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Distribution of the Genus *Leptospira* in Soil and Water

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The distribution of the aerobic spirochetes *Leptospira* in surface waters, soil, and aquatic animals was investigated. Isolates from water and soil exhibited physiological characteristics common to members of the "biflexa complex," none were capable of infecting experimental animals, and leptospires could not be isolated from the eight genera of aquatic animals examined. The isolation frequencies from surface waters were: stream, 100%; lake, 65%; spring, 28%; bog lake, 5%; and marsh, 0%. With the exception of the stream, more isolations were obtained from the soil adjacent to the water than from the water. Leptospires were most frequently associated with soils of high moisture and organic matter content.

The genus *Leptospira* is divided into two complexes, the parasitic complex and the biflexa complex, with *Leptospira interrogans* being the type species (29). Members of the parasitic complex are associated with infection and disease, their prime habitat being the convoluted tubules of the mammalian kidney (27, 29). The isolation of members of the parasitic complex from water (1, 4), and more recently soil (2), has been reported. Water and soil contaminated with the urine of leptospiruric animals is the source of parasitic leptospires, and the role of water as a vehicle of transmission for members of this complex is well known.

In contrast, the members of the biflexa complex are not known to cause a pathological state in mammals and have been isolated from waters of various types (28, 32). For this reason they have commonly been referred to as water or saprophytic leptospires. The existence of members of this complex in soil has been recently established by direct isolation (19) and by immunofluorescent staining methods (12).

Although reports on the isolation of leptospires of one or the other complex from water are numerous, little direct information is available on their actual distribution in various bodies of water and in the soil and on the relative density of these organisms in these habitats. Such information, gained by a study of the distribution of members of the biflexa complex, could be of value in elucidating the survival of the parasitic complex in nature outside the host and provide a greater understanding of the biology of the genus as a whole.

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MATERIALS AND METHODS

Study site. This study was conducted on the Cedar Creek Natural History Area (CCNHA), managed by the University of Minnesota and the Minnesota Academy of Sciences. Within the CCNHA, five bodies of water and the soil adjacent to three of these bodies of water were selected for sampling: a small lake, a marsh, a free-flowing stream, a bog lake, and a spring.

Collection and processing of samples. Water samples were collected and transported in sterile 200- to 500-ml glass bottles. Subsurface samples were obtained by the use of a Kemerrer water sampler (Foerst Mechanical Specialities Co., Chicago, Ill.) and transferred to sterile glass bottles immediately after collection.

Soil from an area of 15 to 20 cm by 4 to 8 cm was taken after all loose surface material had been removed. The soil was immediately placed in a plastic bag, which was then sealed.

All samples were protected against sudden temperature changes during transport to the laboratory and were processed within 12 h.

Temperature and pH were monitored throughout the study. Water and soil temperatures were taken in the field, and pH was determined upon return to the laboratory.

The moisture content of the soil samples was determined by placing a known quantity of the sample (5 to 10 g) in a dry, tared, aluminum dish, and then the sample was dried at 94 to 100°C until the weight remained constant.

Water samples (100 ml) were passed through a sterile 0.45-μm pore size membrane filter, and 1.0 ml was inoculated, in duplicate, into the culture medium.

Soil samples (100 g) were placed in a sterile 1-liter flask with 300 ml of leptospire-free distilled water and mixed by shaking. The suspension was allowed to settle for 5 to 7 min before 50 to 60 ml was centrifuged at 800 to 900 rpm in a clinical table-top centrifuge for 5 to 7 min. The supernatants were filtered using 0.45-μm membranes, and 1.0 ml was inoculated, in duplicate, into the culture medium.

Culturing and the determination of leptospires. The medium used for the isolation of leptospires was the Tween 80-albumin medium of Johnson and Harris (14). The medium was rendered semisolid by the addition of agar to a final concentration of 0.2%, and 5-fluorouracil (5-FU) was incorporated at a concentration of 100 μg/ml to help minimize contamination (17).

