JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1981, p. 714-719
0095-1137/81/040714-06$02.00/0

Vol. 13, No. 4

Isolation of *Legionella* spp. from Environmental Water Samples by Low-pH Treatment and Use of a Selective Medium

CHERYL A. BOPP,* JOHN W. SUMNER, GEORGE K. MORRIS, AND JOY G. WELLS

Bureau of Epidemiology, Bacterial Diseases Division, Centers for Disease Control, Atlanta, Georgia 30333

Received 21 October 1980/Accepted 22 December 1980

A selective medium was developed and used successfully to isolate *Legionella pneumophila* and *Legionella*-like organisms from environmental specimens previously positive by animal inoculation methods. This medium consists of charcoal-yeast extract agar to which have been added cephalothin (4 μg/ml), colistin (16 μg/ml), vancomycin (0.5 μg/ml), and cycloheximide (80 μg/ml). Pretreating of the environmental water samples with an acid buffer (pH 2.2), followed by plating on the selective medium, improved the rate of recovery of both *Legionella* and *Legionella*-like organisms relative to that with direct plating on selective media.

Legionella pneumophila has frequently been isolated from patients with Legionnaires disease and in several instances also from epidemic-related environmental samples. These include air-conditioning cooling towers or evaporative condensers (2, 4, 9; J. D. Band, M. LaVenture, J. P. Davis, G. F. Mallison, P. Skaliy, P. S. Hayes, W. L. Schell, H. Weiss, D. J. Greenberg, and D. W. Fraser, J. Am. Med. Assoc., in press) and hospital showers (2a). It has also been isolated from surface water from a stream, from mud from a stream bottom (15), and from lake waters not associated with epidemics (8).

In addition to *L. pneumophila*, the agent of Legionnaires disease, there are now four other proposed species of *Legionella*: *Legionella micdadei* (*pittsburgensis*) (11), *Legionella dumoffii* (1), *Legionella bozemanii* (1), and *Legionella gormanii* (16). All of these species but *L. gormanii* have been isolated as causative agents for illnesses involving pneumonia (3, 13, 20).

Strains of *L. micdadei* (TATLOCK and HEBA) were isolated from blood from patients in 1943 and 1959, respectively (10-12). *L. micdadei* has also been isolated from patients and shown to be the causative agent of Pittsburgh pneumonia (19, 20). The organism has also been isolated from environmental sources—specifically, nebulizers from respiratory therapy equipment (9a).

L. dumoffii has been isolated from lung tissues of a patient who died of pneumonia (13) and from one environmental source unrelated to that patient. The environmental source was a cooling tower located near the site of an outbreak of Legionnaires disease, but *L. dumoffii* was not implicated in the outbreak (3).

L. bozemanii was isolated from lung tissue of a patient who died of bronchopneumonia in 1958 and a second patient who died of pneumonia in 1978 (3). This species has not been isolated from the environment.

L. gormanii was isolated from soil collected from a creek bank during a Legionnaires disease outbreak investigation (16). The relationship of this organism to Legionnaires disease or other human diseases is not known at this time, although there is evidence that it can cause human as well as animal infections (16).

The Centers for Disease Control have worked with other isolates that bear a morphological and cultural resemblance to *Legionella* spp. but are distinct antigenically and according to gas-liquid chromatography analysis. These isolates, referred to as *Legionella*-like organisms (LLO) (1), are being studied further at the Centers for Disease Control as potentially new species of *Legionella*.

The procedure currently used to isolate *L. pneumophila* and other *Legionella* spp. from environmental specimens is complex and time-consuming and involves inoculating guinea pigs, followed by inoculation of guinea pig necropsy tissue to embryonated eggs (15). The organism can be isolated either from the guinea pig tissues or the egg yolk sacs on charcoal-yeast extract (CYE) agar plating medium (6). In addition to being tedious, this method is costly in terms of laboratory animals, eggs, and time. The procedure may take several weeks and does not allow for rapid processing of specimens, which might be of critical importance during an epidemic

investigation.

This report describes a selective isolation procedure that has been used to isolate *Legionella* directly from environmental samples without animal or egg inoculation. This procedure utilizes a selective medium consisting of CYE agar with four antibiotics added: cephalothin, colistin, vancomycin, and cycloheximide. The procedure also takes advantage of the observation by Wang et al. (23) that *L. pneumophila* is relatively resistant to low pH for a short period of time. Our results show that the rate of recovery of *Legionella* from environmental water samples is improved if samples are treated with acid and then plated on the selective agar medium.

