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Antigenic Relationship of *Brucella ovis* and *Brucella melitensis*

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Immune sera were prepared in rabbits by the injection of living and acetone-killed cells of *Brucella ovis* and smooth and rough *B. melitensis*. The use of whole-cell antigens in agglutination and agglutinin-absorption tests revealed little relationship between *B. ovis* and smooth *B. melitensis*, although there was extensive cross-agglutination between *B. ovis* and rough *B. melitensis*. The use of water-soluble antigens prepared from ultrasonically treated cells of the three strains revealed extensive cross-reactions in indirect hemagglutination, agar gel precipitation, and immunoelectrophoresis tests, as well as in allergic skin tests in rabbits. The most definitive results were obtained with the immunoelectrophoresis technique. *B. ovis* antigen produced at least 11 lines with its homologous serum. All were removed by absorption of the serum with rough *B. melitensis* antigen. All but three were removed by absorption with smooth *B. melitensis* antigen. Smooth *B. melitensis* antigen produced 11 lines with its homologous serum, and all but 3 were removed by absorption with *B. ovis* antigen. Rough *B. melitensis* produced nine lines with its homologous serum, and eight were removed by *B. ovis* antigen. The extensive cross-reactions between soluble antigens of *B. ovis* and *B. melitensis* are added evidence that *B. ovis* belongs in the genus *Brucella*.

The etiological agent of epididymitis in rams was isolated independently in Australia (16) and in New Zealand (4), and was described as a *Brucella*-like organism. It has subsequently been reported in the United States (9) and in other parts of the world. Buddle (3) described the organism as a stable nonsmooth form of *Brucella*, and, on the basis of its cultural characteristics, he assigned it to the genus *Brucella* as a new species, *B. ovis*. Meyer and Cameron (10) suggested that this organism was not properly classified, one of the reasons being that it displayed no serological relationship with smooth *Brucella* antigen in agglutination tests. The Subcommittee on the Taxonomy of Brucella of the International Committee on Bacteriological Nomenclature was not satisfied that the organism was a member of the genus *Brucella* and advised further study (Report, 1966).

Although rough and smooth strains of *Brucella* show little or no serological relationship in agglutination tests, we (R. Diaz and A. Chordi, Bacteriol. Proc., p. 46, 1966) found that soluble antigens prepared from rough or smooth *B. melitensis* when attached to tanned sheep red blood cells had the same titer in indirect hemagglutination tests. Soluble antigens of smooth strains of *B. abortus*, *B. suis*, and *B. melitensis* were shown to be identical or to have only minor quantitative differences on the basis of agar gel diffusion (11) and immunoelectrophoretic analysis (R. Diaz, M.D. Thesis, Univ. of Navarra, Pamplona, Spain, 1965). We wanted to know whether the hemagglutination and immunoelectrophoretic methods would reveal a relationship between *B. ovis* and rough and smooth strains of one of the accepted *Brucella* species. Sera from rabbits infected and hyperimmunized with *B. ovis* and rough and smooth strains of *B. melitensis* were tested in agglutination and agglutinin-absorption tests (with whole-cell antigens), and by use of indirect hemagglutination, agar gel diffusion, and immunoelectrophoretic techniques (with water-soluble antigens). Allergic skin test reactions in infected rabbits were also observed.

MATERIALS AND METHODS

Bacterial cultures. B. ovis 0.64.19, isolated from infected ram semen in South Africa, was obtained from G. C. Van Drimmelen. This strain is of rough colonial morphology and requires an atmosphere of 10% CO_2 for growth. It resembles other *B. ovis* cultures isolated in the United States on the basis of routine typing characteristics and agglutination by sera from rabbits immunized with *B. ovis*.

B. melitensis B115, a rough strain isolated from a

naturally infected goat, was obtained from G. Alton, Malta.

B. melitensis 16M, the FAO/WHO reference strain, was obtained from Central Veterinary Laboratory, Weybridge, England. This strain is of smooth colonial morphology.

The cultures were grown on Trypticase Soy Agar (BBL) for 2 to 3 days at 37 C.

