JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1990, p. 616–618
0095-1137/90/030616-03$02.00/0

Vol. 28, No. 3

Increased Recovery of *Legionella micdadei* and *Legionella bozemanii* on Buffered Charcoal Yeast Extract Agar Supplemented with Albumin

WILLIAM E. MORRILL,* JAMES M. BARBAREE, BARRY S. FIELDS, GARY N. SANDEN, AND WILLIAM T. MARTIN

Respiratory Diseases Epidemic Investigations Laboratory, Division of Bacterial Diseases, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia 30333

Received 22 September 1989/Accepted 6 December 1989

The recovery of *Legionella micdadei* and *L. bozemanii* serogroups 1 and 2 from infected guinea pig spleens was evaluated by using two culture media: buffered charcoal yeast extract agar with 0.1% α-ketoglutarate (BCYEα) and the same medium supplemented with 1% bovine serum albumin (ABCYEα). At the lowest dilution of spleen tissue (10^{-1}), recovery of all strains of *L. micdadei* and *L. bozemanii* was more efficient on ABCYEα than on BCYEα. *L. micdadei* strains had higher recovery rates on ABCYEα after another 10-fold dilution, but recoveries of *L. bozemanii* were similar on both media. Recovery rates for most test strains were comparable on BCYEα and ABCYEα at the highest dilution (10^{-3}) of tissue tested. The presence of albumin in BCYEα increased the recovery rate of *L. micdadei* more than that of *L. bozemanii*. The use of ABCYEα medium in place of BCYEα may improve the recovery of *L. micdadei* and *L. bozemanii* from clinical specimens. Preliminary studies indicate that this medium also enhances recovery of certain *Legionella* spp. from environmental samples.

Although *Legionella micdadei* (TATLOCK) and *L. bozemanii* (WIGA) were first cultured from clinical specimens via embryonated eggs in 1943 and 1959, respectively, they were not successfully grown on an artificial medium until 1980 (6, 9, 11). This medium, charcoal yeast extract agar, has since been modified to buffered charcoal yeast extract agar with 0.1% α-ketoglutarate (BCYEα) and is routinely used for isolating *Legionella* spp. from clinical specimens (3, 4). Despite the improvements in culture media, the recovery of *Legionella* spp. from clinical specimens can be inhibited by the antimicrobial properties of tissue samples, as well as by the antibacterial properties of host defense mechanisms (7). Dilution of tissue inoculum is a proven method for overcoming the effects of tissue toxicity (4, 7), and a 10^{-1} or 10^{-2} dilution is recommended (14). However, this procedure is not practical for samples having $<10^3$ organisms per g of tissue. The incorporation of a neutralizing agent in BCYEα, such as bovine serum albumin, may eliminate the need for dilution of tissue inocula.

Bovine serum albumin has been used successfully as a medium detoxifier and as a substitute for charcoal in yeast extract broth (8). Albumin has also been used to block the toxic effects of starch by-products on *Legionella* spp. and to enhance the recovery of *L. micdadei* from infected guinea pig tissues (1, 5, 10). We compared BCYEα and BCYEα with 1.0% albumin (ABCYEα) for their ability to support the growth of several strains of *L. micdadei* and *L. bozemanii* serogroups 1 and 2.

BCYEα made from dehydrated base (BBL Microbiology Systems, Cockeysville, Md.) was used as the basal medium, and 1% (wt/vol) bovine serum albumin fraction V (ICN Immunobiologicals, Lisle, Ill.) was added to produce ABCYEα medium. Compared to scratch medium, medium made from BBL base has consistently given recovery rates of 95% or greater for *L. pneumophila* (unpublished data). Albumin in deionized water was filter sterilized and aseptically added to the autoclaved medium after the medium was cooled.

Ten strains of *L. micdadei*, four strains of *L. bozemanii*, and one strain of *L. pneumophila* were included in the study (Table 1). Clinical isolates were from human lung tissue (2, 13). After primary isolation, most strains had been suspended in defibrinated sheep blood and stored at −70°C. Strain Pi-12e was isolated in embryonated eggs and stored as an egg yolk sac suspension, while Pi-12s was from stored guinea pig spleen. WIGA and TATLOCK were medium-adapted strains that had been passaged several times before storage.