MATERIALS AND METHODS

Bacterial strains. A stock culture of *L. pneumophila* (Philadelphia 1) that had been passed on artificial media several times was used for plate counts and antibiotic minimum inhibitory concentration determinations. This strain is referred to as the "stock strain" in this report. A 48-h CYE agar culture of the stock strain was suspended in yeast extract broth with 40% glycerol, divided into small aliquots, and frozen at −70°C. A vial was thawed and streaked on CYE agar for use each week.

Another culture of *L. pneumophila* (Philadelphia 1), referred to in this report as the "tissue strain," had never been passed on artificial media. The tissue strain was stored as macerated spleen tissue from a guinea pig infected with the disease. The infected spleen, after having been macerated, was divided into small aliquots and frozen at −70°C. For each experiment, a vial was thawed and diluted 1:5,000 in phosphate-buffered saline before being plated on the media to be tested.

Environmental water samples. The environmental samples used in this study were from various parts of the United States and were specimens from which *Legionella* or LLO had already been isolated with the guinea pig/egg inoculation method. Some of the specimens were collected during epidemic investigations of Legionnaires disease outbreaks but may not have been related to the outbreaks. After being received at the Centers for Disease Control, the samples were stored at 4°C for 2 to 18 months before being used in the study.

Media. The CYE agar used in this study contained 1.0% yeast extract (Difco Laboratories), 1.7% agar (Difco), 0.15% activated charcoal, 0.04% L-cysteine hydrochloride, and 0.025% ferric pyrophosphate (soluble). The pH was adjusted to 6.9 to 7.0 after autoclaving and the addition of L-cysteine and ferric pyrophosphate. Selective media were prepared by adding solutions of antibiotics to autoclaved CYE agar held in a water bath at 50°C until immediately before the plates were poured.

Yeast extract broth consisted of a 1.0% yeast extract (Difco) solution to which 0.04% L-cysteine hydrochloride and 0.025% ferric pyrophosphate were added after autoclaving. The pH of the broth was adjusted to 6.9 to 7.0.

FG agar (Mueller-Hinton [BBL Microbiology Systems] with 0.04% L-cysteine hydrochloride and 0.025% ferric pyrophosphate, pH 6.9 to 7.0) (7) was used to detect the production of brown pigment by isolates presumed to be *Legionella*. Another medium, yeast extract-tyrosine agar, consisting of 1.0% yeast extract (Difco), 1.7% agar (Difco), and 0.04% L-tyrosine, was also used for detection of brown pigment production since many organisms that were difficult to grow on FG agar grew satisfactorily on yeast extract-tyrosine agar.

Direct plating of environmental samples. Two 10-fold dilutions (10^{-1}, 10^{-2}) of each environmental specimen were prepared in phosphate-buffered saline (pH 7.2 to 7.3). A 0.1-ml quantity of each dilution and of the undiluted specimen was spread onto the surface of CYE and selective agar plates in duplicate. The plates were incubated at 35°C in 2.5% CO_2. The plates were read after 5, 6, 7, and 10 days of incubation for colonies with morphology similar to that of *Legionella* (white, glistening, convex, circular, entire, and from 1 to 2 mm in diameter with a "ground glass" appearance under magnification). At the time of each reading, all colonies thought to be *Legionella* were subcultured to fresh CYE agar. A total plate count of all colonies was recorded at 10 days.

Acid buffer treatment of environmental samples. The HCl-KCl buffer used for pretreating water samples was prepared by mixing 3.9 ml of 0.2 M HCl with 25 ml of 0.2 M KCl to yield a buffer solution with a pH of approximately 2.2. Two procedures designated "A" and "B" for acid treatment were used. In procedure A, 10 ml of the water sample was centrifuged at 4,000 rpm for 10 min in aerosol-free centrifuge containers. The supernatant was poured off, and the sediment was resuspended in 1 ml of the original water sample. This suspension was diluted 1:10 with the HCl-KCl buffer. At intervals ranging from 5 to 60 min, 0.1-ml quantities were removed from the acid suspension and were plated on duplicate agar plates of CYE and selective media. After each such interval, a sample was diluted 1:10 with phosphate-buffered saline and then plated in 0.1-ml quantities on duplicate CYE and selective media plates.

