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Glycine-Containing Selective Medium for Isolation of *Legionellaceae* from Environmental Specimens

ROBERT M. WADOWSKY AND ROBERT B. YEE*

Department of Microbiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

Received 4 May 1981/Accepted 15 July 1981

Glycine, at a final concentration of 0.3%, has been shown to be an excellent selective agent for the isolation of *Legionellaceae*. Stock cultures of *Legionella pneumophila* were not inhibited on buffered charcoal-yeast extract agar containing the amino acid. Among the other *Legionellaceae* tested, only one of two strains of *L. dumoffii* and two of six strains of *L. micdadei* were appreciably inhibited. This medium permitted the isolation of *L. pneumophila* from environmental specimens with marked inhibition of many non-*Legionellaceae* bacteria. The selectivity of the medium was subsequently improved by the incorporation of vancomycin (5 μg/ml) and polymyxin B (100 U/ml). This selective medium, glycine-vancomycin-polymyxin B agar, should facilitate the recovery of *Legionellaceae* from environmental sources.

Legionella pneumophila and other members of *Legionellaceae* are present in water (6, 7, 10, 17), water-related (2, 9, 16), and soil (12, 14) environments. Although a simple, excellent medium, buffered charcoal-yeast extract (BCYE) agar, is available for the cultivation of these bacteria (13), their recovery from environmental samples remains difficult. *L. pneumophila* is often present in small numbers in environmental samples harboring high counts of other bacteria, most of which are gram negative. Thus, the legionellae may be masked by these bacteria when direct culturing of specimens is attempted. This problem is compounded by the frequent need to concentrate aquatic specimens by centrifugation to detect *Legionella* spp. (6, 7). The current recommended procedure for isolation entails intraperitoneal inoculation of the concentrate into guinea pigs (11). Although successful, this method is time-consuming and expensive.

Our interest in studying the presence of *Legionellaceae* in the environment led us to consider a selective medium to overcome these problems. Glycine was chosen as a selective agent since this amino acid is effective in tellurite-glycine agar as an inhibitor of gram-negative bacteria (20) and because *L. pneumophila* can grow in a chemically defined medium containing 0.135% glycine (18). This paper reports on the ability of BCYE agar containing glycine, vancomycin, and polymyxin B to detect *Legionellaceae* in environmental specimens by markedly inhibiting other environmental bacteria.

MATERIALS AND METHODS

Bacteria. Centers for Disease Control (CDC)-derived strains and clinical isolates of *Legionellaceae* were obtained from A. W. Pasculle of Presbyterian-University Hospital, Pittsburgh, Pa. (Table 1). The environmental strain of *L. pneumophila* serogroup 1, laboratory designation E-1, was isolated by us from the prefilter of a water deionization system. The stock culture of this isolate had undergone three transfers on BCYE medium. All stock cultures were maintained at −68°C in a yeast extract broth containing 15% (vol/vol) glycerol. The broth was similar to that described by Ristroph et al. (15) except that it also contained 1% (wt/vol) *N*-2-acetamido-2-aminoethanesulfonic acid (ACES; Research Organics, Inc., Cleveland, Ohio) as a buffer.

Medium. BCYE medium was prepared as described by Pasculle et al. (13) except that instead of adjusting the pH to 6.9 with 1 N KOH after the addition and melting of the agar, the pH was adjusted to 6.97 to 6.99 before the addition of the agar. The final pH of the medium after autoclaving and cooling to room temperature was between 6.85 and 6.95. Glycine (Calbiochem Corp., La Jolla, Calif.), when used, was added to BCYE medium before sterilization. Vancomycin-polymyxin B agar was similar to the semiselective medium formulated by Edelstein and Finegold for clinical specimens (4) except that ACES was added as a buffer, and higher concentrations of polymyxin B (Pfizer Inc., New York, N.Y.) and vancomycin (Eli Lilly & Co., Indianapolis, Ind.) were used, 100 U/ml and 5 μg/ml, respectively. Brown (personal communication) had found that higher concentrations of these antibiotics improved the semiselective ability of the medium for environmental samples. The experiments described below led to the formulation of a selective medium which consisted of BCYE agar con-

TABLE 1. *Legionella strains and isolates and their laboratory designations*

Bacterium	No. of strains	Laboratory designation
CDC derived		
L. pneumophila[a]	1	Philadelphia 1
L. bozemanii[b]	1	MI-15, WIGA
L. dumoffii	2	TEX-KL, NY-23
L. gormanii	1	LS-13
Clinical isolate		
L. pneumophila[a]	2	JC-1, K
L. pneumophila[c]	2	LR-1, NT
L. micdadei[b]	6	EK, ML, LR, GL, KR, JC
Environmental isolate (*L. pneumophila*[a])	1	E-1

[a] Serogroup 1.

