Helical Mycoplasmas (Spiroplasmas) from *Ixodes* Ticks

Abstract. *A new spiroplasma isolated from* Ixodes pacificus *collected in Oregon was serologically and morphologically distinct from known spiroplasmas. The new spiroplasma could also be isolated in tick cell cultures. Discovery of a new fastidious mycoplasma in ticks offers opportunities to explore the possible role of these agents in human and animal diseases.*

Spiroplasmas are a group of helical, wall-less prokaryotes associated with arthropods. Early studies of these organisms focused on their role as insect-borne plant pathogens (*1*) or insect pathogens (*2*), whereas more recent studies have stressed their occurrence on flower surfaces (*3*), where they are presumably deposited by pollinating insects. Spiroplasmas have also been found in rabbit ticks (*Haemaphysalis leporispalustris*). The first strains (SMCA and GT-48) obtained from ticks were isolated by inoculation of embryonated chicken eggs with pooled extracts from rabbit ticks collected in Georgia in 1964 (*4*). Although they were initially thought to be slow viruses, they were eventually shown to be spiroplasmas (*5*). Cultivation of the Georgia isolates in an artificial medium (*6*) was followed by serological characterization (*7*) and studies of their genome (*8*). These studies showed that they constitute a distinct group of spiroplasmas that will undoubtedly be recognized as a new *Spiroplasma* species. Laboratory cultivation of the two strains also permitted confirmation of their pathogenicity for vertebrates, in which ocular and neuropathological signs predominate (*6*). A third rabbit tick isolate, strain 277F, was also obtained by inoculation of tick suspensions into chicken embryos (*9*). This strain, isolated from Montana ticks, was also eventually shown to be a spiroplasma (*9*), but the organism was serologically distinct from the SMCA group (*7, 9*) and did not induce disease in vertebrates. More recently, a member of the SMCA serological group was isolated directly in culture medium inoculated with suspensions from rabbit ticks collected in Maryland (*10*). The methods used in this study provided a basis for more thorough searches for spiroplasmas in other tick species.

The vertebrate pathogenicity of spiroplasmas raises questions about their potential pathogenicity for man. Spiroplasmas recovered from rabbit ticks may have limited potential for transmission to humans, since these arthropods infrequently bite man. In contrast, other North American ticks (for example, *Amblyomma, Dermacentor*, and *Ixodes* species) frequently attack man, and spiroplasmas occurring in these ticks could represent a more serious pathogenic potential. Recent association of *Ixodes* species (*11*) with a human disease of unknown etiology, the so-called "Lyme disease" (*12*), directed our attention to the possibility that spiroplasmas may occur in this tick genus. We now report the discovery of a new spiroplasma from *Ixodes*.

Approximately 30 pools of *Ixodes pacificus*, representing about 300 ticks collected in southern Oregon (Douglas, Josephine, and Jackson counties) from December 1979 to May 1980, were transported live to the Rocky Mountain Laboratory (RML). At RML, the pools were ground in 3 ml of phosphate-buffered saline (*p*H 7.0) containing 7.5 percent bovine plasma albumin (PBS-BPA). The suspensions were then passed through a sterile membrane filter (450 nm pore diameter) to remove bacteria, and 0.1 ml of the triturated tick suspension was added to 3 ml of SP-4 spiroplasma culture medium (*6*). The cultures were transmitted rapidly to the Laboratory of Infectious Diseases, where they were incubated at 30°C. Spiroplasmas were recovered from seven pools of either male or female ticks collected between March and May 1980. All isolates caused a moderate acidic shift in the SP-4 broth after an incubation period of 20 to 25 days. Helical and nonhelical forms were readily apparent by dark-field microscopy (magnification, ×1250). All isolates produced turbidity and an acidic shift during repeated passage in SP-4 medium. Such changes were usually noted after 5 to 10 days at 30°C. All seven isolates were also subsequently recovered in the laboratory-maintained RML-15 or RML-19 lines of tick (*Dermacentor variabilis*) cells (*13*). The original tick triturates, which had been stored frozen (−70°C) in PBS-BPA, were thawed, filtered through a sterile 450-nm membrane filter, and inoculated into tick cell cultures maintained at 28°C. Spiroplasmas first appeared, in dark-field examination of culture supernatants, as early as 21, and as late as 56 days after inoculation. The spiroplasmas did not appear to produce cytopathogenic effects on the cells.

