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Isolation of Amoebae and *Pseudomonas* and *Legionella* spp. from Eyewash Stations

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Forty eyewash units were sampled for protozoa, bacteria, and fungi. Total heterotrophic bacterial counts on nutrient agar and R2A agar (Difco Laboratories, Detroit, Mich.) ranged from 0 to 10^5 CFU/ml, with *Pseudomonas* spp. being the most frequently isolated. Total counts of 10^4 and 10^8 cells per ml were obtained with the acridine orange staining procedure. All samples were examined for *Legionella* spp. by direct fluorescent-antibody staining and by culturing on buffered charcoal-yeast extract agar containing α-ketoglutarate and glycine and supplemented with cycloheximide, vancomycin, and polymyxin B. DNA-DNA hybridization was used to confirm identification of the *Legionella* isolates. Legionellae were detected in 35 of 40 (87.5%) samples by direct fluorescent-antibody staining, with 3 samples yielding both *Legionella* spp. and amoebae. Amoebae identified as *Hartmannella*, *Vahlkampfia*, *Acanthamoeba*, and *Cochliopodium* spp. were detected in 19 of 40 (47.5%) samples. Sabouraud dextrose agar was used to obtain a crude estimate of viable fungal populations. pH, hardness, and ammonia, alkalinity, chlorine, copper, and iron contents were recorded for all water samples collected from eyewash stations; 33% of the samples had ≥10 mg of CO_2 per liter. It is concluded that eyewash stations not regularly flushed and/or cleaned and used to flush traumatized eye tissue may be a source of infection and can contaminate laboratory environments via aerosol transmission.

Stationary and portable eyewash stations which go unused for months or years may represent a reservoir of amoebae, heterotrophic bacteria, fungi, and *Legionella* spp. Free-living amoebae, including *Hartmannella*, *Vahlkampfia*, and *Acanthamoeba* spp., have been isolated from eyewash stations (22). The severity of *Acanthamoeba* eye infections is well documented (8, 14–16, 24). Infections caused by *Acanthamoeba* spp. are generally resistant to antibiotic therapy (14, 24).

Pseudomonas spp. are commonly isolated from soil and water samples. Some species, e.g., *Pseudomonas aeruginosa* and *P. cepacia*, are recognized as human pathogens, while others, formerly recognized as saprophytes and commensals, have been incriminated as opportunistic pathogens. The latter have been found to occur in distilled water and to colonize pharmaceutical supplies, disinfectants, and soaps. *P. aeruginosa* is highly invasive and is a causative agent of eye infections. In addition, such eye infections are resistant to antibiotic therapy. In a nosocomial outbreak, *P. aeruginosa* was shown to be responsible for 6 of 10 eye infections, and in 3 of those cases, the patients lost their sight (9).

Legionellae are ubiquitous in freshwater environments and have been isolated from various aquatic habitats, including ponds, lakes, rivers, and both potable and nonpotable water distribution systems (18). Legionellae have frequently been isolated in association with amoebae, which may serve as a host or provide growth factors for the legionellae, as in the case of *Hartmannella* spp. (6, 23). The primary route of infection by legionellae is considered to be via inhalation. If portable, plastic, self-contained eyewash units or safety showers (connected to the same potable water supplies) become contaminated with legionellae, frequent emptying and filling may result in the formation of potentially infectious aerosols.

In light of the above-described information, it was decided to examine stationary and portable eyewash stations for the presence of heterotrophic bacteria which could pose a threat if the stations were used to flush injured eye tissue as well as serve as a potential source or reservoir of *Legionella* spp.

MATERIALS AND METHODS

Sample collection. Water samples (400 ml) were collected in sterile glass bottles (Corning Glass Works, Corning, N.Y.) filled directly from the eyepieces of 40 eyewash stations examined in this study. The collection bottles were returned to the laboratory immediately after collection for bacteriological and chemical examinations.

