JOURNAL OF BACTERIOLOGY, Feb. 1968, p. 618-624
Copyright © 1968 American Society for Microbiology
Vol. 95, No. 2
Printed in U.S.A.

Antigenic Relationship of the Gram-negative Organism Causing Canine Abortion to Smooth and Rough Brucellae

RAMON DIAZ, LOIS M. JONES, AND J. B. WILSON

Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706

Received for publication 9 November 1967

The use of whole-cell antigens in agglutination and agglutinin-absorption tests showed that the organism causing abortion in dogs is similar to rough *Brucella abortus*, *B. melitensis*, and *B. ovis*, but different from smooth *Brucella* cultures. Water-soluble antigens obtained by ultrasonic treatment and examined by immunoelectrophoresis and gel diffusion show extensive cross-reactions within the genus *Brucella*, but little or no cross-reaction with similar antigens from other gram-negative genera in the family *Brucellaceae*. The dog organism showed near identity with rough and smooth *Brucella* cultures on the basis of immuno-gel diffusion tests with water-soluble antigens, but it lacked the lipopolysaccharide-endotoxin associated with the agglutinogen of smooth brucellae. These findings support the proposal of Carmichael and Bruner for the designation of a new species, "*Brucella canis*."

The gram-negative organism causing abortion in dogs (1; L. E. Carmichael and D. W. Bruner, Cornell Vet., *in press*) resembles *Brucella suis* biotype 3 on a bacteriological basis and rough *Brucella* cultures on a serological basis (8). The antigenic relationship between rough *B. ovis* and smooth *B. melitensis*, as demonstrated by the use of water-soluble antigens in indirect hemagglutination and precipitation tests, has been described (6). Similar analysis of the canine organism, reported here, shows the near identity of this organism with rough and smooth *Brucella* cultures. Data are also presented to show that soluble antigens of the *Brucella* species have little or no cross-reaction with similar antigens of some other gram-negative organisms in the family *Brucellaceae*.

MATERIALS AND METHODS

Bacterial cultures. Sources or appropriate references are given for the following cultures employed: *B. melitensis* smooth strain 16M and rough strain B115 (6); *B. abortus* smooth strain 2308, smooth strain 11, rough strain 11 (2); and rough strain 45/20 (11) were obtained from the Bureau of Animal Industry and maintained in the freeze-dried state since 1947. *B. ovis* strain 0.64.19 (6); and strain REO 198, which does not require added CO_2 for growth, was obtained from the National Animal Disease Laboratory, Ames, Iowa. The "dog organism" strain RM 666 was obtained from L. Carmichael, Cornell University, Ithaca, N.Y. *Bordetella pertussis*, *B. parapertussis*, and *Haemophilus influenzae* were obtained from the Wisconsin State Laboratory of Hygiene; *B. bronchiseptica* was a recent isolate from our guinea pig colony (8); *Pasteurella multocida* was obtained from the Dept. of Veterinary Science, Univ. of Wisconsin; *Pasteurella pseudotuberculosis* strain 32 type 4 (4) was obtained from Julius A. Currie, Walter Reed Army Medical Center, Washington, D.C.

Media. Smooth *Brucella* and *B. bronchiseptica* were grown on Trypticase Soy Agar (BBL) for 2 to 3 days at 37 C. Rough *Brucella* organisms and the dog organism grown on this medium were difficult to suspend in saline. The addition of 2% normal rabbit serum to Trypticase Soy Agar resulted in growth which was readily suspended in saline.

P. pseudotuberculosis was grown on Trypticase Soy Agar with 1% yeast extract at 28 C for 3 days, as recommended (4).

B. pertussis and *B. parapertussis* were grown on Bordet Gengou Agar with 20% horse blood added, and *P. multocida* was grown on Brucella agar (Albimi Laboratories, Inc., New York). *H. influenzae* was grown on chocolate-blood-agar.

Antigenic preparations. The preparations for soluble antigens and ether-water extract are the same as those described previously (5).

Antigens for the agglutination test were grown on agar, suspended in saline, heated 1 hr at 60 C, checked for sterility, and then adjusted to the turbidity of the antigen employed in the USDA standard tube agglutination test for brucellosis. Carmichael and Bruner (*in press*) found that incubation in a water bath at 50 C was most satisfactory for agglutination

tests with the dog organism. We used this method for the agglutination of all *Brucella* antigens. Tests were read after 24 hr.

