Comparative Study of Procedures for Isolation and Cultivation of *Legionella pneumophila* from Tap Water in Hospitals

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For the isolation and cultivation of *Legionella pneumophila* from tap water in hospitals, we compared different media and selection techniques. A second part of the study compared the *L. pneumophila* yields from different water samples at identical sites. A total of 210 water samples (500 ml each) were collected from two selected sites in each of 21 hospitals. Warm water samples were collected after flow times of 0, 5, 10, and 15 min; in addition, one cold water sample was collected. Filtration was used to concentrate all samples. Following filtration, 0.1 and 1 ml each of untreated samples, heat-treated samples (3 min, 59°C), and acid-treated samples (pH 2.2, 15 min) were spread onto the selective media MWY (SR 118; Oxoid) and BMPA α (SR 111; Oxoid), and samples from 12 hospitals were also spread onto GVPC medium (SR 152; Oxoid). A total of 72 (34%) of the 210 samples from 12 hospitals were positive. With respect to the positive *Legionella* cultures, there was no significant difference between the selective media MWY, BMPA α , and GVPC. With the BMPA α supplement, more samples were positive following heat treatment ($P < 0.05$) or acid treatment ($P < 0.05$) than without any further treatment. For the maximum yield of *Legionella* colonies with minimum additional microbial flora, acid treatment was the most effective, and by all methods, the GVPC supplement was the most selective. For routine water tests in hospitals for differentiating between systemic and local contamination, acid treatment of the concentrated samples, the use of different selective media, and the correct selection of sampling sites are recommended.

Routine water testing for *Legionella* species in hospitals is recommended by many investigators as a necessary preventive measure (3, 7, 9, 16) and has been a legal requirement in the State of Styria (Austria) for 3 years. This law, however, does not specify sampling and specimen concentration methods, nor does it specify ways of minimizing additional microbial flora. The goal of our investigation was a comparison of the methods of sampling, specimen concentration, and minimization of additional microbial flora in order to select a routine test method in the medical environment which yields optimum test results with a minimum of expense and time.

MATERIALS AND METHODS

Between 1989 and 1992, 210 water samples from 21 hospitals were collected in the course of required routine tests for *Legionella* species. At two sites each, one cold water sample (500 ml) and four warm water samples (500 ml each) were collected after flow times of 0, 5, 10, and 15 min. Immediately following collection, the samples were transported to the laboratory. Filtration was the sole method of specimen concentration. Both polycarbonate (Millipore) and cellulose nitrate (Sartorius) filters (pore sizes, 0.2 μ m) were used. The filters were put into approximately 20 ml of the original water sample under sterile conditions, mixed with a Vortex mixer, and treated with ultrasound for 10 s (240 V; 0.23 A; frequency = 50 Hz; Transsonic 420; Elma). Without further treatment, samples of 0.1 and 1 ml of this treated water were spread onto different selective media. One sam-

ple each received parallel treatment with acid (1:10 in 0.2 M HCl-KCl [pH 2.2] for 15 min) and heat (3 min at $59 \pm 1^\circ\text{C}$), and subsequently, 0.1 and 1 ml of each sample were spread onto different selective media.

The following media were used. Charcoal yeast extract agar (CM 655; Oxoid) (8) with *Legionella*-BCYE α supplement (SR 110; Oxoid) (5) was used with the addition of the following selective supplements. (i) BMPA α medium (SR 111; Oxoid), as described by Edelstein (5), contained polymyxin (80 μ g/ml), anisomycin (80 μ g/ml), and cefamandole (4 μ g/ml). (ii) The medium of Wadowsky and Yee (15) (MWY medium; SR 118; Oxoid), as modified by Edelstein (6), contained polymyxin B (50 U/ml), anisomycin (80 μ g/ml), vancomycin (1 μ g/ml), and glycine (3 mg/ml) as well as bromocresol purple (10 μ g/ml) and bromothymol blue (10 μ g/ml). (iii) The GVPC selective supplement (SR 152; Oxoid) described by Dennis (4), containing glycine (3 mg/ml), vancomycin hydrochloride (1 μ g/ml), polymyxin B sulfate (79.2 units/ml), and cycloheximide (80 μ g/ml), was also used to test water from 12 hospitals.

Preliminary investigations in some Styrian hospitals showed a relatively high level of water contamination with pseudomonads and fungi; therefore, in the present comparative study we did not use the BCYE α medium without the addition of other selective supplements.