Production of immune sera in rabbits. Rabbits were injected with 5×10^9 viable organisms by the intravenous route, two rabbits with each of the three strains. They were bled 5 weeks after infection. One rabbit per strain was skin-tested 8.5 weeks after infection. Three dilutions of each of the soluble antigens were injected intradermally in 0.1-ml amounts. A normal rabbit was similarly injected. The rabbits were examined after 24 and 48 hr, and the reactions were scored on the basis of the area of reddening and thickening of the skin. After 10 days the rabbits were autopsied to determine whether the infection had persisted. Liver, spleen, and lymph nodes were minced and streaked on Trypticase Soy Agar plates. The plates were incubated at 37 C (in the presence of 10% CO_2) for 4 days and examined for *Brucella* colonies.

A second series of rabbits were hyperimmunized with a preparation containing 5 mg of acetone-killed organisms per ml of incomplete Freund adjuvant (Difco). The dose per injection was 1 ml. Rabbits were injected by the subcutaneous route three times per week for 4 weeks and were bled 1 week after the last injection. Two rabbits were immunized with each strain.

Immune sera from both series of rabbits were employed in the agglutination test. Immune sera from hyperimmunized rabbits were most satisfactory for the indirect hemagglutination and immunoelectrophoretic methods, whereas this sera produced too many lines for the agar gel diffusion method. Sera from infected rabbits were used in the latter test.

Agglutination and agglutinin-absorption tests. For the agglutination test, antigens were prepared by washing organisms off agar with phosphate-buffered saline (0.066 M, *p*H 8.4) containing 0.5% phenol as recommended by Buddle (3). Suspensions were heat-killed at 56 C for 1 hr. Doubling dilutions of serum were prepared, and equal volumes of antigen were added. The antigen had been standardized to a density equivalent to the antigen concentration employed in the USDA standard serum tube agglutination test for brucellosis. Dilutions were made in the phosphate-buffered saline at *p*H 8.4. Tubes were incubated at 37 C for 24 hr. All three antigens remained in suspension in control tubes prepared with saline and with normal serum. For agglutinin-absorption, heat-killed cell suspensions were centrifuged, and packed-cell volumes of 0.1 ml were mixed with 1 ml of serum and placed on a shaker at 37 C for 2 hr. The mixture was centrifuged and the serum was tested. If the homologous agglutinins were not entirely removed the absorption was repeated.

Preparation of soluble antigens. Antigens for the hemagglutination and precipitation tests were prepared as follows. Acetone-killed organisms were suspended in distilled water, treated in a Raytheon sonic oscillator for 2 hr, resuspended in distilled water, dialyzed against water at 4 C for 2 days, and centrifuged at $5,500 \times g$ for 1 hr. The supernatant fluid was concentrated by perevaporation (Rotavapor 'R,' Büchi, Switzerland) and stored at −20 C for 3 days. Upon thawing, a precipitate formed which was removed by centrifugation, and the supernatant fluid was lyophilized. The protein concentration of the three soluble antigens varied from 62 to 75% as determined by the Folin test.

Indirect hemagglutination test. The method employed for the indirect hemagglutination test was that of Chordi et al. (6), with some modifications. The sheep red blood cells were tanned at 4 C for 15 min with fresh, cold tannic acid (1:20,000 final concentration) in phosphate-buffered saline (*p*H 7.2), and were washed once. Optimally diluted antigen in phosphate-buffered saline (*p*H 6.4) was added to the tanned cells, and the mixture was incubated for 30 min at 37 C. The optimal concentration of soluble antigen was determined for each strain by titrating against the homologous serum. It was between 100 and 200 μg/ml. The tanned, sensitized cells were washed twice in three volumes of phosphate-buffered saline (*p*H 6.4) containing 0.6% normal rabbit serum, and were resuspended to 2% in this diluent. Plastic trays with cups (2-ml volume) were employed for the test. Doubling dilutions of serum were prepared in the cups, and sensitized cells (0.05 ml of cells to 0.5 ml of serum dilution) were added. The hemagglutination pattern was determined after 3 hr at room temperature.

Precipitation tests. Immunoelectrophoresis was performed according to the micromethod described by Scheidegger (15) with the modifications described by Chordi et al. (6). A Thomas (model 21) electrophoretic chamber was used. Ouchterlony agar gel diffusion reactions were carried out on glass slides [1 by 3 inches (2.54 by 7.62 cm)].

Absorbed sera for immunoelectrophoresis were obtained by mixing 25 mg of lyophilized antigen per ml of serum, placing the mixture on a shaker at 37 C for 2 hr, removing it to 4 C for 18 hr, and centrifuging to remove the precipitated antigen. If the homologous precipitins were not entirely removed, the serum was absorbed with 50 mg of dry antigen per ml.