B. bronchiseptica agglutination antigen was prepared by suspending agar-grown cells in 0.15% Formalin-saline and adjusting to the same turbidity as the *Brucella* antigens. The test was placed on a variable-speed rotator (Clay-Adams, Inc., New York) at room temperature and read after 1 hr, as recommended for *Bordetella* by Lautrop (9).

Production of immune sera. Three types of bacterial preparations were used in the production of immune sera. (i) Acetone-killed organisms in incomplete Freund adjuvant were injected according to the procedure previously described (6). (ii) Soluble antigens obtained from living smooth and rough *B. abortus* strain 11 and suspended in incomplete Freund adjuvant were employed as described (5). (iii) Cell wall antigens were prepared from living *B. ovis*, rough *B. melitensis*, rough *B. abortus*, the dog organism, and acetone-killed smooth *B. melitensis*. The organisms were treated for 2 hr in a 250-w, 10-kc Raytheon sonic oscillator. The aqueous suspension was centrifuged at 5,500 × *g* for 1 hr. The supernatant fluid was centrifuged at 20,000 × *g* for 2 hr, and the pellet was washed six times in distilled water and then lyophilized. Rabbits were immunized with a total of 3 mg of this antigen; the first injection of 1 mg was given intravenously, and on the 3rd and 5th day 1 mg was given intraperitoneally. The rabbits were bled 1 week after the last injection.

Absorption of sera. Absorbed sera were prepared according to the method previously described (6), except that 50 mg of lyophilized soluble antigen was used instead of 25 mg per ml. When heterologous precipitins were not entirely removed, the serum was absorbed with 100 and 200 mg per ml. Sera were also absorbed with living cells. Cell suspensions were centrifuged, the supernatant fluid was discarded, and serum was added in the proportion of 0.5 ml of packed cells to 1 ml of serum.

Immunological methods. These have been described previously (5, 6). Each serum used in the Ouchterlony plate was tested against 10 double dilutions of antigen, starting with 50 mg per ml. This procedure was followed to determine the maximal number of lines detected by this technique. Serum was concentrated by lyophilization, reconstituted in one-third volume of water, and retested in Ouchterlony plates.

RESULTS

Agglutination and agglutinin-absorption tests. In view of suggestions (L. E. Carmichael and D. W. Bruner, *in press*) that the dog organism showed some similarities with *B. bronchiseptica*, the latter organism was included in cross-agglutination tests with antisera prepared against several gram-negative species (Table 1). The dog organism was agglutinated to high titer by its own antisera and by antisera to *B. ovis* and rough *Brucella*, but not by antisera to any other gram-negative cultures. The results with *B. ovis* antigen were similar to those with the dog organism. Smooth *Brucella* were agglutinated to high titer only by antisera to smooth *Brucella*. *B. bronchiseptica* was agglutinated to high titer with its own antiserum and to a lower titer with other antisera and also with preimmunization sera.

Antisera were absorbed with living cells of the various *Brucella* cultures and then retested with the homologous antigen. Table 2 shows that the homologous absorbing cells were usually the only cells able to remove all agglutinins; other cultures of the same colonial type removed most of the agglutinins, and cultures of the other colonial type did not absorb agglutinins.

Agglutination and agglutinin-absorption tests showed that the dog organism has nearly the same surface antigens as rough *Brucella* cultures and little or no cross-reactivity with smooth *Brucella* and other gram-negative organisms.

TABLE 1. *Agglutination titers obtained with antisera from rabbits hyperimmunized with acetone-killed organisms*

Antisera	Antigens			
	Smooth *Brucella melitensis*	*B. ovis*	Dog organism	*Bordetella bronchiseptica*
Smooth *B. melitensis*	1:2,560	—[a]	—	1:80
Rough *B. melitensis*	1:320	1:640	1:640	1:80
B. ovis	—	1:1,280	1:1,280	—
Dog organism	—	1:640	1:1,280	—
B. pertussis	1:160[b]	—	—	1:320[c]
B. parapertussis	—	—	—	1:320[c]
B. bronchiseptica	—	—	—	1:5,120[b]
Pasteurella multocida	—	—	1:20[b]	1:80[c]
P. pseudotuberculosis	—	—	—	—
Haemophilus influenzae	—	—	—	1:160[c]

[a] Negative at 1:20 final serum dilution.
[b] Preimmunization serum was negative with given antigen.
[c] Preimmunization sera gave same titer with given antigen.