Water quality. The pH of each sample was recorded with a compact pH meter (model C-1; Horiba, Kyoto, Japan). The concentrations of NH_4 (10 mg/liter), Fe^{2+} (3 mg/liter), and Cu^{1+} or Cu^{2+} (10 mg/liter) and the total hardness (<50 to >350 mg of $CaCO_3$ per liter) were determined with semiquantitative test strips, the critical detection limits of which are given in parentheses (Merck, Darmstadt, Federal Republic of Germany).

Total and free chlorine (0 to 2.5 mg/liter), carbon dioxide (0 to 100 mg/liter), phenolphthalein alkalinity, and total alkalinity (0 to 300 mg/liter) were measured with titration test kits, the detection ranges of which are given in parentheses (Hanna Instruments, Woonsocket, R.I.).

Subsamples of 10 ml were removed from each sample, fixed in 2% Formalin, and placed in sterile screw-cap tubes for determination of acridine orange direct counts (AODC) by the method of Hobbie et al. (10).

Total viable counts of heterotrophic bacteria and fungi. Counts of the total viable populations of heterotrophic

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bacteria were obtained by plating 0.1-ml and 1.0-ml samples of water collected from the eyewash stations on nutrient agar and R2A agar (Difco Laboratories, Detroit, Mich.) with a sterile glass rod to spread the sample evenly over the agar surface. The inoculated plates were incubated at 20°C for 15 days. Total counts of fungi were determined by plating 0.1-ml and 1.0-ml samples on Sabouraud dextrose agar (Difco) and incubating the inoculated plates at 20°C for 2 weeks, at which time colony counts were tallied.

Identification of isolates. After the heterotrophic bacteria were isolated and purified, the cultures were tested for the Gram and oxidase reactions, the latter with (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine). O/F basal medium (Difco) amended with glucose (11) was used to determine whether the cultures were oxidative or fermentative in their dissimilation of carbohydrates. Other biochemical tests used included gelatin liquefaction, the presence of arginine dihydolase (Thornley's arginine agar), the production of pyoverdin (fluorescein), and growth on MacConkey agar. Growth of the isolates at 4 and 42°C in nutrient broth (Difco) was also examined.

To confirm the identification of bacterial strains isolated from the eyewash water samples, we determined the overall base composition (moles percent G+C content) of the DNA by using the high-pressure liquid chromatography–P1 nuclease method of Tamaoka and Komagata (20) with slight modifications (4). Crude DNA extracted from a 1.5-ml suspension of 10^8 cells per ml was hydrolyzed to nucleosides by the addition of P1 nuclease (Sigma Chemical Co., St. Louis, Mo.) and alkaline phosphatase (Sigma). The nucleoside concentration of the hydrolyzed sample was determined with a high-pressure liquid chromatograph from Beckman Instruments, Inc., Berkeley, Calif. (solvent delivery system, model 110B; integrator, model 427; and variable-wavelength detector, model 165). Nucleosides were eluted with a mixture of 0.2 M $NH_4H_2PO_4$ and acetonitrile (20:1) at a flow rate of 1.5 ml/min and detected at a wavelength of 270 nm.

Direct microscopic enumeration, isolation, and viable counts of *Legionella* spp. Direct fluorescent-antibody (DFA) staining was done with specific conjugates of *Legionella pneumophila* serogroups 1 to 6 and with a conjugate of *L. micdadei*, *L. bozemanii*, *L. longbeachae* serogroups 1 and 2, *L. dumoffii*, *L. gormanii*, and *L. jordanis* (Scimedix, Denville, N.J.). Positive controls of homologous antigens and specific conjugates were included and were stained with each analysis. The staining procedure was as follows. Aliquots (20 μl) were placed in the eight wells of toxoplasmosis slides, air dried, and heat fixed; 20 μl of polyvalent antiserum was added to each well. Slides were incubated in a humid chamber for 30 min at 37°C, washed in phosphate-buffered saline (pH 7.6), rinsed in distilled water, and dried. The specific fluorescence of the preparations was viewed under an epifluorescence microscope (Carl Zeiss, New York, N.Y.).