All enrichment cultures were incubated aerobically at room temperature for 30 days and examined for the presence of leptospires by dark-field microscopy. If leptospires were not detectable after 30 days of incubation, the sample was considered to be negative.

When necessary, leptospires were separated from contaminants by placing a drop of the mixed culture in the center of petri plates containing Tween 80-albumin medium and 1% agar. The leptospires have the ability to migrate through the agar, which is visualized as a veil of growth extending to the periphery of the plate. Plugs of medium from the edge of the veil were inoculated into fresh medium. Isolates of leptospires were examined for their ability to grow in the presence of 8-azaguanine (16), with myristic acid as sole carbon source (15), and at 13°C (14). These procedures have been shown to differentiate between members of the two complexes of the genus.

Aquatic animals for the isolation of leptospires. One hundred and thirty-nine individuals representing eight genera were collected from environments known to contain leptospires, and kidneys, urine, blood, and the turtle cloaca were examined for leptospires. Kidneys were aseptically removed and macerated by passage through a sterile syringe of appropriate size (22). Turtle cloaca samples were obtained with cotton swabs moistened with isolation medium after the anal region was washed sequentially with 1:750 zephiran chloride and 70% ethanol. All samples were placed in 5 ml of isolation medium, and 1:10, 1:100, and 1:1,000 dilutions were made in the medium.

Isolation by direct animal inoculation. Weaning 21-day-old Syrian hamsters (five animals per sample) were injected intraperitoneally with samples ranging from 1 to 2 ml for the water samples and 0.5 to 1.0 ml of the suspension of soil material, similar to the procedure of Baker and Baker (2). Animals were bled by cardiac puncture at 24, 72, and 120 postinoculation, and 0.05 to 0.1 ml of blood was cultured as described above. At 11 to 14 days postinoculation, one kidney per animal was aseptically removed, macerated, and cultured.

RESULTS

Effectiveness of 5-FU in isolation. The effectiveness of 5-FU in minimizing contamination and thereby enhancing the isolation of leptospires in our system was examined (Table 1). 5-FU had previously been shown to be effective in reducing contamination levels in urine cultures (17), but its effectiveness with water and soil material had not been tested. Five water samples and nine soil samples were collected at random, and the filtrates were inoculated into media with and without 100 μg of 5-FU per ml. In medium lacking 5-FU, leptospires were detectable in only two of five (40%) water samples and in one of the nine (11%) soil samples. The negative samples were overgrown with bacteria other than leptospires. Medium with 5-FU yielded isolates in all of the water samples (100%) and in seven of the nine (77%) soil samples.

TABLE 1. *Effect of 5-FU on isolation of leptospires from soil and water*

Medium	No. of positive samples/total samples	
	Water	Soil
Tween-80 albumin plus 5-FU[a]	5/5	7/9
Tween-80 albumin minus 5-FU	2/5	1/9

[a] 5-FU = 100 μg of 5-FU per ml.

Distribution of leptospires in water and soil: lake. The results in Table 2 are a compendium of the data obtained for four sampling stations on the lake consisting of surface and subsurface samples. A total of 126 samples were collected, and 83 (65%) were found to be positive. The heaviest cell density occurred in the lake during the warmer months of the year (June through September). In November an ice cover had formed on the lake, and of the 12 samples collected during the month only 1 was found to be positive. Samples taken in December through March were negative for leptospires, but in April one of eight samples was positive. By June, the cell density was such that all samples were positive, and this pattern held through September. One milliliter of the filtrate was the minimum volume that would result in a positive culture.

The pH of the lake water was within the range reported to be capable of supporting leptospiral viability (24). The temperature, on the other hand, for 6 months, averaged below the minimal growth-supporting temperature (10 to 13°C) reported for these organisms (14).