[b] Garrity et al. (8) have recently proposed new genus designations, *Tatlockia* for *L. micdadei* and *Fluoribacter* for *L. bozemanii*. Also, Pasculle et al. (13) have proposed *L. pittsburgensis* as the species designation for *L. micdadei*.

[c] Serogroup 6.

taining 0.3% (wt/vol) glycine and the above concentrations of vancomycin and polymyxin B. The medium has been designated glycine-vancomycin-polymyxin B (GVP) agar.

Evaluation of glycine-BCYE media. The stock cultures of *Legionellaceae*, maintained at −68°C, were revived for the evaluation by subculturing onto BCYE plates which were then incubated in air at 37°C for 72 h. Isolated colonies of each culture were inoculated into 2 ml of ACES-buffered yeast extract broth which was also incubated in air at 37°C. Broth cultures of the legionellae, which had been incubated for 48 h, were diluted in sterile distilled water, if necessary, to provide suspensions containing approximately 10^5 colony-forming units per ml. Ten microliters of each suspension was spotted onto BCYE medium containing 0.25, 0.3, 0.4, 0.5, and 0.75% glycine. Prior testing had shown that the dilutions used were the highest ones which would result in confluent growth of the inoculum on the glycine-free control plate. The degree of inhibition on the glycine-containing media was estimated by comparison with growth on the glycine-free medium.

Identification of environmental *Legionella* isolates. Colonies suspected of being *Legionella* spp. were identified by morphological, cultural, and immunofluorescent properties as previously described (1, 8, 19). The reagents for identification by the direct immunofluorescence test were supplied by the Biological Products Division of the Bureau of Laboratories at the CDC. In addition, the identification of strain E-1 as *L. pneumophila* was confirmed by deoxyribonucleic acid hybridization and gas-liquid chromatography by George Garrity and Arnold Brown of the Oakland Veterans Administration Hospital, Pittsburgh, Pa.

RESULTS

Initially, we tested the effects of 0.25 to 0.75% glycine in BCYE agar on the growth of stock cultures of *Legionellaceae*. Glycine did not inhibit the CDC-derived strain, the clinical isolates, or the environmental strain of *L. pneumophila*. Strain NY-23 of *L. dumoffii*, isolates JC and KR of *L. micdadei*, and strain LS-13 of *L. gormanii* were the most sensitive legionellae to glycine (Table 2). Strain NY-23 and isolate JC were partially inhibited even at the lowest concentration tested, 0.25%, and were completely inhibited at the highest concentration, 0.75%. The other legionellae were not inhibited by 0.25 to 0.5% glycine. These results indicated that glycine might be an effective selective agent for *Legionellaceae*.

We therefore tested the selective ability of glycine-containing media in isolating *Legionellaceae* from two environmental sources, a prefilter from a water deionization system in a clinical microbiology laboratory and a showerhead from the Oakland Veterans Administration Hospital in our health center. We had previously isolated from a swab specimen of the prefilter one colony of *L. pneumophila* serogroup 1 on BCYE agar with no glycine supplementation. At that time, a careful search under a stereoscopic microscope was required to detect this colony among a heavy growth of non-*Legionellaceae* bacteria. Three subsequent attempts were unsuccessful because of confluent growth of these non-*Legionellaceae* bacteria. The showerhead had previously been reported to contain *L. pneu-*

TABLE 2. *Effect of glycine on the growth of L. bozemanii, L. dumoffii, L. gormanii, and L. micdadei on ACES-BCYE agar*

Strain of *Legionellaceae*	% Inhibition[a] with following concn (%) of glycine in BCYE agar:				
	0.25	0.30	0.40	0.50	0.75
L. bozemanii MI 15	0	0	0	0	25
L. dumoffii					
TEX-KL	0	0	0	0	25
NY-23	25	25	25	25	100
L. gormanii LS-13	0	0	25	75	100
L. micdadei					
EK	0	0	0	0	0
ML	0	0	0	0	25
GL	0	0	0	0	50
LR	0	0	0	0	50
KR	0	25	25	25	25
JC	50	50	50	50	100