RESULTS

Agglutination and agglutinin-absorption tests. The agglutination reactions observed with the three antigens and the sera before and after absorption with the three antigens are shown in Fig. 1. The highest titers were obtained with smooth *B. melitensis* antiserum and antigen. Unabsorbed smooth *B. melitensis* antiserum agglutinated both rough *B. melitensis* and *B. ovis* antigens at a low titer. Absorption of smooth *B. melitensis* antiserum with rough *B. melitensis* or *B. ovis* antigens did not remove agglutinins for smooth *B. melitensis* antigen.

Unabsorbed rough *B. melitensis* antiserum agglutinated rough *B. melitensis* and *B. ovis*

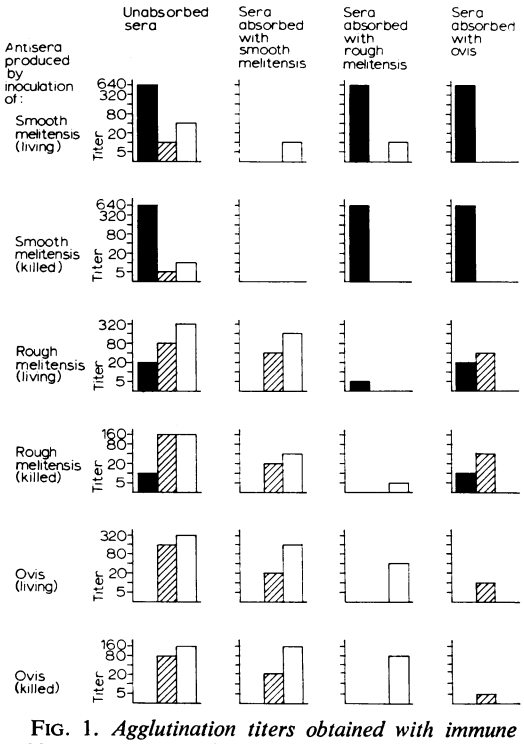

FIG. 1. *Agglutination titers obtained with immune rabbit sera before and after reciprocal absorption with three antigens. Solid bar, titer with smooth Brucella melitensis antigen; hatched bar, titer with rough B. melitensis antigen; open bar, titer with B. ovis antigen.*

antigens at about the same titer, and smooth *B. melitensis* antigen at a low titer. Absorption with smooth *B. melitensis* antigen did not remove agglutinins for rough *B. melitensis* and *B. ovis* antigens, and absorption with *B. ovis* antigen did not remove agglutinins for smooth and rough *B. melitensis* antigens.

Unabsorbed *B. ovis* antiserum agglutinated *B. ovis* antigen at one dilution higher than rough *B. melitensis* antigen but did not agglutinate smooth *B. melitensis* antigen. Absorption of *B. ovis* antiserum with smooth *B. melitensis* antigen reduced the titer to rough *B. melitensis* but not *B. ovis* antigen. Absorption of *B. ovis* antiserum with rough *B. melitensis* slightly reduced the titer to *B. ovis* antigen.

These results suggest that the surface antigens of rough *B. melitensis* and *B. ovis* are similar but not identical, since neither can completely absorb the heterologous agglutinins. Little antigenic relationship was seen between surface antigens of *B. ovis* and smooth *B. melitensis*.

Results with indirect hemagglutination test. Sera obtained from the hyperimmunized rabbits were examined in the indirect hemagglutination test by use of sheep cells sensitized with soluble antigens from the three different strains of brucellae. The results (Table 1) show that the titer of the *B. ovis* antiserum was the same, or within one doubling dilution, with the three antigens. Rough *B. melitensis* antiserum had the same titer with the three antigens. A fourfold difference was observed among antigens with smooth *B. melitensis* antiserum.

Ouchterlony test reactions. Sera obtained 5 weeks after the infection of rabbits with living brucellae were examined in the agar gel diffusion test of Ouchterlony. The sera were concentrated several-fold by lyophilization.

In separate experiments, each serum was set up with varying concentrations of the three soluble antigens to find the optimal concentration of antigen for each serum. Each serum was then set up with the optimal concentrations of the three soluble antigens to determine identity of the lines (Table 2). It should be noted that the lines have been given different series of numbers with each serum, because tests of identity among sera have not been performed.