Lake shore soil. Soil samples were collected along a transect beginning 0.5 m from the water's edge and progressing up a slope for a distance of 10 m. All 15 samples taken within 5 m of the water's edge were positive. None of the soil samples taken beyond 5 m were positive (Table 2). This distribution may be correlated with the moisture content of the soil sample. All soil samples that were positive had a moisture content of 71% or greater. The soil appears to be a more suitable habitat for the leptospires than the water. In November 12 water samples were collected and only 1 was positive, whereas 3 of 3 soil samples were positive. During December none of the 10 water samples collected were

TABLE 2. *Summary of lake and lake shore soil isolations*

Parameter	No. of positive samples/total no. of samples	% Positive	Temp (°C)[a]	pH[a]	% Moisture (range)
Water: sampling period					
January–February	0/8	0	1.00	7.70	
March–April	1/12	8	5.90	7.56	
May–June	11/11	100	25.40	8.91	
July–August	32/32	100	22.14	8.45	
September–October	38/41	92	14.63	7.98	
November–December	1/22	4	3.19	7.72	
Total	83/126	65			
Lake shore soil: location[b]					
<5 m	15/15	100	0.5–18.5[c]		71.5–86.5
>5 m	0/5	0	0.5–17.5[c]		6.8–52.5
Total	15/20	75			

[a] Average values for sampling period.
[b] Distance from water's edge. Sampling period was August through December.
[c] Range.

positive for leptospires, whereas 3 of 4 soil samples were positive.

Stream. Thirty samples were obtained from a stream (creek) which drains the entire CCNHA. The samples were taken in moving water 1 m from the shore. All samples were positive with 1 ml of filtrate regardless of the season (Table 3). Five-tenth- and one-tenth-milliliter amounts of filtrate were also cultured for samples taken in March, June, July, and September. The 0.5-ml volumes were routinely positive, but only two samples, one each in March and June, were positive at 0.1 ml. Since these volumes were negative for samples taken from the lake during the same period, it would indicate that a higher cell density is reached in the stream than in the lake. The pH values for the stream ranged from 7.3 to 8.1, whereas the temperature ranged from 4.5 to 21.5°C.

Bog lake and adjacent soils. Thirty-five water samples were collected consisting of surface and subsurface samples, and only two were found to be positive. These two samples were taken in early June and September. Samples taken in other months were negative. During the sampling period the average pH ranged from 6.7 to 9.2, and the temperature ranged from 0.5 to 26.0°C (Table 3).

Soil samples were collected from three different regions along a transect leading to the bog lake. Basin 1 was the region closest to the lake and basin 2 was the region farthest from the lake. The soil of these regions is high in organic content, peat soils, and moisture content (R. A. Henry, Ph.D. thesis, University of Minnesota, Minneapolis, 1972). The third region, designated the rise, separated the two basins and had a sandy loam soil. Thirty-four samples were collected over the period April through November, and 15 (44%) were positive (Table 3). These 15 positive samples were from basins 1 and 2, which were peat soils with an average moisture content of 74%. Eleven of the 19 negative cultures were from samples of sandy loam soils. None of the eight samples taken in the rise region, which is low in organic matter and moisture content, were positive. It appears that the distribution of leptospires in the soil leading to the bog is related to both moisture and organic matter content.

Comparing the data on the overall isolation percentage for the bog (5%) and its adjacent soil (44%), it is apparent that a higher cell density occurred in the soils surrounding this body of water than in the bog itself.

Spring water and adjacent soil. Samples were collected from two locations at the spring: directly at its origin and at a point 10 m downstream. Of the 28 samples obtained and processed, 8 (28%) yielded leptospires. Only 3 of 17 samples obtained at the mouth of the spring were positive, whereas 5 of 11 of the samples taken downstream yielded leptospires (Table 4). Increased contact with the soil environment may be responsible for the increase in isolations downstream.

