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Oropharyngeal Colonization with *Legionella pneumophila*

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A total of 186 volunteers, including 40 hospital patients, participated in a cross-sectional survey of oropharyngeal colonization with *Legionella pneumophila*. Colonization was defined as the appearance of any *L. pneumophila* organisms on culture or a positive direct fluorescent-antibody (FA) test or both in the absence of signs or symptoms of pneumonia. The direct FA tests were performed on throat swabs, using a polyvalent conjugate directed against *L. pneumophila* serogroups I through IV. Throat swabs were cultured for *L. pneumophila* on a selective medium. Blood specimens were tested for antibody, using an indirect FA test and heat-killed polyvalent antigen for *L. pneumophila* serogroups I through IV. Eight people, none of whom had pneumonia or fever, had positive direct FA tests; no subject had a positive culture for *L. pneumophila*. Whether the positive direct FA results represent colonization cannot be stated with assurance. In any case, the results suggest that colonization occurs infrequently.

Legionnaires disease has been recognized as the cause of sporadic cases and epidemics of pneumonia since at least 1957 (12, 14). With an estimated case fatality rate of 19%, the disease is considered to be a significant cause of morbidity and mortality. The causative agent, *Legionella pneumophila*, is an ubiquitous water microorganism. It is thought to be spread via an airborne route from contaminated potable water sources and air conditioning systems. The mechanism(s) of transfer from the environment to the lungs is not known; one possible mode is for oropharyngeal colonization to occur first, followed by subsequent aspiration. This study was designed to determine whether oropharyngeal colonization exists with *L. pneumophila*.

MATERIALS AND METHODS

Study of swabs and culture media. One strain of *L. pneumophila* serogroup I, obtained from a patient at the Veterans Administration Wadsworth Medical Center (VAWMC), was used for the determination of plating efficiency of various methods of throat swab collection. The strain had been frozen in vials containing skim milk at −70°C. The vials were thawed at room temperature, and the contents were plated on buffered charcoal yeast extract medium supplemented with 0.1% α-ketoglutarate (BCYEα medium); the plates were incubated for 48 h in a 35°C humidified-air incubator (3). Buffered yeast extract broth with 0.1% α-ketoglutarate (BYEα medium) was then inoculated with each isolate from the BCYEα plates and incubated for 16 h in a shaker water bath at 35°C, producing suspensions of ca. 8×10^8 CFU/ml. Dilutions (10^{-6} and 10^{-7}) of the suspensions were prepared in duplicate in sterile distilled water; these were plated on BCYEα medium, using a 0.1-ml inoculum and a spiral inoculation technique.

We first determined the difference in yield of *L. pneumophila* when calcium alginate swabs (Calgiswab, type II; Inolex, Glenwood, Ill.) were dissolved either in 10 ml of Ringer solution with 1% sodium hexametaphosphate (Calgon Ringer Tablets; Oxoid Ltd., Columbia, Md.) or in 10 ml of distilled water and plated on BCYEα medium and on selective BCYEα medium supplemented with cefamandole, polymyxin B, and anisomycin (BMPAα medium) (3). Ringer-Calgon solution reportedly dissolves calcium alginate swabs within 30 min, with higher yields of bacteria from swabs (10). Four previously weighed swabs were inserted in the BYEα broth containing *L. pneumophila*, and each swab was then weighed separately with an analytical balance. The volume of bacterial suspension contained on the swab was calculated by determining the density of these solutions and the wet weight of the swab. Each swab was then placed in 10 ml of either Ringer-Calgon solution or distilled water and shaken at 200 rpm for 60 min in a 35°C water bath. The suspension was then plated in duplicate on BCYEα and BMPAα media, using a 0.1-ml inoculum to determine the viable count. The remaining six swabs were refrigerated at 5°C, and the same plating was repeated 2, 4, and 6 h later to check for differences in bacterial yield over time. All plates were incubated in a 35°C humidified-air incubator for 4 days and examined daily for growth. In a second experiment, calcium alginate swabs and rayon swabs (Culturette; Marion Scientific, Rockford, Ill.) were compared, using the above experimental conditions.