TABLE 1. *Titers obtained in indirect hemagglutination test*

Antisera	Sheep red blood cells sensitized with soluble antigen		
	Brucella ovis	Rough *B. melitensis*	Smooth *B. melitensis*
B. ovis	1:12,800	1:25,600	1:12,800
Rough *B. melitensis*	1:12,800	1:12,800	1:12,800
Smooth *B. melitensis*	1:6,400	1:12,800	1:25,600

TABLE 2. *Ouchterlony test reactions*

Antisera	Soluble antigen	Precipitation lines
Brucella ovis	*B. ovis*	1, 2, 3
	Rough *B. melitensis*	2, 3
	Smooth *B. melitensis*	2, 3
Rough *B. melitensis*	Rough *B. melitensis*	11, 12, 13, 14
	Smooth *B. melitensis*	12, 13, 14
	B. ovis	12, 13, 14
Smooth *B. melitensis*	Smooth *B. melitensis*	21, 22, 23, 24, 25, 26
	Rough *B. melitensis*	22, 23, 24, 25, 26
	B. ovis	23, 24, 25, 26

With a given serum the majority of the lines were common to the three strains, but in each case the homologous system revealed an additional antigen.

Results with immunoelectrophoresis. Table 3 presents a summary of the results obtained in the immunoelectrophoretic analysis of the three soluble antigens by use of unabsorbed and absorbed sera from hyperimmunized rabbits, with references made to the appropriate figure. Table 3 and Fig. 2, 3, and 4 show that the three unabsorbed sera produced 8 to 11 lines with all of the antigens. The homologous antigen-antibody combination usually developed sharper lines and several more lines than heterologous combinations. The exact number of lines was difficult to determine from the photographs, and there was a slight variation in number of lines when reactions were repeated. Reciprocal absorptions were performed to show that the majority of the antigens revealed with immunoelectrophoresis were common to the three cultures. Sufficient antigen was employed to remove all precipitins for the absorbing antigen, but this did not always remove all precipitins for the other antigens (see Table 3). *B. ovis* antigen produced at least 11 lines with its homologous serum. All could be removed by absorption of the serum with rough *B. melitensis* antigen, and all but three could be removed by absorption with smooth *B. melitensis* antigen. Rough *B. melitensis* produced nine lines with its homologous serum, and eight were removed by absorption with *B. ovis* antigen. Smooth *B. melitensis* antigen produced 11 lines with its homologous serum, and all but 3 could be removed by absorption with *B. ovis* antigen. Rough *B. melitensis* antigen removed all precipitins for *B. ovis* antigen from smooth *B. melitensis* antiserum, and smooth *B. melitensis* antigen removed all but three precipitins for *B. ovis* antigen from rough *B. melitensis* antiserum. These reciprocal absorptions suggest a very close antigenic relationship between smooth and rough *B. melitensis*, and between rough *B. melitensis* and *B. ovis*, but the relationship between

TABLE 3. *Summary of antigenic analysis by immunoelectrophoresis*

Antisera	Antigen[a] for absorption	Antigen[a] for test	No. of lines obtained	Photograph of reaction
Brucella ovis	—	*B. ovis*	11	Fig. 2
	—	Smooth	10	Fig. 2
	—	Rough	9	Fig. 2
	Rough	Rough	0	
	Rough	*B. ovis*	0	
	Smooth	Smooth	0	
	Smooth	*B. ovis*	3	
Rough *B. melitensis*	—	Rough	9	Fig. 3
	—	*B. ovis*	8	Fig. 3
	—	Smooth	8	Fig. 3
	Smooth	Smooth	0	
	Smooth	Rough	1	
	B. ovis	*B. ovis*	0	
	B. ovis	Rough	1	
	Smooth	*B. ovis*	3	
Smooth *B. melitensis*	—	Smooth	11	Fig. 4
	—	*B. ovis*	9	Fig. 4
	—	Rough	9	Fig. 4
	Rough	Rough	0	
	Rough	Smooth	1	
	B. ovis	*B. ovis*	0	
	B. ovis	Smooth	3	
	Rough	*B. ovis*	0	

[a] Rough = rough *B. melitensis*, and smooth = smooth *B. melitensis*.

