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 666. 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 through 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 10⁷ 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 10⁵ 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 duplica-
tate 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 homolo-
gous 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+ agglutina-
tion 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 infec-
tion with classical species of smooth Brucella
and to the TA test for B. canis. No precipitins
were observed in GD tests with either saline ex-
tract 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 sensi-
tivity of the GD test using B. canis and B. ovis
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 corre-
lation 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 concentra-
tion was contemplated. The latter antigen was
concentrated two times by Carbowax 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, how-
ever, 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 agglu-
<table>
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<tr>
<th>tination titers*</th>
<th>No. of sera examined</th>
<th>+ B. canis (IgG)</th>
<th>+ B. canis (IgG)</th>
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<tr>
<td></td>
<td></td>
<td>B. ovis (IgG)</td>
<td>B. ovis (IgG)</td>
<td>B. ovis (IgG)</td>
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<td>6</td>
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<td>Total</td>
<td>96</td>
<td>86</td>
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<td>6</td>
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* Reciprocal of titers.

TABLE 1. Agglutination test and immunodiffusion
reactions in sera collected from six dogs at periodic
intervals following intravenous inoculation of 10*
 living B. canis organisms

* 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
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