Isolation of a Spirochete from the Soft Tick, *Ornithodoros coriaceus*: A Possible Agent of Epizootic Bovine Abortion

Abstract. *A* Borrelia*-like spirochete was detected in all three parasitic stages of* Ornithodoros coriaceus, *the soft tick implicated in the epizoology of epizootic bovine abortion. After the spirochete had been isolated, its distinctness from other North American tick-borne borreliae as well as from* Spirochaeta aurantia, Treponema pallidum, *and* Leptospira interrogans *serovar* pomona *was established on the basis of its morphology, protein components, and inability to infect mice. The spirochete is passed trans-stadially and via eggs by ticks, and it is also excreted in coxal fluid after ticks have fed and detached. Circumstantial evidence suggests that the spirochete may be causally related to epizootic bovine abortion.*

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The soft tick, *Ornithodoros coriaceus*, occurs in the western United States and Mexico where it feeds primarily on deer and cattle and occasionally on humans. No known human pathogens, including spirochetes, have been isolated from this tick except the etiologic agent of Q fever (*1*). Moreover, experimental attempts to transmit several tick-borne relapsing fever borreliae with *O. coriaceus* have been largely unsuccessful (*2–4*).

The feeding activities of this tick, however, have been implicated in the epizoology of epizootic bovine abortion (EBA) (*5*), a major disease of rangeland cattle in the western United States. In California, estimated annual calf losses due to EBA vary from 5 to 10 percent and may be responsible for the loss of 30 to 60 percent in individual herds at a cost of $5 to $15 million (*6–8*). Although EBA has apparently occurred in California for more than 60 years (*9*) and has been responsible for heavy calf losses since 1954 (*10*), its etiology has not been established.

We report here the detection, isolation, and partial characterization of a new *Borrelia*-like spirochete from *O. coriaceus*. Initial attempts to find a suitable animal model and to elucidate the tick-spirochete relation are also described. Circumstantial evidence suggests that this spirochete may be associated with EBA.

During vector specificity studies of the Lyme disease spirochete, *Borrelia burgdorferi*, in an endemic area of northern California, unfed *O. coriaceus* ticks were collected in enamelware pitfall traps baited with CO_2 in chaparral and woodland-grass habitats at the University of California Hopland Field Station, Mendocino County, from July to September 1984. Spirochetes were detected by direct immunofluorescence (*11*) in 5 (0.96 percent) of 520 larvae, 10 (8.26 percent) of 121 nymphs, and 31 (24.03 percent) of 129 adult (92 ♂♂, 37 ♀♀) ticks. Infection rates in larvae versus nymphs, and in nymphs versus adults, were significantly different (Fisher exact test, $P < 0.05$, two-tailed), whereas those in males (19.57 percent) versus females (35.14 percent) approached significance ($P = 0.071$). The *O. coriaceus* spirochete (OCS) was found in every type of nymphal and adult tick tissue examined, but no tissue type was infected all the time. Among infected nymphs or adults, tissues that frequently contained spirochetes included the central ganglion (83 to 89 percent), salivary gland (71 to 85 percent), ovary (63 percent), and testis (78 percent). The degree of spirochetal infections in most nymphal tissues was usually light [<1 organism per field of view (×400)] but was generally moderate (1 to 10 organisms per ×400 field) or heavy (>10 organisms per ×400 field) in tissues of adults. Hemolymph was infected in 56 percent of the nymphs ($n = 9$) and 24 percent of the adults ($n = 21$) whose tissues contained spirochetes, though typically such infections were light.

Microscopic examination of wet mounts revealed that the OCS's were highly motile, active organisms capable of rapid rotation, like *Borrelia hermsii*; in contrast, *B. burgdorferi* tends to move sluggishly and rotate slowly (*12*). In addition, the OCS's appear to be more symmetrically helical and stain slightly darker with Giemsa than *B. burgdorferi*.