FIG. 2. *Precipitation lines developed by unabsorbed Brucella ovis antiserum in the three troughs, and the following antigens in wells from top to bottom: B. ovis, smooth B. melitensis, rough B. melitensis, and B. ovis.*

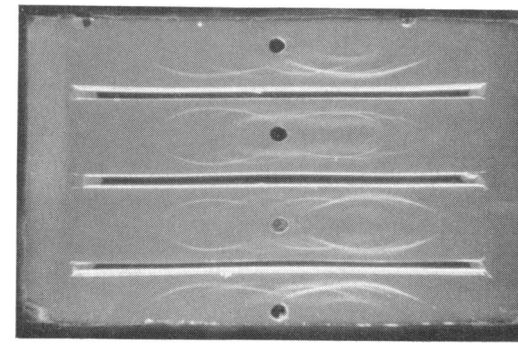

FIG. 3. *Precipitation lines developed by unabsorbed rough Brucella melitensis antiserum in the three troughs, and the following antigens in wells from top to bottom: rough B. melitensis, B. ovis, smooth B. melitensis, and rough B. melitensis.*

smooth *B. melitensis* and *B. ovis* does not appear to be quite as close.

We cannot estimate the exact number of antigens per strain from the present data, but further studies are in progress. Some of the apparent antigenic differences may be merely quantitative as suggested by Olitzki (11). There is, however, at least one qualitative difference in antigens between smooth *B. melitensis* and the other cultures. This is shown by the diffuse line which appears close to the smooth *B. melitensis* antigen well and is developed only by the smooth *B. melitensis* antiserum (see Fig. 4). This line cannot be removed by absorption of smooth *B. melitensis* antiserum with either rough *B. melitensis* or *B. ovis* antigens.

B. ovis antigen was also tested with antisera prepared against *Salmonella typhi*. No lines were produced in the immunoelectrophoresis reaction.

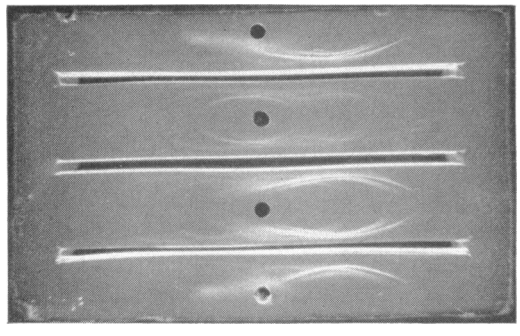

FIG. 4. *Precipitation lines developed by unabsorbed smooth Brucella melitensis antiserum in the three troughs, and the following antigens in wells from top to bottom: smooth B. melitensis, B. ovis, rough B. melitensis, and smooth B. melitensis.*

Skin test reactions. Table 4 gives the allergic skin reactions of rabbits tested 8.5 weeks after infection with living organisms. The rabbit given smooth *B. melitensis* was still heavily infected at the time of the skin test (as shown by the autopsy results 10 days later), and the three doses of the three antigens all stimulated allergic reactions in this rabbit. The other rabbits were less reactive, but some cross-reactivity of the antigens was observed. A normal rabbit given the same injections showed no reactions.

DISCUSSION

Buddle (3) studied the antigenic relationship of New Zealand and Australian strains of *B. ovis* with smooth and nonsmooth *B. melitensis* and *B. abortus*. Immune sera were prepared in rabbits against heat-killed suspensions of all the strains. Using serum agglutination and cross-absorption tests, Buddle showed that *B. ovis* did not possess smooth *B. abortus* or *B. melitensis* antigens but that it did share antigens with rough strains of *B. abortus* and *B. melitensis* and, in addition, had antigenic factors of its own. Renoux and Mahaffey (13) had also shown an antigenic relationship between rough *B. melitensis* and *B. ovis* by agglutinin-absorption tests.

An indirect hemagglutination test was developed by Ris and TePunga (14), in which tanned sheep red blood cells were sensitized with a soluble antigen prepared from ultrasonically treated *B. ovis* organisms. The test was reported to be specific for the detection of antibodies to *B. ovis*.