[a] Compared with amount of growth obtained on medium without added glycine.

mophila serogroup 1 (3). Sediments from these sources were removed with cotton swabs and inoculated directly on BCYE agar that had been supplemented with 0 to 0.75% glycine. Glycine sufficiently inhibited the non-*Legionellaceae* bacteria and, as a result, greatly facilitated the isolation of *L. pneumophila* serogroup 1 from these specimens (Table 3). The bacterium was not isolated from the showerhead specimen on medium containing 0.75% glycine. This result may reflect a sampling error or that the legionellae were sensitive to this concentration of glycine. The medium which contained 0.3% glycine seemed to be suitable for the selective isolation of *Legionellaceae*. A lower concentration of glycine, 0.25%, might not provide adequate inhibition of the non-*Legionellaceae* bacteria, and a higher concentration might be inhibitory for *L. gormanii* (Table 2).

Vancomycin and polymyxin were tested for their ability to enhance the selectivity of BCYE agar containing 0.3% glycine. First, the effect of this medium (GVP agar) on the growth of our stock strains and isolates was determined. With the exception of strain LS-13 of *L. gormanii*, the results were similar to those obtained with the glycine-containing agar. This strain was completely inhibited by GVP agar. The WIGA strain of *L. bozemanii*, which had not been tested on glycine-containing agar, was also included in this experiment. No inhibition was observed. The selected ability of GVP agar was then compared with that of the medium containing 0.3% glycine only and the medium containing vancomycin and polymyxin B only. The above-mentioned prefilter was used for this comparison. *L. pneumophila* serogroups 1 and 6 were isolated on GVP agar with complete inhibition of the non-*Legionellaceae* bacteria (Table 4). The legionellae were also isolated on glycine-containing and vancomycin-polymyxin B-containing BCYE agar. However, the former failed to suppress the growth of a spreading *Bacillus* sp. which was subsequently shown to inhibit *L. pneumophila*. The latter medium permitted moderate growth of other gram-negative bacteria. Although we have not recovered other *Legionellaceae* from environmental samples, we have isolated *L. micdadei* on GVP agar from a clinical specimen. This finding suggests that the medium may be effective in recovering this species from a natural source.

TABLE 3. *Use of various concentrations of glycine as a selective inhibitor in ACES-BCYE agar for the isolation of L. pneumophila*

Source and bacterium isolated	Colony-forming units with glycine at concn (%) of:					
	0	0.25	0.30	0.40	0.50	0.75
Prefilter[a]						
L. pneumophila	0	4	15	8	2	18
Non-*Legionellaceae*	>300	136	60	23	2	0
Showerhead[b]						
L. pneumophila	1	ND[c]	9	4	ND	0
Non-*Legionellaceae*	>300		158	27		3

[a] Prefilter of water deionization system.
[b] Showerhead at Oakland Veterans Administration Hospital, Pittsburgh, Pa.
[c] ND, Not done.

TABLE 4. *Comparison of selective ability of glycine-, vancomycin-polymyxin B-, and GVP-containing media in recovery of L. pneumophila from a prefilter*[a]

Dilution and bacterium isolated	Colony-forming units with following addition to BCYE[b]:			
	None	Glycine	Vancomycin-polymyxin B	GVP
Undiluted				
L. pneumophila	0, 0, 0[c]	0, 0, 0	1, 1, 9	14, 16, 18
Non-*Legionellaceae*	TNTC[d]	21, 28, 36	TNTC	0, 0, 0
10^{-1} Dilution				
L. pneumophila	1, 1, 0	1, 2, 6	1, 2, 5	1, 2, 3
Non-*Legionellaceae*	TNTC	45, 1, 2	TNTC	0, 0, 0

[a] Sediment on a prefilter of a water deionization system was removed with five cotton swabs. The swabs were agitated in 4 ml of sterile distilled water. A 0.1-ml amount of this suspension, undiluted and diluted, was plated out by the spread plate technique.
[b] ACES buffered.
[c] Three plates.
[d] TNTC, Too numerous to count, >300 colony-forming units on each of three plates.

