THE ISOLATION AND IDENTIFICATION OF *LEGIONELLA* SPECIES FROM ENVIRONMENTAL SITES IN ROWAN COUNTY, KENTUCKY

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ABSTRACT

THE ISOLATION AND IDENTIFICATION OF LEGIONELLA SPECIES FROM ENVIRONMENTAL SITES IN ROWAN COUNTY, KENTUCKY

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The incidence and density of Legionella species in 44 water samples from various environmental sites were determined by direct immunofluorescence (DFA) and buffered charcoal-yeast extract (BCYE) culture media. Legionella-like organisms were detected in 33 of 44 (75 percent) samples using DFA, whereas only 10 of 44 (23 percent) samples were positive on culture media. Individual groups of samples were screened to determine the presence of fluorescing Legionella-like cells and colonies. Raw water samples were 75 percent (6 of 8) DFA positive and only 38 percent (3 of 8) culture positive. Air-conditioning cooling towers revealed 57 percent (4 of 7) DFA positive and 29 percent (2 of 7) culture positive samples while condensates showed only 43 percent (3 of 7) DFA positive and 14 percent (1 of 7) culture positive samples. Potable water samples, however, were 77 percent (17 of 22) DFA positive but only 18 percent (4 of 22) culture positive. These positive isolates were morphologically consistent with Legionella organisms.

On further analysis of selected raw water and cooling tower samples, using serospecific reagents, L. pneumophila Serogroups 1, 3, 4, and 6 (Knoxville 1, Bloomington 2, Los Angeles 1, and Chicago 2 respectively) were observed. Knoxville 1 and Bloomington 2 occurred in 45 percent and 64 percent of the samples respectively, whereas Los Angeles 1 and Chicago 2 were observed in only nine percent of the samples. Two other Legionella species, L. gormanii and L. micdadei occurred in 36 percent and 45 percent of the samples respectively. The heterotrophic plate counts revealed a range of about 10^6 to 10^8 colony-forming units/liter raw water sample, whereas the concentrations of colonies in potable water samples were generally reduced 10- to 100-fold. However, the number of cells reacting with Legionella-specific fluorescent antibody conjugates in raw water samples and cooling tower samples ranged from about 10^4 to 10^5 cells/liter.

These findings indicate that Legionella organisms are widespread in nature. They also suggest the need for further studies to identify biocidal agents effective in eliminating Legionella bacteria from air-conditioning cooling towers and potable water. The apparent need for regularly monitoring of environmental sites, especially in hospitals and nursing homes, for Legionella is emphasized by this study.

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LIST OF TABLES

LIST OF TABLES
(Continued)

LIST OF FIGURES

TABLE OF CONTENTS

TABLE OF CONTENTS
(Continued)

CHAPTER I

INTRODUCTION

Historical Significance of Legionnaires' Disease

Legionella pneumophila, a bacterium, is one of the most common causes of Legionnaires' Disease (LD), (Fraser et al., 1977; McDade et al., 1977; Chandler et al., 1977). The disease has been recognized both in the United States and abroad as a pulmonary infection (Fraser et al., 1977). The disease's name was derived from the 1976 pneumonia outbreak among conferees at an American Legion convention in Philadelphia, Pennsylvania.

Since 1976, investigators have attempted to discover an environmental source of the etiological agent and to trace the agent's mode of transmission. To date, the environmental sources of the LD bacterium include wet and moist areas, such as ponds (Fliermans et al., 1979), lakes (Morris et al., 1979; Fliermans et al., 1979 and 1981; Tison et al., 1980), streams, creeks or rivers (Fliermans et al., 1979 and 1981; Morris et al., 1979; Politi et al., 1979), plumbing or water distribution systems in hospitals and hotels (Tobin et al., 1981), soil (Storch et al., 1979; Blaser et al., 1979), and air-conditioning systems (Glick et al., 1978; Morris

et al., 1979; Cordes et al., 1980; Dondero et al., 1980; Band et al., 1981).

