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Protocol for Sampling Environmental Sites for Legionellae

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A protocol for sampling environmental sites was developed and used to identify possible sources of *Legionella* species in support of epidemiologic investigations at two hospitals. In hospital A, legionellae were isolated from 43 of 106 (40%) different sites. Three separate *Legionella pneumophila* serotypes and a previously unrecognized species were present in different combinations in the positive samples. Two of five cooling towers contained the same *L. pneumophila* serogroup 1 monoclonal type (1,2,4,5) as was isolated from patients. The same monoclonal type was also isolated from make-up water for the two cooling towers, a hot water tank, water separators in four main air compressor systems for respiratory therapy, and cold and hot water faucets. In hospital B, 13 of 37 (38%) sample sites contained legionellae, all of which were *L. pneumophila* serogroup 1. The monoclonal type matching isolates from patients (1,2,4,5) was found at the highest concentration in a hot water tank, but it was also present at four other sample sites. Since legionellae not related to disease may be found in many of the sites sampled, an epidemiologic association with the probable source should be established before intervention methods, such as disinfection, are undertaken.

Documentation of the source for the spread of the etiologic agent causing a legionellosis epidemic can be a problem. The agent, *Legionella* species, which is ubiquitous in freshwater environments (9, 10), sometimes causes pneumonia and has been implicated as the agent of Pontiac fever. The primary means for transmission of pneumonia appears to be inhalation of aerosols containing virulent *Legionella* species by susceptible individuals (2, 5, 7). The potential sources of water that could contain legionellae and be aerosolized in an institution such as a hospital make the investigation of the legionellosis outbreak difficult. The selection of sample sites to identify the common sources of legionellae should be based on epidemiologic data proving an association between patients and possible exposure to aerosols containing the agent. However, the microbiological, physical, and chemical conditions of the water may change before epidemiologic data have been collected and analyzed. Thus, it may be appropriate to obtain samples at the outset of an investigation to ensure collection of timely and appropriate specimens.

We developed a protocol as a guide for selecting sample sites at the beginning of a legionellosis investigation. Our selection of sites for the protocol was based on past laboratory experience in legionellosis investigations, sites implicated for the spread of legionellosis by other investigators, and common characteristics of water distribution and air-conditioning systems. The protocol does not include all possible sample sites and contains some sites that are not applicable to all circumstances.

This report describes our results when the protocol was used to establish a profile of positive sites for *Legionella* species in two epidemic investigations.

Background for protocol. Basically, water specimens associated with buildings are in eight categories (Table 1). The first category (A), potable water outside and on the boundary of hospital property, is included to document external sources of municipal water that contain legionellae and may seed the water and plumbing systems of the building. The reports of other investigators such as Voss et al. (17, 18) have indicated that municipal water treatment plants harbor legionellae and emphasize the need to identify external contamination sources. The second category (B), the general potable water system for a hospital or facility, includes sites that can be very important for disease transmission. The presence of legionellae in water heaters and holding tanks is well documented, and these have been implicated as sources in outbreaks of legionellosis (4, 16). The third category (C), pharmacy water, may be important since water for the preparation of sterile solutions used for surgical patients and in respiratory therapy equipment is sometimes supplied by the hospital pharmacy. Legionellosis cases linked to respiratory therapy equipment have been well documented (1). The fourth category (D), air compressor systems, can be important if air for respirators is supplied to patients from such devices at the facility. It is not uncommon for excess water to be observed at times in compressed air lines. Sites listed in the fifth category (E), potable water final distribution outlets, include shower heads, faucets, hemodialysis units, and ice makers. The recovery of legionellae from showers has been documented (6). The sixth category (F), air-conditioning systems, includes parts of air-handling systems such as evaporative condensers and cooling towers. Both have been reported as sources for legionellae causing epidemics (4, 5, 7). The seventh category (G) is included since evidence of the spread of legionellae from whirlpools (hot tubs) has been reported (13). The eighth category (H) is included for consideration in case epidemiologic evidence indicates an association between exposure to aerosols from a source such as a decorative fountain or water from a site such as a creek. Water from a creek or river may be used as make-up water for a cooling tower.