Each of the seven spiroplasma isolates was purified by conventional filtration-cloning techniques (*14*). One of the purified strains (Y32) was selected for further analysis of morphological, biological, and serological characteristics. This strain had been isolated from a pool of 21 male ticks collected at Canyon Creek Road, Douglas County, Oregon, on 4 March 1980 by R. A. Gresbrink. By dark-field microscopy, the organisms were predominantly nonhelical (Fig. 1A), although a few helical forms were observed. Helical forms exhibited typical motility (*1*), but nonhelical organisms displayed only flexional movement. When the organisms were examined by electron microscopy, a few helical cells, some of which were unusually tightly coiled (Fig. 2A) were observed. However, the majority of cells were nonhelical (Fig. 2B). Examination of thin-sectioned pellets of the organisms by electron microscopy showed that the cells were surrounded by a single unit membrane (Fig. 2C). No evidence of cell wall or periplasmic fibrils (axial filaments) was apparent, indicating the organisms were not spirochetes or any true bacterium.

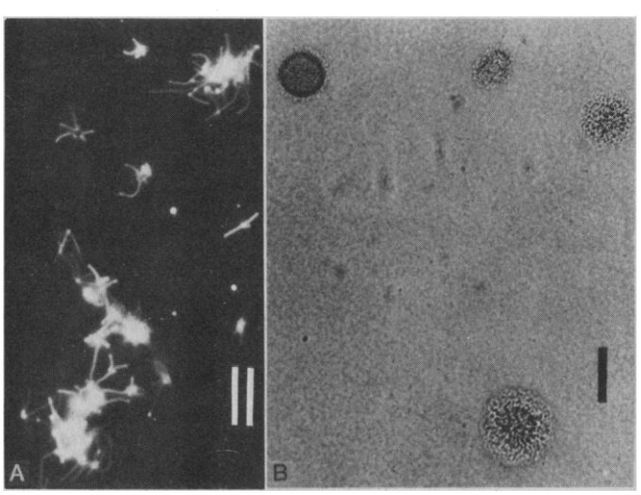

Fig. 1. Morphology of tick spiroplasmas cultured on artificial media. (A) Photograph of dark-field preparation of organisms in SP-4 broth. Scale bar, 1 μm. (B) Spiroplasma colonies on agar. Scale bar, 0.1 mm.

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The Y32 strain produced colonies on 0.8 percent Noble agar plates prepared from the SP-4 formula (Fig. 1B). Classical "fried egg" colonies were apparent after anaerobic incubation at 30°C for 10 to 14 days. None of the isolates grew in conventional mycoplasma broth media. The organisms passed readily through 450-nm membrane filters, generally with no decrease in titer. The organisms also passed through filters with average pore diameters of 300 and 220 nm, but the titer was reduced about 3 to 5 $\log_{10}$ by such filtration.

Serological analysis of the purified spiroplasma isolates was performed with techniques previously used to establish *Spiroplasma* species or serogroups (*15*). Metabolism-inhibition and growth-inhibition tests (*7*) showed that all seven isolates were serologically similar. These serological tests also established that the *Ixodes* isolates were clearly distinct from members of all other spiroplasma serogroups currently available for comparison (Table 1).

Although the *Ixodes* isolates grew in SP-4 medium, they required long incubation periods during primary isolation. Both helical and nonhelical forms were present during most stages of the growth cycle in liquid medium. The predominantly nonhelical structure of the organisms may reflect deficiencies in the SP-4 medium, rather than the development of stable nonhelical variants described in at least one *Spiroplasma citri* strain (*16*). Thus, the SP-4 formulation, which has provided substantial improvement in isolation of several spiroplasmas and mycoplasmas (*17*), probably requires further modification to become optimum for growth of the *Ixodes* spiroplasmas.

Lyme disease of man is a syndrome that usually begins in summer with a characteristic skin lesion, erythema chronicum migrans (*12*). This may be followed weeks to months later by neurologic or cardiac abnormalities, and acute and chronic joint involvement (*11*, *12*). The disease seems to occur in three distinct areas of the United States (*12*). Cases have been observed in the northeastern coastal area, Wisconsin, and in California and Oregon (*11*). The disease syndrome in the first two regions correlates with the distribution of *Ixodes dammini*, whereas cases in California and Oregon have occurred within the range of *Ixodes pacificus* (*11*). The latter species has been implicated in at least one case of Lyme disease in California (*18*). While no evidence is available to relate spiroplasmas to Lyme disease, the isolation of a new spiroplasma from *Ixodes pacificus* provides an opportunity to assess the vertebrate pathogenicity of spiroplasmas and the occurrence of specific antibody in the serum of man and animals. Discovery of the *Ixodes* spiroplasmas also intensifies possible interest in the existence of other similar wall-free prokaryotes in arthropods that attack man.

