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NOTES

Isolation of *Legionella pneumophila* from Nonepidemic-Related Aquatic Habitats

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Continuous centrifugation of large volumes of water from natural southeastern lakes allowed quantitative detection of *Legionella pneumophila* by direct immunofluorescent staining. Positive samples were injected intraperitoneally into guinea pigs, and the *L. pneumophila* were isolated and identified by their morphological, cultural, physiological, and serological characteristics.

The etiological agent of Legionnaires disease has been studied primarily either in the tissues of infected lower animals and humans or in pure cultures obtained from clinical specimens (2, 3, 7, 9). Isolates from air-conditioning cooling towers have been associated with four different outbreaks (1). In an outbreak in Bloomington, Ind., several isolates were obtained from the water or soil of a nearby stream (G. K. Morris, C. M. Patton, J. C. Feeley, S. E. Johnson, G. Gorman, W. T. Martin, P. Skaliy, G. F. Mallison, B. D. Politi, and D. C. Mackel, Ann. Intern. Med., in press). One of these, Bloomington 2, belongs to a new serogroup of *Legionella pneumophila* designated serogroup 3. We are not aware of published reports of the isolation of *L. pneumophila* from natural habitats that were not associated with epidemics. This note describes a technique for detecting the various serogroups of *L. pneumophila* and for isolating the bacterium from aquatic habitats.

Samples (20 liters) were collected from various aquatic habitats and simultaneously measured for temperature, pH, dissolved oxygen, and conductivity. Each sample was concentrated by continuous centrifugation (Sorvall SS-3) at 15,000 rpm at room temperature. Samples were resuspended to a final volume of 40 ml in filter-sterilized water from the appropriate habitat. Subsamples (0.010 ml) were pipetted into predesignated 6-mm-diameter wells on toxoplasmosis slides (Cel-Line Associates, Minotola, N.J.). Slides were air dried, heat fixed, and stained with specific fluorescent antibodies for four serogroups of *L. pneumophila*: 1 (Knoxville 1), 2 (Togus 1), 3 (Bloomington 2), and 4 (Los Angeles 1) (R. M. McKinney, L. Thacker, P. P. Harris, K. R. Lewallen, G. A. Hebert, P. H. Edelstein, and B. M. Thomason, Ann. Intern. Med., in press). Positive and negative controls were included. Negative controls were conjugated sera prepared from the preimmunization

TABLE 1. *Habitat characteristics for isolates of L. pneumophila from nonepidemic sources*[a]

Isolate and sample no.	Depth (m)	Temp (°C)	Conductivity (μmho/cm)	pH	Dissolved O_2 (mg/liter)	No. of cells staining/liter in serogroup:			Serogroup of guinea pig isolates
						Knoxville 1	Togus 1	Control	
SRP-2 (85-13)	2	17.2	62	6.4	5.85	9.6×10^6	BD[b]	Neg.[c]	1
SRP-3 (64-14)	7	28.0	57	6.7	3.80	2.7×10^5	1.8×10^4	Neg.	1
SRP-4 (85-1)	Surf	18.4	24	6.8	9.80	3.0×10^5	BD	Neg.	1
SRP-5 (86-1)	3	17.0	52	6.6	6.90	5.0×10^6	BD	Neg.	1

[a] All specimens were screened with the working dilutions of conjugates for *L. pneumophila* of serogroups 3 (Bloomington 2) and 4 (Los Angeles 1), but none were detected.

[b] BD, Below detectability of 9 *L. pneumophila*/ml.

[c] Neg., Negative.