Thawed suspensions were cultured on BCYEα and incubated for 72 h at 35°C in a humidified 2.5% CO_2 atmosphere. Growth was harvested by using a sterile loop and suspended in sterile, dechlorinated tap water (12). Optical density was measured at 540 nm with a Beckman model 24 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) and adjusted to 0.9. Suspensions were serially diluted to give inocula ranging from 1.3×10^4 CFU/ml (Pi-12 blood) to 8.6×10^6 CFU/ml (WA-3). One milliliter of each suspension was used as an intraperitoneal inoculum for each guinea pig. The suspensions used as inocula were assayed on triplicate plates of BCYEα and ABCYEα and CFU per milliliter were counted. The medium giving best recovery for each strain was used to calculate the actual concentrations inoculated into guinea pigs.

The temperatures of adult male Hartley strain guinea pigs weighing 300 to 600 g were monitored before inoculation and daily for 72 h postinoculation. One guinea pig was used for each strain. Fever was defined as a 1°C rise in temperature.

After 72 h, guinea pig spleens were obtained by necropsy and ground with alumndum and sterile tap water to form a 20% (wt/vol) suspension of tissue. These were plated directly and diluted 10- and 100-fold and cultured in triplicate on BCYEα and ABCYEα.

* Corresponding author.

TABLE 1. Mean CFU of *Legionella* spp. recovered from guinea pig spleens[a]

Species and strain[b]	CFU/ml from infected spleen tissue at dilution:					
	10^{-1}		10^{-2}		10^{-3}	
	BCYEα	ABCYEα	BCYEα	ABCYEα	BCYEα	ABCYEα
L. micdadei						
Pi-12e (E)	0	20	0	26	0	2
Pi-12b (E)	0	>300	0	>300	0	>300
Pi-12s (E)	0	>300	0	>300	0	157
D-1855 (E)	6	294	25	51	6	3
WA891-8 (E)	0	>300	1	41	2	3
D-1768 (C)	0	100	11	162	6	12
WA-1 (C)	50	>300	91	224	16	28
WA-2 (C)	0	20	0	18	1	8
WA-3 (C)	87	200	19	56	5	6
TATLOCK (C)	225	319	13	29	0	0
L. bozemanii						
D-1044 (C)	160	>300	53	50	31	14
WIGA (C)	245	281	35	37	5	5
C-3500 (C)	3	23	9	7	1	2
Toronto 3 (C)[c]	4	187	6	5	4	2
L. pneumophila Phil. 1 (C)	>300	257	51	54	7	7

[a] Based on an average of results from triplicate plates of each medium.
[b] E, Environmental (water) isolate; C, clinical (human lung) isolate.
[c] Toronto 3 is the type strain for *L. bozemanii* serogroup 2.

Unlike BCYEα, ABCYEα grew all *L. micdadei* strains, and recovery was higher on ABCYEα at all dilutions of infected spleen tissue. At the lowest dilution, the recovery rates of the four strains that grew on BCYEα were improved as much as 50-fold on ABCYEα. At the 10^{-2} dilution, recovery rates of the six strains that grew on BCYEα were increased 2- to 40-fold more on ABCYEα (Table 1).

The recovery of *L. bozemanii* serogroup 1 strains was not affected to the same degree as that of *L. micdadei*, although at the 10^{-1} dilution, the recovery rate of the serogroup 2 strain (Toronto 3) increased almost 50-fold on ABCYEα (Table 1).

The use of ABCYEα medium did not enhance the recovery of *L. pneumophila* from guinea pig spleen at any of the dilutions tested.

Albumin-supplemented BCYEα agar enhanced the recovery of both *L. micdadei* and *L. bozemanii* from a low dilution (10^{-1}) of spleen tissue. Recovery rates for ABCYEα and BCYEα were comparable for all *L. bozemanii* test strains at the 10^{-2} dilution, but the *L. micdadei* strains required another 10-fold dilution before recovery was comparable. These findings showed that albumin enhanced the recovery of all *L. micdadei* strains to a greater extent than *L. bozemanii*, and they suggested that *L. micdadei* was more sensitive to tissue toxicity. This phenomenon was most evident with the Pi-12 (environmental) strains. However, the environmental strains D-1855 and WA891-8 were not as sensitive to tissue debris and were recovered on BCYEα.

The favorable effect of tissue dilution on the recovery of *Legionella* spp. has been previously reported by Lattimer et al. (7). They theorized that the growth inhibition produced by undiluted tissue was attributable to either antimicrobial compounds in the specimen or to the natural host defense mechanisms of tissue. Dilution effectively reduces the antibacterial properties of tissue but also decreases the concentration of bacteria present in the specimen. Our results showed that albumin in the growth medium was able to ameliorate the toxic effects of tissue without adversely affecting the recovery of *Legionella* spp. Multiple dilutions were necessary for the recovery of strains on BCYEα but not on ABCYEα.

