Taxonomic Position in the Genus *Brucella* of the Causative Agent of Canine Abortion

LOIS M. JONES, MARILYN ZANARDI, DANIEL LEONG, AND J. B. WILSON

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

Received for publication 9 November 1967

The gram-negative organism causing abortion in dogs was examined in parallel with cultures representative of the *Brucella* species and with *Bordetella bronchiseptica*. The organism fits into the genus *Brucella* and most closely resembles *B. suis* on the basis of its growth characteristics. It is of rough colonial morphology and is agglutinated by antisera prepared against rough *Brucella*. In mouse toxicity tests, no endotoxic activity could be demonstrated. In contrast to most *Brucella* cultures, it does not utilize erythritol. Electron microscopy showed a cell wall structure similar to that of other gram-negative organisms. The question of whether the organism should be designated *Brucella canis*, as proposed by Carmichael and Bruner, or *Brucella suis* biotype 5 is discussed. The authors favor the designation *Brucella canis* because the organism lacks the lipopolysaccharide antigen associated with the smooth agglutinogen and endotoxin, and it does not utilize erythritol.

---

**Materials and Methods**

*Bacterial cultures.* Three cultures which had been isolated from aborted beagle fetuses were obtained from L. E. Carmichael. The first culture isolated, "RM666," has been deposited with the American Type Culture Collection as the type strain. The other cultures, "H966" and "Hoy 1066," gave reactions identical to "RM666." Several *Brucella* cultures were examined from each species, including the type strains (9, 17). These cultures and *B. bronchiseptica* were from our own culture collection.

*Identification methods.* Methods employed in the identification of gram-negative organisms were those recommended by King (*unpublished data*) and Cowan and Steel (5). Tests for differentiation within the genus *Brucella* have been listed by the Subcommittee on Taxonomy of the genus *Brucella* (9, 17) and details of the methods have been given by Alton and Jones (1).

**Studies of growth characteristics.** Standard methods (1) were followed for the observation of colonial morphology and the preparation of selective media for *brucellae*.

Growth studies in broth with and without added erythritol were performed as described by Wyl (M.S. Thesis, University of Wisconsin, 1964). Trypticase Soy Broth (BBL) was prepared as 50, 75, and 100% of the dry material, as recommended by the manufacturer. Each concentration of broth was subdivided into flasks with an attached side arm to permit direct turbidity readings on a Coleman Junior spectrophotometer model 6A. Erythritol was added to each broth concentration in the amount of 0.1 and 1 mg per ml final concentration. Flasks were inoculated with log-phase broth cultures of the dog organism (RM666) and smooth virulent *Brucella abortus* strain 2308.

**Susceptibility to lysis by lysozyme.** Trypticase Soy Broth cultures in the early log phase were employed. The addition of both glycerine and ethylenediaminetetraacetic acid at final concentrations of 0.3 M and 0.0013 M, respectively, was required for rapid lysis of *Brucella* cells by lysozyme (Jones and Wilson, *unpublished data*). The effect of lysozyme on cultures of the dog organism was observed in parallel with the effect of rough and smooth *Brucella* cultures. The time required for the optical density to be reduced 50% was the value taken to express the susceptibility of the culture to lysozyme (final concentration, 333 μg/ml).

**Mouse toxicity tests.** The lethal effect of the dog organism was compared with that of rough *B. abortus* (strain 45/20) and smooth *B. abortus* (strain 2308) by the intraperitoneal inoculation of 10-fold dilutions of saline suspensions of the living organisms into groups of white Swiss mice, six mice in each group.
Mice which did not die within 48 hr were killed after 7 days and the spleens from each group were removed, pooled, weighed, homogenized, diluted in saline, and colony counts were made.

The procedure described by Baker and Wilson (4) was followed for the serum iron assay of endotoxin. A dose of 10⁸ organisms was injected intraperitoneally into each mouse. Five replications, consisting of five mice each, were employed for each of the three strains, i.e., RM666, 45/20, and 2308. Serum was removed 12 hr after injection and examined for iron.

Electron microscopy. The methods described below were found most satisfactory in a study of the fine structure of B. abortus (Zanardi, unpublished data). They are modifications of procedures described by Kay (10), Pease (13), and Newcomb (E. H. Newcomb, personal communication, 1965).