Subject selection. A cross-sectional survey, performed from September to December 1982, was designed to determine the prevalence of colonization in the study population. The study was performed ca. 1 year after the eradication of endemic Legionnaires

disease from Building 500 of VAWMC. Four groups were chosen to make up a potential study population of 250 people, aged 45 to 59 years. The groups included employees working in the West Los Angeles Federal Building, employees working in Building 218 of VAWMC, and patients housed and nurses working in Building 500 of VAWMC. Building 500 is the main hospital of VAWMC. Building 218 is ca. 0.5 mile (ca. 0.8045 km) north of Building 500 on the medical center grounds and does not house patients. The West Los Angeles Federal Building is situated ca. 1 mile (ca. 1.609 km) east of Building 500 and lies outside the medical center grounds.

The groups were chosen based on differences in variables thought to be related to the risk of colonization, such as health status and exposure to potable water containing *L. pneumophila*. The West Los Angeles Federal Building was chosen because of its location near VAWMC and because of the good health of its employees. Water samples taken from the building previously were culture negative for *L. pneumophila*. Building 218 of VAWMC was chosen because water samples taken from it previously were culture positive for *L. pneumophila*. Employees working in Building 218 were thought to be healthy; however, it was hypothesized that they may have been at an increased risk of colonization due to their exposure to the potable water in the building. Water samples taken previously from Building 500 of VAWMC were culture negative for *L. pneumophila*. Only potable water in Building 500 was hyperchlorinated; no cases of Legionnaires disease had been recognized in occupants of the other buildings.

Sample collection and analysis. The third stage of the study involved sampling and specimen processing. Five milliliters of venous blood were drawn for antibody studies. Throats were then swabbed twice with a calcium alginate swab. The first swab was used to prepare two slides for direct fluorescent-antibody (FA) examination. The second swab was emulsified in 2.5 ml of sterile distilled water and kept refrigerated at 5°C until plated, which was always done less than 4 h after collection. Suspensions were vortexed vigorously using a mixer (Scientific Products, Inc., Bohemia, N.Y.); 0.1 ml was inoculated onto BMPAα medium. BMPAα plates were incubated at 35°C in a humidified-air incubator for 10 days and examined daily for growth of *L. pneumophila*. Specimens were processed on a daily basis. Blood samples were centrifuged, and serum was separated into freezer vials and frozen at −70°C until antibody testing could be performed. The two throat swab slides were heat and Formalin fixed and held for direct FA testing for *L. pneumophila*.

The direct FA test was performed on one of the two Formalin-fixed throat swab slides, using standard techniques (1) and a polyvalent conjugate, obtained from the Centers for Disease Control, Atlanta, Ga., directed against *L. pneumophila* serogroups I through IV (15). This test has a lower limit of detectability of ca. 10^4 bacteria per ml in our laboratory. Positive slides were defined by the appearance of any fluorescing *Legionella*-like organisms. The second Formalin-fixed slide was used for serogrouping any positive polyvalent slides. Serogrouping was performed using monovalent conjugates.

Levels of antibody directed against *L. pneumophila* were determined by an indirect FA assay. The indirect FA test was performed using the frozen serum and standard techniques (16). All specimens were screened at a dilution of 1:128 in combination with a heat-killed polyvalent antigen for *L. pneumophila* serogroups I through IV (7). When specimens showed at least 2+ fluorescence at a titer of 128, the FA test was repeated, using dilutions of 1:32 to 1:1,024 and heat-killed monovalent antigens for *L. pneumophila* serogroups I through IV. For the purpose of calculating geometric mean titers, a value of less than 32 was calculated as 16.

Colonization was defined as the appearance of *L. pneumophila* on culture or direct FA examination or both in the absence of signs or symptoms of pneumonia.

Water cultures. Environmental water samples were taken from each of the three buildings during the period of throat sampling. Nine water samples were taken from Building 500, four were taken from Building 218, and seven were taken from the West Los Angeles Federal Building. The specimen collection and processing were performed using techniques previously described (4).