We isolated the OCS by inoculating 1.0 ml of a suspension containing the

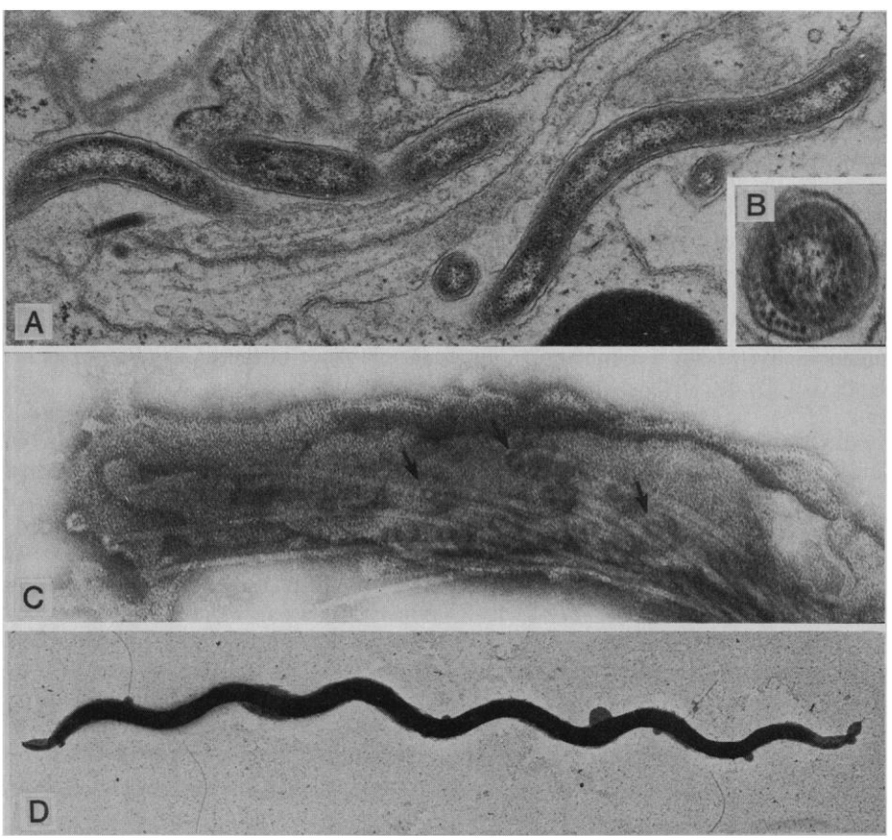

Fig. 1. Electron micrographs of *O. coriaceus* spirochetes. (A) Association with duct cells of a salivary gland acinus (×26,000). (B) Cross section of the 11 or more periplasmic flagella (11 to 14 nm wide) within the asymmetric region (×85,000). (C) Negatively stained spirochete (2 percent ammonium molybdate, *p*H 6.5), showing insertion points (arrows) of several periplasmic flagella (×128,000). (D) Negatively stained, dividing whole organism (×10,300).

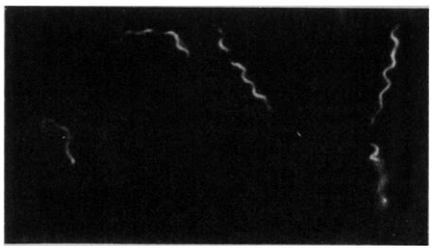

Fig. 2. Indirect immunofluorescence reaction of anti-*Borrelia* monoclonal antibody (H9724) against the *O. coriaceus* spirochete cultivated in vitro (×400).

triturated tissues of a male tick into 9 ml of modified Kelly's (BSK II) medium (*13*). Viable spirochetes were detected in culture tubes after 17 days of incubation at 35°C and were cultured every 2 weeks thereafter. The isolate of strain Co53 also grew in BSK II medium in which fetal calf serum had been substituted for rabbit serum.