Thirty-seven soil samples were taken in the area of the spring and 22 (59%) were positive. Thirteen out of 15 (86%) of the samples taken within 5 m of the mouth of the spring and its stream were positive (Table 4). The percentage of positive cultures decreased with increasing

TABLE 3. *Summary of isolation data for the stream, bog lake, and bog soils*

Parameter	No. of positive samples/total no. of samples	% Positive	Temp (°C)[a]	pH[a]	% Moisture[a]
Stream: sampling period					
January–February	3/3	100	4.5	7.45	
March–April	5/5	100	5.5	7.47	
May–June	7/7	100	21.0	7.40	
July–August	7/7	100	20.5	8.05	
September–October	4/4	100	11.5	7.92	
November–December	4/4	100	4.5	7.60	
Total	30/30	100			
Bog water: sampling period					
February–November	2/35	5	0.5–26.0[b]	6.72–9.20[b]	
Bog soils: location[c]					
Basin 1	8/15	53	0.5–17.0[b]	6.6–6.8[b]	74.9
Rise	0/8	0	0.5–18.0[b]	6.3–6.6[b]	26.9
Basin 2	7/11	64	0.5–18.0[b]	6.4–6.7[b]	73.2
Total	15/34	44			

[a] Average values for sampling period.
[b] Range.
[c] Sampling period was April through November.

TABLE 4. *Summary of isolation data for the spring area*

Location	No. of positive samples/total no. of samples	% Positive	Temp (°C)[a]	pH[a]	% Moisture[a]
Water[b]					
Mouth	3/17	17	8.67	7.55	
Downstream	5/11	45	8.77	7.69	
Total	8/28	28			
Soil[c]					
0–5 m	13/15	86	13.5	7.27	81.67
10–15 m	8/13	61	13.7	6.13	79.88
15–25 m	1/4	25	12.5	5.45	61.30
>25 m	0/5	0	14.8	5.40	24.65
Total	22/37	59			

[a] Average values.
[b] Sampling period was February through December.
[c] Distance from the spring. Sampling period was April through December.

distance from the spring. Only 61% of the samples taken 10 to 15 m from the spring or its stream were positive, and only 25% were positive in the region of 15 to 25 m. Beyond 25 m no positive cultures were obtained. Changes in the moisture, organic content, and pH of the soil were noted with increasing distance from the spring. Peat soils (Henry, Ph.D. thesis) were encountered from 0 to 10 m from the spring, with a moisture content of 79 to 81%. A transition zone followed such that at a distance of 25 m the soil was loamy sand with a pH of 5.4 and a moisture content of 24%. A higher frequency of isolation from the soil (59%) than from the water (28%) occurred at the spring, which is similar to the observations made at the bog location.

Marsh. Adjacent to the lake was a marsh with a small body of open water. Thirty-six samples were taken during the period April through December, and no samples were found to be positive. The average pH values ranged from 5.9 to 6.3, and the temperature ranged from 1.0 to 24.5°C. The consistently low pH values for this body of water may be responsible for the apparent low cell density (24).

Influence of soil moisture. The possible influence of soil moisture content on the distri-

bution or survival of leptospires in soil may be seen in Table 5. Eighty-three percent of the samples with a moisture content of 65% or greater were positive, whereas in samples with a moisture content of less than 65%, only 13% were found to be positive.

Attempts at isolation of leptospires from aquatic animals. Members of the biflexa complex have been referred to as saprophytic or water leptospires, chiefly because they are readily isolated from water and are not known to be pathogenic for higher mammals (28, 32). Several reports have dealt with the isolation of this group from aquatic animals, suggesting a possible role other than saprophytic (R. D. Andrews, Ph.D. thesis, University of Illinois, Urbana, 1966; 5, 20). We attempted to determine the role of this group in the environment under study. The following aquatic animals (number of individuals, common name, genus, species) were taken from areas known to be positive for leptospires, and their kidneys and body fluids were cultured for the presence of leptospires: 44—frogs, *Rana pipiens, R. catesbiana;* 33—turtles, *Chrysemys picta, Chelydra sepentina*; 6—mud puppies, *Necturus maculosus*; 11—salamanders, *Ambystoma tigrinum, A. maculatum*; 45—fish, *Umbra limi, Eucalia inconstans, Ictalurus* sp. A total of 135 kidneys were cultured and all were negative for leptospires, as were 15 turtle cloaca samples. Attempts to infect frogs, salamanders, and mud puppies with water isolates from CCHNA all failed. The leptospires could not be isolated from the blood of the animals 72 h postinoculation or the kidneys 10 to 14 days postinoculation.

