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# Comparison of Agar-Gel Immunodiffusion with Other Serological Tests for Diagnosis of Bovine Brucellosis

K. J. McMahon, Ann Gersten and Jean Coull

In recent years, brucellosis has remained a zoonosis of world-wide public health and economic importance (1). Although there has been a marked reduction in the prevalence of brucellosis in cattle in the United States, from approximately 11% of all cattle tested in 1935 to less than 1% in 1977, the U.S. Department of Agriculture (USDA) has estimated that the disease still costs American livestock producers in excess of \$30 million annually (24). Also, there was a resurgence of human brucellosis in 1974, with 246 cases reported to the Center for Disease Control, an increase of 71 cases over the 175 reported in 1973 (5). This increase continued in 1975 with 328 cases reported; and, for the first time since 1959, the number of cases acquired from cattle exceeded the number acquired from swine (5). In 1976, 282 cases of human brucellosis were reported (6), and the decline continued with 241 and 172 cases reported in 1977 and 1978 (7).

Isolation and identification of the organism provides definite evidence of *Brucella* infection; but, because this is often difficult, serological tests are relied upon for routine diagnosis of brucellosis (1). The agglutination test has been the principal serological method used in the past and continues to be the most useful for human and bovine brucellosis (1). The tube- and plate-agglutination tests are most commonly used in the United States as standard tests, and all laboratories use the same antigens, the same test procedures and the same criteria for interpreting the results (24). However, where the incidence is low, these tests lack the sensitivity and specificity to accurately indicate the presence or absence of brucellosis in some cattle (24). The agglutination test frequently gives low titers in infected animals and also in animals from which no organisms can be isolated (2). The card test is a rapid, simple agglutination screening test, but may yield false-positive reactions so positive sera are subjected to confirmatory tests (1).

Various supplemental tests have been devised in an effort to distinguish reactions caused by field infections from those caused by vaccination with Strain 19. The complement-fixation test, when conducted by methods approved by National Veterinary Services Laboratories (NVSL), is an official test (26), and it is used as the standard definitive test in Australia, Great Britain, Ireland and some European countries (24). However, in the United States it has not been well-standardized, and other tests yield similar information and are easier to perform (24).

In 1965 Reddin *et al.* (23) found that the IgG-type immunoglobulin was the distinguishing feature of acute and chronic forms of human brucellosis. This type of immunoglobulin is indicative of infection in cattle also (12, 16, 24). The rivanol test detects IgG agglutinins (18), and it is an official test when conducted in State-Federal laboratories (26). The 2-mercaptoethanol (2-ME)-agglutination test is a supplemental test that also detects IgG agglutinins (18).

In 1976, a radioimmunoassay was suggested for use in diagnosis of bovine brucellosis, but its usefulness in routine diagnosis has yet to be determined (8).

In 1978, Byrd *et al.* (3) reported that the sensitivity of the enzyme-linked immunosorbent assay (ELISA) was comparable to the complement-fixation and rivanol tests for detecting *Brucella* antibodies in bovine serum. They concluded, however, that standardization of reagents and procedures should be established before the method is proposed for diagnostic application. An enzyme immunoassay has been developed for detecting *Brucella* antibodies in milk of cows infected with *Brucella abortus* (25).

It has been suggested that serum precipitins for *Brucella* may be a better indication of active infection than serum agglutinins (13). Corbel evaluated an immunodiffusion test for detection of *Brucella* antibodies in bovine sera and concluded that it may be useful for sera that give equivocal results in standard tests (9). An agar-gel immunodiffusion (AGID) technique for ram epididymitis caused by *B. ovis* was

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described by Myers and Siniuk (21), and results were shown to be similar to those obtained with the complement-fixation test. The technique was also recommended by Muncz *et al.* as a screening test for *B. ovis* antibodies (19).