All inoculated culture media were incubated at $36 \pm 1^\circ\text{C}$ for a maximum of 10 to 14 days at 90% relative humidity without CO₂. The first evaluation was started after 72 h. *L. pneumophila* and other microbial flora were identified by Gram staining and with the API- and Vitek systems (Vitek AMS/IMS Labor System; Api bioMérieux). In order to verify the presence of *L. pneumophila*, colonies were inoculated onto blood and cysteine-free BCYE α agar. Colonies

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FIG. 1. Detection of legionellae from tap water after various flow times. □, 0 min; ▤, 5 min; ▥, 10 min; ■, 15 min.

that grew only on BYCE α agar were identified by an agglutination test (latex slide agglutination test; Serobac/Mercia Diagnostics) and a direct immunofluorescence test (fluorescein isothiocyanate conjugate; *L. pneumophila* serogroups 1 to 6 [polyvalent and monovalent]; serogroups 7 to 14 [polyvalent]; non-*L. pneumophila* [polyvalent]: *L. bozemanii*, *L. dumoffii*, *L. longbeachae*, *L. gormanii*, *L. micdadei*, and *L. jordanii*; Hofmann-Pharma/Salzburg). Nonidentifiable non-*L. pneumophila* strains were sent to reference laboratories (G. Wewalka, Vienna, Austria, and London). A comparison with all three selective media was carried out with the samples ($n = 50$) from five *L. pneumophila*-positive hospitals. For these samples, the exact number and species of the additional microbial flora were also determined. For the calculation of descriptive statistics and for the comparison of groups, the programs SPSS (SPSS Inc., Chicago, Ill.) and SYSTAT (SYSTAT Inc., Evanston, Ill.) were used.

RESULTS

Of 210 samples collected from 21 hospitals, *L. pneumophila* could be isolated from 72 (34%) samples from 12 (57%) hospitals. *L. pneumophila* counts were between <10 and a maximum of 1.6×10^4 per liter. In 10 of 12 hospitals where *L. pneumophila* was found, *L. pneumophila* could also be isolated from cold water; however, the count was less than 20 CFU/liter in all cases. The most frequently isolated serogroups were serogroup 1 (62%), serogroup 6 (51%), and serogroup 3 (36%). Other serogroups that were isolated included serogroup 5 (22%), serogroup 2 (6.7%), and serogroups 7 to 14 (6.7%). Only 1.8% of all isolates were found to be non-*L. pneumophila*. The media used did not reveal a significant difference regarding the number of different serogroups. Of 12 hospitals in which *L. pneumophila* was found, 8 had the highest count at 0 min of flow time, three hospitals had the highest count after 5 min of flow time, and one hospital had the highest count after 15 min of flow time (Fig. 1). For all 12 hospitals, *L. pneumophila* was found in the first sample (0 min flow time). Detection of *L. pneumophila* in the 12 hospitals was possible by all of the methods (filtration, filtration with acid treatment, and filtration with heat treat-

TABLE 1. *L. pneumophila*-positive cultures from five hospitals compared by the various methods to minimize additional microbial flora

Sample treatment	No. of <i>L. pneumophila</i> -positive cultures on the following selective medium ^a :		
	MWY	BMPA α	GVPC
None	15	13	19
Heating	18	23	23
Acid	23	23	20

^a A total of 50 cultures were tested. For no treatment versus heat treatment on BMPA α , $P < 0.05$. For no treatment versus acid treatment on BMPA α , $P < 0.05$.

ment) and with all supplements (MWY, BMPA α , GVPC). With respect to the positive *Legionella* cultures from five hospitals ($n = 50$), there were no significant differences between the selective media MWY, BMPA α , and GVPC. However, looking at the BMPA α supplement alone, a significant difference between the three methods was found; a higher number of samples were positive for *L. pneumophila* following heat ($P < 0.05$) and acid ($P < 0.05$) treatments than following filtration without further treatment (Table 1).

The number of colonies determined from all *L. pneumophila*-positive samples ($n = 50$) from five hospitals showed a significant advantage of filtration with acid treatment compared with filtration with heat treatment or without further treatment, as determined by the Wilcoxon signed rank test (10). In each case, the GVPC supplement was the most selective. The highest median values of *Legionella* colonies from these samples were obtained by using the GVPC supplement after acid treatment, and the lowest median values were obtained by using the BMPA α and MWY supplements after filtration only. The median values of the additional microbial flora were inversely proportional (Table 2 and 3).