The first immunization procedure used to control *B. ovis* infection in rams consisted of the simultaneous inoculation of rams at different sites with living *B. abortus* strain 19 vaccine and

TABLE 4. *Skin test reactions of infected rabbits*

Rabbits infected with	Antigen	Skin reactions[a] obtained with antigen dose			Autopsy results 10 days later
		2,500 μg	25 μg	0.25 μg	
Smooth *Brucella melitensis*	Smooth *B. melitensis*	++++	+	+	Heavily infected with smooth *B. melitensis*
	Rough *B. melitensis*	++	+	+	
	B. ovis	++	+	+	
Rough *B. melitensis*	Smooth *B. melitensis*	+	±	−	No organisms recovered
	Rough *B. melitensis*	+	±	−	
	B. ovis	±	−	−	
B. ovis	Smooth *B. melitensis*	+	−	−	A few *B. ovis* cells recovered from mammary lymph nodes
	Rough *B. melitensis*	−	−	−	
	B. ovis	++	−	−	

[a] Symbols: ±, less than 10 by 10 mm area of redness which disappeared within 48 hr; +, about 10 by 10 mm area of redness which persisted for 48 hr; ++, about 15 by 15 mm area of redness which persisted for 48 hr; ++++, more than 20 by 20 mm area of redness and thickness which persisted for 48 hr; −, no reaction.

killed *B. ovis* organisms in a saline-in-oil emulsion (2). Neither vaccine given as a single inoculation was effective. Biberstein et al. (1) reported that a combined vaccination procedure consisting of living *B. abortus* strain 19 and killed *B. ovis* organisms in aluminum hydroxide adjuvant conferred solid immunity, whereas the use of the adjuvant vaccine alone gave unreliable results. Buddle (5) later showed that two inoculations of *B. ovis* saline-in-oil vaccine administered several months apart were as effective as the simultaneous inoculation with *B. abortus* strain 19.

In South Africa, a different procedure has been used to control ram epididymitis. A live attenuated strain of *B. melitensis*, Rev. 1, developed by Elberg and Faunce (8) for the control of *B. melitensis* infection in sheep and goats, has been shown to be highly effective against *B. ovis* infection (17–19).

The specificity of the immunity conferred by the living strains 19 and Rev. 1 would be in question if there were no antigenic relationship among *B. ovis*, *B. melitensis*, and *B. abortus* as claimed by some workers.

In a previous study (Diaz and Chordi, Bacteriol. Proc., p. 46, 1966), a close antigenic relationship was shown between the water-soluble antigens of rough and smooth strains of *B. melitensis* by the use of the indirect hemagglutination test. Sera from rabbits immunized with rough or smooth strains of *B. melitensis* had the same titer, and there was no difference in titer whether the red blood cells were sensitized with soluble antigens prepared from rough or from smooth *B. melitensis*. Olitzki (11) had shown by the agar gel diffusion method that soluble antigens extracted from *B. abortus*, *B. suis*, and *B. melitensis* were probably identical, as absorption with soluble antigens from one species removed precipitins for the other species. An immunoelectrophoretic analysis of soluble antigens extracted from the WHO/FAO reference strains, *B. abortus* 544, *B. suis* 1330, and *B. melitensis* 16M, revealed at least 19 antigens in common in the three species (R. Diaz, M.D. Thesis, Univ. of Navarra, Pamplona, Spain, 1965); methods similar to those described in the present paper were used, except that rabbits were hyperimmunized for 2 months instead of 1 month. It appeared that only minor quantitative differences existed among the soluble antigens of the three *Brucella* species, and, therefore, we did not think it necessary to include *B. abortus* and *B. suis* antigens in the present study. We observed that the majority of the soluble antigens of *B. ovis* and *B. melitensis* were identical.

It might be thought that soluble antigens are less specific than whole-cell antigens and that cross-reactions between brucellae and other gram-negative organisms would be expected. Olitzki and Godinger (12) tested the antigenic relationship of 60 strains of *Brucellaceae*, *Enterobacteriaceae*, and *Pseudomonadaceae* by the agar gel precipitation technique. Rabbits were immunized with soluble antigens prepared from acetone-killed, ultrasonically treated cells of each strain. Three *B. abortus* cultures and one culture each of *B. melitensis* and *B. suis* were studied. Organisms which are reputed to cross-react with *Brucella* were included (e.g., *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Vibrio comma*, *Pasteurella tularensis*, and *Bordetella pertussis*). The *Brucella* antigens formed six lines with their own sera but none with any other sera. The *Brucella* antisera did not produce lines with any heterologous antigens, with the exception of two lines formed with *B. pertussis*. Olitzki and Godinger (12) concluded that the members of the genus *Brucella* have little antigenic relationship with other gram-negative bacteria.