Procedure B did not involve centrifugation but involved adding 0.5 ml of the water sample to 4.5 ml of HCl-KCl buffer. At intervals of 5 to 60 min, 0.1-ml quantities of undiluted and diluted (1:10 in phosphate-buffered saline) acid-treated water sample were plated onto CYE and selective media. This procedure resulted in plating a 10^{-1} and a 10^{-2} dilution of the water sample, which was 10-fold more dilute when plated than samples prepared according to procedure A.

The plates inoculated with the acid-treated suspensions were incubated, read, and counted as described above for direct plating of water samples.

Screening suspect *Legionella* isolates. Each colony thought to be *Legionella* was streaked for isolation to a CYE agar plate (containing no antibiotics) and incubated for 2 to 5 days. From each such plate, the isolate was streaked onto yeast extract-tyrosine agar (or FG) and 5% sheep blood agar. If growth did

not occur on blood agar after 5 days of incubation (*Legionella* characteristically fails to grow on blood agar) or if the isolate produced browning of the yeast extract-tyrosine agar or FG agar, the isolate was examined by the fluorescent-antibody (FA) staining method (14) with conjugated antisera to each of the five species of *Legionella*. Isolates that were positive by FA in the polyvalent *L. pneumophila* conjugate were then further examined by FA with type-specific conjugates to *L. pneumophila* serogroups 1 to 6. If the FA results indicated a *Legionella* species, the isolate was examined for the cellular fatty acids characteristic of the *Legionella* species to confirm the identification (17, 18). Isolates whose FA results were negative were also tested by gas-liquid chromatography. If the fatty acid profile demonstrated a pattern of a high percentage of branched-chain cellular fatty acids, and if the isolate did not grow on blood agar or produce browning on FG or yeast extract-tyrosine agar, was gram negative, and was otherwise similar to *Legionella*, the isolate was considered to be an LLO.

Selective media experiments. To determine their suitability for inclusion in a selective medium, colistin and cephalothin were added separately to CYE agar in several different levels. Plate counts of the *L. pneumophila* stock strain were performed on these selective media and on CYE agar with no antibiotics. In addition, 10 environmental samples were direct-plated on these media as described above. Subsequently, cephalothin at a level of 4 μg/ml was combined in CYE agar with different levels of colistin (2, 4, 8, 16, and 32 μg/ml) to determine the optimum concentration. The *L. pneumophila* stock strain and two environmental specimens were plated on these five selective media with cephalothin and colistin and on CYE agar without antibiotics. The medium containing 4 μg of cephalothin and 16 μg of colistin per ml was selected as optimal, and to this medium were added two concentrations of vancomycin (3 and 0.5 μg/ml). On these two selective media, and also on CYE medium, plate counts of the stock strain of *L. pneumophila* were done. Using cephalothin-colistin-vancomycin (CCV) agar and CYE agar, five environmental water samples were plated by using the method described.

Edelstein-Finegold (E-F) medium (5) (CYE agar with 40 U of polymyxin B and 0.5 μg of vancomycin per ml) was compared with CCV medium (CYE agar with 4 μg of cephalothin, 16 μg of colistin, and 0.5 μg of vancomycin per ml) for isolation of *Legionella* and suppression of non-*Legionella* organisms. Ten environmental water samples from which *Legionella* had been isolated by guinea pig/egg inoculation were used. Samples were diluted and plated as described above on three media: E-F medium, CCV medium, and CYE medium without antibiotics. These plates were read and picked for *Legionella* colonies, and total plate counts were determined to assess the inhibition of total flora.

In a final attempt to improve the selectivity of CCV medium, we added cycloheximide (an antifungal agent) because the prolonged incubation time (10 days) necessary to recover *Legionella* can sometimes result in overgrowth by fungi. A comparison of plate counts of the stock and tissue strains of *L. pneumophila* on CCV medium containing various concentrations of cycloheximide resulted in the selection of 80 μg of cycloheximide per ml as the optimum level. Thirteen environmental water samples that had already been shown to be positive for *Legionella* and LLO were plated on CYE agar with, per ml, 4 μg of cephalothin, 16 μg of colistin, 0.5 μg of vancomycin, and 80 μg of cycloheximide (CCVC medium) and on CYE agar without antibiotics.