TABLE 2. *Agglutination titers obtained with antisera tested with homologous antigen before and after absorption with living cells*

Absorbing antigen (living cells)	Antisera				
	Smooth *Brucella melitensis*[a]	Smooth *B. abortus*[b]	Rough *B. abortus*[b]	*B. ovis*[a]	Dog organism[a]
None	1:1,280	1:1,280	1:320	1:2,560	1:1,280
Smooth *B. melitensis*	—[c]	1:40	1:320	1:2,560	1:640
Smooth *B. abortus*	1:20	—	1:320	1:1,280	1:1,280
Rough *B. abortus*	1:1,280	1:640	—	1:320	1:160
B. ovis	1:1,280	1:640	1:20	1:20	1:20
Dog organism	1:1,280	1:1,280	—	1:320	—

[a] Antisera prepared with acetone-killed organisms.
[b] Antisera prepared with cell walls.
[c] Negative at 1:20 final serum dilution.

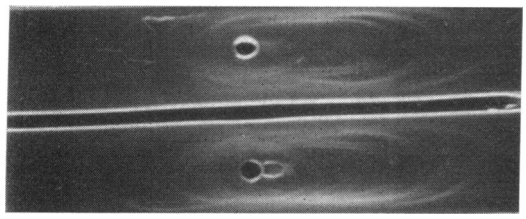

FIG. 1. *Immunoelectrophoresis of soluble antigen from dog organism (in wells) reacted against antisera prepared with acetone-killed smooth Brucella melitensis, dog organism, and rough B. melitensis, respectively, in troughs from top to bottom.*

Gel diffusion and immunoelectrophoresis of water-soluble extracts of sonically treated brucellae. The number of lines obtained in the immunoelectrophoresis of *Brucella* antigens reacted against their homologous antisera varied from 9 to 15. When the soluble antigen from the dog organism was tested with these *Brucella* antisera, 10 to 15 lines were produced with each antiserum (Fig. 1). Absorption of the antisera with soluble antigens from rough and smooth *Brucella* and the dog organism removed most or all of the precipitins for the homologous antigen (Table 3). This shows that the majority of the antigens revealed with immunoelectrophoresis are common to all the cultures and that the dog organism fits into the genus *Brucella* on an antigenic basis.

As reported previously (6), there is one characteristic diffuse line which appears close to the antigen well in the immunoelectrophoresis of soluble antigen from smooth *B. melitensis*, but this line does not appear with soluble antigen from smooth *B. abortus*. This diffuse line is developed by smooth *B. abortus* and *B. melitensis* antisera, but not by rough brucellae antisera or

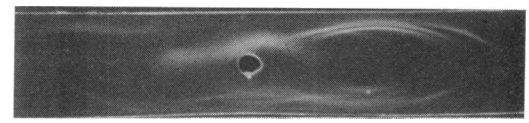

FIG. 2. *Immunoelectrophoresis of soluble antigen from smooth Brucella melitensis (in well) reacted against antisera prepared with acetone-killed smooth B. melitensis (top trough) and dog organism (bottom trough).*

by antiserum to the dog organism (Fig. 2). Soluble antigen from smooth *B. melitensis* and *B. abortus* can absorb the precipitins to this diffuse line, but soluble antigens from rough brucellae, including the dog organism, cannot (Table 3).

To determine whether soluble antigens from gram-negative species show cross-reactivity, antisera were prepared in rabbits against acetone-killed organisms of six gram-negative species. These antisera were reacted against their own soluble antigens and the *Brucella* antigens in immunoelectrophoresis (Table 4). None of the sera developed lines against *Brucella* antigens. All developed 12 to 17 lines in the homologous reaction, which is italicized in Table 4. The three *Bordetella* species revealed extensive cross-reactions within the genus *Bordetella* but little or no cross-reactions with the other organisms. In contrast, *P. multocida* and *P. pseudotuberculosis* showed little cross-reactivity. *H. influenzae* did not cross-react to a significant extent with any of the cultures.

