of the FIAX test should be examined with care. All 90 cattle used in calculating specificity were challenged with virulent

TABLE 3. Comparison of serological and bacteriological results of 90 animals challenged with strain 2308

Test	Result	Bacteriological results[a] (%)	
		Positive	Negative
FIAX	Positive	46.7	6.7
	Negative	16.7	30.0
ST	Positive	43.3	1.1
	Negative	20.0	35.6
RIV	Positive	43.3	1.1
	Negative	20.0	35.6
CF	Positive	38.9	0.0
	Negative	24.4	36.7

[a] Numerous lymph nodes, spleen, uterine washings, and each quarter of the mammary gland were cultured. Culture results were defined as positive when *B. abortus* S-2308 was isolated.

B. abortus 2308, and the greater number of false-positive (FIAX-positive–culture-negative) cattle detected may indicate the ability of the FIAX test to detect cattle harboring low numbers of the organism. The greater number also may have been due to the more sensitive FIAX test identifying residual vaccine titers. Five of the six sera that were false-positive by the FIAX test were from cattle that had been vaccinated, but because of the small sample size, statistical significance ($0.05 < P < 0.06$) could not be demonstrated. Further evidence incriminating residual titers as the cause of FIAX false-positive reactions were seen. When the mean titers of vaccinated and challenged cattle were compared with mean titers of nonvaccinated and challenged cattle, only the FIAX test did not show a significantly lower mean titer in the vaccinated group.

The FIAX, ST, RIV, and CF tests all demonstrated a significant ability to differentiate, on the basis of mean titer, challenged from vaccinated, challenged from control, and vaccinated from control cattle. But because of the overlap of titers among these groups, the FIAX test did not appear any more advantageous than the ST, RIV, or CF test in determining serologically whether an individual animal was infected with virulent strain 2308.

Agreement among the FIAX, ST, RIV, and CF tests on classification of an animal as positive or negative was significant on all 285 serum samples. Based on correlation coefficients obtained, there may have been a slightly higher agreement between the FIAX and RIV tests than between FIAX and each of the other two assays. This would be logical because the FIAX and RIV tests primarily detect an IgG response to *B. abortus*, whereas the ST and CF tests would detect IgM responses as well (1, 12).

TABLE 4. Comparison of linear correlations among serological tests

Test comparison	Correlation coefficient (*r* value)
FIAX vs CF	0.600
FIAX vs RIV	0.722
FIAX vs ST	0.601
CF vs RIV	0.456
CF vs ST	0.681
CF vs FIAX	0.600
RIV vs ST	0.762
RIV vs CF	0.456
RIV vs FIAX	0.722
ST vs FIAX	0.610
ST vs RIV	0.762
ST vs CF	0.681

TABLE 5. Comparison of relative sensitivity and specificity of four serological tests

Test	Sensitivity[a] (%)	Specificity[b] (%)
FIAX	79.2	84.6
ST	76.0	97.1
RIV	76.0	97.1
CF	72.2	100.0

[a] Sensitivity = ([number of true positives]/[number of true positives + number of false negatives]) × 100%.

[b] Specificity = ([number of true negatives]/[number of true negatives + number of false positives]) × 100%.

From these studies, it can be concluded that there are several advantages to the FIAX test as used in brucellosis serology. A nonlogarithmic endpoint titer can be achieved with one working dilution of serum. The test is rapid and relatively simple to perform. Consistency is maintained in the evaluation of results as no subjective measurements are required. With alterations in reagents, measurement of other classes or subclasses of immunoglobulin would be possible. The FIAX test appears to have greater sensitivity than the ST, RIV, and CF tests.

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