Table 1. Serological relationships of *Ixodes pacificus* (Y32 strain) spiroplasmas to other spiroplasmas. Antibodies to triply cloned organisms were produced in each of two rabbits as described (*15*). The metabolism-inhibition test with strain Y32 was performed essentially as described (*7*), except guinea pig complement was omitted from the system. Results are expressed as reciprocals of dilution end points. Homologous metabolism-inhibiting antibody titers with antiserum listed here varied from 1:13,000 to 1:1,000,000 [see (*7*)]. Growth-inhibition test results are expressed as zones of inhibition (in millimeters) around paper disks impregnated with undiluted antiserum. Homologous growth-inhibition zones varied from 6 to 18 mm. Serogroup designations are based on earlier data (*8*); N.D., not done.

Antiserum (strain designation)	Serogroup	Metabolism-inhibition with Y32 antigen	Growth-inhibition with Y32 antigen
Y32	VI	4374	16
Spiroplasma citri (Maroc)	I-1	<18	0
Honey bee (BC3)	I-2	<18	0
Corn stunt (E-275)	I-3	<18	0
277F (tick)	I-4	<18	0
Sex ratio (WSRO)	II	<18	N.D.
Flower (OBMG)	III	<18	0
Flower (PPS1)	IV	<18	0
SMCA (tick)	V	<18	0

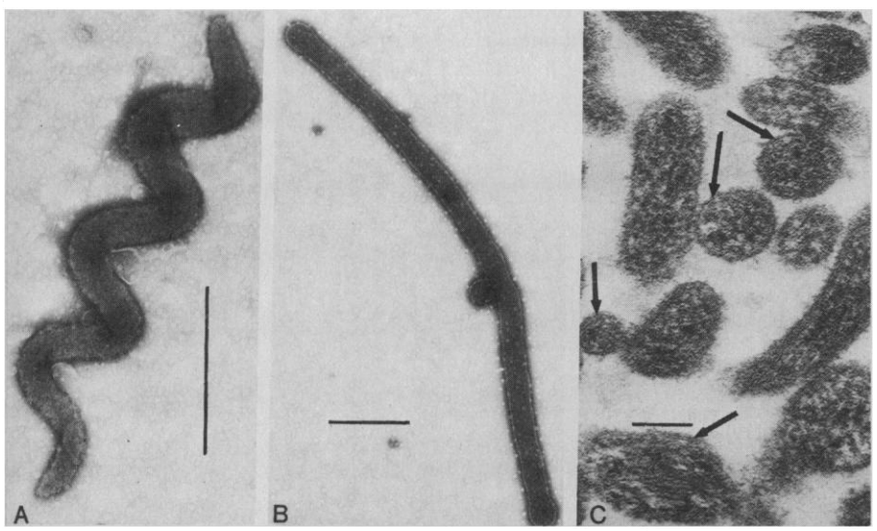

Fig. 2. Ultrastructural appearance of tick spiroplasma (strain Y32). (A) Electron micrograph of negatively stained helical filament. Scale bar, 0.5 μm. (B) Electron micrograph of negatively stained nonhelical filament. Scale bar, 1 μm. (C) Electron micrograph of thin-sectioned pellet of spiroplasmas, showing unit membrane (arrows). Scale bar, 0.1 μm. For electron microscopy details, see (*5*).

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14. Broth cultures of the organisms were filtered through a sterile membrane filter (220 nm pore diameter) and diluted filtrates were plated onto solid SP-4 agar medium, containing 0.8 percent Noble agar (Difco). The inoculated plates were incubated in a Gaspak anaerobic jar (BBL Microbiology Systems) at 30°C. Individual colonies were picked after 10 to 14 days, placed in SP-4 broth, and the tubes incubated for 10 to 14 days. This purification procedure was repeated twice and the triply cloned spiroplasma isolates in SP-4 broth were then lyophilized.
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Salt Glands in the Tongue of the Estuarine Crocodile *Crocodylus porosus*

Abstract. *The apparent absence of salt glands in marine and estuarine Crocodilia has long been a puzzle. However, we have identified glands in the tongue of* Crocodylus porosus *which exude a concentrated secretion of sodium chloride. The glands are similar in ultrastructure to other reptilian salt glands and undoubtedly play a major role in electrolyte regulation.*

In the estuarine crocodile, *Crocodylus porosus*, a clear fluid exudes from numerous pores scattered over the upper surface of the tongue (*1*). The secretion is stimulated by injection of methacholine chloride, is hyperosmotic to the body fluids, and contains high concentrations of both sodium and chloride, pointing to the existence of a major and hitherto unknown salt gland in the Crocodilia.