Some of the preimmunization sera developed one or two lines against some of the gram-negative antigens. This was not unexpected, since *B. bronchiseptica*, *P. multocida*, and *P. pseudotuberculosis* are enzootic in laboratory animals. Even when the preimmunization sera

TABLE 3. *Number of lines obtained in immunoelectrophoresis of Brucella antigens reacted against their homologous antisera before and after absorption with various Brucella antigens*

Absorbing antigen (soluble antigen)	Antisera					
	Smooth *B. melitensis*[a]	Rough *B. melitensis*[a]	Smooth *B. abortus*[b]	Rough *B. abortus*[b]	*B. ovis*[a]	Dog organism[a]
None	9	9	12	13	14	13–15
Smooth *B. melitensis*	0	0	0	1	2	0
Rough *B. melitensis*	1[c]	0	0	1	2	0
Smooth *B. abortus*	1	1	0	0	3	2
Rough *B. abortus*	3[c]	0	0	0	3	0
B. ovis	2[c]	1	2–3	4	0	1
Dog organism	2[c]	0	0	0	1	0

[a] Antisera prepared with acetone-killed organisms.
[b] Antisera prepared with soluble antigen.
[c] The diffuse line characteristic of smooth *B. melitensis* antigen was not removed by absorption with rough antigens.

TABLE 4. *Number of lines obtained in immunoelectrophoresis of soluble antigens from gram-negative species reacted against antiserum prepared in rabbits hyperimmunized with acetone-killed organisms*

Antisera	Antigens											
	Bordetella pertussis	*B. parapertussis*	*B. bronchiseptica*	*Pasteurella multocida*	*P. pseudotuberculosis*	*Haemophilus influenzae*	Smooth *Brucella melitensis*	Rough *B. melitensis*	Smooth *B. abortus*	Rough *B. abortus*	*B. ovis*	Dog organism
B. pertussis	*16–17*[a]	13–14[a]	13–14[b]	5–6[c]	0	3[b]	0	0	0	0	0	0
B. parapertussis	13–14[a]	*16–17*[a]	13–14[a]	5–6[c]	1	3–4[d]	0	0	0	0	0	0
B. bronchiseptica	11–12[a]	11–12	*14–15*	2[b]	0	1–2[b]	0	0	0	0	0	0
Pasteurella multocida	0[a]	0[a]	0[b]	*13–15*[b]	1[a]	1	0	0	0	0	0	0
P. pseudotuberculosis	0	0	1	2[b]	*13–14*	0	0	0	0	0	0	0
Haemophilus influenzae	2[a]	1–2	0	2–3[b]	1	*12–13*	0	0	0	0	0	0

[a] Rabbit serum before hyperimmunization gave one line.
[b] Rabbit serum before hyperimmuniztion gave no lines.
[c] Rabbit serum before hyperimmunization gave two lines.
[d] Rabbit serum before hyperimmunization gave same number of lines as after immunization.

were negative, there was the possibility of infection developing during the course of the immunization.

Because the gel diffusion test is more sensitive than immunoelectrophoresis, these antisera to gram-negative organisms were tested against *Brucella* antigens to see if lines would be revealed by the Ouchterlony method. Table 5 shows that, even when the serum was concentrated threefold, only one line, at the most, was produced and this was often faint and diffuse. When *Brucella* antisera were tested against the gram-negative antigens (Table 6), the results were similar, with one or no line appearing. Five to six poorly defined lines appeared with *P. multocida* antigen and two of the *Brucella* antisera. Sera taken from one of these rabbits prior to immunization produced the same number of lines with *P. multocida* antigen. Examination of a number of rabbit sera revealed the common occurrence of precipitins to *P. multocida* and clinical symptoms of "snuffles" were not uncommon. No significance can therefore be attached to the reaction of *Brucella* antisera and *P. multocida* antigen. No line was produced in the reciprocal reaction of *Brucella* antigen and

TABLE 5. *Number of lines obtained in gel diffusion tests with Brucella antigens reacted against antisera prepared against other gram-negative species*

Antisera	Antigens					
	Smooth *Brucella melitensis*	Rough *B. melitensis*	Smooth *B. abortus*	Rough *B. abortus*	*B. ovis*	Dog organism
Bordetella pertussis	1	1	0	0	1	1
B. parapertussis	0	0	0	0	0	1?
B. bronchiseptica	1	1	1	0	0	1
Pasteurella multocida	0	0	0	0	0	0
P. pseudotuberculosis	1	0[a]	1	1	0[a]	1
Haemophilus influenzae	0	0	0[a]	0[a]	0	0

[a] All antisera were concentrated threefold and retested. One line appeared in the indicated combinations.

