Journal of Clinical Microbiology, Dec. 1990, p. 2828–2829
0095-1137/90/122828-02$02.00/0
Copyright © 1990, American Society for Microbiology
Vol. 28, No. 12

Detection of Hippurate Hydrolysis by *Legionella* spp. by Using a Rapid High-Performance Liquid Chromatography Method

D. MARMET,* N. BORNSTEIN, and J. FLEURETTE

Laboratoire National de la Santé, Centre National de Référence des Légionelloses, Faculté de Médecine Alexis Carrel, Rue Guillaume Paradin, 69372 Lyon Cédex 08, France

Received 4 June 1990/Accepted 20 September 1990

A rapid high-performance liquid chromatography method was developed to determine hippurate hydrolysis by *Legionella* spp. Benzoic acid, an end product of enzymatic activity, was directly detected by high-performance liquid chromatography after 1 and 24 h of incubation in 1% sodium hippurate. Because of its sensitivity, this procedure offers more precise identification of some *Legionella* spp.

A hippurate hydrolase that hydrolyzes hippuric acid to benzoic acid and glycine has been shown to be one of the rare biochemical characteristics that differentiate the various legionellae (3). The conventional method of Hwang and Ederer detects glycine by a colorimetric reaction with ninhydrin (4). For legionellae, reading is done after 24 h of incubation and is not easy to interpret for strains with weak enzymatic activity (3). Ishizaki et al. have developed a high-performance liquid chromatography (HPLC) method to determine the levels of sodium benzoate in plasma and its urinary nitrogen excretion in the form of hippurate (5). We applied the technique to determination of hippurate hydrolysis in legionellae. Benzoic acid was detected directly with neither addition of chromogenic reagents (2) nor chemical derivatization before analysis (6).

Legionella colonies were emulsified in 0.4 ml of a 1% solution of sodium hippurate. Dense suspensions (no. 8 McFarland turbidity standard) were agitated at 37°C for 1 and 24 h. After addition of 0.4 ml of acetonitrile and centrifugation, the supernatant was analyzed by HPLC on a C18 column (Merck AG, Darmstadt, Federal Republic of Germany) and eluted at a flow rate of 1 ml/min with acetonitrile-water-acetic acid (250:730:20) at pH 2.6. Detection was effected by means of a UV spectrophotometer at a wavelength of 235 nm.

Benzoic acid was quantified by measuring the peak areas. The results were expressed as a percentage ratio of the benzoic acid peak area of the sample minus the benzoic acid peak area of the negative control in the hippuric acid peak area.

The controls included a hippurate hydrolase-positive strain of *Streptococcus agalactiae*, a hippurate hydrolase-negative strain of *S. pyogenes*, a *Legionella pneumophila* 1 suspension in sterile distilled water, and an uninoculated sodium hippurate solution for each series of tests; inclusion of the uninoculated control allowed us to measure the trace amounts of benzoic acid present in the initial hippurate solution and to obtain a negative control.

HPLC analysis of five different samples of an *L. pneumophila* 1 culture demonstrated good reproducibility of the technique (1.46, 1.37, 1.44, 1.60, and 1.70). One of the five samples that were injected five times yielded identical results (1.37, 1.38, 1.36, 1.36, and 1.38).

The HPLC detection limit for benzoic acid was 0.5 μg/ml (4 nmol/ml). Sensitivity was evaluated by using a standard scale prepared from dilutions ranging from 1/10 to 1/20,000 of a 1% benzoic acid solution in a 50:50 mixture of water and acetonitrile.

Of 84 legionella strains tested, 61 were reference strains; 22 of them belonged to different *L. pneumophila* serogroups, and 39 belonged to other previously described legionella species and serogroups. The strains of five new, unclassified species were also tested: "*L. geestiae*," "*L. londoniensis*" 1 and 2, "*L. nautarum*," "*L. quateriensis*," and "*L. worsliensis*." Furthermore, 23 legionellae isolated from the environment and from patients were also selected: 2 *L. pneumophila* serogroup 4 strains to confirm the heterogeneity of the hippuric acid activity of this serogroup; 3 *L. pneumophila* strains with antigenic cross-reactions in serogroups 4 and 5; 2 *L. pneumophila* strains with antigenic cross-reactions in serogroups 4, 5, 8, and 10; 1 *L. feeleii* serogroup 1 strain; and 15 strains for which interpretation posed problems when ninhydrin colorimetry was used (*L. pneumophila* serogroup 6 [1 strain], *L. longbeachae* serogroup 1 [1 strain], *L. londoniensis* [3 strains], *L. bozemanii* serogroup 1 [1 strain], *L. dumoffii* [1 strain], *L. oakridgensis* [3 strains], and *Legionella* sp. [5 strains]).

Each strain was tested after 1 and 24 h of incubation by the conventional ninhydrin method. The results were expressed as the means obtained for each strain after three or four tests with 1 h of incubation and two tests with 24 h of incubation.

The results of legionella hippurate hydrolysis tests are shown in Table 1.