Since environmental and epidemiologic circumstances may dictate priorities on the selection of sample sites, the protocol was written as a guide for selecting sample sites and not as a master list of sites that must all be sampled.

MATERIALS AND METHODS

Outbreak sites. The protocol (Table 1) was used at two hospitals designated as hospital A and hospital B. Hospital A

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TABLE 1. Protocol for sampling environment sites for legionellae

Site and description	Approx no. of samples	Vol of samples
A. Potable water outside or on boundary of hospital property		
1. Treatment plant (raw and refined water)	2	10 liters
2. Guard house or outlying facility if water is not fed there from hospital	1	1 liter
3. Fire hydrant(s)	2	1 liter
B. General potable water system for hospital		
4. Incoming water pipe(s)	2	10 liters
5. Water softener (pre and post)	2	1 liter
6. Preheater (discharge side)	1	1 liter
7. Primary heater (discharge side)	1	1 liter
8. Circulating pump(s)	2	1 liter
9. Holding tanks (cold water, discharge side)	2	1 liter
10. Expansion tank for hot water (if possible)	1	1 liter
11. Back drain on sprinkler system(s) (trap to prevent backflushing may be present and should be sampled)	2	1 liter
12. Fireline where it branches off main system (may be multiple)	1	1 liter
C. Pharmacy		
13. Water used for respiratory therapy equipment	2	≥1 liter
D. Air compressor system		
14. Vacuum water source	1	≥100 ml
Positive pressure equipment side		
15. Condensate from tank(s)	3	≥100 ml
16. Water separator(s) (directly off compressors)	4	≥100 ml
17. Water source(s) near air intake(s)	4	≥100 ml
18. Air samples where patients were ill with legionellosis	3	NA[a]
E. Potable water final distribution outlets		
Hemodialysis water source		
19. Before demineralizer	1	≥1 liter
20. After demineralizer	1	≥1 liter
Intensive care units		
21. Respiratory therapy (patient rooms)	2	1 liter
22. Cardiac	2	1 liter
23. Services with different geographical locations	7	1 liter
24. Ice maker (entry water)		≥1 liter
F. Air-conditioning system		
25. Air handling unit to service where disease occurred (drain pan)	2	≤100 ml
Cooling towers		
26. Blowdown	3	≥1 liter
27. Water supply	1	1 liter
G. Whirlpools		
28. Whirlpool (one nearest air intake system)	1	1 liter
29. Whirlpool drain	1	Wet swab
H. Other		
30. Decorative fountain(s)	1	1 liter
31. Creeks, ponds, and sites of stagnant water	4	>1 liter

[a] NA, Not applicable.

is an acute care facility in the New England Area with approximately 700 beds and 28 buildings. A total of 12 of 15 legionellosis cases were from one of six main buildings, and all isolates from patients were *Legionella pneumophila* serogroup 1 with a monoclonal antibody reactivity pattern to antigens 1,2,4,5 (11). There were at least four municipal water lines leading into the hospital plumbing system. The water was not chlorinated in the hospital system. Hospital B is a northern midwest pediatric hospital with approximately 300 beds. There were three municipal water intake pipes, and a residual chlorine level of <0.5 ppm (<0.5 μg/ml) was present in some of the water samples. At the time of the investigation, there were three confirmed cases of legionellosis. Isolates from patients were also *L. pneumophila* serogroup 1 that reacted with monoclonal antibodies to antigens 1,2,4,5.

Collection and treatment of water samples. The number of samples taken at each site was limited owing to resources and epidemiologic considerations. However, a large number (106) of different sites were sampled in association with hospital A, and 37 sites were sampled at hospital B. Each of the five cooling towers at hospital A was sampled two to three times.

