Comparative Sensitivity of Gel-Diffusion and Tube Agglutination Tests for the Detection of *Brucella canis* Antibodies in Experimentally Infected Dogs

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The signs of canine brucellosis caused by *Brucella canis* are similar to those seen in *Brucella ovis* infection in sheep. These include abortions and embryonic deaths in the female and epididymitis and testicular atrophy in infected males (1). Little is known of the incidence or distribution of the infection, although, like ovine brucellosis, it appears to be widespread. The disease has frequently been associated with the beagle, although infection has been reported in other breeds of dogs and more recently also in humans (6, 11).

The gel-diffusion (GD) method reported by Myers and Siniuk (9) was shown to be a simple, rapid, and reliable technique for the diagnosis of *B. ovis* infection of sheep. Results of GD test correlated very well with those obtained with the complement fixation test. It was further demonstrated by Myers et al. (8), and others (2, 3), that a hot saline-extracted antigen from rough phase "R" *Brucella* reacts only with sera from animals infected or vaccinated with organisms containing the specific R surface antigen characteristic of *B. ovis* and *B. canis* and not with the specific smooth "S" *Brucella* surface antigens. These findings suggested that the microslide gel-diffusion technique using the saline-extract R antigen would be of value in the diagnosis of *B. canis* infection.

The present study was designed to compare the results obtained with the agar gel-diffusion test using *B. canis* and *B. ovis* R antigens and the standard *B. canis* tube agglutination (TA) test (1). Sera from dogs experimentally infected with *B. canis* and from a random sample of dog sera with unknown histories of exposure to this organism were used.

**MATERIALS AND METHODS**

**Antigens.** Saline extracts of *B. canis* and *B. ovis* R antigen were prepared as described previously by Myers et al. (8) using a strain of *B. ovis* isolated at this Center and the *B. canis* strain RM 606. The antigens were freeze-dried or distributed in 1-ml amounts and stored at -25°C for use in GD tests.

Portions of these saline extracts were concentrated with Carbowax and fractionated in 2-ml amounts with Sephadex G-200 columns (2 by 90 cm) equilibrated with 0.125 M phosphate buffer (pH 7.0) containing 0.02% sodium azide. The optical density of the column eluates was determined spectrophotometrically at 280 nm and examined without concentration by GD tests against rabbit antisera to *B. canis* and *B. ovis*. The same *B. canis* strain was used to prepare antigen for the TA test as described by Carmichael and Kenney (1). This antigen was stored at 5°C until ready for use.

**Source of sera.** Six male mix-breed dogs were experimentally infected with 10⁹ living *B. canis* organisms by intravenous inoculation (group 1). Two other dogs were similarly inoculated with the same number of heat-killed (56°C for 15 min) *B. canis* organisms (group 2). Two other dogs were administered 10⁹ living *B. ovis* organisms by the intravenous route (group 3), and two additional animals were employed as unexposed controls (group 4).

All animals were separately housed by groups according to the inoculum received. Prior to exposure, all dogs received prophylactic vaccinations against distemper, canine hepatitis, and rabies, and treatment for possible arthropod or gastrointestinal parasites.

Blood and serum samples were collected 7 days
after the dogs had been exposed, then weekly for 3 months and again three times at monthly intervals.

In an attempt to isolate Brucella organisms, portions of the clotted blood were inoculated into Albimi Broth (Pfizer Co.) and incubated at 37°C in an atmosphere of 10 to 20% carbon dioxide. After 1 and 2 weeks of incubation, subcultures were made in duplicate Brucella Albimi agar plates containing 10% rabbit serum. Culture plates were examined daily for 2 weeks for the presence of characteristic Brucella colonies. Sera were stored at -25°C for subsequent serological testing.

Sera collected from 276 mix-breed dogs, purchased locally for other purposes from 1968 to 1972, were examined for antibodies to Brucella by GD and TA tests.

Serological tests. Sera were examined in the microslide GD test as previously described (8, 9). The tests were carried out at room temperature, and precipitin reactions were recorded at 24, 48, and 72 h. Selected serum samples were absorbed with homologous and heterologous Brucella antigens as described previously (8).

The TA test for B. canis was the one recommended by Carmichael and Kenney (1) in which sera were serially diluted in phosphate-buffered saline (pH 7.2) from 1:50 to 1:6,400. Tests were incubated in a water bath at 50 to 52°C and examined visually at 24 and 48 h. The highest dilution showing a 3+ agglutination was considered the end point, and the titer was expressed as the reciprocal of this dilution.

RESULTS

Pre-inoculation serological examinations were negative to the regular TA test for infection with classical species of smooth Brucella and to the TA test for B. canis. No precipitins were observed in GD tests with either saline extract of B. canis or B. ovis R antigens. The same serological results were observed in unexposed dogs (group 4) throughout the entire study period.