Acid treatment experiments. To evaluate the effects of low pH on survival of *Legionella* over a period of time, we exposed the stock strain of *L. pneumophila* to the HCl-KCl buffer (pH 2.0) for periods of 5, 15, 30, and 60 min. Plate counts of the acid suspension of the stock strain were performed after each time interval.

Five water samples already shown to be positive for *L. pneumophila* by the guinea pig/egg inoculation method were subjected to the HCl-KCl buffer treatment for 30 min according to procedure A, and attempts were made to recover *Legionella*. Two naturally contaminated environmental water samples, LS-4 and MI-56, were retested using 0-, 5-, 15-, and 30-min exposure times to HCl-KCl buffer to determine the most appropriate exposure interval for acid buffer treatment.

In the last experiment the acid buffer treatment (procedures A and B), with an exposure time of 5 min and with samples directly plated onto CYE medium and CCVC selective medium, were compared for efficacy in isolating *Legionella* from 11 environmental

TABLE 1. *Comparison of total plate counts of all organisms (legionellae and non-legionellae) from 10 environmental specimens*

Environmental specimen	Total plate count on:		
	CYE medium with no antibiotics	E-F medium	CCV medium
CH-1	2.7×10^3	1.4×10^3	1.4×10^2
CH-4	2.5×10^4	1.8×10^3	1.0×10^2
CH-55	9.0×10^4	9.1×10^4	1.6×10^5
CH-47	$>3.0 \times 10^5$	$>3.0 \times 10^5$	2.0×10^4 [a]
ME-11	8.5×10^3	9.6×10^2	3.3×10^2
MI-56	1.6×10^4	1.4×10^4	2.6×10^3 [b]
JA-19	3.7×10^4	7.3×10^3	4.4×10^2 [a]
WA-104	$>3.0 \times 10^5$	8.3×10^4	2.4×10^4
NY-23	3.0×10^3	2.9×10^3	1.2×10^2 [c]
PI-12	$>3.0 \times 10^5$	2.1×10^5	4.2×10^4
L. pneumophila stock strain	2.2×10^8	2.6×10^8	2.5×10^8

[a] LLO were isolated from these plates. These strains have characteristics similar to *Legionella* but were not identified by FA and gas-liquid chromatography results to be one of the five species of *Legionella*. They are presently being evaluated as potentially new species of *Legionella*.

[b] *L. pneumophila* serogroup 1 isolated.

[c] *L. dumoffii* isolated.

water samples already shown to be positive for *Legionella* by the guinea pig/egg inoculation method.

RESULTS

The growth of the *L. pneumophila* stock strain was inhibited on CYE medium containing 8 or 16 μg of cephalothin per ml, although the minimum inhibitory concentration of cephalothin for *L. pneumophila* has been reported to be 16 μg/ml (21, 22).

Although the minimum inhibitory concentration of colistin was reported to be 3.6 μg/ml (21), colistin did not inhibit growth of *L. pneumophila* (stock and tissue strain) even at a level of 7.2 μg/ml in our study. If an antibiotic is to be an effective agent in selective media it must inhibit bacteria other than *Legionella*; however, colistin at 7.2 μg/ml did not sufficiently reduce the total plate counts of 10 environmental specimens as compared to the total plate counts on CYE without colistin. The average total plate count with no antibiotic was 7.7×10^5, and that with 7.2 μg of colistin per ml was 2.5×10^5.

Using cephalothin-colistin medium (CYE agar with 4 μg of cephalothin and 16 μg of colistin per ml), we isolated *L. pneumophila* group 1 from 1 fo 2 environmental samples, but the average amount of suppression of competing non-*Legionella* flora was less than 1 log.

CCV medium (CYE agar with, per ml, 4 μg of cephalothin, 16 μg of colistin, and 0.5 μg of vancomycin) was more inhibitory for gram-positive spreading colonies than cephalothin-colistin medium and showed little suppression of the *L. pneumophila* stock strain. Three of five water samples cultured on CCV agar yielded *L. pneumophila*; one sample yielded two serogroups of *L. pneumophila*.