TABLE 5. *Comparison of soil isolations with percent moisture content of the samples*

% Moisture	No. of positive samples/total no. of samples	% Positive
>65	53/64	82
<65	3/23	13

Attempted isolation of leptospires by direct animal inoculation. We attempted to determine if virulent members of the parasitic complex were present in the area by direct animal inoculation. Twelve samples were tested from the spring water and spring soils, six from the stream, four from the lake, and one each from the bog and adjacent soils. All samples failed to yield leptospires by the direct animal inoculation procedure, and all but one sample, bog water, did yield leptospires by our direct culture method.

Characterization of isolates. Direct animal inoculation indicated the absence of virulent members of the parasitic complex, suggesting that our cultures were members of the biflexa complex or nonvirulent members of the parasitic complex. In vitro characterization of pure culture isolates was carried out as described by Johnson et al. (14–16). Two nonvirulent serovars of the parasitic complex, and two of the biflexa complex, were cultured simultaneously with randomly selected isolates. As has been reported (14–16), the serovars of the parasitic complex failed to proliferate under each of these conditions, whereas the members of the biflexa complex did (Table 6). Seven water and four soil isolates, representing each sampling area at CCNHA, possessed the characteristics associated with the biflexa complex.

DISCUSSION

We attempted in this study to examine the distribution of leptospires in water and soil, within a given area, by an enrichment culture method using a standard volume of sample material. It is clear from the results presented that

TABLE 6. *Characterization of field isolations*

Serovar, strain	Increase in no. of organisms/ml × 10^7					
	8-Azaguanine		Myristic acid		Temp	
	0 μg/ml	200 μg/ml	Control	3×10^{-4} M	26°C	13°C
Parasitic complex						
copenhageni M20	37	2	47	0	38	3
pomona Wickard	26	5	48	0	36	1
Biflexa complex						
patoc Patoc I	59	48	70	31	58	45
semaranga Veldrat S173	50	47	63	29	49	35
Isolates						
Water[a]	47	43	61	41	61	59
Soil[b]	38	38	67	34	65	52

[a] Representative of seven water isolates.
[b] Representative of four soil isolates.

the density of leptospires from one body of water to the other is quite variable. The frequency of isolation was highest for the stream (100%) followed by the lake (65%), the spring (28%), the bog lake (5%), and, finally, the marsh (0%).

Noticeable changes in the cell density of leptospires occurred in the lake with seasonal variation. The maximum positive isolation frequency occurred during the months of May through September. This peak period was followed by an interval, October through November, in which the number of isolations decreased, reaching zero for the months December through March. Similar seasonal variations have been recorded for the total bacterial flora (7, 21) and the periphytic bacterial population (10) of various lakes. It has been reported that the minimal growth-supporting temperature for leptospires is 10 to 13°C (14), and temperature changes within a lake have been correlated to variations in distribution (11), in numbers of specific organisms (33), and to biochemical activity (31). The decrease in the leptospiral density which occurred in the lake studied appears to be correlated to decreasing temperatures. For the months May through October, when the average temperatures ranged from 10 to 25°C, the isolation frequency was 100 to 92%. When the water temperature averaged below 10°C, November through April, only 1 sample out of 42 was positive for leptospires.

The results obtained for the stream are similar to those previously reported (6). Leptospires were isolated from this body of water no matter the season. For 7 months the temperature of the stream was below the minimal growth-supporting temperature (14), yet leptospires were consistently isolated. There may be two possible explanations for this, and they may be interrelated. The stream may have reached a higher cell density during the summer months as compared to the lake; thus, the decrease in temperature and its effect on the population would not be as evident as in the lake. It is also possible that the persistence of leptospires in the stream is correlated with the constant contact of the water with a reservoir of leptospires. Such a reservoir could be the soil.