Electron microscopy (*14*) of the salivary gland from an infected male tick revealed spirochetes in close association with cells of the salivary ducts and tracheoles and in the intercellular spaces of the gland's acini (Fig. 1A). Fine structural details of the OCS are similar to those of *Borrelia* (*15*). Eleven to 15 periplasmic flagella (11 to 14 nm in diameter) are inserted in a random matrix of two to four rows along the long axis of the cell between the outer and periplasmic membranes (Fig. 1, B and C). The negatively stained OCS is 8 to 10 μm long and ~0.3 to ~0.4 μm in diameter and has only three to five serpentine waves per organism. Its ends vary from pointed to blunt and truncated (Fig. 1D).

The relatedness of the OCS to the genus *Borrelia* was indicated by its immunoreactivity with a monoclonal antibody (H9724) directed against *B. hermsii* (Fig. 2). In the indirect immunofluorescence test, H9724 bound with OCS isolate Co53 as well as with isolates of *B. hermsii*, *B. parkeri*, *B. turicatae*, *B. anserina*, and *B. burgdorferi*, but not with those of *Spirochaeta aurantia*, *Treponema pallidum*, or *Leptospira interrogans* serovar *pomona* (*16*).

Comparison of the Coomassie blue–stained proteins of whole cell lysates of *B. burgdorferi*, *B. hermsii*, and isolate Co53 subjected to polyacrylamide gel electrophoresis demonstrated several differences in the major bands (Fig. 3). The number of proteins that differed among the three isolates was greater than the number of distinguishing cellular proteins among several strains of *B. burgdorferi* (*16*). Although we did not compare isolate Co53 with isolates of *B. parkeri* and *B. turicatae*, DNA homology studies indicate that the latter organisms may be conspecific with *B. hermsii* (*17*); our animal inoculation studies (see below) suggest that the OCS, unlike *B. parkeri* and *B. turicatae*, does not readily infect mice. These findings indicate that the OCS represents a hitherto undescribed *Borrelia*-like spirochete whose relatedness to other spirochetes must await characterization of its DNA and physiological properties.

OCS could not be detected in blood samples from 11 infant Swiss White mice or 10 infant BALB/c mice inoculated intraperitoneally with infected tick suspensions or first-passaged OCS in culture medium, respectively (*18*). Mice are easily infected with all previously described North American tick-borne borreliae except *B. burgdorferi* (*2, 4, 19*). Similarly, OCS was not found in the blood of six adult New Zealand White rabbits, each of which had been fed upon by at least one to eight infected *O. coriaceus* ticks (*18*). Also, 83 normal *O. coriaceus* larvae were fed xenodiagnostically on two of the rabbits for up to 2 weeks after infected ticks had fed on them. After detachment and molting to nymphs, the central ganglion and midgut of each tick were tested by direct immunofluorescence for OCS with negative results.

Of two ovarially infected female ticks whose F_1 progenies were examined by direct immunofluorescence (*11*), 14 percent of the larvae from one female and none from the other contained OCS. One of 21 blood-fed nymphs that were dissected after molting was found to contain OCS. Transovarial and trans-stadial passage of the OCS can therefore occur, but the efficiency of these processes for maintenance of the parasite requires investigation.

Borreliae were also detected by direct immunofluorescence in coxal fluid of nymphs (18.75 percent, 6 of 32), males (14.3 percent, 1 of 7), and females (21.1 percent, 4 of 19) that had recently fed. This finding probably has little epizoologic significance because all such ticks apparently excreted coxal fluid only after they had completed feeding and left the host.

We suggest that the OCS may be causally related to EBA because of (i) the high percentage of infected adult *O. coriaceus* at the Hopland Field Station, a recognized EBA area; (ii) the presence of OCS in the salivary glands of most infected nymphal and adult ticks; and (iii) the association of feeding by this tick with development of EBA (*5*). Moreover, the fact that prophylactic treatment of cattle with chlortetracycline reduces the rate of EBA under field conditions (*20*) indicates that an antibiotic-sensitive organism (possibly a spirochete) may be the abortifacient agent. Chlamydiae have been proposed as the cause of EBA (*7*), but a vaccine prepared from this agent and field-tested in EBA enzootic areas did not protect cattle (*20*). The only spirochete known to cause disease in cattle is *Borrelia theileri*, which produces a mild infection characterized by fever, inappetence, weakness, and anemia (*21*). On the other hand, pregnant women infected with Borreliae that cause epidemic relapsing fever frequently abort (*22*). Moreover, pathologic lesions observed in human borreliosis resemble those seen in EBA fetuses; these lesions typically affect several organs and systems including the liver, central nervous system, and vascular system (*21, 23*). These observations indicate the need for further studies to establish unequivocally whether the OCS is involved in the etiology of EBA.