Table 1 lists the isolates of *Legionella* and LLO and gives the results of the total plate counts of all organisms for the comparison of inhibitory characteristics of CCV, E-F, and CYE media. CCV medium was superior to E-F and CYE media in inhibiting competing flora in the environmental specimens. In addition, *Legionella* and LLO were isolated from 4 of the 10 samples on CCV medium, whereas no isolations were made from E-F or CYE media.

When CCV plus 80 μg of cycloheximide per ml (CCVC medium) was plated with 13 environmental water samples that had already been shown to be positive for Legionella and LLO, 7 were positive on CCVC medium, and only 3 were positive on CYE without antibiotics. However, two of the three isolates on CYE were recovered from chlorinated water samples relatively free of competing flora.

In the experiments done to develop the acid buffer method, the *L. pneumophila* stock strain was shown to be resistant to the acid for up to 30 min. The plate count of the stock strain was reduced from 6.3×10^8 colony-forming units per ml at 0 min of acid exposure to 6.1×10^8 at 30 min and to 6.8×10^7 at 60 min. When five environmental samples were exposed to the acid buffer from 30 min by procedure A (see Materials and Methods), *L. pneumophila* group 1 was recovered from two of the five samples. One of the three samples that did not yield *L. pneumophila* yielded an LLO isolate. However, two samples tested later yielded isolates of *L. pneumophila* at 5 min but not at 30 min of exposure to the acid buffer. The total microbial flora for both samples in this instance was reduced by about 2 logs after 5 min of exposure to HCl-KCl buffer, indicating that 5 min is an effective exposure interval for acid buffer treatment.

Table 2 lists the results of the comparison of acid buffer procedures A and B with direct plating onto CYE medium and CCV selective medium. Direct plating yielded isolates of *L. pneumophila* group 5 from one water sample (MI-56) on both CYE and CCVC. Five of the water samples yielded LLO after direct plating. When procedure B was used, 2 of 11 samples yielded *Legionella*, and 3 yielded only LLO. When procedure A was used, five water samples yielded *Legionella* and five yielded LLO.

Analysis of the isolates from 15 environmental samples, tested by both the guinea pig/egg inoculation method and the acid pretreatment/plating media method discussed above, revealed that multiple species and serogroups of *Legionella* were isolated by both methods (Table 3). The most obvious difference in the groups or species was that LLO was isolated from six samples cultured directly on plating media, but only one LLO isolate was obtained with the guinea pig/egg inoculation method.

DISCUSSION

Procedure A was superior to both direct plating on CYE and CCVC and to procedure B for isolating *Legionella*. After the acid buffer treatment, CCVC selective medium used for plating gave slightly better results than CYE medium. However, one *L. pneumophila* isolate was recovered on CYE that was not recovered on CCVC medium. This may have been coincidence rather than the result of inhibition of *L. pneumophila* by the selective medium, but because it occurred we recommend that both CYE and CCVC media be used when samples are pretreated with acid buffer.

At this time no isolation method for *Legionella* can completely replace the guinea pig/egg

TABLE 2. *Comparison of three methods for efficacy in isolating Legionella from water samples known to be positive for Legionella*

Water sample	Isolates yielded by method of isolation[a]					
	Direct plating		Acid buffer, procedure B (5 min)		Acid buffer, procedure A (5 min)	
	CYE	CCV	CYE	CCVC	CYE	CCVC
MI-56	5	5	—	—	5	—
WA-176	LLO	LLO	4	1	1, 4, LLO	1, 4, LLO
WA-180	—	LLO	—	1, LLO	1, 4, LLO	1, 4, LLO
WA-104	—	LLO	LLO	—	LLO	4, LLO
WA-103	LLO	—	—	LLO	LLO	LLO
JA-19	—	LLO	LLO	—	LLO	LLO
NY-23	—	—	—	—	—	*L. dumoffii*
CH-4	—	—	—	—	—	—
CH-6	—	—	—	—	—	—
ME-11	—	—	—	—	—	—
LS-4	—	—	—	—	—	—

[a] Numbers indicate *L. pneumophila* serogroup. LLO, LLO isolated; see footnote *a*, Table 1. —, No isolates.