The extensive cross-reactions which we have shown between the soluble antigens of *B. melitensis* and *B. ovis* are added evidence that *B. ovis* belongs in the genus *Brucella* as first described by Buddle (3). An investigation is underway in which we are comparing the soluble antigens of *Brucella* with similarly prepared antigens from other genera in the family *Brucellaceae*.

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LITERATURE CITED

1. BIBERSTEIN, E. L., B. MCGOWAN, JR., E. A. ROBINSON, AND D. R. HARROLD. 1962. Epididymitis in rams. Studies on immunity. Cornell Vet. **52**:214–227.
2. BUDDLE, M. B. 1954. Production of immunity against ovine brucellosis. New Zealand Vet. J. **2**:99–109.
3. BUDDLE, M. B. 1956. Studies on *Brucella ovis* (n. sp.). A cause of genital disease of sheep in New Zealand and Australia. J. Hyg. **54**: 351–364.
4. BUDDLE, M. B., AND B. W. BOYES. 1953. A brucella mutant causing genital disease of sheep in New Zealand. Australian Vet. J. **29**:145–153.
5. BUDDLE, M. B., F. K. CALVERLEY, AND B. W. BOYES. 1963. *Brucella ovis* vaccination of rams. New Zealand Vet. J. **11**:90–93.
6. CHORDI, A., K. W. WALLS, AND I. G. KAGAN. 1964. Studies on the specificity of the indirect

hemagglutination test for toxoplasmosis. J. Immunol. **93**:1024–1033.
7. CHORDI, A., K. W. WALLS, AND I. G. KAGAN. 1964. Analysis of *Toxoplasma gondii* antigens by agar diffusion methods. J. Immunol. **93**: 1034–1044.
8. ELBERG, S. S., AND K. FAUNCE, JR. 1957. Immunization against brucella infection. VI. Immunity conferred on goats by a nondependent mutant from a streptomycin-dependent mutant strain of *Brucella melitensis*. J. Bacteriol. **73**:211–217.
9. MCGOWAN, B., AND G. SHULTZ. 1956. Epididymitis of rams. I. Clinical description and field aspects. Cornell Vet. **56**:277–281.
10. MEYER, M. E., AND H. S. CAMERON. 1956. Studies on the etiological agent of epididymitis in rams. Am. J. Vet. Res. **17**:495–497.
11. OLITZKI, A. L. 1959. Differences between antigenic specificity of nonsoluble particles and soluble extracts prepared from brucellae by disintegration. Proc. Soc. Exptl. Biol. Med. **101**:388–390.
12. OLITZKI, A. L., AND D. GODINGER. 1963. Interfamiliar antigenic relationships between Enterobacteriaceae, Brucellaceae and Pseudomonadaceae revealed by the agar gel precipitation technique. Boll. Ist. Sieroterap. Milan. **42**:213–232.
13. RENOUX, G., AND L. W. MAHAFFEY. 1955. Sur l'existence probable de nouveaux antigènes des *Brucella*, avec un nouveau schéma proposé pour représenter la répartition de antigènes. Ann. Inst. Pasteur **88**:528–532.
14. RIS, D. R., AND W. A. TE PUNGA. 1963. An indirect haemagglutination test for the detection of *Brucella ovis* antibodies. 1. Development of the test. New Zealand Vet. J. **11**:94–97.
15. SCHEIDEGGER, J. J. 1955. Une micro-méthode de l'immuno-électrophorèse. Intern. Arch. Allergy Appl. Immunol. **7**:103–110.
16. SIMMONS, G. C., AND W. T. K. HALL. 1953. Epididymitis of rams. Australian Vet. J. **29**: 33–40.
17. VAN DRIMMELEN, G. C. 1960. Control of brucellosis in sheep and goats by means of vaccination. J. S. African Vet. Med. Assoc. **31**:129–138.
18. VAN HEERDEN, K. M. 1964. Results obtained by the use of Rev. 1 vaccine in sheep against infectious infertility suspected to be caused by ovine brucellosis. Bull. Offic. Intern. Epizoot. **62**:52.
19. VAN HEERDEN, K. M., AND S. W. J. VAN RENSBURG. 1962. The immunization of rams against ovine brucellosis. J. S. African Vet. Med. Assoc. **33**:143–148.