With the rapid 1-h incubation method, 11 of 14 *L. pneumophila* subgroups and the Lansing 3 strain were positive; 2 serogroups (2 and 7) gave weakly positive reactions. Only a few serogroup 4 strains failed to hydrolyze hippurate, as observed in 1981 by Hebert (3). The heterogeneity of serogroup 4 is consistent with the classification of Brenner et al. (1). The Portland 1 strain, which hydrolyzed hippurate, belongs to subgroup *L. pneumophila* subsp. *pneumophila*, whose type strain is Philadelphia, while the hippurate-negative Los Angeles 1 and San Francisco 3 strains belong to subgroup *L. pneumophila* subsp. *fraseri*. All of the other legionella species were negative after 1 h of incubation, except *L. israelensis* and *L. spiritensis*, which were weakly positive. As a whole, these results confirm those described in the literature, which were based on detection of glycine by ninhydrin following 24 h of incubation. Only *L. israelensis* and Lansing 3 have been described as negative, and conversely, the *L. feeleii* strains tested were completely negative, although they are described as giving variable hippurate reactions. The sensitivity of the method enables discrimina-

* Corresponding author.

TABLE 1. Hippurate hydrolysis by legionellae

Species, serogroups, and strains	HPLC results with incubation for: 1 h		HPLC results with incubation for: 24 h		Ninhydrin test result
	Benzoic acid concn	Interpretation	Benzoic acid concn	Interpretation	
L. pneumophila					
Serogroups 1, 3, 5, 6, and 8–14; strain Lansing 3; serogroups 4 and 5 or 4, 5, 8, and 10	1.1–2	+	13–27	+	+
Serogroups 2 and 7	0.5, 0.6	+	6, 6.9	+	+
Serogroup 4 strains					
Portland 1	1.3	+	8	+	+
810207 LNS	1.7	+	20	+	+
Nancy II no. 2 LNS	1.1	+	18	+	+
Los Angeles 2	0.02	−	0.2	W[a]	−
San Francisco 3	0.02	−	0.3	W	−
83 20 164 LNS	0.02	−	0.2	W	−
Other *Legionella* spp.					
L. anisa, *L. birminghamensis*, *L. bozemanii* 1 and 2, *L. brunensis*, *L. cherrii*, *L. dumoffii*, *L. feeleii* 1 and 2, "*L. geestiae*," *L. gormanii*, *L. gratiana*, *L. hackeliae* 1 and 2, *L. jamestowniensis*, *L. jordanis*, "*L. londoniensis*," *L. longbeachae* 1 and 2, *L. maceachernii*, *L. micdadei*, "*L. nautarum*," *L. oakridgensis*, *L. parisiensis*, *L. rubrilucens*, *L. sainthelensi*, *L. santicrucis*, *L. steigerwaltii*, *L. tucsonensis*, *L. wadsworthii*, "*L. worsliensis*," *Legionella* spp.	0.01–0.04	−	0.01–0.04	−	−
L. cincinnatiensis, *L. moravica*, *L. quinlivanii*, "*L. quateriensis*"	0.01–0.03	−	0.08–0.12	W	−
L. israelensis, *L. spiritensis*	0.09–0.3	W	1.9–2.7	+	−
Controls					
S. agalactiae	27	+	96	+	+
S. pyogenes	0.02	−	0.04	−	−
L. pneumophila 1 in sterile distilled water	0.00	−	0.02	−	−
Unseeded sodium hippurate	0.01–0.04	−	0.01–0.04	−	−

[a] W, Weak.

tion of *L. pneumophila* serogroups 2 and 7, which have weak enzymatic activity.

When incubation was extended to 24 h, the *L. pneumophila* serogroup 4 strains that were negative demonstrated weak hippurate hydrolysis activity. Moreover, this method allowed us to discriminate four other legionella species which were weakly positive, i.e., *L. cincinnatiensis*, *L. moravica*, *L. quinlivanii*, and "*L. quateriensis*."

In conclusion, HPLC determination of hippurate hydrolysis is a reproducible and rapid method that requires no chemical derivatization; it is specific, since the benzoic acid detected is an end product of hydrolysis (6). Because of its sensitivity, the technique is particularly useful for organisms with weak enzymatic activity. The rapid 1-h incubation test confirms previous results by revealing slightly differing enzymatic activities within the different *L. pneumophila* serogroups. Incubation for 24 h provides more precise identification of some of the other species.

LITERATURE CITED

1. **Brenner, D. J., A. G. Steigerwalt, P. Epple, W. F. Bibb, R. M. McKinney, R. W. Starnes, J. M. Colville, R. K. Selander, P. H. Edelstein, and C. W. Moss.** 1988. *Legionella pneumophila* serogroup Lansing 3 isolated from a patient with fatal pneumonia, and descriptions of *L. pneumophila* subsp. *pneumophila* subsp. nov., *L. pneumophila* subsp. *fraseri* subsp. nov., and *L. pneumophila* subsp. *pascullei* subsp. nov. J. Clin. Microbiol. **26:**1695–1703.
2. **Edberg, S. C., and S. Samuels.** 1976. Rapid, colorimetric test for the determination of hippurate hydrolysis by group B *Streptococcus*. J. Clin. Microbiol. **3:**49–50.
3. **Hebert, G. A.** 1981. Hippurate hydrolysis by *Legionella pneumophila*. J. Clin. Microbiol. **13:**240–242.
4. **Hwang, M. N., and G. M. Ederer.** 1975. Rapid hippurate hydrolysis method for presumptive identification of group B streptococci. J. Clin. Microbiol. **1:**114–115.
5. **Kubota, K., Y. Horai, K. Kushida, and T. Ishizaki.** 1988. Determination of benzoic acid and hippuric acid in human plasma and urine by high performance liquid chromatography. J. Chromatogr. Biomed. Appl. **425:**67–75.
6. **Wallace, P. L., C. M. Patton, and C. W. Moss.** 1987. Determination of hippurate hydrolysis by gas-liquid chromatography. J. Clin. Microbiol. **25:**1766–1768.