Total viable counts of legionellae were determined by the following method. After centrifugation (7,000 × *g* for 20 min) of 200 ml of sample water, the precipitate was resuspended in 2 ml of sterile distilled water and selected samples were pretreated with acid (1). Following pretreatment, 0.1-ml portions were spread on duplicate plates of buffered charcoal-yeast extract agar containing α-ketoglutarate and glycine (3 g/liter) and supplemented with the antibiotics cycloheximide (80 mg/liter), vancomycin (5 mg/liter), and polymyxin B (100 IU/ml) (BCYEαGCVP); the plates were incubated at 37°C in a humid 4% CO_2 environment for 5 to 7 days. Colonies consistent with *Legionella* morphology were examined by DFA staining and tested by microplate DNA-DNA hybridization.

Microplate DNA-DNA hybridization. To confirm the identification of *Legionella* spp., we used the microplate DNA-DNA hybridization method combined with biotin labeling and enzymatic colorimetry (3). A suspension of 10^8 cells per ml was prepared from harvested *Legionella* cells (before whole DNA was extracted) and labeled with photobiotin (Vector Labs, Burlingame, Calif.). Microplates containing immobilized DNA prepared from each of 24 *Legionella* species were obtained from Kobayashi Pharmaceutical Co., Osaka, Japan. Colorimetric detection of annealed DNA was done with peroxidase and tetramethylbenzidine as the substrates. Developed microplates were read quantitatively with an automated microplate reader (Lambda Reader; Perkin-Elmer Instrument Division, Norwalk, Conn.).

Isolation of protozoa. To detect bacterivorous protozoa, we used a culture method involving a bacterial suspension as a food source. Subsamples (10 ml) of eyewash water samples were placed in sterile tissue culture flasks, and 100 μl of a suspension of ca. 10^8 cells of *Escherichia coli* per ml in sterile distilled water was added. Flasks were observed daily for 10 days under an inverted phase-contrast microscope. Liquid from positive flasks was placed on non-nutrient agar plates inoculated with a feeder layer of *E. coli* and was examined for protozoa. Amoebae and flagellates from the water samples were identified by characteristic trophozite and cyst morphology with a phase-contrast microscope (Nikon Instrument Group, Garden City, N.Y.). Cysts were identified by direct microscopic observations with oil immersion (magnification, ×100), and amoebae were identified in wet mounts (magnification, ×40).

RESULTS AND DISCUSSION

Water samples were collected from 40 eyewash stations (37 stationary and 3 portable) during the months of September and November 1989. The eyewash stations surveyed were purchased from at least eight different manufacturers and included some models with a single nozzle (hand held) and fountain models with two or six nozzles. No single manufacturer's model could be judged superior to the others when chemical and bacteriological parameters were compared, since water samples from the eyewash stations yielded results which correlated with the number of joints in the copper plumbing and the amount of use rather than the type of fixture. Visible debris or coloration of the water was observed in 27 of 40 (67.5%) first-draw samples from individual stations.

Results of chemical analyses (Table 1) revealed concentrations of Fe^{2+} and Cu^{1+} or Cu^{2+} below the detectable limits of the method used. Similarly, ammonium was not detected in 39 of the 40 water samples tested. Free chlorine was not detectable in 38 of 40 (95%) eyewash station water samples, and total chlorine was not detectable in 35 of 40 (87.5%). Total hardness was 125 mg/liter (as $CaCO_3$) in 92.5% of the samples, and the pH of the water samples ranged from 6.4 to 9.8. The majority (75%) of the samples were in the pH range of 6.4 to 7.4. A total of 13 of 40 (33%) samples tested had CO_2 concentrations higher than 10 mg/liter.

