Use of an Enzyme Immunoassay Test for Characterizing the A and M Antigens of *Brucella*

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An enzyme immunoassay test was developed for detecting the A and M antigens of brucellae. One hundred fifteen isolates, including 96 strains of *Brucella abortus*, 5 strains of *B. suis*, 1 strain of *B. melitensis* and 1 strain of *B. neotomae*, were accurately serotyped with the enzyme immunoassay test. No reactions were observed with 11 isolates of *B. canis* and 1 isolate of *B. ovis*. This rapid serological test provides for considerable savings in time and materials as compared with the standard tube agglutination test.

The standard tube agglutination test has provided a useful procedure for identifying the presence of A and M antigens in brucellae (1). Serotyping is used in characterizing various biotypes and has provided information that has proven to be useful in epidemiological investigations on brucellosis in cattle and swine (3).

Recent changes in the National Brucellosis Eradication Program, including the use of adult vaccination in high-incidence herds, has resulted in an increased interest in bacteriological examinations. The increase in number of reference cultures and routine submissions to National Veterinary Services Laboratories has stimulated efforts to develop rapid, simplified procedures for identifying brucella isolates. The purpose of this study was to evaluate an enzyme immunoassay test (EIA) for identifying the presence of A and M antigens of brucella species.

MATERIALS AND METHODS

Antigens. Subcultures of 115 strains of brucella were made on tryptose agar slants and incubated at 37°C in the presence of 5% CO₂ for 4 days (Table 1). The cultures were harvested by washing the slants with physiological saline. Cell suspensions were killed by autoclaving at 121°C for 15 min.

EIA conjugate. Goat anti-rabbit gamma globulin (Miles Laboratories, Elkhart, Ind.) was labeled with horseradish peroxidase (Sigma Chemical Co., St. Louis, Mo.), using a modification of the method of Nakane and Kawaoi (4). Sephadex column filtration was not used. Five milligrams of horseradish peroxidase was used to label 20 mg of gamma globulin. The sterile filtered conjugate was stored at 4°C. In preliminary evaluations, two dilutions of conjugate (1:100

and 1:200) were compared. Dilutions were made using 0.5 M NaCl containing 1% Tween 80 (Fisher Scientific, Fair Lawn, N.J.) adjusted to pH 7.5 with 0.1 M K₂HPO₄.

Monospecific antisera. Three rabbits in each of two groups were infected with a suspension of brucella. Each rabbit in group I was given approximately 10⁹ *Brucella abortus* strain 2308 intravenously and each rabbit in group II was given approximately 10⁹ *B. melitensis* strain 16M intravenously. After 6 days the rabbits were bled and the serum was recovered. Heat-killed cells of *B. abortus* strain 1119-3 were used to absorb the serum from rabbits inoculated with *B. melitensis* strain 16M, and heat-killed cells of *B. melitensis* strain 16M were used to absorb the serum from rabbits infected with *B. abortus* strain 2308. Duplicate serial dilutions of the absorbed sera were set up in a tube test, using homologous and heterologous antigens to evaluate absorption procedures and determine when the maximum amount of heterologous antibody had been removed with a minimum reduction in the homologous antibody titer.

Substrate. A working solution of substrate was prepared using hydrogen peroxide and 2,2'-azino-di-(3-ethyl benthiozoline 6-sulfonate) in citric acid (ABTS) (M. L. Bartlett, unpublished data).

EIA test protocol. The EIA test was conducted by a modification of a procedure described previously (5, 6). A 0.05-ml volume of a 2.5% solution of calf serum in saline was placed in each well of a microtiter tray (type IS-FB-96TC, Linbro Scientific, Hamden, Conn.). The trays were allowed to air dry, and then 0.05 ml of a 0.25% glutaraldehyde in phosphate-buffered saline solution (pH 7.2) was added to each well. After 30 min, the trays were washed four times with sterile distilled water. After drying, 0.05 ml of a cell suspension of the unknown test culture was added to each of four microtiter wells. The plates were allowed to dry at about 22°C for 16 h or dried under a heat lamp for 45 min and then were washed with saline.