Whether or not salt glands are present in Crocodilia has long been debated. Their presence was suggested more than 20 years ago (*2*) and doubted 10 years ago (*3*); recent reviews (*4, 5*) suggest that the marine or estuarine *C. porosus* and *Crocodylus acutus* probably lack significant extrarenal salt glands. However, the mechanism by which hatchling *C. porosus* are able to survive and grow in seawater (*6*) needs explanation and a resolution of the salt gland question is crucial to any understanding of their salt and water economy.

The search for salt glands has concentrated on the head because all known reptilian salt glands are cephalic. Because "crocodile tears" have been on record for at least 800 years (*7*) and because of the lack of any other visible secretions, there has been inevitable speculation that there may be orbital salt glands such as those in marine turtles (*2, 8*). However, the results of the only reported investigation (*3*) of orbital secretions were equivocal. Tears collected from both *C. porosus* and *C. acutus* showed some increase in sodium concentration after the animals were exposed to seawater, but the highest observed concentrations were considerably lower than any reported for salt gland secretions from other marine reptiles. That study has been used, with some reservations, both to accept (*8*) and to reject (*3–5*) the hypothesis that crocodiles possess functional orbital salt glands.

After our initial observation of salty secretions from the tongue, physiological and ultrastructural studies showed unequivocally that the lingual glands of *C. porosus* are salt glands comparable in structure and function to those of other marine reptiles.

Observations were made on 29 crocodiles (0.08 to 4.7 kg). Nine had been maintained in the laboratory in Sydney for 6 months or more. Twenty were captured freshly from salinities of 20 to 36 parts per thousand in the Liverpool-Tomkinson river system in northern Australia and were studied at Maningrida, Northern Territory. Of the nine laboratory animals, five had been kept in seawater for approximately 3 months, three with and two without fresh water to drink. The remainder had been kept in fresh water.

To observe and collect lingual gland secretions, crocodiles were washed thoroughly and restrained with the mouth propped open. The oral cavity was washed with distilled water and blotted dry. A watch for spontaneous secretory activity was maintained for 15 to 60 minutes. Secretion was seen in animals exposed to both fresh water and salt water, but the rates were too low to allow collection. Attempts, in some animals, to induce secretion by oral or intraperitoneal administration of 1*M* NaCl, at 10 mmole/kg, were unsuccessful. Accordingly, each animal was injected intraperitoneally with methacholine chloride (1 to 2 mg/kg), which initiates salt gland secretion in other marine reptiles (*2*). Secretion began within 3 to 15 minutes. In the 9 laboratory animals and 12 of the field animals, secretions were collected into a syringe as they welled up continuously from pores on the tongue. Tears were collected from four of the laboratory animals and plasma from eight of them. Osmotic pressure was measured with a Knauer Semimicro osmometer, Na and K were determined by flame photometry, and Cl was measured by coulometric titration. In eight field animals, Na secretory rates were measured by collecting secretions into filter papers placed on the tongue sequentially for measured time intervals. Papers were then soaked in 5 ml of distilled water to extract Na for analysis.

Lingual gland secretions from both laboratory and field animals were consistently three to five times more concentrated than the plasma and consisted principally of Na and Cl (Table 1). Among the laboratory animals, the secretion concentrations were somewhat higher in the two animals denied access to fresh water than in the remaining seven animals, but the difference was not statistically significant, and therefore the data were pooled (Table 1). In the field animals, there was no significant correlation between concentration and body weight or salinity at the capture site, and therefore these data were pooled also. Electrolyte concentrations were considerably higher in secretions from field animals compared to laboratory animals, almost certainly reflecting a greater degree of adaptation to salt water in the former group. However, some of the difference could be attributed to the smaller size of the field animals, which led to lower absolute secretory rates and the likelihood of more substantial evapo-

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