Duplicate tap water samples were collected from three of the buildings which housed some of the eyewash stations to serve as controls. The results for the tap water samples (mean values) were as follows: free chlorine, 1.0 mg/ml; total chlorine, 1.0 mg/ml; pH 7.08; CO_2, 6 mg/liter; hardness,

TABLE 1. Environmental parameters measured for eyewash water samples[a]

Parameter	Mean ± SD (SE)	Minimum	Maximum
pH	7.36 ± 0.64 (0.10)	6.42	9.83
NH_4	0.25 ± 1.58 (0.25)	0.0	10.0
Fe^{2+}	0.00 ± 0.00 (0.00)	0.0	0.0
Cu	0.00 ± 0.00 (0.00)	0.0	0.0
Hardness	129.37 ± 30.42 (4.81)	50.0	250.0
Alkalinity			
Phenolphthalein	3.2 ± 7.3 (1.16)	0.0	30.0
Total	63.9 ± 26.0 (4.12)	18.0	162.0
CO_2	10.0 ± 11.6 (1.83)	0.0	56.0
Chlorine			
Free	0.0 ± 0.1 (0.02)	0.0	1.0
Total	0.0 ± 0.2 (0.03)	0.0	1.0

[a] Data are reported in ppm, except for pH.

>125 mg/liter (as $CaCO_3$); and alkalinity, 66 mg/liter (as $CaCO_3$). Ammonium, copper, and iron were not detectable. DFA staining and culturing on Sabouraud dextrose agar, R2A agar, nutrient agar, and BCYEαGCVP were uniformly negative. AODC for the tap water control samples ranged from 10^1 and 10^2 cells per ml.

AODC for the eyewash water samples were 10^4 to 10^9 cells per ml, while bacterial counts on R2A and nutrient agars ranged from 0 to 10^6 CFU/ml (Table 2). AODC showed a significant correlation ($P < 0.05$, 95% confidence limit) with total viable counts on nutrient, R2A, and Sabouraud dextrose agars (Table 3). Total fungal counts ranged from 0 to 10^4 CFU/ml on Sabouraud dextrose agar, with 17 of 40 (42.5%) samples yielding fungi. The fungi were not identified, since the fungal analysis was limited to enumeration, i.e., a crude estimate of the viable fungal population.

Heterotrophic bacteria isolated in the study were identified with Gram and oxidase reactions, utilization of glucose in O/F medium, gelatin liquefication, production of fluorescein, arginine hydrolysis, growth at 4 and 42°C, growth on MacConkey agar, and G+C content as the determining features. The isolates were pigmented, gram-negative rods, with the majority of the samples producing no change in O/F medium. Oxidase-positive, rod-shaped bacteria with G+C contents of 60 to 69% represented the majority (104 of 142) of the isolates, whereas the G+C contents were 70 to 75% for 15 isolates, 50 to 59% for 11, and 37 to 49% for the remainder. *P. aeruginosa* was not identified in any of the samples. However, on the basis of biochemical characterizations, the majority of the isolates were identified as *Pseudomonas* spp., including *P. putida* and *P. alcaligenes*.

TABLE 2. Results of microbiological analyses of eyewash water samples[a]

Analysis	Mean ± SD (SE)	Minimum	Maximum
AODC	6.17 ± 1.00 (0.15)	4.24	8.66
Viable counts on:			
Nutrient agar	3.55 ± 1.56 (0.25)	0.00	6.38
R2A agar	4.39 ± 1.25 (0.19)	0.00	5.88
Fungal counts on Sabourad dextrose agar	0.88 ± 1.22 (0.19)	0.00	4.49
Legionellae determined by:			
Viable counts	0.16 ± 0.59 (0.09)	0.00	2.72
DFA staining (Lp)	3.47 ± 1.91 (0.30)	0.00	6.79
DFA staining (poly)	1.37 ± 1.82 (0.28)	0.00	4.56

[a] Data are reported as log values. Lp, Sera for *L. pneumophila* serogroups 1 to 6; poly, sera for *L. bozemanii*, *L. micdadei*, *L. longbeachae*, *L. dumoffii*, *L. gormanii*, and *L. jordanis*.

Hartmannella spp. were identified in 12 of the 19 eyewash stations positive for amoebae and often occurred with *Vahlkampfia* spp. *Cochliopodium* and *Acanthamoeba* spp. were detected only once in the different samples. Three species of amoebae remained unidentified, since they failed to form cysts or exhibit typical trophozite morphology. Only three samples contained flagellates, and these were identified as *Monas* sp. and another, as-yet-unidentified, species. Two of three eyewash stations positive for flagellates were also positive for amoebae (Fig. 1). Ciliates were not detected in any of the samples.