Seven days post-inoculation with living B. canis organisms, sera from all six dogs (group 1) showed agglutination titers ranging from 1:400 to 1:1,600 and positive GD reactions with both B. ovis and B. canis antigens.

From the second week to the third month postexposure, all animals were serologically positive to the three antigens, and B. canis was recovered two times by blood culture from one dog. None of the animals showed clinical signs of infection. TA titers began to decline from the third to sixth month postexposure, as did the number of positive GD reactions.

To determine the comparative diagnostic sensitivity of the GD test using B. canis and B. ovis R antigens and the TA test, the results obtained with the 96 sera in group 1 were tabulated as shown in Table 1. An 88.8% agreement was observed between the GD test using B. canis antigen and the TA test. Differences in the sensitivity of these tests were confined to sera showing TA titers of 1:100 to 1:400.

When the GD test was performed employing B. ovis saline-extract antigen, however, a correlation of 95.8% was obtained with the TA test results. Only 4.1% of the sera was negative to the GD test with either antigen, although these showed agglutinin activity at levels of 1:100 or 1:200 (Table 1).

The possibility that the higher sensitivity of the B. ovis antigen in the GD test was due to a difference in the B. canis R antigen concentration was contemplated. The latter antigen was concentrated two times by Carbowaax and used to retest the six sera which were positive to B. ovis and negative to B. canis in GD tests. Three of these sera were then found to be positive to the GD test with this concentrated B. canis antigen. Two of these had TA titers of 1:200, and the third one had a titer of 1:400.

One week after the inoculation of two dogs with killed B. canis organisms (group 2), their sera were positive to the GD test with both B. canis and B. ovis antigens and had TA titers of 1:200. By the second week, the GD test using B. canis antigen was negative. These sera, however, were positive to the B. ovis antigen at this time and became negative by the third week. TA titers of 1:100 were observed in the sera of these animals until the third week and were negative thereafter.

The two dogs inoculated with living B. ovis organisms (group 3) were positive to the GD test using the homologous antigen, and one of them had a TA titer of 1:100 by the second week post-inoculation. By the third week, the GD test was positive to the B. canis and B. ovis anti-

| B. canis agglutination titers* | No. of sera examined | + | + | + | + |
|-------------------------------|----------------------|---|---|---|---|
|                               |                      | B. canis | B. ovis | B. canis | B. ovis |
| 100                           | 4                    | 0         | 0     | 2        | 2       |
| 200                           | 8                    | 3         | 0     | 3        | 2       |
| 400                           | 20                   | 19        | 0     | 1        | 0       |
| 800                           | 38                   | 38        | 0     | 0        | 0       |
| 1,600                         | 20                   | 20        | 0     | 0        | 0       |
| 3,200                         | 6                    | 6         | 0     | 0        | 0       |
| Total                         | 96                   | 86        | 0     | 6        | 4       |

* Reciprocal of titers.
gens, and both dogs showed a serum agglutinin titer of 1:200. Thereafter, the number of positive GD and TA tests gradually declined.

Eight of the randomly collected dog serum samples showed a positive GD reaction with B. canis and B. ovis antigens. As shown in Table 2, six sera were TA-test positive when the B. canis whole cell antigen was used. Three of these were also positive at low titers, using smooth B. abortus antigen. Samples 193 and 199 absorbed with B. canis, and B. ovis R antigen removed the precipitins to these antigens without affecting the GD reaction to antigens prepared from smooth Brucella. In contrast, when the two sera were absorbed with smooth Brucella antigen, GD reactions were observed only with the R antigen of B. canis and B. ovis.

Both the B. canis and B. ovis saline-extract antigens, characterized by gel-filtration using Sephadex G-200, showed identical elution patterns. The single peak in the exclusion volume of the column was then used to study the antigenic relationship of the B. canis and B. ovis R antigens. As illustrated in Fig. 1, the B. ovis eluate showed a single precipitin line of identity in GD tests with homologous and B. canis rabbit antisera and with sera from dogs 193 and 199. The same reaction pattern of antigenic identity was observed when the B. canis eluate and the same sera were employed in GD tests.

**DISCUSSION**

Low level nonspecific agglutinins in the B. canis TA test are attributed to cross-reactions with other gram-negative bacteria (1, 10) or the use of hemolyzed serum samples (5), whereas titers varying from 1:100 to 1:400 or greater have been considered as indicative of active B. canis infection (1, 4, 5).

In the present study, the sensitivity of the GD test using the B. ovis saline-extract antigen in the serological diagnosis of experimental B. canis infection of dogs was found to be comparable to that of the TA test of Carmichael and Kenney (1) which is currently employed for this purpose by many laboratories. Minor differences in sensitivity between both tests, however, were observed only in sera showing low levels of agglutinin activity. In sera with TA titers of 1:100 to 1:200, the B. ovis antigen was more sensitive than the B. canis antigen in GD tests. This was also observed in the sera from dogs inoculated with killed B. canis cells or with living B. ovis organisms. This suggested that, although both organisms show antigenic identity by immunodiffusion tests, quantitative differences in the amount of the R surface antigen in the two strains may account for this variation.