Diaz *et al.* (11) made a comparative study of diagnostic tests with sera from patients with acute brucellosis and found a close correlation among results obtained with the agglutination and Rose Bengal tests and the AGID test using *Brucella* A & M antigen. McMahon *et al.* (17) used a standard *B. abortus* antigen in an AGID test on human serum and reported 97% agreement with the tube-agglutination test when the titer was 1:160 or higher.

Iannelli *et al.* (15) evaluated the single radial diffusion technique for identification of *B. abortus* antibodies in cattle sera and reported that it was as specific as the complement-fixation and Rose Bengal tests but less sensitive than those tests. A radial immunodiffusion (RID) technique using *Brucella* polysaccharide B antigen has been suggested as an initial confirmatory test on card-positive bovine sera by Diaz *et al.* (10).

The purpose of the present study was to determine the diagnostic value of the AGID test for bovine brucellosis in comparison with the tube-agglutination, rivanol and 2-ME-agglutination tests.

## MATERIALS AND METHODS

### Antigens

All antigens used were obtained from NVSL, Ames, IA. The antigen used for each test was the one designated for the specific test with the exception of the AGID test. Because there is no standard antigen for the AGID test, *B. abortus* strain 1119-3 plate antigen was used. It was modified as described for use in detection of *Brucella* antibodies in human serum (17). Ten-milliliter amounts were sonicated for 15 minutes in an ice bath with the standard probe of the Biosonik IV sonic oscillator (VWR Scientific, Inc., San Francisco, CA) at 20 W with settings of high range and minimum output.

### Sera

Tube-agglutination and AGID tests were applied to 125 card-negative bovine sera. Those that produced agglutination in the tube-agglutination test were also examined in the 2-ME-agglutination test. Of the 125 sera, 62 were obtained from vaccinated dairy cows in the North Dakota State University dairy herd. Forty-three were obtained from cattle delivered for slaughter to Flavorland Industries, West Fargo, ND, and 20 were submitted to the cooperating State-Federal laboratory, Pierre, SD.

The AGID, rivanol and 2-ME-agglutination tests were applied to 270 card-positive bovine sera that showed a titer of incomplete 1:50 or higher in the tube-agglutination test. These sera were submitted to the cooperating State-Federal laboratories in Huron or Pierre, SD, for serological examination for *Brucella* antibodies. Forty of the sera were obtained from bison.

### Card test

The USDA method (28) using the brucellosis card-test kit from Hynson, Wescott and Dunning, Inc. (Baltimore, MA), was employed. With a capillary tube attached to a rubber bulb, 0.03 ml of serum was delivered to the test area of the card. Two drops (0.015 ml in each drop) of card-test antigen were placed adjacent to the drop of serum. The serum and antigen were mixed with a stirrer over the surface of the "tear-drop" area. The card was rocked for 4 min with a rocking machine at approximately 10 to 12 rocks per min. A positive serum showed moderate to large clumps, and a negative one showed dispersed particles without characteristic clumps or showed no clumping.

### Tube-agglutination test

The technique used was the USDA decimal-dilution method (27). With a 0.2-ml pipette, 0.08 ml of serum was delivered to the first tube in a series, and 0.04, 0.02, 0.01 ml and 0.005 ml were delivered to tubes two through five. Two milliliters of properly diluted tube antigen was added to each tube. This resulted in dilutions of 1:25, 1:50, 1:100, 1:200 and 1:400. Racks of tubes were shaken and incubated in a covered water bath at 37°C for 48 hr. In reading results, tubes in which the serum-antigen mixture was clear, and gentle shaking did not disrupt the flocculi, were considered positive. An incomplete reaction was recorded when the serum-antigen mixture was only partially clear, and gentle shaking did not disrupt the flocculi.