DFA staining for *L. pneumophila* serogroups 1 to 6 revealed that 32 of 40 (80%) samples were positive, yielding counts of up to 10^6 cells per ml. However, *L. pneumophila* serogroups 1 to 6 were not recovered by culturing. DFA staining for polyvalent serogroups of legionellae, including *L. micdadei*, *L. bozemanii*, *L. gormanii*, *L. jordanis*, *L. longbeachae* serogroups 1 and 2, and *L. dumoffii*, revealed that 15 of 40 (37.5%) samples were positive, with counts of up to 10^4 cells per ml, but only 3 eyewash station water samples yielded positive cultures (Fig. 1). DFA staining of the water samples culture positive for *L. gormanii* stained with antisera specific for *L. gormanii* revealed approximately 10^5 cells per ml. Nevertheless, upon culturing, only 10^2 CFU/100 ml was recovered. Two of the isolates were identified as *L. gormanii*, while the third isolate was identified as *L. feeleii*, based on DNA-DNA hybridizations.

From the data obtained in this study, it is concluded that stationary and portable eyewash stations, unless regularly maintained by periodic flushing and careful monitoring of the microbiological water quality, can be a source of infection for the potential user. The alarmingly high bacterial counts and frequent isolations of amoebae which were obtained suggest that the same standards that are applied to drinking water should also be considered for eyewash water. It is important to remember that, because these stations are used only in emergencies, the source water may stand in the incoming pipes at room temperature for a year or more if the station is not flushed weekly, as recommended by the American National Standards Institute (Z358-1981).

Carbon dioxide concentrations are often monitored as indicators of polluted or stagnant waters. Lakes and rivers generally have CO_2 levels lower than 10 mg/liter. In this study, 33% of the eyewash water samples yielded more than 10 mg of CO_2 per liter. Stagnant water can often generate large amounts of CO_2 as a result of organic or mineral decomposition. Periodic flushing serves several functions, including physical movement of water, which cleans the pipes, and maintenance of appropriate chlorine concentrations and pH in the plumbing system, both of which may act to control contamination by *Legionella* spp. (19). When the practice of weekly flushing of an eyewash station is not followed after installation of the system, heterotrophic bacteria may be detected in the system, mainly because of biofilm formation along the walls of the pipes and inside the eyewash fixture. Biofilm formation was detected in this study by swabbing with sterile cotton swabs and culturing on R2A and nutrient agars (data not shown).

Small-volume (500-ml), hand-held eyewash bottles were also examined. The data obtained in this study indicated that unused bottles of sterile water remained sterile but that bottles which were used and replaced were contaminated (data not shown). To avoid the potential risk of infection of injured eye tissue, small bottles of sterile eyewash solution

TABLE 3. Correlation among environmental factors measured for eyewash water samples[a]

Factor	Correlation with factor:								
	1	2	3	4	5	6	7	8	9
1									
2	**0.771**								
3	0.021	0.242							
4	**0.329**	**0.462**	**0.353**						
5	−0.041	−0.024	−0.105	0.104					
6	−0.162	0.079	0.017	**0.358**	0.066				
7	−0.111	0.090	0.124	0.148	**0.404**	0.049			
8	0.081	0.272	**0.320**	**0.418**	−0.294	0.128	−0.001		
9	0.077	0.156	0.156	**0.498**	−0.033	0.062	0.053	**0.582**	
10	0.057	0.057	0.266	**0.308**	0.110	0.070	−0.022	0.270	0.184

[a] 1, Hardness; 2, alkalinity; 3, CO_2; 4, AODC; 5, *Legionella* viable counts; 6, DFA staining with sera for *L. pneumophila* serogroups 1 to 6; 6, DFA staining with sera for *L. bozemanii*, *L. micdadei*, *L. longbeachae*, *L. dumoffii*, *L. gormanii*, and *L. jordanis*; 8, viable counts on nutrient agar; 9, viable counts on R2A agar; 10, fungal counts on Sabouraud dextrose agar. Data in the table represent correlations. Boldface type indicates a significant value at $P < 0.05$ (95% confidence).

may be superior to eyewash stations, which use water from the buildings potable water supply. However, a disadvantage of the eyewash bottles is the small volume. When chemical spills to the eyes are involved, copious amounts of water are required to flush the injured area.