TABLE 3. *Comparison of types of Legionella isolates by the animal inoculation/egg method and direct media isolation method*

Legionella isolate	No. of samples isolated from:	
	Animal/egg	Media
L. pneumophila serogroup 1	4	5
L. pneumophila serogroup 3	0	1
L. pneumophila serogroup 4	4	2
L. pneumophila serogroup 5	3	1
L. pneumophila serogroup 6	4	3
L. pneumophila nontypable	1	0
L. dumoffii	1	1
L. micdadei	1	0
LLO	1	6

inoculation method. Since our environmental specimens contained numerous and diverse flora, no single combination of antibiotics tested was effective in suppressing competing flora in all specimens. Even the acid buffer procedure used in combination with CCVC selective medium and CYE medium led to the recovery of *Legionella* from only 45% of water samples known to contain *Legionella*. However, it had been as long as a year in some cases between the time *Legionella* had been isolated from guinea pigs or eggs and the time we did this study. *Legionella* may not have been still viable in some of the water samples, and the acid pretreatment/media method may be more effective than our results indicate.

Direct plating without acid buffer treatment on the selective medium, CCVC agar, led to a recovery rate of up to 36% for *Legionella* from environmental samples used in our study. It does appear to be more sensitive than animal inoculation for isolation of LLO, possibly indicating that this group of organisms is less pathogenic for guinea pigs (Table 3). These organisms are being considered as possible new *Legionella* species since they have many characteristics similar to other *Legionella*.

The utility of CCVC medium for clinical isolation of *L. pneumophila* and other *Legionella* species remains to be evaluated. E-F medium, which in our study was not as effective as CCV medium for environmental isolation of *Legionella*, has been demonstrated to be effective for isolation of *L. pneumophila* from clinical specimens, although its efficacy for isolation of other species of *Legionella* is unknown.

ACKNOWLEDGMENTS

We thank William T. Martin, Linda Corcoran, George W. Gorman, and William Bibb for their laboratory assistance. We also thank Martin J. Blaser and John V. Bennett for their suggestions and encouragement.

LITERATURE CITED

1. **Brenner, D. J., A. G. Steigerwalt, G. W. Gorman, R. E. Weaver, J. C. Feeley, L. G. Cordes, H. W. Wilkinson, C. Patton, B. M. Thomason, and K. R. Lewallen Sasseville.** 1980. *Legionella bozemanii* species nova and *Legionella dumoffii* species nova: classification of two additional species of *Legionella* associated with human pneumonia. Curr. Microbiol. **4**:115-120.
2. **Cordes, L. G., D. W. Fraser, P. Skaliy, C. A. Perlino, W. R. Elsea, G. F. Mallison, and P. S. Hayes.** 1980. Legionnaires' disease outbreak at an Atlanta, Georgia, country club: evidence for spread from an evaporative condenser. Am. J. Epidemiol. **111**:425-431.

2a. **Cordes, L. G., A. M. Wiesenthal, G. W. Gorman, J. T. Phair, H. M. Sommers, A. Brown, V. L. Yu, M. H. Magnussen, R. D. Meyer, J. S. Wolf, K. N. Shands, and D. W. Fraser.** 1981. Are showers a source of nosocomial Legionnaires' disease? Ann. Intern. Med. **94**:195-197.

3. **Cordes, L. G., H. W. Wilkinson, G. W. Gorman, B. J. Fikes, and D. W. Fraser.** 1979. Atypical *Legionella*-

