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A simple gel-diffusion technique is described for the diagnosis of ram epididymitis caused by *Brucella ovis*. The results are shown to be very similar to those obtained by the complement-fixation test, which is currently the standard method of diagnosis. The method is suitable for use in the field and is expected to facilitate the control of ram epididymitis in areas where laboratory facilities are not available.

Ram epididymitis caused by *Brucella ovis* was first reported in 1953 in Australia (8) and New Zealand (2). It has subsequently been reported in many sheep-raising countries including the United States (5) and Argentina (9). The disease is characterized by epididymitis in male sheep but is also capable of causing genital infection in females, occasionally producing abortion.

Flocks are often heavily infected and pose problems in diagnosis. Neither physical examination of the testes for signs of epididymitis nor cultural examination of semen for the isolation of *B. ovis* can be relied upon to identify all infected male animals. The complement-fixation (CF) test described by Clapp (3, 4) and Biberstein and McGowan (1) has come to be accepted as an effective diagnostic test for individual animals upon which control measures can be based but is complicated and requires highly trained personnel and laboratory equipment. This paper describes preliminary results obtained with a simple microslide adaptation of the agar-gel diffusion method described by Ouchterlony (7). The results obtained in testing sheep sera for *B. ovis* infection by this method are compared with those obtained with the CF test.

**MATERIALS AND METHODS**

Antigens. The same soluble antigen, similar to that described by Biberstein and McGowan (1), was used for both the agar-gel precipitin method and the CF test. A strain of *B. ovis* isolated at this Center was cultured on Brucella Agar (Albini Laboratories Inc., Brooklyn, N.Y.) fortified with 10% rabbit or bovine serum in eight Roux bottles for 72 hr at 37 °C in an atmosphere containing an added 10 to 20% carbon dioxide. The resulting growth was harvested with Sorensen's phosphate-buffered saline (pH 7.2), keeping the final volume to approximately 100 ml. This heavy cellular suspension was centrifuged at 15,000 X g for 15 min, the supernatant fluid being discarded, and the packed cellular material was resuspended in 100 ml of buffered saline. This centrifugation process was repeated once more, and the resulting suspension was placed in a boiling-water bath for 15 min, allowed to cool, and then subjected for 20 min to sonic vibration in a Raytheon 10-ke megasonic oscillator. Particulate matter consisting of the cellular debris was removed by centrifugation at 15,000 X g for 15 min. The resulting supernatant liquid, which was opalescent, constituted the stock antigen; it was distributed in 1-ml amounts in stoppered tubes and stored at −25 °C until used. The frozen stored antigen was found to be stable for at least 1 year.

Sera. (i) Fourteen male sheep were artificially infected with *B. ovis* by intratesticular inoculation and subsequently bled at regular intervals. (ii) Twenty sera from sheep known to be free from *B. ovis* infection were kindly supplied by Dr. Fife, Walter Reed Army Medical Center, Washington D.C. (iii) A total of 524 sera were obtained from male sheep on two farms whose infection status had been unknown previously, but many reactors were found to the two tests and some rams were found to show symptoms of epididymitis. (iv) Ten pig sera, nine goat sera, and six cow sera, all positive to the regular agglutination test for infection with classical species of smooth *Brucella*, were also tested. Sera were stored at −25 °C until used. Microslide gel diffusion test. A gel-diffusion chamber was constructed by first rubbing the surface of a microglass slide (75 by 50 mm) with 1.25% melted agar (Difco) in buffered saline containing 1:10,000 Merthiolate to prevent contamination. When the slides were dry, 8 ml of the same agar solution was dispensed with a pipette, first around the borders of the slide and then gradually working towards the center. Bubbles were removed with an appli-
cator stick, and the agar was allowed to solidify at room temperature, after which the slides were placed in sterile petri dishes in the refrigerator for approximately 30 min or longer. A central horizontal trench was cut approximately 60 mm in length and 4 mm in width with a razor blade, and on each side of the trench a row of five holes was made with a 6-mm cork borer; the holes were 3 mm from the trench and 4 mm apart. The punched agar was removed from the trench and holes by the use of a hypodermic needle. The slides were returned to the petri dishes, and a degree of humidity was maintained by the addition of a small amount of sterile buffered saline solution to the bottom of the dish.