All water samples of ≤1 liter were collected in sterile 1-liter or 250-ml polypropylene containers. Larger samples, such as water from the city treatment plant, were collected in 5-gallon (19-liter) plastic containers. Potable water from faucets was collected after allowing the faucet to run steadily for approximately 2 min. All water samples were shipped in large insulated containers and were received unrefrigerated in the laboratory for processing within 48 h of collection. Potable water samples concentrated by filtration and in some cases (when many nonlegionellae were present) were acid treated as described by Gorman et al. (12). When swabs were used, they were sterile cotton swabs dipped in sterile water before use.

Media and isolation procedures. The following media were inoculated (0.1-ml inoculum per plate) with each concen-

TABLE 2. Results with the environmental sample site protocol

Sample site category	Hospital A		Hospital B	
	Sites positive/ sites sampled	Sites sampled	Sites positive/ sites sampled[a]	Sites sampled
Potable water outside or on the boundary of hospital property	0/8	5 outside fire hydrants Raw, aerated, and refined water at the water treatment plant	0/4	2 fire hydrants Raw and refined water from the municipal water treatment plant
General potable water system for hospital	4/19	6 incoming pipes 6 hot water heaters 3 high zone pumps 2 hot water tank drains 1 hot water heater inlet 1 water line before cooling tower	1/6	3 sites where water enters plumbing system for 3 buildings (1 site/ building) 2 hot water heaters 1 fireline connector
Potable water final distribution outlets	24/43	43 cold or hot water faucets	11/18	5 showers 5 aerators 11 faucets
Air compressor system	5/19	5 compressed air lines 4 water separators 5 compressor water supply lines 2 excess water lines from air compressors Air compressor condensates	0/1	1 air compressor receiver tank
Air-conditioning system	9/10	5 cooling tower sumps (1 tower/ building) Make-up water	1/7	4 cooling tower sumps Make-up water for 3 of the 4 cooling towers
Whirlpools and other	1/7	3 aerators 2 whirlpool drains 1 whirlpool 1 operating room spray humidifier	0/1	Water supply for a dialysis unit

[a] All legionellae isolated from sample sites at hospital B were *L. pneumophila* serogroup 1.

trated or nonconcentrated water sample: two plates of buffered charcoal-yeast extract agar containing alpha-ketoglutarate (αBCYE); two plates of glycine-polymyxin B-anisomycin-vancomycin medium (GPAV), which is BCYE supplemented with glycine (0.3%), polymyxin B (100 units/ml), anisomycin, (80 μg/ml), and vancomycin (5 μg/ml); one plate of αBCYE without L-cysteine; and one plate of GPAV without L-cysteine. The last two plates were used as negative controls. An adjustment was made for dilution or concentration when the CFU of legionella-like organisms were calculated; 100% recovery was assumed after filtration. Several or all legionella-like organism colonies from at least one plate of GPAV per sample were confirmed as legionellae based on colony morphology, L-cysteine requirement, and direct fluorescent-antibody tests (3).

Reactivity to *L. pneumophila* serogroup 1 monoclonal antibodies. *L. pneumophila* serogroup 1 cultures were tested by Roger McKinney (Immunology Laboratory, Meningitis and Special Pathogens Branch, Division of Bacterial Diseases, Centers for Disease Control) for reactions to monoclonal antibodies (15). Reactivity patterns to the antibodies were used to subgroup types.

Immunoautoradiographic procedure. The immunoautoradiographic procedure reported by Martin et al. (14) was used to detect colonies of *L. pneumophila* serogroup 1 containing monoclonal antigen 2. Ascites fluid containing monoclonal antibody to antigen 2 was provided by R. McKinney.

Air-sampling methods. A six-stage Andersen air sampler containing GPAV as the medium for impact of air was used for air sampling at a rate of 0.0283 m^3/min. Under normal circumstances, a selective medium should not be used with the Andersen air sampler, but it was used in this case to avoid overgrowth by nonlegionellae. Air was sampled for 24 and 10 mins, respectively, from air compressor lines and ambient air in five legionellosis patient rooms in hospital A. Also, an all-glass impinger containing 0.25% aqueous yeast extract was used to collect air at a rate of 0.0108 m^3/min for 10 min in each of the five rooms. The broth was stained by the direct fluorescent-antibody procedure and plated (six times) onto αBCYE agar. Plates were incubated for >4 days at 35°C in a humid atmosphere of air plus 2.5% CO_2 and examined periodically for legionella-like organisms.