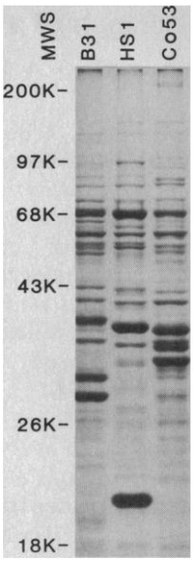

Fig. 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of whole cell lysates of *Borrelia burgdorferi*, strain B31 (ATCC 35209); *B. hermsii*, strain HS1, serotype C (ATCC 35210); and the *Ornithodoros coriaceus* spirochete, strain Co53. Spirochetes were grown in BSK II medium; acrylamide concentration 12.5 percent. The molecular weight standards were as follows: myosin H-chain [200,000 (200K)], phosphorylase B (97K), bovine serum albumin (68K), ovalbumin (43K), γ-chymotrypsinogen (26K), and β-lactoglobulin (18K).

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11. Larval ticks were crushed, and smears were prepared from their body contents. After hemolymph specimens had been obtained from most nymphal and adult ticks [W. Burgdorfer, *Am. J. Trop. Med. Hyg.* **19**, 1010 (1970)], they were dissected and smears were prepared from gut, central ganglion, and (in adults) ovary or testis as well. All specimens were air-dried, fixed in

acetone, and stained with fluorescein isothiocyanate–labeled rabbit anti–*Borrelia burgdorferi* conjugate, which cross-reacted with the *O. coriaceus* spirochete and *B. hermsii*. The antiserum was prepared against whole cells of *B. burgdorferi*, and the gamma globulin fraction was conjugated by the methods of M. Peacock, W. Burgdorfer, and R. A. Ormsbee [*Infect. Immun.* **3**, 355 (1971)]. If fluorescence microscopy revealed spirochetes, then Malpighian tubules, salivary glands, and occasionally coxal organs were examined in the same manner and wet mounts of infected tissues were inspected by dark-field microscopy.
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16. The monoclonal antibody H9724 was the product of a fusion of NS-1 myeloma cells with the spleen cells of a mouse that had been immunized with *B. hermsii* spirochetes. The antibody was found to react with isolated periplasmic flagella of *B. hermsii* by immune electron microscopy. Further details regarding this monoclonal antibody will be presented elsewhere (A. G. Barbour, S. F. Hayes, M. Schrumpf, in preparation). The indirect immunofluorescence test procedure follows that of A. G. Barbour, S. L. Tessier, and S. F. Hayes [*Infect. Immun.* **45**, 94 (1984)].
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18. Four Swiss mice were inoculated with 0.2 ml of a suspension containing infected tick tissues triturated in 1.5 ml of phosphate-buffered saline (pH 7.4); seven mice from a second litter were inoculated intraperitoneally with 0.05 ml of a suspension consisting of the infected tissues of two ticks triturated in 2 ml of BSK II medium. They were bled on days 4 through 14 after inoculation, and thick smears were stained with fluorescein isothiocyanate–labeled anti–*B. burgdorferi* conjugate. Each of the BALB/c mice was injected with $\sim 10^7$ organisms and examined similarly for spirochetes. The rabbits were fed upon by 11 to 43 ($\bar{x}$ = 19.3) nymphal or adult ticks, and thick and thin (Giemsa-stained) blood smears were prepared daily for 14 to 21 days. Infected ticks were identified by a direct immunofluorescence study of multiple tissue smears or coxal fluid.
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24. We thank A. H. Murphy for use of the facilities at the Hopland Field Station, J. A. Howarth and Y. Hokama for pertinent literature, V. H. Resh for reviewing an earlier version of the manuscript, and S. A. Manweiler and L. Bohlmann for technical assistance. This investigation was supported in part by BRSG grant 2-S07-RR07006 from the Biomedical Research Support Program, Division of Research Resources, National Institutes of Health.