A similar situation was observed at the spring. The isolation frequency for the spring was 28%, and the maximum temperature measured was 9.5°C. This fact, coupled with the minimal nutrient levels measured (Henry, Ph.D. thesis) and usually associated with such waters, would severely limit the population density achieved. A higher percentage of positive samples was achieved downstream (45%) than at the source itself (17%), which would suggest that increased contact with the soil, since other factors remained nearly identical, may be important in the population reached in the stream originating from the spring.

In this study 91 soil samples were examined for the presence of leptospires and 52 (57%) were positive. This is in contrast to a 42% isolation frequency for water samples taken during the same period and in the same areas. The percentage of positive soil samples was 31% higher than for water samples from the spring location and 39% higher for the soil adjacent to the bog lake. Such evidence indicates a high population of leptospires in the soil surrounding these bodies of water rather than in the body of water itself. This information coupled with two other reports of the isolation of leptospires from soil (2, 19) would indicate that the blanket term "water leptospires" may no longer be totally valid.

As noted, samples taken downstream at the spring had a higher isolation percentage than those taken at the source. With the high population density recorded in the immediately adjacent soil (86% of the soil samples taken within 5 m of the source or stream were positive), it seems clear that the interaction of the water and soil is responsible for the increase in the number of isolations observed downstream. It may be possible to extend this to the stream (creek) proper. It had the highest consistent population of leptospires, and it would be feasible to conclude that this is, in part, due to its constant interaction with the soil and that it serves as the drainage route for the entire area.

In November and December the population of leptospires in the lake had decreased to a point at which only 1 sample out of 22 was positive. For these same months, 100% of the soil samples taken at the shore location within 5 m were positive. Apparently the population change that occurred in the lake was not reflected in the soil of its shore or the change was delayed. The change in population density that occurred in the lake during the winter months may not be due solely to a retarded growth rate caused by low temperature, but also to a lack of interaction between the water and the soil of its shore line.

In the laboratory (23, 25) and recently under natural conditions (18), the amount of soil moisture has been shown to be a factor in the survival of leptospires of the parasitic complex outside the host. Fluctuations in the bacterial populations of soils have also been associated with variations in moisture and organic content (8, 9, 13). These two factors appear to play a role in the distribution of leptospires that we observed. The greatest number of positive soil samples occurred for those soils in which the moisture

content was 65% or greater, 83% compared to 13% for those with less than 65% moisture. The largest number of positive soil samples were obtained from peat soils high not only in moisture content but also in organic matter (Henry, Ph.D. thesis). Twenty-one out of 28 (75%) samples of peat soil in the spring area were positive, and 15 out of 26 (57%) in the bog area were positive. No samples were positive from soils strictly sandy loam in texture from these two areas. Supporting the fact that soil organic matter is important to the survival of leptospires is the report of an endemic focus for leptospirosis in Israel, which is characterized by soils high in organic matter (26).

To determine the nature of the leptospiral population at CCNHA, direct animal inoculations and in vitro characterization tests were conducted. Twenty-four samples representing each of the study locations were tested for members of the parasitic complex by direct animal inoculation, and all were negative. This indicated either the absence of members of the complex or that the organisms, if present, were avirulent. In vitro characterization tests on randomly selected isolates gave results consistent with members of the biflexa complex.

In contrast to various reports in the literature (5, 19, 30) dealing with the isolation of leptospires from aquatic animals, we were not able to isolate the organism from the kidneys of 135 animals representing eight genera, nor from 15 turtle cloaca samples. Sera collected from the turtles and frogs examined in this study were found to possess antibody-mediated antileptospiral activity against both complexes of the genus (3). Our results suggest that the members of the biflexa complex are primarily saprophytic, since none were found to parasitize lower forms.

This study has established that not all aquatic bodies of water are equally capable of supporting a leptospiral population, that consisderble variation may exist within a given area, and that the soil, particularly that high in organic matter, must be considered to be a prime habitat for members of the biflexa complex. Of importance is whether or not those habitats capable of maintaining a high population of free-living leptospires are also those in which members of the parasitic complex survive best outside the host and constitute a source of infection.

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