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Factors affecting the recovery of *Legionella pneumophila* serogroup 1 from cooling tower water systems

Hsu-Feng Lu[1], Mei-Fen Tsou[2], Shau-Yen Huang[3], Wen-Cherng Tsai[4], Jing-Gung Chung[5], Ken-Sheng Cheng[2]

[1]Department of Clinical Pathology, Cheng Hsin Rehabilitation and Medical Center, Taipei; [2]Department of Clinical Laboratory, China Medical College Medical Center, Taichung; [3]College of Human Ecology, Fu-Jen University Ecology, Taipei; [4]Department of Microbiology and Immunology, National Yang-Ming University, Taipei; and [5]Department of Microbiology, China Medical College, Taichung, Taiwan, ROC

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A total of 20 water samples collected from the cooling towers at 20 different sites were analyzed under various conditions for the presence of *Legionella pneumophila* serogroup 1. A comparative assessment was performed to evaluate methods of sample collection (spray drops, beneath water at 20- to 40-cm depth, and water outlet), concentration (filtration and centrifugation), acid buffer treatment (no treatment, treatment for 3, 5, and 15 min), and CO_2 incubation or candle jar incubation. The reduction in viable colonies and false negative rate were compared for the different factors. No quantitative differences in isolation of *L. pneumophila* serogroup 1 was found among samples collected from water at a depth of 20 to 40 cm, from water outlet, and from spray drops. Treatment in an acid buffer for 15 min significantly reduced the recovery rate, with a reduction in bacterial counts of about 40%, compared with a 3-min (12%) or a 5-min (25%) treatment. Acid buffer treatment for 3 or 5 min reduced the overgrowth of commensal flora. This treatment improved the selectivity but not the sensitivity for *L. pneumophila* serogroup 1. Colonies on plates incubated at 37°C in a candle jar with a humidified atmosphere grew better than those incubated at 35°C with 5% CO_2. These results demonstrate that methods of sample collection, concentration, and incubation, but not collection site, can affect the isolation rate for *L. pneumophila* serogroup 1.

Key words: Filtration, *Legionella pneumophila* serogroup 1, selectivity, sensitivity

Legionella pneumophila was first isolated in 1977 when it was epidemiologically linked to an outbreak of pneumonia among delegates from the American Legion who attended a convention in Philadelphia [1]. *L. pneumophila* outbreaks are now thought to be seasonally independent. The bacterium is recognized as a very important pathogen that causes nosocomial and community-acquired pneumonia [2]. Legionel -losis is a systemic infectious disease that primarily involves the lungs with multisystemic extrapulmonary manifestations, and is caused by exposure to contaminated residential water sources [3-11]. Patients who have impaired immunity or have undergone surgery recently are at high risk of acquiring legionellosis. Pulmonary and extrapulmonary complications of legionellosis include respiratory failure, lung abscess, and transitory renal impairment. Though uncommon, acute renal failure can also result from legionellosis, and its mortality rate is high [12,13]. Cooling tower systems have been shown to be both the amplifiers and disseminators of *Legionella*, which is the causative organisms of legionnaires' disease [14]. Various procedures, which included filtration and centrifugation methods, have been developed for isolating *L. pneumophila* serogroup 1 from cooling tower water systems [15-22]. Methods for sample collection and isolation of *L. pneumophila* serogroup 1 from cooling tower water systems, however, have not been standardized. Establishing a standardized method to isolate *L. pneumophila* serogroup 1 from cooling tower water systems would improve the efficiency of identification, and this may be done by evaluating the feasibility and sensitivity of the various methods in detecting the presence of *L. pneumophila* serogroup 1. Few studies have compared the various methods for isolating and collecting *L. pneumophila* serogroup 1 from cooling towers. This study was thus designed to determine the followings: (1) whether filtration versus

Corresponding author: Ken-Sheng Cheng, Department of Clinical Laboratory, China Medical College Hospital, 2, Yuh-Der Road, Taichung 404, Taiwan, ROC.