RESULTS

Recovery efficiency results are shown in Tables 1 and 2. *L. pneumophila* recovery rates were higher on BCYEα than on BMPAα plates. Calcium alginate swabs emulsified in distilled water gave higher recovery percentages than did the same swabs emulsified in Ringer-Calgon solution. The calcium alginate swabs which had been emulsified in Ringer-Calgon solution had not dissolved significantly after 30 min of vigorous shaking. The same was true for swabs held for 6 h. Calcium alginate swabs and rayon swabs that were dissolved in distilled water produced essentially equal recovery percentages. The difference in bacterial recovery seen when swabs were refrigerated at 5°C for 0 to 6 h was minimal. From these results, the decision was made to use calcium alginate swabs and 2.5 ml of sterile

TABLE 1. Percent recovery of *L. pneumophila* (82-063A_1) on calcium alginate swabs in Ringer-Calgon solution and in sterile distilled water, held at 5°C for various times and inoculated on BCYEα and BMPAα media

Time (h)	% Recovery on calcium alginate swab in:[a]			
	Ringer-Calgon solution		Distilled water	
	BCYEα[b]	BMPAα[c]	BCYEα[b]	BMPAα[c]
0	11	2	58	8
2	21	2	44	5
4	11	3	40	5
6	14	3	44	7

[a] Initial inoculum size, 2.54×10^9 CFU/ml of solution.
[b] $P = 0.007$ by paired-t test, two tailed.
[c] $P = 0.016$ by paired-t test, two tailed.

TABLE 2. Percent recovery of *L. pneumophila* (82-063A_1) on calcium alginate swabs and rayon swabs in sterile distilled water, held at 5°C for various times and inoculated on BCYEα and BMPAα media

Time (h)	% Recovery in sterile distilled water on:[a]			
	Calcium alginate swab		Rayon swab	
	BCYEα[b]	BMPAα[c]	BCYEα[b]	BMPAα[c]
0	89	27	146	30
2	77	21	122	36
4	120	28	114	29
6	74	26	67	22

[a] Initial inoculum size, 2.06×10^9 CFU/ml of solution.
[b] $P = 0.27$ by paired-t test, two tailed.
[c] $P = 0.42$ by paired-t test, two tailed.

distilled water as an emulsifying agent when taking throat cultures.

Fifty-nine volunteers from the West Los Angeles Federal Building were enrolled in the study between 29 September and 22 October. Fifteen Building 218 employees were enrolled on 4 November; this represented a low response rate of 35.7%, which could be attributed to the lack of participation by one department. Of 19 people invited to participate from this department, only 1 person participated. Between 26 October and 19 November, 40 patients were enrolled in the study. Seventy-two nurses volunteered for the study between 1 and 15 December.

All 186 throat specimens were culture negative for *L. pneumophila*. The negative results could not be explained by overgrowth of the BMPAα plates with other oropharyngeal flora. Generally, the BMPAα plates appeared to be effective in inhibiting most microorganisms.

The results of direct FA testing can be seen in Table 3. Of the 186 slides tested using the polyvalent conjugate, 8 (4.3%) were positive. Positive direct FA tests were found in 1 of 59 (1.7%) West Los Angeles Federal Building employees, no Building 218 employees, and 4 of 40 (10%) patients and 3 of 72 (4.2%) nurses from Building 500. No fluorescent organisms could be detected using the second slide and monovalent conjugates for *L. pneumophila* serogroups I through IV.

Two of the eight subjects who had positive direct FA smears reported current symptoms of rhinorrhea, nasal stuffiness, or both. One person reported current symptoms of myalgia, headache, dyspnea, and sputum production; he was well enough to work. Two additional people had chest symptoms believed to be due to recently diagnosed carcinoma of the lung. None of these eight subjects reported fevers or chills. One of the eight was receiving co-trimoxazole, given for surgical prophylaxis, at the time of sampling. No positive-smear subject was receiving immunosuppressive drugs or radiotherapy.

The results of the indirect FA test, using the polyvalent antigen preparation for *L. pneumophila* serogroups I through IV, showed geometric mean titers of 41.6 for the West Los Angeles Federal Building group, 111.8 for the Building 500 group of nurses, 51.2 for the Building 218 group, and 60.2 for the Building 500 patient group.

All nine water samples taken from Building 500 were culture negative for *L. pneumophila*. Three of the four water samples taken from Building 218 were culture positive for *L. pneumophila* serogroups I and IV. Sources of positive specimens included the basement hot water heater main drain and two sinks. The seven water samples from the Federal Building were culture negative for *L. pneumophila*.

