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Hippurate Hydrolysis by *Legionella pneumophila*

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Strains of *Legionella pneumophila* were shown to hydrolyze sodium hippurate in an overnight test system, but strains of *L. bozemanii*, *L. micdadei*, *L. dumoffii*, and some other organisms similar to the legionellae did not. Although only a small number of strains of legionellae other than *L. pneumophila* have been classified and tested, the results indicate that the hippurate hydrolysis test may prove useful for differentiating among *Legionella* species.

The phenotypic characteristics of the four named species of *Legionella* (2, 3, 8) are very similar. They are all gram-negative bacilli that are not acid fast with the Ziehl-Neelsen stain for mycobacteria; a polar flagellum has been seen on most strains of each species. The legionellae do not ferment carbohydrates, reduce nitrate to nitrite, or produce urease, but they do liquify gelatin, utilize starch, and produce catalase. Three phenotypic characteristics are useful for differentiating the species: (i) the ability of the organisms to grow on supplemented Mueller-Hinton agar and Feeley-Gorman agar, (ii) the fluorescence of growth on charcoal yeast extract agar when exposed to long-wavelength ultraviolet excitation, and (iii) the production of a beta-lactamase. Before an isolate can be identified to the species level, however, specific direct fluorescent antibody-staining reactions and cellular fatty acid composition must be determined. Additional simple and rapid tests are needed to identify these organisms.

Bacterial hydrolysis of sodium hippurate to benzoic acid and glycine was observed as early as 1864 by Van Tieghem (12). Since then the reaction has been observed with many bacilli and cocci. In 1922 Ayers and Rupp (1) used hippurate hydrolysis to separate hemolytic streptococci of human and bovine origin; they incorporated sodium hippurate in a broth medium, incubated the culture for 7 days, and then tested for benzoic acid. A rapid hippurate hydrolysis test was described in 1975 by Hwang and Ederer (9) to differentiate group B from other beta-hemolytic streptococci. A heavy cell suspension of growth from agar plates was prepared in an aqueous solution of sodium hippurate and incubated for 2 h at 37°C. Hydrolysis was determined by adding ninhydrin reagent to detect glycine. Recently, Harvey (7) reported using the rapid procedure to differentiate between *Campylobacter fetus* subsp. *jejuni* and *C. fetus* subsp. *intestinalis*. The procedure used in this study of legionellae required overnight incubation in aqueous sodium hippurate; glycine was then detected in 10 min with a ninhydrin reagent.

A 1% solution of sodium hippurate was prepared in sterile distilled water, dispensed in 0.4-ml amounts into screw-capped tubes (13 by 100 mm), and frozen at −20°C until used. A 3.5% solution of ninhydrin was prepared in a 1:1 mixture of acetone-butanol and stored in the dark at room temperature. In testing the legionellae cultures, a loopful of 24- to 96-h growth from charcoal-yeast extract agar was emulsified in a thawed tube of 1% sodium hippurate. The screw caps were tightened, and the dense suspensions were placed in an incubator at 35°C. After 18 to 20 h of incubation, 0.2 ml of the ninhydrin solution was added to each tube. The screw caps were tightened, the contents were mixed well by

TABLE 1. *Hippurate reactions of Legionella species and some Legionella-like organisms*

Culture tested	No. of strains	Hippurate hydrolysis	
		No. positive	No. negative
L. pneumophila	102	100	2
serogroup 1	61	61	0
serogroup 2	8	8	0
serogroup 3	5	5	0
serogroup 4	10	8	2[a]
serogroup 5	5	5	0
serogroup 6	13	13	0
L. bozemanii	3	0	3
L. micdadei	5	0	5
L. dumoffii	2	0	2
Legionella-like organisms[b]	2	0	2

[a] Strains Los Angeles 1 and San Francisco 6.
[b] Environmental isolates LS 13 (5) and BL 540 (4).