DFA staining revealed 10^5 cells of *L. gormanii* per ml, but viable count determinations on BCYEαGCVP yielded only 10^2 CFU/100 ml. The significant difference between the results of DFA staining and total viable count determinations for *L. gormanii* can be attributed to several factors, e.g., an inhibitory effect of antibiotics in the recovery medium on this *Legionella* species and/or a loss of viability caused by acid treatment prior to plating on an antibiotic medium, a step routinely used in the selective isolation of *Legionella* spp. However, the difference observed between the results of total viable count determinations and DFA staining cannot be explained fully by physiological effects. One must always consider the possibility that dead but antigenically intact *Legionella* cells are detected by DFA staining or that a cross-reaction with nonlegionella cells can influence the total direct count. However, it is also possible that unfavorable environmental conditions induce the cells to enter into a dormant, i.e., viable but nonculturable, phase (12).

It is interesting to note that, of the three *Legionella* strains isolated, two were recovered from stationary eyewash stations connected to the potable water supply of the building in which the stations were located. In contrast, *L. feeleii* was recovered from a 5-gal (ca. 19-liter) eyewash unit constructed of molded plastic. The water from this reservoir was emptied and replaced biweekly, providing the potential for infection via inhalation of contaminated aerosols.

The first case of *L. gormanii* infection in humans was

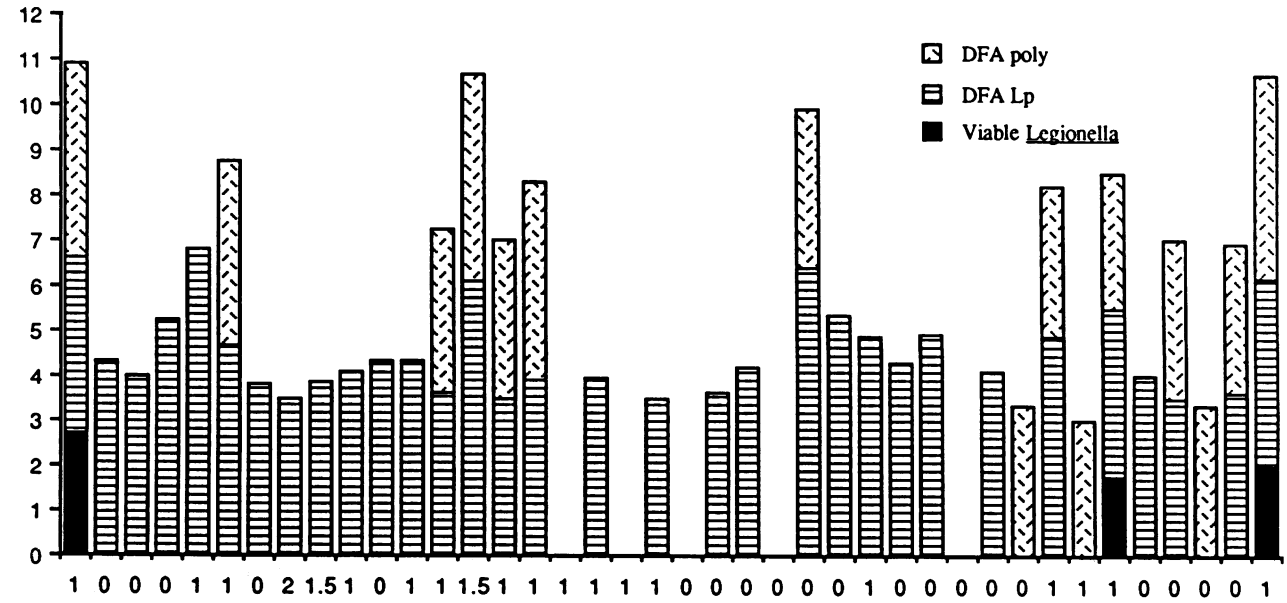

FIG. 1. Results of viable count determinations for *Legionella* spp. compared with results of DFA staining for *Legionella* spp. and observations of protozoa for all 40 samples. On the *x* axis, 1 indicates the presence of amoebae in that sample, 1.5 indicates the presence of amoebae and flagellates, 2.0 indicates the presence of flagellates only, and 0 indicates that no protozoa were detected. Log values are given on the *y* axis. poly, Sera for *L. bozemanii*, *L. micdadei*, *L. longbeachae*, *L. dumoffii*, *L. gormanii*, and *L. jordanis*, Lp, sera for *L. pneumophila* serogroups 1 to 6.

reported in 1988 (7), and that of *L. feeleii* was reported in 1985 (17, 21). That both of these organisms are potentially pathogenic is not in dispute. What has been demonstrated in this study is that there is yet another source of legionellae, one which has insidious implications, since the victims of a possible infection from this source may be individuals already suffering a traumatic injury.

In conclusion, contaminated eyewash stations constitute a potential environmental hazard, since 95% of the stations examined in this study yielded viable counts of heterotrophic bacteria ranging from 10^2 to 10^5 CFU/ml on R2A agar. In addition, 47.5% were positive for amoebae, 42.5% were positive for fungi, and 7.5% were culture positive for *Legionella* spp.

When eyewash stations are used, they create aerosols, and inhalation of aerosols contaminated with *Legionella* spp. has been shown to cause legionellosis. Documented cases of Legionnaires disease and Pontiac fever (a nonpneumonic form of legionellosis) have been attributed to contaminated faucets (2), shower heads (18), a decorative fountain in a hotel lobby (5), and a vegetable misting machine in a supermarket (13). Thus, an airborne route of transmission has been documented for *Legionella* spp., but other routes should not be excluded.

