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Detection of *Leptospira* in Soil and Water by Immunofluorescence Staining

RONALD A. HENRY, RUSSELL C. JOHNSON, B. B. BOHLOOL, AND E. L. SCHMIDT
Department of Microbiology, University of Minnesota, Minneapolis, Minnesota 55455

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Specific immunofluorescence staining was used for the detection of leptospires in soil and water under laboratory and field conditions.

Reports documenting the isolation of leptospires from soil (1, 6) and studies on their survival in soil under laboratory conditions (8, 11) are few. Accordingly, the importance of soil as a reservoir or habitat for leptospires remains largely unknown.

In our studies on leptospires in nature, we have been able to isolate the organism from 73% of the soil samples taken (peat soils, *unpublished data*). The cultural method used for isolation of leptospires from soil is laborious and time-consuming and another method was sought to aid in their detection and to study their ecology.

Specific immunofluorescence staining coupled with the buried slide technique has been a recent advance in microbial ecology studies. This technique has proven valuable for the direct microscopic detection of *Aspergillus* (9), *Bacillus* (4), *Rhizobium* (3), and *Azotobacter* (12) in the soil and has in some instances pointed out interesting relationships between the organism and its immediate surroundings.

This note deals with the application of the contact (buried) slide technique coupled with specific immunofluorescence staining for the detection of leptospires in soil (i) under selected conditions in the laboratory to test its feasibility and (ii) in a pilot study under field (natural) conditions.

The organism used in the laboratory studies was serotype *patoc* Patoc I, a saprophyte, which was grown and maintained in the modified Tween 80-albumin medium (5).

For the production of immune sera, New Zealand rabbits were injected intraperitoneally with 25 ml of viable washed leptospires (2×10^8 to 6×10^8 leptospires per ml) twice at 10-day intervals. The rabbits were bled 10 days after the final injection, and the sera were harvested. The agglutinin titer of the antiserum was determined (>1:10,000) before fractionation [50% $(NH_4)_2SO_4$] and conjugation. The fractionated antiserum as a 1% protein solution in buffer was conjugated with fluorescein isothiocyanate (FITC) by method I of Lewis et al. (7). The cross-reactive pattern of the FITC antiserum for various leptospiral serotypes was determined. The FITC antiserum reacted with a plus two or greater fluorescence with eight serotypes of the biflexa complex and five serotypes of the parasitic complex. The total cross-reaction pattern of this FITC antiserum within the genus *Leptospira* has not been established.

Previously sterilized soil preparation (peats, approximately 84% organic matter and 70% moisture) were inoculated with a washed suspension of leptospires (4-day culture) to a concentration of approximately 10^7 cells per g of moist soil. Pairs of sterile microscope slides, back to back, were introduced into the soil preparations approximately 2.5 to 3.0 cm from the site of leptospire inoculation. The slides were removed every 7th day for a period of 21 days. The slides were heat-fixed; a gelatin-rhodamine counterstain (2) was applied to avoid nonspecific staining of the soil matrix, followed by the specific FITC antiserum. They were allowed to react for 40 min at room temperature and were then rinsed for 10 min in phosphate-buffered saline (*p*H 7.2). The equipment and film for photomicrographs have been described previously (10).

The leptospires on the soil slides that were incubated in the laboratory fluoresced a bright apple green and were clearly distinguishable. The dull orange-brown fluorescence imparted to the soil particles by the gelatin-rhodamine counterstain provided excellent contrast. As seen on the photomicrographs, the leptospires appear to be structurally intact with characteristic morphology (Fig. 1).

The organisms appeared on the slides from the inoculated soils both as well-separated individuals and as clusters near or on the soil particles. The latter situation appeared to be

common. An increase was observed in the density of leptospires on the slides as a function of increasing incubation time, but it is not known whether this resulted from growth in the soil or from migration to the slides from sites of inoculation. The recovery of viable leptospires from these soils after 21 days was not attempted.