The dog organism was grown for 24 hr on a slant of Trypticase Soy Agar with 2% rabbit serum. Growth was scraped from the slant and suspended in 6% glutaraldehyde in phosphate buffer (pH 6.8). After 1 hr at room temperature, the suspension was centrifuged, the pellet was resuspended in melted 2% agar containing 1% glycerine in phosphate buffer, and it was allowed to solidify. The specimen was minced; small cubes of agar were washed for 2 hr in several changes of phosphate buffer, placed in 2% osmium tetroxide in phosphate buffer for 2 hr, and then dehydrated in a graded series of acetone. The cubes of agar were embedded in a plastic mixture consisting of Araldite 6005, Epon 812, dodeceny1 succinic anhydride, and 2,4,6-tridimethylaminomethyl phenol. Thin sections were cut with a diamond knife on a Porter-Blum MT-2 Ultramicrotome and stained with uranyl acetate for 15 min, followed by lead citrate for 10 min.

Bacteria were negatively stained by mixing equal volumes of an aqueous suspension of the organism and 2% sodium phosphotungstate (pH 7.0) and placing a drop on a collodion-coated copper grid. The excess was blotted dry and the grid was examined immediately.

Bacteria were shadow-cast in a Kinney high-vacuum evaporator with uranium at an angle of 60°. The specimen was an aqueous suspension placed on a collodion-coated grid attached to a glass slide. It was freeze-dried in the vacuum chamber of an Edwards Model 5PS Freeze Dryer for 2 hr before shadow-casting.

All preparations were examined with a Zeiss 9A electron microscope.

RESULTS

The organism isolated from dogs is a short gram-negative rod which does not ferment glucose. Serum or enrichment materials are not required for growth. The tests commonly used to identify organisms in this category are given in Table 1; results were obtained with Brucella species and Bordetella bronchiseptica. The canine organism fits into the genus Brucella on the basis of these tests.

Table 2 gives the results obtained in tests employed in this laboratory for the identification of Brucella species and their biotypes. The canine organism most closely resembles B. suis biotype 3 in growth characteristics, although it is more sensitive to basic fuchsin than biotype 3. It is unaffected by brucellaphage concentrations as high as 10⁸ times routine test dilution. Broth cultures of the dog organism were cross-dropped on lawns of B. abortus, B. suis, and B. melitensis to see if the cultures were carrying a phage, but no evidence was obtained in this regard.

Cultures streaked on Trypticase Soy Agar (BBL) and incubated in air at 37 C attained a colony size of 1 mm in 3 days, similar to that of Brucella cultures but smaller than that observed with B. bronchiseptica. Observation of colonies by obliquely transmitted light with the aid of a stereoscopic binocular microscope revealed blue-gray, somewhat granular colonies with an entire margin. These colonies resembled B. ovis and a rough B. melitensis culture isolated from goat's milk in Malta in that they were less dry and

### Table 1. Tests for identification of the genus Brucella

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Brucella abortus</th>
<th>B. suis biotype 1</th>
<th>B. suis biotype 3</th>
<th>B. melitensis</th>
<th>B. neotomae</th>
<th>B. ovis</th>
<th>Bordetella bronchiseptica</th>
<th>Dog organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grows on MacConkey</td>
<td>+ or -</td>
<td>+ or -</td>
<td>+ or -</td>
<td>+ or -</td>
<td>+ or -</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Catalase produced</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase produced</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate utilized</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduced</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Urea hydrolyzed</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>H₂S produced</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Litmus milk change</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Alkaline</td>
<td>-</td>
</tr>
<tr>
<td>No. of cultures examined</td>
<td>10</td>
<td>2</td>
<td>2</td>
<td>9</td>
<td>3</td>
<td>8</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

* The following tests were negative with all cultures: glucose fermentation, gelatin hydrolyzation, indole production, methyl red, and Voges Proskauer tests.
<table>
<thead>
<tr>
<th>Species</th>
<th>Bio-type</th>
<th>CO₂ requirement</th>
<th>H₂S production</th>
<th>Urease production</th>
<th>Growth on Trypticase Soy Agar containing</th>
<th>Colonial morphology</th>
<th>Lysisa by brucellaphage</th>
<th>Agglutinated by antisera:</th>
<th>Host</th>
<th>No. of cultures examined</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. abortus . . . . . .</td>
<td>1</td>
<td>+ or −</td>
<td>+</td>
<td>−</td>
<td>Basic fuchsinb</td>
<td>Thioninc</td>
<td>Saffron O</td>
<td>Thionin bluec</td>
<td>Ethyl Violetd</td>
<td>Penicillin</td>
</tr>
<tr>
<td>B. suis . . . . . . .</td>
<td>1</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B. suis . . . . . . .</td>
<td>3</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Dog organism</td>
<td>1</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>B. melitensis . . .</td>
<td>1</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>B. neotomae . . . .</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>B. ovis . . . . . .</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