DISCUSSION

Sampling. In all 12 hospitals, *Legionella* contamination was determined in the first sample (500 ml without heat treatment of the water pipe). Although in one case the largest number of bacteria was obtained after of flow time of 15 min, routine tests for *L. pneumophila* in hospitals can be limited to one warm water sample (250 to 500 ml) per sampling site. However, the sampling sites must be chosen in such a way as to be able to differentiate between local and systemic contamination. In buildings without risk groups (i.e., immunosuppressed patients) the choice of sampling sites can be limited to the central warm water systems and certain peripheral sites (e.g., showers) (7).

Minimizing additional microbial flora. The results show

TABLE 2. Numbers of *Legionella* colonies

Sample treatment ^a	No. of <i>L. pneumophila</i> colonies on the following selective medium ^b :		
	MWY	BMPA α	GVPC
None	20 (730)	10 (1,680)	190 (460)
Heat	80 (40)	210 (340)	320 (130)
Acid	600 (1)	600 (3)	750 (<1)

^a A total of 50 samples were subjected to each treatment.

^b Values in parentheses are median numbers of non-*Legionella* colonies.

TABLE 3. Comparison of median *Legionella* spp. counts by the Wilcoxon test

Sample treatment	Result for the following selective medium and treatment:								
	MWY (88) ^a	BMPA α (67)	GVPC (592)	Heat, MWY (323)	Heat, BMPA α (304)	Heat, GVPC (651)	Acid, MWY (653)	Acid, BMPA α (700)	Acid, GVPC (976)
MWY	—								
BMPA α	NS ^b								
GVPC	+ ^c	+							
Heat, MWY	NS	NS	NS						
Heat, BMPA α	++ ^d	+++ ^e	NS	NS					
Heat, GVPC	++	+++	NS	NS	NS				
Acid, MWY	+++	+++	NS	NS	NS	NS			
Acid, BMPA α	+++	+++	NS	+	+	NS	NS		
Acid, GVPC	+++	+++	++	++	+++	+	NS	NS	

^a Values in parentheses are median numbers of *Legionella* colonies detected.

^b NS, not significant.

^c $P < 0.05$.

^d $P < 0.01$.

^e $P < 0.001$.

that most *L. pneumophila*-positive samples were found after filtration and then acid treatment and by using the MWY and BMPA α supplements or after filtration and heat treatment and by using the GVPC supplement. Overall, the greatest reduction in the additional microbial flora and the highest number of *Legionella* colonies were found after acid treatment. Bornstein (2) also found an advantage of acid treatment with various supplements (BYCE α ; BYCE α plus cephalothin, colistin, and vancomycin; BCYE α plus glycine, colistin, and vancomycin; and BYCE α plus α -ketoglutarate, colistin, and vancomycin); following acid treatment, recovery was greatest with regard to positive cultures, the number of isolated *L. pneumophila* organisms, and the frequency of strain isolation. The method of Bopp et al. (1) yielded a higher number of *L. pneumophila* isolates by using acid treatment and the CCVC supplement (cephalothin, colistin, vancomycin, and cycloheximide). Other investigators (11), however, point out an overly strong inhibition by this selective medium.

In a comparison of BCYE α , MWY, and BMPA α , Edelstein (6) summarized that MWY medium is the best medium for isolating *L. pneumophila* from potable water specimens. We found no significant difference between MWY and BMPA α in the rate of isolation by all of the methods used in the present study. GVPC was the most selective supplement (followed by BMPA α and MWY) regarding the reduction of the CFU of the additional microbial flora and provided a higher number of *Legionella* colonies. The cefamandole-containing supplement BMPA α is known to inhibit those *Legionella* isolates that do not form cephalosporinase (e.g., *L. micdadei* [6]); also, growth of *L. pneumophila* serogroup 1 subtype Bellingham is inhibited (14).

These different results regarding the isolation rates of *L. pneumophila* with various supplements and the different number of unwanted bacteria in water require the use of several different selective media in routine tests as well; by using two selective media, Bornstein (2) detected *L. pneumophila* at a rate of 80%; by using three selective media, Bornstein (2) detected *L. pneumophila* at a rate of 90%.

The sequential culturing methods suggested by Shahamat et al. (12) for improved *Legionella* recovery or new molecular biological methods for rapid identification of *Legionella* cultures (13) do not seem feasible for routine water tests at present.

For standardized sampling and preparation in routine

water tests for *L. pneumophila* in hospitals, the following recommendations are made. (i) Sampling sites should be carefully selected to differentiate between local and systemic contamination, (ii) 250 to 500 ml of warm water should be tested, (iii) polycarbonate or cellulose nitrate filters (pore size, 0.2 μ m) should be used, and (iv) water samples should be treated with acid and selective supplements (e.g., GVPC) should be used.

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