TABLE 2. *Differential phenotypic characteristics for Legionella species*

Species	Fluorescence on CYE agar[a]	Growth on F-G and MH-IH agars[b]	Production of beta-lactamase[c]	Hippurate hydrolysis[d]
L. pneumophila	Dull yellow	Primary	+	+
L. micdadei	Dull yellow	Adapted	−	−
L. bozemanii	Blue-white	Adapted	+	−
L. dumoffii	Blue-white	Adapted	+	−

[a] Under long-wavelength ultraviolet excitation. CYE, Charcoal-yeast extract.
[b] F-G, Feeley-Gorman agar; MH-IH, supplemented Mueller-Hinton agar; primary, supports growth for primary isolation; adapted, supports growth only after several successive transfers on CYE agar.
[c] Chromogenic cephalosporin test of 24 hour growth from CYE agar.
[d] During 18 to 20 h of incubation in 1% aqueous sodium hippurate.

shaking, and the tubes were returned to the incubator for 10 min. The tubes were then removed and observed for color development within 20 min; all shades of purple were read as a positive reaction, a very light purple was designated as weakly positive, and shades of gray or a very light yellow were reported as negative for hippurate hydrolysis. Controls included positive and negative legionellae from the previous day, a hippurate-positive *C. fetus* subsp. *jejuni*, a hippurate-negative *C. fetus* subsp. *intestinalis*, an uninoculated tube of sodium hippurate, an uninoculated tube of water, and suspensions of some of the test strains of legionellae prepared in 0.4 ml of sterile distilled water instead of sodium hippurate.

Strains of *Legionella pneumophila*, *L. bozemanii*, *L. micdadei*, *L. dumoffii*, and *Legionella*-like organisms have been examined by this test for hippurate hydrolysis (Table 1). Of the 102 *L. pneumophila* strains tested, 100 strains, or 98%, were positive. These hippurate-positive strains represented all six of the defined serogroups of *L. pneumophila* (6, 10, 11). Both of the hippurate-negative strains were in serogroup 4 and were isolated in California—one (Los Angeles 1) from lung tissue from a hospitalized pneumonia patient and the other (San Francisco 6) from water from an air conditioning cooling tower. All known strains of *L. bozemanii*, *L. micdadei*, and *L. dumoffii* and the *Legionella*-like strains LS 13 (5) and BL 540 (4) were negative for hippurate hydrolysis by this procedure.

A few strains of each species were tested for hippurate hydrolysis after 2, 3, 4, 5, 6, and 18 h of incubation in the sodium hippurate solution. After 18 h of incubation in hippurate, the *L. pneumophila* strains developed purple reactions during 10 min of incubation with the ninhydrin reagent. The same strains tested after 2, 3, 4, 5, and 6 h of incubation in hippurate were negative. All tests of the other *Legionella* species were negative. Numerous strains of *L. pneumophila* were tested without substrate (hippurate) to determine whether the positive ninhydrin reaction might be the result of leakage of intracellular amino acids or cellular autolysis. One loopful of growth from charcoal-yeast extract agar was emulsified in 0.4 ml of 1% sodium hippurate; a second loopful of growth from the same agar slant or plate was emulsified in 0.4 ml of sterile distilled water. Both tubes were incubated for 18 to 20 h at 35°C and then tested with the ninhydrin reagent. All strains incubated with hippurate gave a distinct positive reaction, but those incubated in water without hippurate were completely negative. Uninoculated hippurate, uninoculated water, and strains of the other *Legionella* species incubated with and without hippurate were negative.

With the test conditions and procedures described, essentially all of the *L. pneumophila* demonstrated an ability to hydrolyze hippurate, but the other species of *Legionella* did not. Although only a small number of strains of legionellae other than *L. pneumophila* have been classified and tested, the results indicate that the hippurate hydrolysis test may prove to be a useful additional criterion (Table 2) in differentiating among the species.

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