TABLE 6. *Number of lines obtained in gel diffusion tests with Brucella antisera reacted against soluble antigens prepared from other gram-negative species*

Antisera	Antigens					
	Bordetella pertussis	*B. parapertussis*	*B. bronchiseptica*	*Pasteurella multocida*	*P. pseudo-tuberculosis*	*Haemophilus influenzae*
Smooth *Brucella melitensis*	1	1	1	5–6	1	1
Rough *B. melitensis*	1	1	1	0	1	1
Smooth *B. abortus*	1[a]	1[a]	1[a]	5–6[b]	1[a]	1[a]
Rough *B. abortus*	1[a]	1[a]	1[a]	0	0	1[a]
B. ovis	0	0	0	0	0	0
Dog organism	1	1	1	0	0	0

[a] Rabbit serum before hyperimmunization gave one line.
[b] Rabbit serum before and after immunization gave same number of lines.

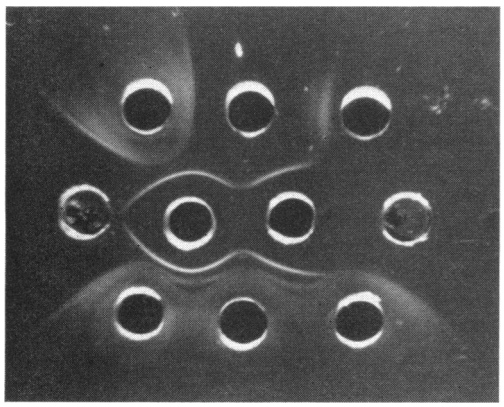

FIG. 3. *Gel diffusion test of ether-water extract of smooth Brucella melitensis reacted against antiserum prepared with acetone-killed smooth B. melitensis. Wells in top row contain from left to right: unabsorbed antiserum, antiserum absorbed with soluble antigen from rough B. melitensis, antiserum absorbed with soluble antigen from smooth B. melitensis. Wells in middle row contain soluble antigen from rough B. melitensis in first well on left, ether-water extract from smooth B. melitensis in remaining wells. Wells in bottom row contain from left to right: unabsorbed antiserum, antiserum absorbed with living rough B. melitensis, antiserum absorbed with living smooth B. melitensis.*

P. multocida antiserum (Table 5), and, therefore, an antigenic relationship between these organisms was not apparent by these methods.

Gel diffusion and immunoelectrophoresis of ether-water extracts of brucellae. Although the majority of lines developed with water-soluble antigens were common to both rough and smooth brucellae, the use of an ether-water extract (5) showed a clear difference between rough and smooth *B. abortus*. These findings were extended to *B. melitensis*. Figure 3 shows the gel diffusion pattern obtained with an ether-water extract of smooth *B. melitensis*, prepared according to the method of Ribi et al. (15), tested with antiserum prepared against acetone-killed cells of smooth *B. melitensis*. Absorption of the antiserum with smooth antigens removed the sharp, clear band closest to the antigen well and also removed the

agglutinins to smooth cells. Absorption with rough antigens did not remove the clear band or the smooth agglutinins. The ether-water extracts of smooth *B. abortus* and *B. melitensis* were examined in gel diffusion and immunoelectrophoresis with antiserum prepared against a number of rough and smooth brucellae, including the dog organism. In all absorption experiments, the dog organism behaved like the rough *B. melitensis*, illustrated in Fig. 3, in its inability to absorb the smooth agglutinin or the precipitin developing the clear band.

The relationship of the sharp, clear band produced by the ether-water extract of smooth brucellae and the diffuse band close to the antigen well, characteristic of soluble antigen from smooth *B. melitensis*, will be reported elsewhere.

Attempts were made to extract an antigen from the dog organism by the ether-water method, but these were unsuccessful (8).

DISCUSSION

Rough and smooth brucellae show little or no antigenic relationship in agglutination tests. The dog organism was agglutinated by antiserum prepared against rough *B. melitensis*, rough *B. abortus*, and *B. ovis*, and was able to adsorb agglutinins from these sera but not from antisera prepared against smooth brucellae.

An ether-water extract of smooth *B. abortus* contained a lipopolysaccharide with endotoxic properties (2, 10) and, in immuno-gel diffusion, produced several lines, one of which could be associated with the smooth agglutinogen (5). It was not possible to extract a similar substance from the dog organism by the ether-water method.

In contrast, water-soluble extracts of sonically disintegrated *Brucella* cells have yielded gel-diffusible antigens which are nearly identical in all *Brucella* species and colonial forms (12). These extracts are composed of 62 to 75% protein (6) and attach to tanned red blood cells. Water-soluble extracts of the dog organism show near identity with *Brucella* antigens in immuno-gel diffusion.