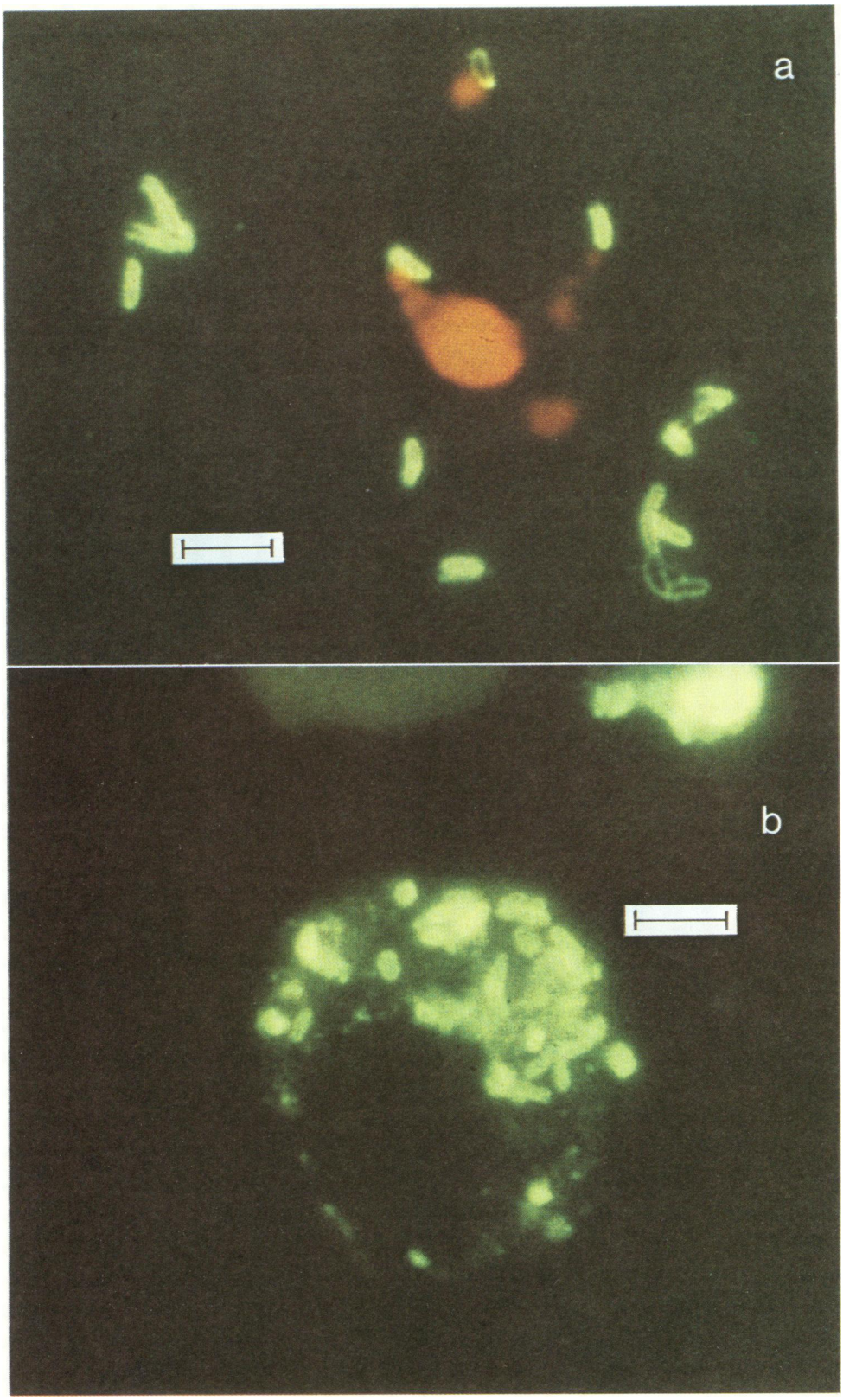

FIG. 1. *(a) Photomicrograph of L. pneumophila from a concentrated water sample, 85-1, stained with the serogroup 1 (Knoxville 1) conjugate. Green fluorescence is L. pneumophila staining 4+; red-orange fluorescence is chlorophyll-containing algae. Bar, 2 μm. (b) Photomicrograph of L. pneumophila in a peritoneal smear from a guinea pig injected with water sample 85-1 stained with serogroup 1 (Knoxville 1) conjugate.*

sera of rabbits later immunized with the Knoxville serogroup of *L. pneumophila*. Samples were viewed by epifluorescence microscopy as previously described (6). The number of fluorescing cells with morphological characteristics of *L. pneumophila* (3, 7) was counted in 100 fields at ×1,600 magnification, and the number of staining *L. pneumophila* per liter of unconcentrated sample was determined. Photomicrographs were taken with GAF film ASA 200 with 30-s exposure at ×1,600 magnification.