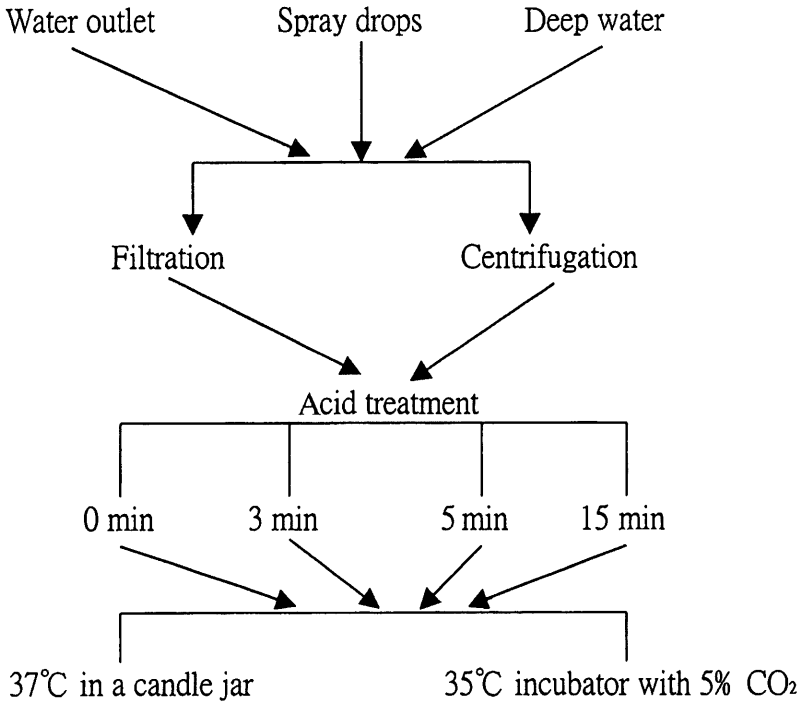

Fig. 1. Flow chart of sample collection and procedure.

centrifugation and candle jar versus CO_2 incubator results in better isolation sensitivity; (2) whether recovery is affected by the duration of acid pretreatment; and (3) which of the available sampling methods provide the most efficient and effective means for the monitoring of cooling tower water systems.

Materials and Methods

Bacterial strains

A total of 20 positive samples of *L. pneumophila* serogroup 1 were collected from cooling towers, which included 4 isolates each from electronic companies, apartment blocks, hospitals, 3 each from restaurants and military bases, and 2 from cinemas. The flow chart of sample collection and processing are shown in Fig. 1.

Culture procedure

Water samples of 200 mL were collected from each of the following 3 sites: water outlets (labeled A), 20 to 40 cm beneath water (labeled B), and spray drops (labeled D). Samples were concentrated by a filtration machine (model WS-666) through a disposable monitor system with 0.45 μm-pore size polycarbon-filter (Difco Laboratories, Detroit, MI, US). The filters were decontaminated by going through 3 min of acid treatment (0.2 M HCl-KCl, pH 2.2), and then rinsed with disinfected water. Buffered charcoal yeast extract with α-ketoglutarate (BCYEα) broth selectively amplified *L. pneumophila* serogroup 1. A 0.1-mL volume of the solution was inoculated on the BCYEα with cephalothin 4 mg/L, colistin 16 mg/L, vancomycin 0.5 mg/L, and cycloheximide 80 mg/L (CCVC) [23]. The plate was incubated at 37°C in a candle jar with humidified atmosphere.

Identification procedures

The suspected *L. pneumophila* serogroup 1 colonies were subcultured on blood agar plate and BCYEα (without CCVC) for verification after 7 days. The suspected colonies that grow on BCYEα with L-cysteine but not on blood agar plate and BCYEα (without L-cysteine) were considered to be potential *L. pneumophila* serogroup 1, which was then determined in an agglutination test (Difco) as well as by screening with hippurate, oxidase, and catalase tests.