### Rivanol test

Following the USDA plate method (28), 0.4 ml of 1% rivanol solution was added to a tube for each sample to be tested. An equal quantity of serum was added to the tube. It was shaken, held at room temperature for 5 min and centrifuged at 1000 x *g* for 15 min. With a 0.2-ml pipette, 0.08, 0.04, 0.02 and 0.01 ml quantities of supernatant fluid were placed on squares on a glass plate. One drop (0.03 ml) of rivanol antigen was added to each quantity of supernatant fluid and mixed with a stirrer. The plate was rotated four times and placed in a holding box. After 6 min, the plate was rotated as before and incubated another 6 min. Then the plate was rotated

four times. In reading results, a complete reaction was recorded if most of the cells were agglutinated. If intermediate degrees of reaction occurred, an incomplete reaction was recorded. Complete agglutination in the 1:25 dilution or higher was considered positive, and less than complete agglutination in the 1:25 dilution was considered negative (26).

### 2-ME-agglutination test

For each serum to be tested, the quantity added to each of five tubes was the same as for the tube-agglutination test. One milliliter of 0.1-M solution of 2-ME was delivered to each tube. The 2-ME solution was prepared in 0.85% sodium chloride that did not contain phenol. After addition of 2-ME, 1.0 ml of double-strength tube antigen without phenol was added to each tube. Tubes were incubated and read in the same manner as the tube-agglutination test. Complete agglutination in the 1:25 dilution or higher was considered positive. Less than complete agglutination in the 1:25 dilution was considered negative.

### AGID test

The technique developed for detection of antibodies in human serum was used (17). It was a slide technique using 50 x 75 mm slides containing 10 ml of 1% Noble agar (Difco). The buffer was borate saline, pH 8.3 (4) with ethylmercurithiosalicylic acid-sodium salt (0.1%) added to prevent bacterial growth. The melted agar was added to slides with a pipette and solidified at 4°C for 30 min. Wells 7 mm in diameter and 3 mm apart were made with an Auto-Gel T/M cutting instrument (Grafer Corp., Detroit, MI) in a hexagonal pattern with a center well. The bottom of each well was sealed with 10 microliters of agar before the addition of serum or antigen to prevent possible seepage of reactants. Seventy microliters

of sonicated antigen was placed in the center well with an Eppendorf pipet (Brinkman Instruments, Inc., Westbury, NY), and equal amounts of undiluted sera were added to the outer wells. Slides were incubated in humidity chamber at 37°C and examined after 6, 8, 24, 48 and 72 hr for precipitin lines. A magnifying hand lens was used to facilitate reading of results.

## RESULTS

All 125 card-negative sera were negative in the AGID test; however, 24 produced complete or incomplete agglutination in the 1:25 dilution and six in the 1:50 dilution in the tube-agglutination test. None of these was positive in the 2-ME-agglutination test.

Results obtained in the AGID, rivanol and 2-ME-agglutination tests on the 270 card-positive sera that showed a tube-agglutination titer of incomplete 1:50 or higher are compared in Table 1. Of the 270 sera, 237 were positive in the AGID test, 200 were positive in the rivanol test and 196 were positive in the 2-ME-agglutination test.

The positive and negative reactions of the three tests on each of the sera are shown in Table 2. Of the 270 sera, 179 were positive and 24 were negative in all three tests. Of the additional 58 sera that were positive in the AGID test, 15 were positive in the rivanol test and negative in the 2-ME-agglutination test, 14 were positive in the 2-ME-agglutination test and negative in the rivanol test, and 29 were negative in both the rivanol and 2-ME-agglutination tests. Six sera that were negative in the AGID and 2-ME-agglutination tests were positive in the rivanol test, and three sera that were negative in the AGID and rivanol tests were positive in the 2-ME-agglutination tests. No serum was negative in the AGID test and positive in both of the other tests.

Table 1. Comparison of the brucellosis standard tube-agglutination titer with results obtained in the AGID, rivanol and 2-ME-agglutination tests on 270 card-positive bovine sera.