like organisms: fastidious water-associated bacteria pathogenic for man. Lancet ii:927-930.
4. **Dondero, T. J., R. C. Rendtorff, G. F. Mallison, R. M. Weeks, J. S. Levy, E. W. Wong, and W. Schaffner.** 1980. An outbreak of Legionnaires' disease associated with a contaminated air conditioning cooling tower. N. Engl. J. Med. **302**:365-370.
5. **Edelstein, P. H., and S. M. Finegold.** 1979. Use of a semiselective medium to culture *Legionella pneumophila* from contaminated lung specimens. J. Clin. Microbiol. **10**:141-143.
6. **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.
7. **Feeley, J. C., G. W. Gorman, R. E. Weaver, D. C. Mackel, and H. W. Smith.** 1978. Primary isolation media for Legionnaires' disease bacterium. J. Clin. Microbiol. **8**:320-325.
8. **Fliermans, C. B., W. B. Cherry, L. H. Orrison, and L. Thacker.** 1979. Isolation of *Legionella pneumophila* from nonepidemic-related aquatic habitats. Appl. Environ. Microbiol. **37**:1239-1242.
9. **Glick, T. H., M. B. Gregg, B. Berman, G. Mallison, W. W. Rhodes, and I. Kassanoff.** 1978. Pontiac fever: an epidemic of unknown etiology in a health department. I. Clinical and epidemiological aspects. Am. J. Epidemiol. **107**:149-160.
9a. **Gorman, G. W., V. L. Yu, A. Brown, J. A. Hall, L. K. Corcoran, W. T. Martin, W. F. Bibb, G. K. Morris, M. H. Magnussen, and D. W. Fraser.** 1980. Isolation of Pittsburgh pneumonia agent from nebulizers used in respiratory therapy. Ann. Intern. Med. **93**:572-573.
10. **Hebert, G. A., C. W. Moss, L. K. McDougal, F. M. Bozeman, R. M. McKinney, and D. J. Brenner.** 1980. The rickettsia-like organisms TATLOCK (1943) and HEBA (1959): bacteria phenotypically similar to but genetically distinct frox *Legionella pneumophila* and the WIGA bacterium. Ann. Intern. Med. **92**:45-52.
11. **Hebert, G. A., A. G. Steigerwalt, and D. J. Brenner.** 1980. *Legionella micdadei* species nova: classification of a third species of *Legionella*associated with human pneumonia. Curr. Microbiol. **3**:255-257.
12. **Hebert, G. A., B. M. Thomason, P. P. Harris, M. D. Hicklin, and R. M. McKinney.** 1980. "Pittsburgh pneumonia agent": a bacterium phenotypically similar to *Legionella pneumophila* and identical to the TATLOCK bacterium. Ann. Intern. Med. **92**:53-54.
13. **Lewallen, K. R., R. M. McKinney, D. J. Brenner, C. W. Moss, D. H. Dail, B. M. Thomason, and R. A. Bright.** 1979. A newly identified bacterium phenotypically resembling, but genetically distinct from *Legionella pneumophila*: an isolate in a case of pneumonia. Ann. Intern. Med. **91**:831-834.
14. **McKinney, R. M., L. Thacker, P. P. Harris, K. R. Lewallen, G. A. Hebert, P. H. Edelstein, and B. M. Thomason.** 1979. Four serogroups of Legionnaires' disease bacteria defined by direct immunofluorescence. Ann. Intern. Med. **90**:621-624.
15. **Morris, G. K., 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.** 1979. Isolation of the Legionnaires' disease bacterium from environmental samples. Ann. Intern. Med. **90**:664-666.
16. **Morris, G. K., A. Steigerwalt, J. C. Feeley, E. S. Wong, W. T. Martin, C. M. Patton, and D. J. Brenner.** 1980. *Legionella gormanii* sp. nov. J. Clin. Microbiol. **12**:718-721.
17. **Moss, C. W., and S. B. Dees.** 1979. Further studies of the cellular fatty acid composition of Legionnaires disease bacteria. J. Clin. Microbiol. **9**:648-649.
18. **Moss, C. W., and S. B. Dees.** 1979. Cellular fatty acid composition of WIGA, a rickettsia-like agent similar to Legionnaires disease bacterium. J. Clin. Microbiol. **10**: 390-391.
19. **Myerowitz, R. L., A. W. Pasculle, J. N. Dowling, G. J. Pazin, M. Puerzer, R. B. Yee, C. R. Rinaldo, and T. R. Hakala.** 1979. Opportunistic lung infection due to "Pittsburgh pneumonia agent." N. Engl. J. Med. **301**: 953-958.
20. **Pasculle, A. W., R. L. Myerowitz, and C. R. Rinaldo, Jr.** 1979. New bacterial agent of pneumonia isolated from renal-transplant recipients. Lancet ii:58-61.
21. **Thornsberry, C., C. N. Baker, and L. A. Kirven.** 1978. In vitro activity of antimicrobial agents on the Legionnaires disease bacterium. Antimicrob. Agents Chemother. **13**:78-80.
22. **Thornsberry, C., and L. A. Kirven.** 1978. β-Lactamase of the Legionnaires' bacterium. Curr. Microbiol. **1**:51-54.
23. **Wang, W. L. L., M. J. Blaser, J. Cravens, and M. A. Johnson.** 1979. Growth, survival, and resistance of the Legionnaires' disease bacterium. Ann. Intern. Med. **90**: 614-618.