Comparison of the water-soluble antigens of *Brucella* with similarly prepared antigens from other genera in the family *Brucellaceae* has confirmed the findings of Olitzki and Godinger (13), that bacteria in the genus *Brucella* have little antigenic relationship with other gram-negative bacteria. Prince and Smith (14) compared *B. suis* and *P. multocida* antigens in gel diffusion and immunoelectrophoresis and were unable to show cross-reactions. Despite reports that *Brucella* have some antigenic determinant groups in common with *Pasteurella*, *Salmonella*, and *Vibrio* (3, 7), these cross-reactions are only occasionally demonstrable. It appears that both the surface and internal antigens of *Brucella* cultures have a distinctive character which shows little or no cross-reaction with other genera.

These studies have shown that the gram-negative organism causing abortion in dogs belongs in the genus *Brucella* on the basis of immuno-gel diffusion tests with water-soluble antigens. It deserves separate species rank, "*Brucella canis*," as proposed by Carmichael and Bruner (*in press*), because it lacks the lipopolysaccharide-endotoxin associated with the agglutinogen of smooth brucellae.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant AI 06161 from the National Institute of Allergy and Infectious Diseases, and by a World Health Organization research grant. The senior author was on an International Postdoctoral Research Fellowship awarded by the National Institutes of Health.

LITERATURE CITED

1. ANON. 1966. Abortions in 200 beagles. J. Am. Vet. Med. Assoc. **149**:1126.
2. BAKER, P. J., AND J. B. WILSON. 1965. Chemical composition and biological properties of the endotoxin of *Brucella abortus*. J. Bacteriol. **90**:895–902.
3. BERMAN, D. T. 1957. The effect of bacterins containing *Pasteurella multocida* on agglutinins for Brucella in cattle. Proc. Ann. Meeting U.S. Livestock Sanitary Assoc., 60th, p. 97–103.
4. CURRIE, J. A., J. D. MARSHALL, JR., AND D. CROZIER. 1966. The detection of *Pasteurella pseudotuberculosis* antibodies by the micro-hemagglutination test. J. Infect. Diseases **116**: 117–122.
5. DIAZ, R., L. M. JONES, D. LEONG, AND J. B. WILSON. 1967. Differences between *Brucella* antigens involved in indirect hemagglutination tests with normal and tanned red blood cells. J. Bacteriol. **94**:499–505.
6. DIAZ, R., L. M. JONES, AND J. B. WILSON. 1967. Antigenic relationship of *Brucella ovis* and *Brucella melitensis*. J. Bacteriol. **93**:1262–1268.
7. ELBERG, S. S. 1965. *In* R. J. Dubos and J. G. Hirsch [ed.], Bacterial and mycotic infections of man, p. 706. J. B. Lippincott Co., Philadelphia.
8. JONES, L. M., M. ZANARDI, D. LEONG, AND J. B. WILSON. 1968. Taxonomic position in the genus *Brucella* of the causative agent of canine abortion. J. Bacteriol. **95**:625–630.
9. LAUTROP, H. 1960. Laboratory diagnosis of whooping-cough or *Bordetella* infections. Bull. World Health Organ. **23**:15–35.
10. LEONG, D., R. DIAZ, AND J. B. WILSON. 1968. Identification of the toxic component of *Bru-*

cella abortus endotoxin and its labeling with radioactive chromate. J. Bacteriol. **95**:612–617.

11. MCEWEN, A. D., AND F. W. PRIESTLEY. 1938. Experiments on contagious abortion. Immunization studies with vaccines of graded virulence. Vet. Rec. **50**:1097–1106.
12. OLITZKI, A. L. 1960. The antigenic structure of the Brucellae. Boll. Ist. Sieroterap. Milan. **39**: 97–104.
13. 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.
14. PRINCE, G. H., AND J. E. SMITH. 1966. Antigenic studies on *Pasteurella multocida* using immunodiffusion techniques. II. Relationships with other gram-negative species. J. Comp. Pathol. Therap. **76**:315–320.
15. RIBI, E., K. C. MILNER, AND T. D. PERRINE. 1959. Endotoxic and antigenic fractions from the cell wall of *Salmonella enteritidis*. Methods for separation and some biologic activities. J. Immunol. **82**:75–84.