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Intercontinental Spread of a New Antibiotic Resistance Gene on an Epidemic Plasmid

Abstract. *Bacteria of different genera isolated at nine medical centers in different parts of the United States and at one center in Venezuela during the first decade of gentamicin usage carried the gentamicin resistance gene 2″-aminoglycoside nucleotidyltransferase on the same transferable plasmid. Such widespread dissemination of a newly observed resistance gene on one plasmid suggests that a new resistance gene may emerge once on a single plasmid, which then carries it to other centers and other plasmids. The resistance gene might, therefore, be contained if detected early.*

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A gene encoding resistance to an antibiotic may begin to appear in bacteria of many genera at a succession of medical centers only after the antibiotic has been used for several years (*1–8*). Presumably, the antibiotic is eventually used in the presence of an obscure bacterial strain carrying the resistance gene or its ancestor, and the overgrowth of this strain increases the chances of the gene's becoming inserted into a plasmid that can transfer between bacteria (*9–11*). The gentamicin resistance gene, 2″-aminoglycoside nucleotidyltransferase [ANT(2″)], was observed early in its appearance and was found on different plasmids in different centers (*4–6, 8, 12, 13*). These observations were consistent with a separate emergence of the resistance gene at each center. We have now examined isolates from many centers and find that ANT(2″) was on the same plasmid in various genera at nine widely separated centers. This suggests that ANT(2″) had emerged once on this plasmid and had then been widely disseminated by it (*14*).

We studied collections of gentamicin-resistant isolates of enteric bacteria from the Brigham and Women's Hospital, Boston (BW); Hospital Centro Medico, Caracas, Venezuela (CM); and the West Roxbury Veteran's Administration Hospital, Boston (WR); as well as isolates collected from various centers by Schering Laboratories (SL) and a single isolate from the Seattle Veteran's Administration Hospital (SV). The isolates had been stored in agar in sealed tubes at room temperature or at 4°C. Isolates were mated with a strain of *Escherichia coli* K-12 (SY 663; *trpΔ met his nal*^{r} *rif*^{r}). Isolate susceptibility to antibacterials was tested by disk diffusion. Plasmid DNA was extracted by an alkaline method (*15*) and digested by restriction endonucleases Eco RI or Bgl II. Digest fragments, subjected to electrophoresis in 0.7 percent agarose gel, were stained with ethidium bromide and photographed under ultraviolet light.

Eleven isolates from these collections transferred gentamicin resistance on plasmids that shared identical Eco RI fragment sizes (Table 1 and Fig. 1). The similarity of these fragments to those of the previously reported pLST1000 (*6*) led us to adopt that designation for these plasmids. Six of these 11 isolates had been among 38 from the SL collection chosen for mating because they were resistant to the moderate levels of kanamycin, gentamicin, and tobramycin characterizing ANT(2″) (*16*). Another 21 of these 38 isolates also transferred gentamicin resistance, but their plasmid restriction fragments differed, and as far as we could judge no two from different centers were the same. Finding ANT(2″) on a different plasmid in each of a majority of these centers was in agreement with previous reports from individual centers (*4–6, 8, 12, 13*) and contrasts with our finding it on pLST1000 in nine widely separated centers in the United States and one center in Venezuela.

Each of the pLST1000 plasmids also carried resistance to streptomycin and to ampicillin. Enzyme assays performed on cell-free extracts of a pLST1000 transconjugant gave the substrate profile of ANT(2″) (*17*). The pLST1000 plasmid hybridized with a gene probe for ANT(2″) and with another for the 3″-aminoglycoside nucleotidyltransferase [ANT(3″)] (*18*), which produces resistance to streptomycin. Isoelectric focusing studies showed that each of the clini-

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