The 1976 Legionnaires' Disease outbreak affected 182 Legionnaires at their annual convention in Philadelphia (Fraser et al., 1977; McDade et al., 1977; Chandler et al., 1977). This new disease entity caused 147 (81 percent) hospitalizations and 29 (16 percent) deaths (Fraser et al., 1977; McDade et al., 1977).

By November, 1978, the Center for Disease Control (CDC) in Atlanta, Georgia had confirmed 558 cases associated with outbreaks of LD as well as 496 sporadic cases in the United States. The disease has occurred in 43 states and the District of Columbia, as well as in other countries, including Canada, Scotland, England, Wales, the Netherlands, Sweden, Denmark, Spain, Italy, Israel, and Austria (Lawson et al., 1978; Macrae et al., 1978; Reid et al., 1978; Meyer et al., 1980). Retrospective evidence for the existence of LD predates the outbreak of 1976, the earliest documented case having occurred in 1947 (McDade et al., 1979; Meyer and Finegold, 1980).

Legionnaires' Disease can range in severity from a mild respiratory illness without pneumonia to a severe multisystem disease involving the lungs, liver, intestines, kidneys, and the central nervous system

(Beaty et al., 1978; Fliermans et al., 1978; Kirby et al., 1978). Epidemiologic and laboratory investigations yield considerable information concerning LD. It has been estimated that 25,000 of the 800,000 annual cases of undiagnosed pneumonia in the United States are cases of Legionnaires' Disease (Fraser and McDade, 1979; Fraser, 1980). Continued epidemiologic investigation may reveal, not only the source of epidemic outbreaks, but also, the potential for outbreaks of legionellosis.

Need for the Study

In view of the epidemic outbreaks and sporadic cases of LD in both the United States (Meyer, 1980) and the United Kingdom (Macrae, 1977), the pathogenesis of the bacterium and a high case-fatality ratio (discussed in Chapter II), there is a need to know more about L. pneumophila, its ecology, mode of transmission, and infection, as well as incidence. A review of the literature (Chapter II) indicates that efforts to isolate the organism from environmental sites in Kentucky have not been reported. Therefore, it would be important to ascertain whether or not the bacterium can be isolated from environmental sites in the state. If it is found in Rowan County, Kentucky (a site selected for convenience), it may be necessary to develop procedures to prevent a

possible outbreak of legionellosis, and to alert area physicians to the prevalence of this organism.

Area physicians and pathologists have expressed an interest in documenting the presence of L. pneumophila associated with environmental sites. Cultures are not usually taken from pulmonary patients to determine the etiological agent responsible for their pneumonia. All such patients are routinely treated with erythromycin or other broad spectrum antibiotics (Clark, 1983). Therefore, it would prove helpful to physicians to be able to relate the prevalence of environmental Legionella to the incidence of LD in the Gateway Area Health District. In future studies, it may be possible to correlate this information with the incidence of Legionella in other areas of Kentucky.

Objectives of the Study

The objectives of this study are:

1. To document the presence of Legionella pneumophila in Rowan County environmental sites and to identify those conditions which might be conducive to transmission and sporadic outbreaks.

2. To identify habitats where Legionella pneumophila is present and to better understand the ecology of the bacterium.

3. To determine the effectiveness of typical water treatment procedures in eliminating Legionella from source water.

4. To influence future medical and public health procedures by making area physicians more cognizant of the presence, or absence, of Legionella pneumophila.

5. To become familiar with Legionella sampling methods, and isolation and identification techniques that will provide information potentially useful in future epidemiologic investigations.

6. To encourage local physicians and pathologists to participate in short term programs designed to determine the past and future incidence of legionellosis in Rowan County and surrounding areas.