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REFERENCES

1. **Bopp, C. A., J. W. Summer, G. K. Morris, and J. G. Wells.** 1981. Isolation of *Legionella* sp. from environmental water samples by low-pH treatment and use of a selective medium. J. Clin. Microbiol. **13**:714–719.
2. **Botzenhart, K., W. Heizman, S. Sedaghat, P. Heeg, and T. Hahn.** 1986. Bacterial colonization and occurrence of *Legionella pneumophila* in warm water faucet aerators and in drains of hospitals. Zentralbl. Bakteriol. Mikrobiol. Hyg. Ser. A **183**:79–85.
3. **Ezaki, T., Y. Hashimoto, H. Yamamoto, M. L. Lucida, S. L. Liu, S. Kusunoki, K. Asano, and E. Yabuuchi.** 1990. Evaluation of the microplate hybridization method for rapid identification of *Legionella* species. Eur. J. Clin. Microbiol. Infect. Dis. **9**:214–217.
4. **Ezaki, T., S. M. Saidi, S.-L. Liu, Y. Hashimoto, and E. Yabuuchi.** 1990. Rapid procedure to determine the DNA base composition from small amounts of Gram-positive bacteria. FEMS Microbiol. Lett. **67**:127–130.
5. **Fenstersheib, M. D., M. Miller, C. Diggins, S. Liska, L. Dewiler, S. B. Werner, D. Linquist, W. L. Thacker, and R. F. Benson.** 1990. Epidemiology: outbreak of Pontiac fever due to *Legionella anisa*. Lancet **336**:35–37.
6. **Fields, B. S., G. N. Sanden, J. M. Barbaree, W. E. Merrill, R. M. Wadowsky, E. H. White, and J. C. Feeley.** 1989. Intracellular multiplication of *Legionella* in amoebae isolated from hot water tanks. Curr. Microbiol. **18**:131–137.
7. **Griffith, M. E., D. S. Lindquist, R. F. Benson, W. L. Thacker, D. J. Brenner, and H. W. Wilkinson.** 1988. First isolation of *Legionella gormanii* from human disease. J. Clin. Microbiol. **26**:380–381.
8. **Hamburg, A., and J. F. DeJonckheere.** 1980. Amoebic keratitis. Ophthalmologica **181**:75–80.
9. **Hilton, E., A. A. Adams, A. Uliss, M. L. Lesser, S. Samuels, and F. D. Lowy.** 1983. Nosocomial bacterial eye infections in intensive-care units. Lancet **i**:1318–1320.
10. **Hobbie, J. E., R. J. Daley, and S. Jasper.** 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33**:1225–1228.
11. **Hugh, R., and E. Leifson.** 1953. The taxonomic signifigance of fermentative versus oxidative metabolism of carbohydrates by various gram-negative bacteria. J. Bacteriol. **66**:24–26.
12. **Hussong, D., R. R. Colwell, M. O'Brien, A. Weiss, A. D. Person, R. Weiner, and W. Burge.** 1987. Viable *Legionella pneumophila* not detectable by culture on agar media. Bio/Technology **5**:947–950.