Comparison of filtration and centrifugation methods in monitoring *Legionella pneumophila* serogroup 1

For the filtration method, a water sample of 200 mL was collected from a depth of 20 to 40 cm beneath water (labeled B) and was concentrated by a filtration machine. The filter papers were decontaminated by going through 3 min of acid treatment. *L. pneumophila* serogroup 1 was selectively amplified by using BCYEα

Table 1. Factors affecting the isolation rate of *L. pneumophila* serogroup

Items surveyed	Factor surveyed	Colony count (CFU/plate)	*p*
Collection site	Water outlet	278 ± 90	0.62[a]
	Deep water	281 ± 84	
	Spray drops	272 ± 84	
Time treatment	0 min	319 ± 83	<0.05[a]
	3 min	281 ± 84	
	5 min	240 ± 79	
	15 min	189 ± 72	
Extract method	Filtration	281 ± 84	<0.05[b]
	Centrifugation	125 ± 43	
Incubation condition	5% CO_2 incubator	266 ± 84	<0.05[b]
	Candle jar	281 ± 84	

[a]Regression analysis of repeated measure (mixed model).
[b]Paired *t* test.

broth. A 0.1-mL volume of the solution was inoculated onto a BCYEα plate with CCVC. The plate was incubated at 37°C in a candle jar with humidified atmosphere. The suspected *L. pneumophila* serogroup 1 colonies were checked using identification procedures stated above.

For the centrifugation method, a water sample of 500 mL was collected from a depth of 20 to 40 cm, and then centrifuged in a KUBOTA (Model 5800; KUBOTA Corp, Tokyo, Japan) with RA-500/6 angle rotor at 6600 rpm for 10 min to reach 6000 x *g*. All but 1 mL of supernatant was removed, and the tube content was treated with HCl-KCl for 3 min with the sample vortexed for around 30 sec. A 0.1-mL volume of the vortexed solution was then plated on the BCYEα with CCVC.

Effect of acid buffer treatment on the recovery of *Legionella pneumophila* serogroup 1

A water sample of 800 mL was collected from a depth of 20 to 40 cm and then placed in 200-mL bottles. All 4 samples were concentrated using filtration. The collected samples were decontaminated in acid buffer (0.2 M HCl-KCl, pH 2.2) for different durations (0, 3, 5, and 15 min) [9]. The acid buffer was prepared by mixing 3.9 mL of 0.2 M HCl into 25 mL of 0.2 M KCl to yield a buffer solution. The filters were then washed to release the bacteria with sterile water.

Influences of incubation on the recovery of *Legionella pneumophila* serogroup 1

Water samples of 400 mL were collected from a depth of 20 to 40 cm and were put into 2 bottles of 200-mL volume. These samples were then concentrated by filtration. The filter papers were decontaminated by acid treatment for 3 min. A 0.1-mL volume of the washings was inoculated onto BCYEα with CCVC. Two plates were inoculated for each sample. One was incubated at 37°C in a candle jar with humidified atmosphere, and the other was incubated at 35°C in an incubator with 5% CO_2.

Statistics

All procedures were performed by the same operator to eliminate personal variations. The means of the 2 incubation conditions and the 2 extract methods were compared using paired *t* tests. All *p* values given were based on 2-tailed testing. All analytical statistical calculations were performed by using Microsoft Excel software (version 97). The 4 time-treatment periods and the 3 collection sites were compared using regression analysis of the repeated measurement (mixed model). Data were entered into and analyzed by the SAS system (Version 6.12, US). A *p* value of less than 0.05 was taken as statistically significant.