Successful demonstration of immunofluorescence detection of leptospires in soil under laboratory conditions and the broad cross-

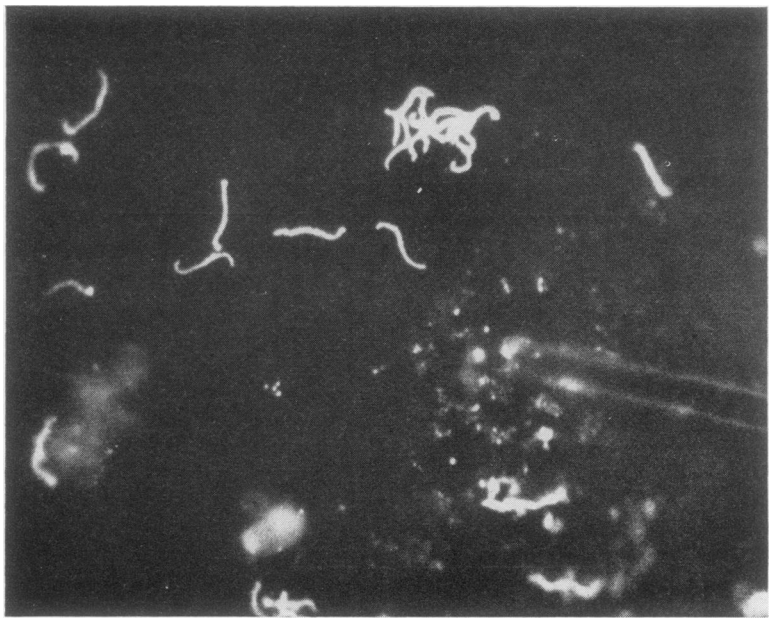

FIG. 1. *Leptospires found on slide buried in peat soil incubated in the laboratory (14 days, 97 × oil, reproduced from color slide).*

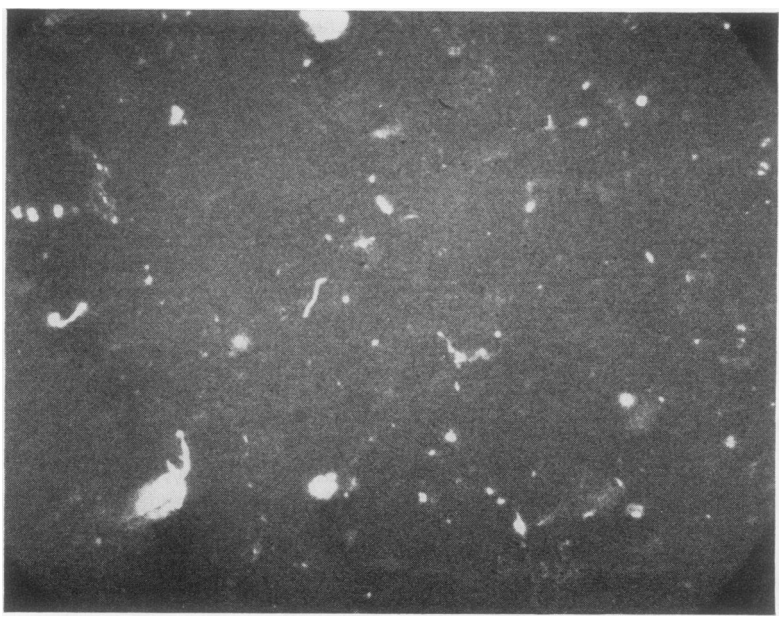

FIG. 2. *Specific fluorescing cell(s) found on slide buried in soil 3 m from lake water edge (28 days, 97 × oil, reproduced from color slide).*

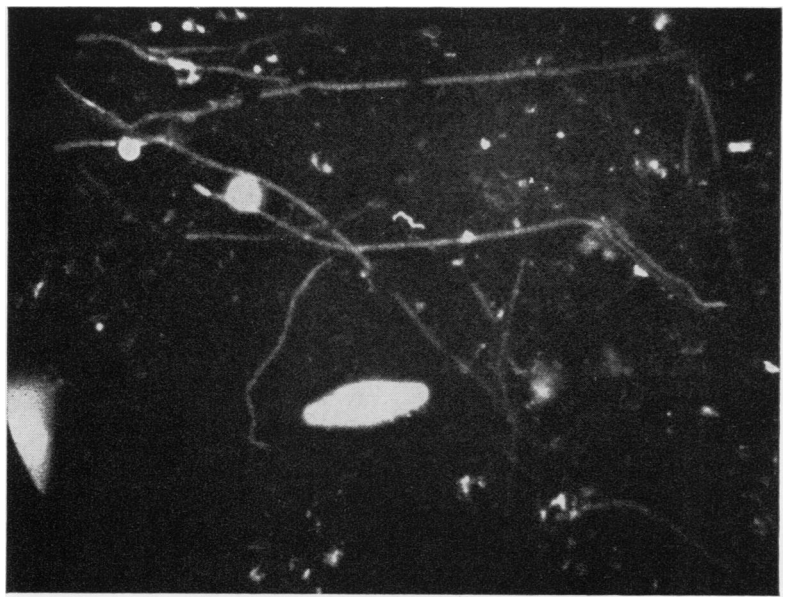

FIG. 3. *Specific fluorescing cell found on slide placed in stream formed by a free-flowing spring (14 days, 97 × oil, reproduced from color slide).*