13. **LaMaire, W., H. Jackson, and L. McFarland.** 1990. Legionnaires' disease outbreak associated with a grocery store mist machine—Louisiana, 1989. Morbid. Mortal. Weekly Rep. **39**: 108–110.
14. **Ma, P., E. Willert, and K. B. Juechter.** 1981. A case of keratitis due to *Acanthamoeba* in New York and features of 10 cases. J. Infect. Dis. **143**:662–667.
15. **Martinez, A. J.** 1985. Free-living amoebas: natural history, prevention, diagnosis, pathology and treatment of the disease. CRC Press, Inc., Boca Raton, Fla.
16. **Nagington, J., P. G. Watson, T. J. Playfair, J. McGill, B. R. Jones, and A. D. M. Steele.** 1974. Amoebic infection of the eye. Lancet **ii**:1537–1540.
17. **Palutke, W. A., L. R. Crane, B. B. Wentworth, J. G. Geiger, L. Carddozo, A. Singhakowinta, J. Bartley, and B. E. Borbinson.** 1986. *Legionella feeleii*-associated pneumonia in humans. Am. J. Clin. Pathol. **86**:348–351.
18. **Ribero, C. D., S. H. Burge, S. R. Palmer, J. O. Tobin, and I. D. Watkins.** 1987. *Legionella pneumophila* in a hospital water system following a nosocomial outbreak: prevalence, monoclonal antibody subgrouping and effect of control measures. Epidemiol. Infect. **98**:253–262.
19. **States, S. J., C. F. Conley, J. M. Kuchta, R. S. Wolford, R. M. Wadowsky, and R. B. Yee.** 1989. Chlorine, pH, and control of *Legionella* in hospital plumbing systems. J. Am. Med. Assoc. **261**:1882–1884.
20. **Tamaoka, J., and K. Komagata.** 1984. Determination of DNA base composition by reverse-phase high-performance liquid chromatography. FEMS Microbiol. Lett. **25**:125–128.
21. **Thacker, W. L., H. W. Wilkinson, B. B. Plikaytis, A. G. Steigerwalt, W. R. Mayberry, C. W. Moss, and D. J. Brenner.** 1985. Second serogroup of *Legionella feeleii* strains isolated from humans. J. Clin. Microbiol. **22**:1–4.
22. **Tyndall, R. L., M. M. Lyle, and K. S. Ironside.** 1987. The presence of free-living amoebae in portable and stationary eye wash stations. Am. Ind. Hyg. Assoc. J. **48**:933–934.
23. **Wadowsky, R. M., L. J. Butler, M. K. Cook, S. M. Verma, M. A. Paul, B. S. Fields, G. Keleti, J. L. Sykora, and R. B. Yee.** 1988. Growth-supporting activity for *Legionella pneumophila* in tap water cultures and implication of hartmannellid amoebae as growth factors. Appl. Environ. Microbiol. **54**:2677–2682.
24. **Yeoh, R., D. C. Warhurst, and M. G. Falcon.** 1987. Acanthamoeba keratitis. Br. J. Ophthalmol. **71**:500–503.