Preliminary studies have shown that ABCYEα medium was also more useful than BCYEα for the recovery of an environmental strain of *L. anisa*. The recovery of this isolate from water was enhanced 60% by culturing on ABCYEα rather than on BCYEα. The possibility that some *Legionella* spp. may derive nutritional value from albumin cannot be excluded. Further testing may reveal additional *Legionella* spp. whose recovery is improved on ABCYEα medium.

LITERATURE CITED

1. **Bortner, C. A., R. D. Miller, and R. R. Arnold.** 1983. Effects of α-amylase on in vitro growth of *Legionella pneumophila*. Infect. Immun. **41**:44–49.
2. **Brenner, D. J., A. G. Steigerwalt, G. W. Gorman, H. W. Wilkinson, W. F. Bibb, M. Hackel, R. L. Tyndall, J. Campbell, J. C. Feeley, W. L. Thacker, P. Skaliy, W. T. Martin, B. J. Brake, B. S. Fields, H. V. McEachern, and L. K. Corcoran.** 1985. Ten new species of *Legionella*. Int. J. Syst. Bacteriol. **35**:50–59.
3. **Edelstein, P. H.** 1981. Improved semiselective medium for isolation of *Legionella pneumophila* from contaminated clinical and environmental specimens. J. Clin. Microbiol. **14**:298–303.
4. **Feeley, J. C., R. J. Gibson, G. W. Gorman, N. C. Langford, J. K. Rasheed, D. C. Mackel, and W. B. Baine.** 1979. Charcoal-yeast extract agar: primary isolation medium for *Legionella pneumophila*. J. Clin. Microbiol. **10**:437–441.
5. **Fields, B. S., J. M. Barbaree, E. B. Shotts, Jr., J. C. Feeley, W. E. Morrill, G. N. Sanden, and M. J. Dykstra.** 1986. Comparison of guinea pig and protozoan models for determining virulence of *Legionella* species. Infect. Immun. **53**:553–559.
6. **Hebert, G. A., C. W. Moss, L. K. McDougal, F. M. Bozeman, R. M. McKinney, and D. J. Brenner.** 1980. Rickettsia-like organisms TATLOCK (1943) and HEBA (1959): bacteria phenotypically similar to but genetically distinct from *Legionella pneumophila* and the WIGA bacterium. Ann. Intern. Med. **92**:45–52.
7. **Lattimer, G. L., L. V. Rhodes III, J. F. Salventi, and B. R. Cepil.**

1980. Isolation of *Legionella pneumophila* from clinical specimens: salutary effects of lung tissue dilution. Am. Rev. Respir. Dis. **122**:101–105.

8. **Muller, D., M. L. Edwards, and D. W. Smith.** 1983. Changes in iron and transferrin levels and body temperature in experimental airborne legionellosis. J. Infect. Dis. **147**:302–307.
9. **Myerowitz, R. L., A. W. Pasculle, J. N. Dowling, G. J. Pazin, M. Puerzer, R. B. Yee, C. R. Rinaldo, Jr., and T. R. Hakala.** 1979. Opportunistic lung infection due to "Pittsburgh pneumonia agent." N. Engl. J. Med. **301**:953–958.
10. **Pasculle, A. W., J. N. Dowling, F. N. Frola, D. A. McDevitt, and M. A. Levi.** 1985. Antimicrobial therapy of experimental *Legionella micdadei* pneumonia in guinea pigs. Antimicrob. Agents Chemother. **28**:730–734.
11. **Pasculle, A. W., J. C. Feeley, R. J. Gibson, L. G. Cordes, R. L. Myerowitz, C. M. Patton, G. W. Gorman, C. L. Carmack, J. W. Ezzell, and J. N. Dowling.** 1980. Pittsburgh pneumonia agent: direct isolation from human lung tissue. J. Infect. Dis. **141**: 727–732.
12. **Skaliy, P., and H. V. McEachern.** 1979. Survival of the Legionnaire's disease bacterium in water. Ann. Intern. Med. **90**: 662–663.
13. **Tang, P. W., S. Toma, C. W. Moss, A. G. Steigerwalt, T. G. Cooligan, and D. J. Brenner.** 1984. *Legionella bozemanii* serogroup 2: a new etiological agent. J. Clin. Microbiol. **19**:30–33.
14. **Wilkinson, H. W.** 1987. Hospital-laboratory diagnosis of *Legionella* infections, p. 5–7. Centers for Disease Control, Atlanta.