a + = confluent lysis; I = partial lysis or inhibition; − = no reaction.
b Concentrations were: a = 1:25,000; b = 1:50,000; and c = 1:100,000.
c Concentrations were: 1:5,000 with Saffron O; 1:500,000 with Thionin blue; 1:100,000 with Ethyl Violet; and 5 μ per ml with penicillin.
granular than rough mutants observed in laboratory cultures. The canine organism gave the characteristic rough reaction to the acriflavine and crystal violet staining methods. In slide agglutination tests, no agglutination occurred with smooth \textit{B. abortus} or \textit{B. melitensis} antisera, but immediate agglutination occurred with rough \textit{B. abortus}, \textit{B. melitensis}, and \textit{B. ovis} antisera.

Selective media used for isolation of \textit{Brucella} were examined for their ability to initiate growth of the dog organism. Crystal Violet (1.4 mg per liter) or 1\% normal serum, and cycloheximide, bacitracin, and polymyxin B (1) were added to Brucella agar (Albimi Laboratories, Inc., New York). The dog organism grew well on the selective medium with serum and antibiotics, but did not grow on the medium with Crystal Violet and antibiotics. Incubation of plates in 10\% added CO\textsubscript{2} did not inhibit the growth on serum-antibiotic medium.

The size and numbers of colonies of the dog organism were about the same on Trypticase Soy Agar with and without the addition of 2\% normal rabbit serum, but growth on medium without serum was very difficult to suspend in saline. For the preparation of antigen suspensions, Roux flasks of Trypticase Soy Agar with 2\% rabbit serum resulted in growth which was easily suspended in normal saline. Tween 40 added to media in a final concentration of 0.1 or 0.5\% can be substituted for serum for the growth of serum-requiring \textit{B. abortus} biotype 2 (1), but this medium did not produce growth of the dog organism which could be suspended in saline.

The addition of erythritol (1 mg per ml) to Trypticase Soy Agar did not stimulate or inhibit the growth of the dog organism. Growth studies in several concentrations of Trypticase Soy Broth with and without added erythritol gave further evidence that the dog organism is unable to utilize erythritol. The growth curves of "RM666" were identical at any given broth concentration, whether erythritol had been added or not. In contrast, the growth of \textit{B. abortus} strain 2308 was stimulated by the addition of both concentrations of erythritol in all concentrations of broth.

Although some reports (8, 14, 20) have indicated that rough cultures are more sensitive to lysis by lysozyme than are smooth cultures, we have not observed this correlation in a study of 50 \textit{Brucella} cultures. The time required for a 50\% drop in turbidity of the dog organism was 50 min, whereas smooth cultures varied in time from 20 to 120 min.

\textbf{Results of mouse toxicity tests.} A dose of 2 \times 10\(^8\) living organisms of smooth \textit{B. abortus} strain 2308 killed mice within 48 hr, whereas this dose of rough \textit{B. abortus} strain 45/20 and the dog organism, strain RM666, did not kill mice. Higher doses were not tested. Groups given 2 \times 10\(^8\) organisms were killed 7 days later, and the following bacterial counts per gram of pooled spleens were obtained: for 2308, 4.2 \times 10\(^8\) organisms; for RM666, 4.2 \times 10\(^8\) organisms; for 45/20, 3.6 \times 10\(^8\) organisms. This shows that the rough organisms were able to multiply in the mouse tissues, although not to as great an extent as the smooth strain 2308.

An attempt was made to extract endotoxin from the dog organism by the ether-water procedure of Ribi, Milner, and Perrine (15), but no material with endotoxic activity was obtained. Heat-killed cells were then employed in an attempt to demonstrate endotoxic activity by the serum iron assay method (4). A dose of 10\(^8\) heat-killed cells of strain 2308 caused a mean reduction in serum iron of 162 \mu g per 100 ml, which is in the range obtained with this strain (3). The same dose of RM666 and 45/20 cells caused no reduction in serum iron.

\textbf{Results of electron microscopy.} The thin sections of the dog organism (Fig. 1) were very similar to what we have observed with similar sections of \textit{B. abortus}. A multilayered envelope was observed consisting of an outer triple-layered cell wall, a middle homogeneous layer of variable thickness, and an inner membrane. Preparations which were negatively stained or shadow-cast did not reveal any structure suggestive of an unusual surface layer or a capsule.