In the test proper, 0.1 ml of undiluted serum under test was placed in each hole and the trench was charged with the undiluted antigen (0.5 ml). During the cooler parts of the year, the plates were incubated at room temperature (25°C), but during abnormally hot weather they were placed in the refrigerator (4°C). Sera having a high CF titer usually showed precipitin band formation at 24 hr, and those with lower titers showed precipitin band formation at 48 to 72 hr; further incubation did not affect the results.

Precipitation zones, bands, or lines developing in the agar medium were read either against a lighted background or preferably against a dark background illuminated by side lighting and by use of a hand lens. Precipitates can be recorded by copying with a pencil on paper, or the slide can be stained by the technique described by Uriel (6) and stored for future reference.

CF test. The technique used was based on that described by Biberstein and McGowan (1). The antigen, prepared as described above, was titrated in the presence of complement, and the dilution selected for the test (usually 1:50) was that which showed the greatest sensitivity towards positive sera while still permitting complete lysis with negative sera. Serum samples to be tested were diluted 1:10 in Sorensen's buffered saline and inactivated at 56°C for 30 min. A dilution of 1:10 was the only one tested, and samples showing complete fixation at this dilution were considered positive, whereas those showing only partial fixation or complete lysis were considered to be negative.

RESULTS

Agar-gel precipitin lines and positive CF reactions were demonstrated on the first postinoculation bleeding at 12 days in all of the artificially infected animals. All 14 sheep gave positive reactions to both tests on serum samples obtained weekly up to the 124th day postinoculation. Evidence of infection was demonstrated by semen culture isolations of *B. ovis* in 11 of the inoculated animals; samples from the remaining 3 animals were highly contaminated and *B. ovis* could not be isolated. Blood samples taken at monthly intervals thereafter until the 335th day postinoculation also showed agreement between the two test methods. At this last bleeding, four of the animals were negative to the CF test and these sera also did not produce a precipitin line by agar-gel diffusion. The sera from the 20 male sheep known to be free from ram epididymitis were negative to both the gel-diffusion and the CF tests.

The results obtained with the 524 serum samples coming from the two farms are presented in Table 1. Almost half of them were positive to one or other of the tests, 209 samples were positive to both tests, 30 were positive to the CF test only, and 10 were positive to the gel-diffusion test only. The percentage of agreement between the two tests were 92.3 (Fig. 1).

The 10 pig sera, 9 goat sera, and 6 cow sera that were positive to the agglutination test for classical brucellosis were all negative to the gel-diffusion test for *B. ovis* infection.

A preliminary analysis of the immunoelectrophoretic pattern of the soluble *B. ovis* antigen

<table>
<thead>
<tr>
<th>Source of serum samples</th>
<th>Reaction to CF test</th>
<th>Reaction to agar-gel diffusion test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Sera from two herds, natural infection</td>
<td>285</td>
<td>239</td>
</tr>
<tr>
<td>Sera from artificially infected rams</td>
<td>5</td>
<td>114</td>
</tr>
<tr>
<td>Sera from known negative rams</td>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 1. Precipitin lines formed with sheep sera containing complement-fixing antibodies and negative reactions with those not having complement-fixing antibodies.
concentrated 100 times showed that it was a mixture of lipoproteins. These lipoproteins were easily separated into three fractions with different electrophoretic migration characteristics. These fractions could be characterized as (i) a rapidly moving one which migrates like albumin; (ii) a slower fraction, also migrating in the zone of albumin; and (iii) a slow fraction that migrates in the zone corresponding to the beta globulins. These fractions are now under study to determine whether a purified antigen can be prepared in the freeze-dried form.

DISCUSSION

The data presented show that, in the diagnosis of ram epididymitis, the simple microslide gel-diffusion technique described gives results very similar to those obtained with the CF technique, which is currently accepted as a reliable method of diagnosis.

If these results can be confirmed by field use, the new technique should extend the possibility of testing large numbers of sheep to areas where laboratory facilities do not exist for carrying out the CF test.

The precipitin reaction in almost all of the artificially infected animals and sheep infected under natural field conditions demonstrated a single band in gel diffusion. The presence of a single band indicates that the antigen is relatively pure. Only in occasional positive sera were two or three bands observed. At present, the significance of these additional band formations is not clearly understood, but their presence did not interfere with the interpretation of the results.

Preliminary results with electrophoresis suggested that there should be no difficulty in preparing the soluble antigen in freeze-dried form for distribution to the field along with the other materials required for carrying out the gel-diffusion test. The test could then be performed in the field by personnel who possess only a minimum of laboratory training; it would have the additional advantages of being inexpensive and not particularly time-consuming.

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LITERATURE CITED