Recently, we used GVP agar to carry out a relatively rapid culture survey of sink faucet aerators and drinking water fountains in a nonhospital environment. As a result we were able to show that some of the aerators and faucets and a drinking fountain were contaminated with *L. pneumophila*. The survey also showed that some environmental sources contained non-*Legionellaceae* bacteria which were resistant to the selective agents in GVP agar. The culturing of specimens from 2 of 16 sources sampled yielded moderate to heavy growth of these microorganisms, i.e., 200 to >300 colonies per plate. The results of this environmental survey will be published elsewhere (R. M. Wadowsky, R. B. Yee, L. Mezmar, E. P. Wing, and J. N. Dowling, submitted for publication).

DISCUSSION

The direct culturing of environmental water and water-related samples on a nonselective me-

dium for the isolation of *Legionellaceae* is an insensitive method. These bacteria may comprise a small minority of the total bacterial flora and exhibit an extended lag period for growth, requiring at least 3 days to produce visible colonies on ACES-BCYE agar. Thus, the *Legionellaceae* may be inhibited (5) or masked by the abundant growth of the other bacteria. Even when colonies of *Legionellaceae* are present, a tedious and careful examination of the plate must be made under a stereoscopic microscope to discern their presence. Thus, the inoculation of samples into guinea pigs is the recommended procedure for the isolation of these bacteria from environmental specimens. It is understandable that the detection of these bacteria in environmental samples has been limited to a few laboratories, and the processing of a large number of specimens has not been feasible.

Our study shows that these limitations can be overcome by using GVP agar as a selective medium for the direct culturing of environmental specimens. Using this medium, we have been able to isolate *L. pneumophila* from water-related environments. Since the non-*Legionellaceae* bacteria were either completely or partially inhibited, colonies of these legionellae were easily discerned. We also have evidence that the medium can support the growth of naturally occurring *L. micdadei* in addition to stock strains. Although we have not recovered *L. micdadei* from environmental samples, we have isolated it on GVP agar from a tissue specimen. These findings demonstrate that GVP agar is suitable for primary plating of environmental specimens for the isolation of *L. pneumophila* and, most likely, *L. micdadei*. The results with stock cultures suggest that the medium may also be effective for the recovery of other members of *Legionellaceae* from the environment, with the exception of *L. gormanii*. A single strain, LS-13, of this species was tested, and it was completely inhibited by the medium.

The efficiency of plating for *Legionellaceae* on the medium has not been determined. Thus, colony counts of different environmental samples on GVP agar should be considered as representing relative, and not absolute, numbers of *Legionellaceae* present.

We propose that samples initially be screened for *Legionellaceae* by plating on GVP agar. Colonies which appear on this medium after 3 days of incubation and have the characteristic cut-glass appearance and purple or green iridescence can be presumptively identified as being *Legionellaceae* and confirmed by the usual procedures (1, 8, 19). In our experience, presumptive identification by these criteria has always been confirmed. GVP plates can be read quickly since frequently no or only a few colonies of non-*Legionellaceae* bacteria are present. Specimens which do not yield *Legionellaceae* by direct culture can be examined by the direct immunofluorescent-antibody test. The specimens which are positive by this test can then be assayed by inoculation into guinea pigs. This last procedure may be necessary, since we have not compared the sensitivity of GVP agar and guinea pig inoculation in detecting *Legionellaceae*. The expense and time-consuming nature of inoculation of samples into guinea pigs have precluded our carrying out a comparison of the procedure.

We have encountered specimens of water environments which contained gram-negative bacteria that were not inhibited by GVP agar. Fortunately, a small minority of the specimens contained these bacteria in large numbers, which negated the effectiveness of the selective medium. Work is in progress to identify compounds which will inhibit these bacteria.

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ADDENDUM

Immediately before the submission of this paper for publication, the work of Bopp et al. (J. Clin. Microbiol. **13**:714–719, 1981) was published. They describe the use of low-pH treatment and a selective medium for the isolation of *Legionellaceae* from environmental water samples. They were able to achieve selectivity by supplementing charcoal-yeast extract agar with cephalothin, colistin, vancomycin, and cycloheximide. Thus, two selective media are now available for the recovery of *Legionellaceae* from environmental water samples.

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