For routine tests, the antisera prepared in rabbits were diluted 1:32 and 1:64 in 0.5 M NaCl containing 1% Tween 80 (pH 7.5). Added to each test well was

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TABLE 1. Comparison of EIA and agglutination test results on 115 *Brucella* isolants from animals

<i>Brucella</i> sp.	Animal source	No. isolated	No. showing:			
			Agglutination in monospecific serum		EIA test reactions	
			A	M	A	M
<i>B. abortus</i> biotype						
1	Cattle	38	38	0	38	0
1	Mink	4	4	0	4	0
1	Deer	1	1	0	1	0
2	Cattle	25	25	0	25	0
4	Cattle	27	0	27	0	27
4	Chicken	1	0	1	0	1
<i>B. suis</i> biotype						
1	Swine	3	3	0	3	0
3	Swine	1	1	0	1	0
4	Reindeer	1	1	1	1	1
<i>B. melitensis</i> biotype						
1	Goat	1	0	1	0	1
<i>B. neotomae</i>	Desert wood rat	1	1	0	1	0
<i>B. canis</i>	Dogs	10	0	0	0	0
	Human	1	0	0	0	0
<i>B. ovis</i>	Sheep	1	0	0	0	0

0.05 ml of diluted serum. The sera were incubated for 8 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. 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 10 min at 22°C. The color developed by the reaction was observed and graded from 0 to 4 for intensity of green color developed in the reaction mixture. No color change (0) was considered negative.

RESULTS

Either the 1:32 or the 1:64 dilution of the monospecific serum provided suitable concentrations in the EIA test for identifying A and M antigens. No important cross-reactions were observed using these concentrations with any of the 115 *brucella* isolates examined.

The results of comparisons of conjugate concentration at 1:100 and 1:200 dilutions, respectively, revealed that either concentration provides for definitive identification of A and M antigens. However, the 1:200 dilution of conjugate was selected for final testing because the intensity of green color which developed provided for suitable identification of positive reactions at 10, 20, and 30 min. Therefore, using a 1:200 dilution of conjugate permitted the testing of greater numbers of isolates during the work day. Minor cross-reactions (2+) were observed at a 1:100 dilution of conjugate; none of these strains showed cross-reactions using a 1:200 dilution of conjugate.

The A and M antigens of 96 isolates of *B. abortus*, including 43 strains of biotype 1, 25 strains of biotype 2, and 28 strains of biotype 4, were correctly identified by the EIA test. Five strains of *B. suis* representing biotypes 1, 3, and 4 were also characterized by the EIA test. The test accurately identified the A and M antigens of reference strains of *B. melitensis* and *B. neotomae*. No reactions were observed in the EIA test using 11 strains of *B. canis* and a reference culture of *B. ovis* as the test antigens.

DISCUSSION

The EIA test has been used for identifying antibody present in sera of animals with viral, bacterial, and parasitic infections (5, 6). However, there are no reports on its use in characterizing bacteria. The rapid EIA test described here provided for the accurate identification of the predominant antigen (A or M) of 115 strains of *brucella* isolated from various animals in different geographical regions of the United States. The strains examined were representative of the isolates obtained at National Veterinary Services Laboratories (2). Studies should be made using biotypes commonly isolated in other countries.

The EIA test provides for considerable savings in materials and time as compared with the standard tube agglutination test. Moreover, test results are available for reporting in 90 min, as compared to 48 h for the tube test. Additional studies should be made to determine the efficiency of the EIA test for the characterizing of

rough strains. Although rough strains are not frequently isolated from animals, there are reports of such isolations. The test could be useful in characterizing isolates which may have dissociated after repeated subculture on laboratory media.

The EIA test is a rapid, simple procedure which can be readily automated to allow for screening large numbers of cultures for antigenic characteristics. The use of the modified EIA test in typing other microorganisms, including certain bacteria, fungi, and parasites, should be investigated.

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