Results

The colony counts of the 20 positive samples treated with different procedures were compared (Fig. 1) to determine which factors significantly affect the recovery of *L. pneumophila* serogroup 1 (Table 1). The numbers of *L. pneumophila* serogroup 1 colonies recovered from samples collected from water outlets (278 ± 90 colony-forming unit [CFU] /plate), at 20 to 40 cm beneath water (281 ± 84 CFU/plate), and from the spray drops (272 ± 84 CFU/plate) did not differ significantly (p=0.62, regression analysis of repeated measurement, mixed model) (Tables 1 and 2). Filtration (281 ± 84 CFU/plate) was much more effective than centrifugation (125 ± 23 CFU/plate) in recovering *Legionella* organisms (p<0.05, paired *t* test) (Table 2). When CFU/mL was considered, however, centrifugation (50 ± 17.2 CFU/mL) had a recovery rate of almost 80% higher than

Table 2. Regression analysis results of repeated measurement (mixed model) for collection sites and acid buffer treatments

Statistical items	Statistical factor	Estimate	Standard error (±)	*p*	*p*
Collection sites	INTERCEPT	271.5	18.7	0.0001	0.6227
	Spray drops	0			
	Under water	9.9	10.8	0.3714	
	Tower outlet	6.95	13.2	0.6053	
Acid buffer treatment	INTERCEPT	319	18.6	0.0001	0.0001
	0 min	0			
	3 min	-37.6	4.0	0.0001	
	5 min	-79.05	4.4	0.0001	
	15 min	-130.3	7.8	0.0001	

filtration (28 ± 8.4 CFU/mL).

Tables 1 and 2 show that samples that did not go through acid buffer treatment have the highest mean number (in CFU/plate) of *L. pneumophila* serogroup 1 ($p<0.05$, regression analysis of repeated measurement, mixed model). The mean plate count for *L. pneumophila* serogroup 1 reduced from 319 ± 83 CFU/plate at 0 min of acid exposure to 189 ± 72 CFU/plate at 15 min (Table 1). The colony loss rates for 3, 5, and 15 min were 12%, 25%, and 40%, respectively. The effects of acid buffer treatment on the recovery rate, the colony loss, and the false negative rate for *L. pneumophila* serogroup 1 were significantly different among the 3-, 5-, and 15-min treatment (Tables 1, 2, and 3). A false negative result was defined as a finding of over 10 CFU/mL after 0-min acid treatment, or a finding of less than 10 CFU/mL after treatment for 3, 5, or 15 min.

When the plating efficiency of incubation conditions was compared, the mean plate count of *L. pneumophila* serogroup 1 reduced from 281 ± 84 CFU/plate for incubation in a candle jar at 37°C to 266 ± 84 CFU/plate at 35°C incubator with 5% CO_2. The colony loss rates for the 35°C-incubator with 5% CO_2 was 5.3%. A significant difference in the recovery of *L. pneumophila* serogroup 1 between the 2 methods is thus resulted ($p<0.05$, paired *t* test) (Table 1), with colonies on plates incubated in 37°C in a candle jar growing better.

Discussion

The 3 collection sites (water outlets, 20 to 40 cm beneath water, and spray drops) showed no significant differences in isolation rates. If convenience was the only consideration, the collection method at 20 to 40 cm beneath water should be used because it is simple and speedy. This is especially true when water samples of 500 mL or more are collected, which can be time consuming if other methods are used. Nevertheless, although cooling towers are routinely cleaned, the decontamination agents were not able to completely eliminate *L. pneumophila*. Under normal circumstances, isolation of *L. pneumophila* is only possible in samples collected at water outlets. Thus, despite the fact that sample collection at a depth of 20 to 40 cm can save time, collecting samples from water outlets would seem to be the best method. Schulz-Robbecke *et al* [24] have shown that the peripheral areas of hot water systems are especially colonized by *L. pneumophila*. Marrie *et al* [25] concluded that each water outlet represents a unique ecological niche for *L. pneumophila*. Further studies are necessary to define the factors that make each outlet unique.

In this study, a comparison was made between membrane filtration and centrifugation for the isolation of *L. pneumophila* from water samples collected at cooling towers. Filtration was much more effective than centrifugation. On average, filtration recovers more than twice the number of *Legionella* organisms than centrifugation, which indicates that some organisms are lost in the process of centrifugation. When CFU/mL was considered, however, centrifugation showed a recovery rate nearly 80% higher than filtration. That the BCYEα broth is not able to grow all the *L. pneumophila* on the filter is a major cause of concern, which explains why the CFU/mL and CFU/plate results are not consistent. In this study, 200-mL rather than

Table 3. Effects of acid buffer treatment on recovery rate, colony lose, and false negative rate of *L. pneumophila* serogroup 1

Duration of acid treatment	False negative[a] (%)	Colony loss (%)	Recovery rate (%)
3 min	0.8	12	88
5 min	3	25	75
15 min	16.7	41	59

[a]0-min acid treatment as 100% recovery rate.