\textbf{DISCUSSION}

The ultrastructure of the dog organism is similar to that of \textit{B. abortus} and other gram-negative bacteria. DePetris, Karlsbad, and Kessel (6) have examined rough and smooth \textit{B. abortus} and found no evidence that colonial variation was associated with morphological differences in surface structures and no indication of the existence of a capsule. It has been pointed out by Tomcik (19), however, that fixation and staining invariably leads to shrinkage and, because of the high water content of capsules, electron microscopy probably does not give a true picture of the morphology of a capsulated cell.

Smith et al. (16) suggested that, in brucellosis of pregnant cows, the predilection of \textit{B. abortus} for fetal rather than adult tissue is due to the presence of erythritol in the uterus. Erythritol serves as a growth stimulatory factor both in vivo and in vitro and could be responsible for the rapid multiplication of \textit{B. abortus} within the uterus and the expulsion of the fetus due to accumulation of endotoxin. In further studies (11), the presence of erythritol in the placentae, seminal vesicles, and testes of goats, sheep, and pigs, and
the growth stimulation of *B. melitensis* and *B. suis* by erythritol in vitro, was offered as an explanation for the localization of brucellae in these animal species as well. Although the clinical and pathological findings of abortion in dogs, as reported by Carmichael (*personal communication*), are quite similar to those in cattle, sheep, and pigs, the hypothesis that fetal erythritol is a cause of localization and abortion does not fit the situation in canine abortion, since the growth of this organism is not stimulated by erythritol and since endotoxin is not demonstrable. The presence of erythritol in the uterus of dogs has not been reported to our knowledge.

Carmichael (*personal communication*) has pointed out that, in previous reports of canine brucellosis, transmission of *B. abortus*, *B. suis*, or *B. melitensis* from domestic animals to dogs occurred, but the dog appeared to be the terminal host. In contrast, this organism has been observed only in dogs, mainly in beagles, is highly contagious among dogs, but has not been reported in other species. Present information suggests this agent may be more host-specific than *B. abortus*, *B. suis*, or *B. melitensis*.

Serological analysis (7) showed that the surface antigen of the canine organism is similar to that of rough *B. abortus*, rough *B. melitensis*, and the rough species *B. ovis*; the soluble antigens, as revealed by gel diffusion and immunoelectrophoresis, are similar to all *Brucella* cultures, whether rough or smooth, but bear no resemblance to soluble antigens of other gram-negative genera in the family *Brucellaceae* (i.e., *Bordetella*, *Pasteurella*, and *Haemophilus*). On the basis of growth characteristics used for differentiation within the genus *Brucella*, the organism resembles *B. suis* biotype 3 fairly closely. The Subcommittee on Taxonomy of the genus *Brucella* (17) recommended the use of oxidative metabolic tests in the characterization of unusual biotypes. The three cultures were sent to the Central Veterinary Laboratory, Weybridge, England, a reference laboratory equipped to do these tests. W. J. B. Morgan reported (*unpublished data*) that the canine organism had the metabolic pattern of *B. suis* biotypes 3 and 4, except that it did not oxidize erythritol, which *B. abortus*, *B. suis*, *B. melitensis*, and *Brucella neotomae* cultures oxidize but *B. ovis* cultures do not.

At the last meeting of the Subcommittee on Taxonomy of *Brucella* (9), the *Brucella* organism causing abortion in reindeer was given the taxonomic position of *B. suis* biotype 4. This organism is identical to *B. suis* biotype 3 on the basis of its biochemical and metabolic tests, but it is antigenically different from biotype 3, since

---

**Fig. 1.** Thin sections of the dog organism. (a) × 60,000; (b) × 260,000 of area indicated with arrow in (A) Marker represents 0.1 μ.
it is agglutinated by both monospecific abortus and melitensis antisera.

The question of whether the canine organism should be designated B. suis biotype 5, or Brucella canis, as proposed by Carmichael and Bruner (in press), will be discussed by the Subcommittee. The authors favor the designation of Brucella canis, because the organism lacks the lipopolysaccharide antigen associated with the smooth agglutinogen and endotoxin and because it does not utilize erythritol.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant AI 06161 from the National Institute of Allergy and Infectious Diseases, by Public Health Service training grant PHS-S-T1-GM-686 from the Division of General Medical Sciences, and by a World Health Organization research grant.

LITERATURE CITED