Tube agglutination titer (I or +)	No. of sera	No. of sera giving positive reactions with:		
		AGID	Rivanol (+ 1:25 or higher)	2-ME agglutination (+ 1:25 or higher)
1:50	59	39	23	28
1:100	68	60	46	39
1:200	58	54	47	46
1:400	85	84	84	83
TOTAL	270	237	200	196
% positive		87.8	74.1	72.6

**Table 2. Positive and negative reactions in AGID, rivanol and 2-ME-agglutination tests on each of the 270 card-positive bovine sera with a tube-agglutination titer of incomplete 1:50 or higher.**

AGID	Reactions		No. of sera	% of total
	Rivanol	2-ME		
+	+	+	179	66.3
+	+	-	15	5.6
+	-	+	14	5.2
+	-	-	29	10.7
-	+	+	0	0.0
-	+	-	6	2.2
-	-	+	3	1.1
-	-	-	24	8.9

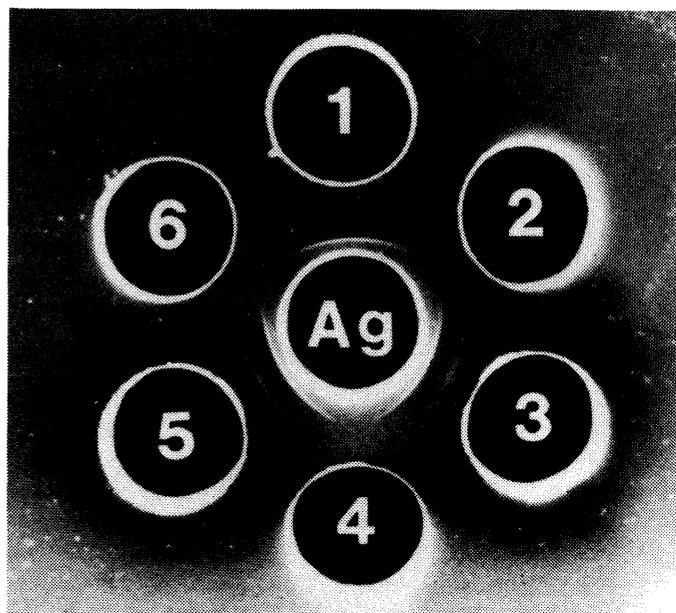
In the AGID test, slides were observed for precipitin lines after 6, 8, 24, 48 and 72 hr of incubation. The number of sera that required each of the incubation times before visible lines were formed is shown in Table 3. All 48 sera that produced precipitin lines within 8 hr were positive in both the rivanol and 2-ME-agglutination tests. Of the 135 sera that formed precipitin lines within 24 hr, 110 were positive and 11 were negative in both of the other tests. Of the remaining 14 sera, seven were positive in the rivanol test and negative in the 2-ME-agglutination test, and seven were positive in the 2-ME-agglutination test and negative in the rivanol test. Of the 48 sera that formed precipitin lines within 48 hr, 19 were positive and 15 were negative in both of the other tests. Of the remaining 14 sera, seven were positive in the rivanol test and negative in the 2-ME-agglutination test, and seven were positive in the 2-ME-agglutination test and negative in the rivanol test.

Only six sera required 72 hr of incubation before precipitin lines were formed. Two of these were positive, and three were negative in both of the other tests. One serum was positive in the 2-ME-agglutination test and negative in the rivanol test.

More than one precipitin line was formed by 78 of the 237 AGID-positive sera. Most frequently a single line appeared first, and the second and third lines formed upon continued incubation. Double lines observed with some sera are shown in Fig. 1.