reactivity pattern of the antiserum led to a pilot field study to test the feasibility of the approach in nature. Slides were buried (two back to back) at various points in both soil and water (University of Minnesota Cedar Creek Natural History Area) known to be positive for leptospires by cultural methods. The slides were removed after specified timed intervals and stained. One slide was used as an autofluorescent control.

Specifically fluorescing cells comparable in morphology and size to cultured leptospires were found scattered on slides that had been buried in soils or in bodies of water of various types. Individual cells associated with the soil particles seemed to predominate; few clusters of cells were seen on the slides examined to date (Fig. 2 and 3). On slides left in place for extended periods of time (28 to 30 days) there appeared, in addition to intact cells, evidence of cells in stages of decay or disruption. Such cellular debris was not observed on slides left in place for shorter time periods (4 to 14 days). In this study, approximately 5% of the fields (microscopic) examined contained specifically fluorescing cells.

The presence of leptospires in soil and water as detected by direct fluorescent-antibody microscopy correlated well in these preliminary studies with data obtained earlier from the same sites by cultural methods. Although the fluorescent-antibody method is not a highly quantitative one, it should permit the examination of various natural environments to study the biology of the leptospires. Investigations are in progress to evaluate the effectiveness and specificity of additional fluorescent-antibody preparations.

The application of this technique to such questions as the role of various soil factors such as moisture, organic content, and clay content in the survival of leptospires is anticipated. Such information may provide a clearer understanding of the role of the soil, or soil type, in the transmission of leptospirosis.

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LITERATURE CITED

1. Baker, M. F., and H. J. Baker. 1970. Pathogenic *Leptospira* in Malaysian surface waters. I. A method of survey for *Leptospira* in natural waters and soils. Amer. J. Trop. Med. Hyg. 19:485–492.
2. Bohlool, B. B., and E. L. Schmidt. 1968. Nonspecific staining: its control in immunofluorescence examination of soil. Science 162:1012–1014.
3. Bohlool, B. B., and E. L. Schmidt. 1970. Immunofluorescent detection of *Rhizobium japonicum* in soils. Soil Sci. 110: 229–236.
4. Hill, I. R., and T. R. G. Gray. 1967. Application of the fluorescent-antibody technique to an ecological study of bacteria in soil. J. Bacteriol. 93:1888–1896.
5. Johnson, R. C., and V. G. Harris. 1967. Differentiation of pathogenic and saprophytic leptospires. I. Growth at low temperatures. J. Bacteriol. 94:27–31.

6. Kingscote, B. F. 1970. Correlation of bedrock type with the geography of leptospirosis. Can. J. Comp. Med. 34:31–37.
7. Lewis, V. J., W. L. Jones, J. B. Brooks, and W. B. Cherry. 1964. Technical considerations in the preparation of fluorescent-antibody conjugates. Appl. Microbiol. 12:343–348.
8. Okazaki, W., and L. M. Ringen. 1957. Some effects of various environmental conditions on the survival of *Leptospira pomona*. Amer. J. Vet. Res. 18:219–223.
9. Schmidt, E. L., and R. O. Bankole. 1962. Detection of *Aspergillus flavus* in soil by immunofluorescent staining. Science 136:776–777.
10. Schmidt, E. L., R. O. Bankole, and B. B. Bohlool. 1968. Fluorescent-antibody approach to study of rhizobia in soil. J. Bacteriol. 95:1987–1992.
11. Smith, D. J. W., and H. R. M. Self. 1955. Observations on the survival of *Leptospira australis A* in soil and water. J. Hyg. 53:436–444.
12. Tchan, Y. T., and R. R. DeVille. 1970. L'application de l'immunofluorescence a l'etude des *Azotobacter* du sol. Ann. Inst. Pasteur. 118:665–673.