500-mL samples were collected to allow better standardization of sample volume. In terms of the concentration of cooling tower water, filtration is limited by its high sand and soil content–it becomes difficult when samples are highly contaminated with such material, or when they contain certain biocides, because these types of samples do not filter very rapidly. If 500 mL of the water samples were filtered successfully, the recovery of *L. pneumophila* serogroup 1 would be 5 to 6 times that of the centrifugation method, with higher number of colonies per plate (CFU/plate) but colony counts (CFU/mL) would remain constant.

There are widely different standards of quality between drinking water and water used in cooling towers. According to the American Pathogen Association Disinfection Rules, isolation of less than 10 CFU/mL is allowed for cooling tower, but only a much more stringent 0 CFU/mL is allowed for drinking water. Methods for the sampling of drinking water have not been addressed in this study, but we suggest that the filtration method would be much better than the centrifugation method because of the need to detect lower colony counts in drinking water. Filtration can process high volumes of drinking water but not cooling tower water. Thus, filtration is a good method in terms of sensitivity but not quantification. When we compare the positive isolation rate between filtration and centrifugation methods in this study, all samples that were positive isolation by filtration were also positive by centrifugation. Thus the filtration method can lead to a higher false negative rate than centrifugation. One of the factors in the variation of colonization rates between different studies may be centrifuge speed. Many laboratory centrifuges do not reach 6000 x *g*, and this may have caused the colonization rates to vary from one study to another.

Among the different procedures, the duration of acid treatment was the most critical factor in determining the level of organism colonization in cooling tower water. Compared with this factor, the method of concentration, the sampling sites, and the incubation conditions did not affect the recovery rate as much. The colony loss rates for 3, 5, and 15 min of acid treatment were 12%, 25%, and 40%, respectively. A 0-min acid treatment with 100% contamination, however, was not effective in isolating *L. pneumophila* serogroup 1. Calculated of the correlation between duration of acid treatment (X [min]) and recovery rate (Y) yielded the formula $Y = 0.0037X2 - 0.094X + 1.1048$.

The acid buffer treatment, incubation conditions, methods for extract, and collection sites had significantly different effects on the false negative rate for *L. pneumophila* serogroup 1. The procedures w-f-3-37, s-f-3-37, d-f-3-35, d-f-3-37, d-f-0-37, d-c-3-37, d-f-5-37, and d-f-15-37 had false negative rates of 0%, 5%, 0%, 0%, 0%, 0%, 5%, and 15%, respectively. To reach a 90% true positive rate for samples obtained from cooling tower water systems, the samples should not be treated with acid for more than 5 min. Exposure times of 3 or 5 min are effective; *L. pneumophila* serogroup 1 is fairly resistant to the acid for up to 5 min.

Before any guidelines can be applied, the standardized protocols for isolating *L. pneumophila* serogroup 1 should be implemented, because different isolation techniques will yield different colony counts. Based on the findings in this study, we recommend that cooling tower water samples be concentrated by filtration. Filtration but not cen-trifugation can be carried out at site, because it is not possible to transport a centrifuge for such a purpose. Filtration also allows standardization of the sampling method for drinking water, which requires large volumes, which cannot be easily centrifuged. We also recommend a 3-min acid buffer treatment to reduce overgrowth of commensal flora, which, although it does not improve sensitivity, does improve the selectivity for *L. pneumophila* serogroup 1. These results clearly demonstrate that methods of sample collection, concentration, and incubation, but not collection site, can affect the isolation rate for *L. pneumophila* serogroup 1.

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