## DISCUSSION

Results obtained in the present study are in general agreement with the commonly held view that high-agglutination titers usually indicate infection, but low



**Fig. 1. Immunodiffusion of sonicated *B. abortus* antigen (Ag, center well) and bovine sera with agglutination titers of 1:100 (well 1), 1:200 (well 3) and 1:400 (well 5). Known negative sera were in wells 2, 4 and 6.**

titers or negative reactions do not exclude it (14, 16). Of the 85 sera with a titer of 1:400 (highest dilution tested), 83 were positive in the AGID test and both supplemental tests, and 84 were positive in the AGID and rivanol tests. Of the 59 sera with a titer of only 1:50, the numbers of positive reactions in the AGID, 2-ME-agglutination and rivanol tests were 39 (66%), 28 (48%) and 23 (39%) respectively. Although the rivanol test is an official test (26), in the present study, the 2-ME-agglutination test appeared to be equally effective. Of the 270 card-positive sera tested, 200 were positive in the rivanol test and 196 were positive in the 2-ME-agglutination test. When the reactions of the two tests on the same sera were compared, the agreement was 86% (Table 2).

The rivanol and 2-ME-agglutination tests detect IgG-type antibody which is indicative of *Brucella* infection (18). Pike (22) concluded that IgG antibodies were more effective precipitins than IgM antibodies. The ELISA test has been used in our labora-

**Table 3. Time required for formation of precipitin lines by 237 AGID-positive bovine sera.**

Length of incubation (hr)	No. of sera
6	26
8	22
24	135
48	48
72	6

tory to detect IgG *Brucella* antibodies in human serum, and results correlated well with those obtained with the AGID test (manuscript in preparation). Of the 237 sera that were positive in the AGID test, 208 (87.8%) were positive in either the rivanol or 2-ME-agglutination tests or in both tests. Twenty-nine sera that were positive in the AGID test were negative in both the rivanol and 2-ME-agglutination tests. In comparing the AGID and 2-ME-agglutination tests for detection of *Brucella* antibodies in human serum, McMahon *et al.* (17) reported that sera from two patients with bacteriologically proven brucellosis and eight sera from abattoir employees with suspected but not bacteriologically proven brucellosis were positive in the AGID test and negative in the 2-ME-agglutination test.

The AGID test has been reported to be as sensitive as the complement-fixation test and more practical for the diagnosis of *B. ovis* infection (20). Recently, Diaz *et al.* (10) have suggested that the RID test with polysaccharide B antigen could be employed as an initial confirmatory test on card-positive bovine sera, and results could be available the same day. If a serum were positive, they would consider the corresponding animal infected. If the RID test were negative, the serum should be tested by complement fixation or other confirmatory tests.

Of the 270 card-positive sera tested in the present study, 203 produced identical reactions in the AGID, rivanol and 2-ME-agglutination tests. Of those, 179 sera were positive in all tests, and 24 were negative in all tests. With 38 additional sera, there was agreement between results obtained with the AGID test and either the rivanol or 2-ME-agglutination tests.

Results obtained suggest that the AGID test described merits further consideration as a possible confirmatory test on card-positive bovine sera. It is a simple procedure using a standardized antigen modified only by sonication.

If precipitin lines appear within 8 hr, further testing should not be necessary because all sera that formed lines by that time were positive in both of the supplemental tests. Of the 135 sera that formed precipitin lines within 24 hr, 110 were positive and 11 were negative in both of the other tests. The remaining 14 sera were positive in either the rivanol or the 2-ME-agglutination tests but not in both tests. Further study may help to determine if sera that form precipitin lines in 24 hr should be subjected to other confirmatory tests. Sera that require 48 hr or longer to form precipitin lines should be subjected to other confirmatory tests.

## SUMMARY

A comparison was made of results obtained with the agar-gel immunodiffusion (AGID) test and the rivanol and 2-ME-agglutination tests on 270 card-positive bovine sera that showed a tube-agglutination titer of incomplete 1:50 or higher. Of the 270 sera, 237 (87.8%) were positive in the AGID test, 200 (74.1%) were positive in the rivanol test and 196 (72.6%) were positive in the 2-ME-agglutination test. The time required for formation of precipitin lines in the AGID test varied from 6 to 72 hr, with 183 of the 237 positive sera forming lines within 6 to 24 hr. Of the 183 sera, 158 were positive in both the rivanol and 2-ME-agglutination tests. All 125 card-negative sera were negative in the AGID test.

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