RESULTS

Table 2 summarizes results from using the sample site protocol (Table 1) as a guide at both hospitals. More samples were taken and were positive at hospital A (43 of 106) than at hospital B (13 of 37).

A variety of legionellae were recovered at hospital A (Tables 2 and 3). In the first category (A), samples of municipal water before it entered a hospital building were negative for legionellae. In the general potable water system (category B), *L. pneumophila* serogroup 1 monoclonal group 1,2,4,5 was isolated from a water tank in building E. In building B, three tanks contained *L. pneumophila*. Serogroups 1 and 3 were present in two tanks, and serogroup 3 was present in the third one. Pharmacy water (category C) was not used for respiratory therapy and was not sampled. In category D, legionellae were not recovered in air samples of the compressed air lines used for respiratory therapy in five patient rooms, but the water separators in the air compressors did contain *L. pneumophila*. Extensive sampling of the potable water final distribution outlets (category E) showed combinations of *L. pneumophila* serogroups 1 and 3 and an unrecognized species in some of the hot and cold water plumbing systems in five buildings (Table 3). In the air-conditioning system (category F), two of five cooling towers contained *L. pneumophila* serogroup 1 monoclonal

TABLE 3. Legionellae isolated from sample sites at hospital A

Category of sample site	Sites with *L. pneumophila*[a]			Other legionellae
	SG-1	SG-3	SG-1 and SG-3	
Air-conditioning system	Cooling towers 2[b], 4[c], and 5[c]		Cooling towers 1[b] and 3[c]	*L. pneumophila* SG-5 in cooling tower 3
	Make-up water for cooling towers 2[b] and 4[c]		Make up water for cooling tower 1	*L. pneumophila* and a previously unrecognized species in make-up water for cooling tower 3
General potable water system	Building E, hot water tank[b]	Building B, low-zone hot water tank no. 2	Building B,[c] low-zone hot water tanks no. 1 and 3	
Cold water—final distribution outlets	Sink faucet in 1 room in building A[b] 1 sink faucet in each of 3 rooms in building B[b,c]		2 sink faucets,[b] one each in a room in buildings A and B	Unrecognized species from sink faucet in 1 room each in buildings B and C
Hot water—final distribution outlets	Water discharge line from hot water heater in building D[b]	Sink faucet in each of 3 rooms in building B and 1 room in building C	Sink faucet[d] in each of 6 rooms in building B and 1 room in building C	
Air compressor system	Air compressor water separators in buildings B[b,c] and C[d]			
Whirlpools, pharmacy, other			Whirlpool[c] Hemodializer water	Unrecognized species also in whirlpool and hemodializer water

[a] SG, Serogroup.
[b] *L. pneumophila* serogroup 1 monoclonal group 1,2,4,5.
[c] *L. pneumophila* serogroup 1 monoclonal group not containing antigen 2.
[d] *L. pneumophila* serogroup 1 undetermined monoclonal group.

type 1,2,4,5. *L. pneumophila* serogroup 5 was found in only one cooling tower. In category G, a whirlpool and water being used with a hemodializer contained a combination of *L. pneumophila* serogroups 1 and 3 and a previously unrecognized *Legionella* species. No samples were taken from sites covered in category H.

L. pneumophila serogroup 1 was isolated from all 13 positive sites at hospital B. The highest numbers of legionellae found in the potable water system were in the hot water tank (4.5×10^7 CFU/liter). Of the final distribution outlets sampled, three aerators and water from one shower head and seven faucets contained *L. pneumophila*. Water outlets in 10 of the 14 patient rooms (71%) were positive for *L. pneumophila*. *L. pneumophila* serogroup 1 monoclonal type 1,2,4,5 was isolated from one shower head, three faucets, and a hot water tank (Table 4). One of four cooling towers used in the air-conditioning system contained *L. pneumophila* serogroup 1. *Legionella* species were not isolated from the water supply for one dialysis unit.