CHAPTER II

LITERATURE REVIEW

Background

Legionnaires' Disease, since first reported as a new disease entity in the summer of 1976 (Sharror et al., 1976), has been recognized as a pulmonary infection caused by a bacterial pathogen (Fraser et al., 1977; Australia, 1978; Macrae et al., 1978). The causative agent has been identified as Legionella pneumophila (Australia, 1978; and Sanford, 1979), a faintly staining, thin, pleomorphic gram-negative bacillus that slowly multiplies in aerobic conditions (McDade et al., 1977). The bacterium typically measures 0.3 to 0.9 μm by 2 to 4 μm. However, some cells have been reported to measure up to 20 μm in length (McDade et al., 1977; Chandler et al., 1979; Fraser et al., 1981). It is weakly oxidase-positive, catalase-positive and gelatin positive (Finegold and Martin, 1982). This fastidious bacterium requires cysteine and ferric salts for growth, has an optimum growth temperature of 35°C and thrives in a pH from 6.9 - 7.0, under an atmosphere of 2.5 - 5.0 percent carbon dioxide (Brenner et al., 1978; Alexander and Dismukes, 1979).

Antibodies have been detected, retrospectively, in stored serum, indicating that L. pneumophila was responsible for several earlier outbreaks of previously unexplained acute febrile pulmonary infections (McDade et al., 1977; Glick et al., 1977; Terranova et al., 1978; Lawson et al., 1978; Fraser et al., 1978; Osterholm et al., 1983).

Definition and Manifestation of the Disease

Legionellosis is an acute infection of humans occurring in at least two clinically and epidemiologically distinct syndromes: (1) Legionnaires' Disease, is characterized by pneumonia and a high case-fatality ratio, with an incubation period of two to 10 days [mean of 5.5 days] (Fraser et al., 1977; Kirby et al., 1978; Tsai et al.; 1979; Band and Fraser, 1981), and (2) Pontiac Fever, a non-pneumonic, self-limited, acute febrile illness without associated deaths (Band and Fraser, 1981). Pontiac Fever is characterized by a short incubation period (one-to-two days) and equally short duration, lasting two to five days, and does not cause permanent sequella (Kaufmann et al., 1981).

Tsai et al. (1979), reported that LD has a broader manifestation range, varying from a mild grippe to a fulminant multisystem disease. The typical patient has

an abrupt onset of illness with anorexia, malaise, diffuse myalgias, and non-productive cough, recurrent chills and fever, and a temperature ranging from 102° to 105°F (38.9 to 40.6°C). Most commonly, there are localized or diffused pulmonary rales with or without signs of pleural effusion. Usually, dyspnea and pleuritic pain are present and the patient appears toxic and seriously ill. A characteristic group of extrapulmonary symptoms and signs, which also tend to aid in diagnosis, may be present. They include headache, delirium, vomiting, diarrhea and abdominal pain (Fraser et al., 1977; Kirby et al., 1978; Lattimer et al., 1978; Alexander et al., 1979; Evans, 1981).

Laboratory findings for LD patients have included a mild to moderate leukocytosis ($\leq$20,000 wbc/mm^3) with a predominance of polymorphonuclear cells, elevated liver enzymes, hyponatremia, hypophosphatemia, proteinuria and microscopic hematuria (Fraser et al., 1977; Tsai et al., 1979).

Outbreaks and Sporadic Cases

Numerous outbreaks of LD (Fraser et al., 1976) and one of Pontiac Fever (Glick et al., 1968) have been recognized since the benchmark outbreaks. Outbreaks have ranged in size from eight to 221 cases. Attack rates

have typically been 0.5 to 5.0 percent in outbreaks of LD and 95 percent to 100 percent in outbreaks of Pontiac Fever (Fraser, 1981). The 1976 Philadelphia outbreak was the most severe. Alexander et al. (1979), Fraser et al. (1979), and Fraser and McDade (1979), reported that the 182 persons who contracted LD either attended the convention or had other business at, or near, the Bellevue - Stratford Hotel. A total of 221 people became ill; 34 died of pneumonia or related complications. Environmental investigations at the time failed to detect the source of the infecting organism (Fraser et al., 1977).