DISCUSSION

In investigating the use of swabs for culturing the respiratory tract, other investigators have reported that bacterial plating efficiencies are dependent on the specific types of swabs used and on the use of swab suspensions for plating (11, 13). We determined that calcium alginate

TABLE 3. Positive direct FA tests, using polyvalent conjugate for *L. pneumophila* serogroups I through IV, and corresponding indirect FA titers, using polyvalent antigen (PV) and monovalent antigen (MV) for *L. pneumophila* serogroups I through IV

Specimen no.	Respondent location (building)	Direct FA (organisms per smear)	Indirect FA titer				
			PV	MV for serogroup:			
				I	II	III	IV
23	Federal	1	<32				
61	500 (patient)	8	32				
66	500 (patient)	47	256	256	128	64	32
67	500 (patient)	7	32				
106	500 (patient)	1	64				
117	500 (nurse)	1	32				
154	500 (nurse)	3	32				
189	500 (nurse)	2	64				

swabs suspended in distilled water, rather than in Ringer-Calgon solution, were preferable for the recovery of *L. pneumophila*, the reason for which may be inhibition by salt solutions (8). The plating efficiency of calcium alginate swabs suspended in distilled water and plated on BMPAα medium was shown to be between 5 and 30%. These low results were probably due to the adherence of some of the organisms to the swab and to the inhibitory effect of BMPAα medium on *L. pneumophila*. Despite its inhibitory effect, we decided to use this semiselective medium for culturing oropharyngeal specimens to maximize the chance of recovering *L. pneumophila* by preventing its overgrowth by oropharyngeal bacteria.

Using one of our definitions, 4.3% of subjects were colonized with *L. pneumophila*. Due to the low prevalence of colonization, efforts to compare the four groups by the hypothesized risk factors proved unfruitful. The low participation rate from the Building 218 group was unfortunate. This group may have been at an increased risk of demonstrating colonization due to their exposure to potable water containing *Legionella* organisms; however, with an estimated prevalence rate of 4.3%, colonization would probably have been missed in a group of only 15.

Different criteria have been applied for the interpretation of direct FA smears for *L. pneumophila*. The Centers for Disease Control criterion for a positive sputum smear were five or more typical fluorescent forms per smear (*Legionella* Direct Fluorescent Antibody Reagents, product brochure B77, 1982, Biological Products Program, Centers for Disease Control, Atlanta, Ga.). This contrasts with our criterion of one typical fluorescent form per smear, which we have found to be more sensitive, and no less specific, than the higher criterion (6). If the Centers for Disease Control criterion for smear interpretation were used, the frequency of colonization would drop to 1.6%; this lower figure does not change the interpretation of the observations.

Whether the positive direct FA smears were true- or false-positives is open to question for several reasons. One is that none of the direct FA-positive specimens were culture positive. In this laboratory, during the 2-year period of 1981 to 1982, we analyzed only patients not treated with erythromycin; all 58 lower respiratory tract specimens which were direct FA positive were culture positive for *L. pneumophila*. The two exceptions were due to cross-reacting non-*Legionella* organisms. The estimated specificity in our laboratory of direct FA testing for *L. pneumophila* from lower respiratory tract specimens, including sputum, is 99.9%, and the sensitivity is 70 to 75%. Thus, even if disease (colonization) prevalence was as low as 1%, the positive predictive accuracy of a direct FA test would be 88%. This high specificity and positive predictive accuracy have also been found in other clinical and laboratory studies (2, 6, 15).

Another problematic issue is our inability to serogroup the positive direct FA specimens. Cross-reactive organisms capable of causing a falsely positive direct FA test have been reported, although this is a very rare laboratory phenomenon (2, 5, 6, 15). These cross-reactive organisms have been serogroup specific in our unpublished observations, as well as in published reports (2, 5, 15).

Failure to obtain positive cultures probably cannot be explained on the basis of too few organisms present in the sample. One organism per smear represents about 10^4 bacteria per ml of sample. Even when the swab dilution factor (0.08), the volume plated (0.1 ml), and the plating efficiency (5 to 30%) are considered, 10 to 50 CFU should have been observed on the culture plate. This argument assumes that the behavior of plate-passaged organisms is the same as the behavior of organisms found in the oropharynx. This may not be true because nonpassaged organisms appear to be more fastidious (8) and because of inhibition of *L. pneumophila* by oropharyngeal secretions and organisms (9; C. A. Bortner, R. D. Miller, and R. R. Arnold, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, B151, p. 43). This discrepancy might be better explained by a sampling error, consistent with the rare organisms seen on the original smears, rather than by cross-reactive organisms.

Whether the positive FA results represent colonization cannot be stated with assurance. Regardless, the results show that if colonization does exist, it is a rare event.

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