DISCUSSION

Although legionellae were not isolated from water before it entered either hospital property, periodic sampling would be needed to rule out intermittent entry of legionellae from outside sources such as municipal water reservoirs. Legionellae have been isolated from water treatment plants (17, 18).

It is interesting that three serotypes of *L. pneumophila* and another species were coexisting at different sites at hospital A, yet the cases were caused only by one monoclonal type of *L. pneumophila* serogroup 1. Only one of five cooling towers contained serogroup 5 as the predominant type, whereas serogroup 1 was predominant in the other four. Perhaps the microbial flora and environmental conditions were more optimal for serogroup 5 in tower 3, or the contaminating source of legionellae was different for that tower. The predominance of *L. pneumophila* among other legionellae in the two towers implicated as the source for spread of legionellosis may be evidence of its superior ability to survive, compete with other microbial flora, and multiply to a concentration necessary for the transmission of legionellosis.

Fliermans (8) reported that *L. pneumophila* serogroup 1 (Knoxville strain) multiplied and survived longer than representative strains of serogroups 2, 3, and 4 in diffusion chambers at 55°C. Thus, some legionellae have equivalent virulence characteristics, but respond differently to environmental conditions. The ability of a *Legionella* strain to compete with other bacteria and multiply in places such as cooling towers and hot water tanks can be a crucial factor in the transmission of legionellosis.

Use of the protocol revealed that *L. pneumophila* serogroup 1 monoclonal type 1,2,4,5 was in the make-up

TABLE 4. Monoclonal reactivity patterns of *L. pneumophila* serogroup 1 isolates from hospital B

Monoclonal reactivity	Source of sample[a]
1,2,4,5	1 showerhead
	3 faucets[b]
	1 hot water tank
1,4	1 shower
	3 faucets[c]
1,4,6	1 faucet
1,4,5,6,7,8,9	1 shower[d]
No reactivity	1 shower
	3 faucets

[a] Each source is a different sample site unless noted otherwise.
[b] One isolate was from an aerator.
[c] One isolate was from an aerator washer.
[d] This shower is the same one also containing *L. pneumophila* serogroup 1 with a 1,4 reactivity pattern.

water for towers 1 and 2 and presumably was responsible for seeding the towers. These two towers were reported as the source for the outbreak at hospital A (11).

Although no legionellae were isolated from compressed air lines that were supplying air for respiratory therapy equipment, the presence of legionellae in the water separator in the compressed air system poses a potential hazard if a malfunction allows the entry of aerosols from the water separators.

Limited use of the protocol at hospital B did show that *L. pneumophila* serogroup 1 monoclonal type 1,2,4,5 was probably being distributed from the hot water tank to outlets, such as the shower heads. Epidemiologic data had implicated the showers as sources of exposure to the agent.

The isolation of a variety of legionellae at hospital A demonstrated that random sampling without an epidemiologic evaluation and comparing isolates from the environment and from patients could lead to false conclusions about sources of epidemic strains.

The protocol should be streamlined to have only five categories, with water from the pharmacy, whirlpools, and other sites as one category. Also, category A should include a well, and humidifiers should be added to category F. High priority for sampling should be given to the hot water tanks, outlets in the rooms of legionellosis patients, cooling tower water (if it is possible that aerosols of this water could have been breathed by patients), respiratory therapy equipment, and holding tanks for water. The lack of legionellosis cases caused by legionellae other than the case isolate types (*L. pneumophila* serogroup monoclonal type 1,2,4,5) present in the water reinforces the need for documenting an epidemiologic association before disinfection measures are undertaken. Prudent use of the protocol to gather samples in a timely manner should support data from an epidemiologic study.

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