This consideration seems to be supported by the observation that sera which were positive only to B. ovis in GD tests also became reactive to the B. canis antigen when it was used at 2× concentration. Furthermore, the intensity of the precipitin lines produced by B. ovis against homologous and heterologous hyperimmune rabbit sera was more pronounced than when B. canis antigen was used. Using the concentrated (2×) B. canis antigen, however, the intensity of the precipitin lines against both hyperimmune sera was also observed to increase.

Using the GD test with B. ovis and B. canis antigens, specific antibodies to rough Brucella were observed in the sera of eight of 276

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**Table 2. Comparison of positive results obtained by agglutination and gel-diffusion tests in sera from eight randomly selected dogs with unknown exposure to Brucella**

| Serum identification no. | Agglutination titers* | Gel-diffusion reactions with B. canis | Gel-diffusion reactions with B. ovis R antigen |
|--------------------------|----------------------|-------------------------------------|--------------------------------------|
|                          | B. canis | B. abortus | B. canis and B. ovis | R antigen |
| 199                      | 800      | 100        | +                     |            |
| 196                      | 400      | 0          | +                     |            |
| 194                      | 800      | 25         | +                     |            |
| 195                      | 800      | 25         | +                     |            |
| 160                      | 100      | 0          | +                     |            |
| 76                       | 0        | 0          | +                     |            |
| 71                       | 100      | 0          | +                     |            |
| 16                       | 0        | 0          | +                     |            |

*Reciprocal of titers.
randomly selected dogs. To our knowledge, this is the first report of rough *Brucella* antibodies demonstrated in dogs in Latin America.

Because a freshly isolated culture of *B. canis* was not available for the experimental infection of dogs, animals in the present study were infected with the reference *B. canis* strain RM 666. Evidence of infection was confirmed by blood culture isolation from one dog as late as 2 months after inoculation with this organism. Although a higher recovery rate of *B. canis* organisms was anticipated, a reduction in the virulence of the inoculated strain, as a result of repeated subculturing and/or the culture method employed, may account for their low recovery rate from the experimentally infected dogs. Animals receiving this infection, however, maintained a high antibody level throughout the entire 6-month study period, in contrast to dogs receiving the same number of killed organisms which showed an antibody response only until the third week post-inoculation.

Previous work has shown (7) that the *B. ovis* saline-extracted antigen is of value in the diagnosis of ram epididymitis caused by *B. ovis*. The present findings, using the GD test, indicate that this antigen preparation is also adequate for the serological diagnosis of canine brucellosis caused by *B. canis*. This antigen may be easily prepared and maintained in freeze-dried form for ready use in the field and does not have the difficulties associated with the antigen employed in the TA test for the diagnosis of *B. canis* infections (3).

**LITERATURE CITED**

1. Carmichael, L. E., and R. M. Kenney. 1968. Canine abortion caused by *Brucella canis*. J. Amer. Vet. Med. Ass. 152:605--616.
2. Díaz, R., and N. Bossery. 1973. Identification d'un campé de *Brucella* de la phase rugueuse (R des *Brucella*). Ann. Rech. Vét. (Paris) 4:283--292.
3. Díaz, R., and L. M. Jones. 1973. The immuno-diffusion method for the identification of cattle vaccinated with *Brucella abortus* strain 45/20. Vet. Rec. 93:300--302.
4. Moore, J. A. 1969. *Brucella canis* infection in dogs. J. Amer. Vet. Med. Ass. 153:2034--2037.
5. Moore, J. A., B. N. Gupta, and G. H. Conner. 1968. Eradication of *Brucella canis* infection from a dog colony. J. Amer. Vet. Med. Ass. 153:523--527.
6. Morrisset, R., and W. W. Spink. 1969. Epidemic canine brucellosis due to a new species, *Brucella canis*. Lancet 2:1000--1002.
7. Myers, D. M. 1973. Field evaluation of the gel-diffusion test for the diagnosis of ram epididymitis caused by *Brucella ovis*. Appl. Microbiol. 26:855--857.
8. Myers, D. M., L. M. Jones, and V. M. Varela-Díaz. 1972. Studies of antigens for complement fixation and gel diffusion tests in the diagnosis of infections caused by *Brucella ovis* and other *Brucella*. Appl. Microbiol. 23:894--902.
9. Myers, D. M., and A. A. Siniuk. 1970. Preliminary report on the development of a diffusion-in-gel method for the diagnosis of ram epididymitis. Appl. Microbiol. 19:335--337.
10. Pickerill, P. A. 1970. Comments on epizootiology and control of canine brucellosis. J. Amer. Vet. Med. Ass. 156:1741--1742.
11. Swenson, R. M., L. E. Carmichael, and K. R. Cundy. 1972. Human infection with *Brucella canis*. Ann. Intern. Med. 76:435--438.