Those samples with bacteria, morphologically similar to *L. pneumophila*, which reacted with any of the four serogroup conjugates were considered positive for the presence of *L. pneumophila*. Uncompromised guinea pigs (Abyssinia or Hartley breed, 2 to 3 months old) whose base line body temperatures had been established were injected intraperitoneally with 3.0 ml of the concentrated sample and were observed for increases in temperature and for illness as demonstrated by lethargy, ruffled fur, watery eyes, etc. Animals whose temperatures increased as much as 0.6°C above base line for 2 consecutive days or those which had a temperature rise with any of the above symptoms during the 10 days after injection were sacrificed and necropsied. Peritoneal swabs and liver and spleen smears were stained with the four serogroup conjugates. Positive direct staining of bacteria which morphologically resembled *L. pneumophila* was considered presumptive evidence of *L. pneumophila*.

The peritoneal swabs and organ tissues were cultured on charcoal-yeast extract agar as described by Feeley et al. (4, 5). Cultures were incubated at 35°C in candle extinction jars. Typical *L. pneumophila* colonies were transferred to slants of charcoal-yeast extract agar and repurified through two additional successive platings. The cultures contained slender, gram-negative rods with the morphological and tinctorial properties of *L. pneumophila*. They grew only on supplemented Mueller-Hinton agar or on Feeley-Gorman and charcoal-yeast extract agar containing cysteine and iron (11). The cultures uniformly produced a brown soluble pigment, liquefied gelatin, and were catalase positive. The Kovacs oxidase test performed on filter paper impregnated with the tetramethyl *p*-phenylenediamine dihydrochloride reagent and a loopful of cells from a young culture was negative (no color change within 10 s). Some clinical isolates of *L. pneumophila* were also negative when tested under the same conditions. The *L. pneumophila* cultures utilized starch slowly but did not reduce nitrates (11).

Using the penicillin medium described by Martin and Lewis (8), we tested the typical *L. pneumophila* isolates for production of β-lactamase as reported by Weaver (11). All isolates inactivated the penicillin and allowed *Sarcina lutea* to grow. The qualitative and quantitative gas-liquid chromatograms of the cellular fatty acids of the isolates were consistent with those reported earlier (10). After these tests and positive direct fluorescent-antibody staining, the isolate was considered a confirmed *L. pneumophila*. Four isolates have been obtained and are deposited with the Center for Disease Control in Atlanta, Ga.

Presently, we have screened over 200 different water samples from 23 different lakes in Georgia and South Carolina. Serospecific fluorescent antibodies were used to detect all four known serogroups of *L. pneumophila*. Over 90% of the samples contained fluorescing cells of *L. pneumophila* morphology (unpublished data). The summary of data for the four habitats confirmed to contain *L. pneumophila* is shown in Table 1; these lakes are typical of the small soft-water ponds in eastern South Carolina. The remaining habitats are still being tested.

Organisms staining with the serogroup 1 conjugate were present in pond waters at concentrations ranging from 2.7×10^5 to 9.6×10^6 per liter and were isolated from guinea pigs inoculated with these samples (Table 1). Although only serogroup 1 strains have been isolated, staining *L. pneumophila* of serogroup 2 (Togus 1) were also observed by fluorescent-antibody staining at the level of 1.8×10^4 per liter in water from one of the ponds (Table 1).

Figure 1a is a photomicrograph of *L. pneumophila* in a concentrated water sample stained with fluorescent antibodies for serogroup 1 (Knoxville 1). Figure 1b shows *L. pneumophila* in a peritoneal smear from a guinea pig infected by an injection of the same water sample. No gross difference in morphology was observed.

To our knowledge, these are the first isolates that have been obtained from lakes and the first isolates of *L. pneumophila* reported from habitats that have not been associated with outbreaks of Legionnaires disease. The described techniques or simple modifications thereof may allow the detection and isolation of *L. pneumophila* from a wide range of habitats. Such techniques can be used to identify habitats where *L. pneumophila* are present and facilitate the understanding of the ecology of the bacterium.

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