In retrospect, there had been at least three major outbreaks of pulmonary illness resulting from exposure to Legionella in the United States prior to July, 1976. In two instances, diagnosis was made on sera collected from affected patients and stored for more than eight years. The sera yielded evidence of infection with Legionella (McDade et al., 1977; Glick et al., 1978). The first of these outbreaks occurred in the summer of 1965 at the St. Elizabeth Psychiatric Hospital, with 17 deaths (Thacker et al., 1978; Band and Fraser, 1981). The second outbreak occurred in the summer of 1968 among 144 persons who either worked in, or visited the county health department building in Pontiac, Michigan (Glick et al., 1978). Serologic and cultural studies conducted at the

time failed to identify an etiological agent. However, Band and Fraser (1981), reported that only those persons who were in the health department building when the central air-conditioning system was in operation became ill. The illness had an unusually high attack rate (95 percent) among exposed individuals. Nevertheless, there were no deaths and this outbreak of self-limiting, mild febrile illness has since been referred to as "Pontiac Fever" (Alexander et al., 1979; Kaufmann et al., 1981). Table 1 compares Legionaires' Disease and Pontiac Fever.

The third outbreak (Terranova et al., 1978) occurred in Philadelphia in September, 1974. It involved attendees at an Independent Order of Odd Fellows Convention. The convention was held in the same hotel which two years later hosted the Legionnaires.

Retrospective serologic analysis confirmed that Legionella had caused the illness among the conventioneers. Alexander et al. (1979), also reported that the clinical feature of the pulmonary illness and the case-fatality rate were nearly identical to those observed among the Legionnaires in July, 1976. However, Meyer and Finegold (1980), showed that LD occurred in 2.9 percent of 392 respondents. This was lower than the 4.0 percent overall attack rate in the 1976 outbreak (Fraser et al., 1977).

TABLE 1

Comparison of Legionnaires' Disease and Pontiac Fever*

Parameters	Legionnaires' Disease	Pontiac Fever
Attack rate	1-5 percent	95-100 percent
Incubation period	2-10 days	1-2 days
Symptoms	Fever, cough, muscle ache, chills, headaches, chest pain, sputum, diarrhea, confusion	Fever, cough, muscle ache, chills, headaches, chest pain, confusion
Effects on lung	Pneumonia Pleural effusion	No pneumonia Pleuritis
Other affected organ systems	Kidney, liver, gastro-intestinal tract, nervous system	None
Case-fatality ratio	15-20 percent	No fatalities

*Modified from Fraser and McDade, (1979).

By the end of 1978, nine additional epidemic outbreaks had been recorded, resulting in 550 confirmed cases of LD and 70 deaths (CDC, 1978). At three of the outbreak sites, L. pneumophila was isolated from either air-conditioning cooling towers or evaporative condenser water. Epidemiological data from these outbreaks suggested that the cooling tower was the source of organism dissemination (Fraser et al., 1980). A summary of legionellosis outbreaks is shown in Table 2.

Other sporadic outbreaks and case clusters have been documented. Nine cases of LD, associated with exposure in two hospitals with recent excavation (Meyer et al., 1980), were identified in Columbus, Ohio, in 1977. Meyer also reported that in Kingsport, Tennessee, in August and September, 1977, 33 cases were identified in a hospital, and in the community. In Bloomington, Indiana, 39 cases were identified, with four deaths between May, 1977 and August, 1978 (Politi et al., 1979). In 1977, Burlington, Vermont recorded 69 confirmed and presumptive cases of LD, with 17 deaths (Broome et al., 1979). A cluster of eight cases was reported in an Atlanta, Georgia, Country Club in 1978. Another cluster of approximately 33 confirmed and presumptive cases was recorded in a Memphis, Tennessee hospital and the nearby area (Meyer et al., 1980). They also reported that

TABLE 2

Summary of Legionnaires' Disease and Pontiac Fever Outbreaks*

Location of Outbreak	Date	Number of Cases	Incubation Period	Syndrome	Case-Fatality Ratio (%)
Hospital; Washington, D.C.	1965	81	-	LD	17
Health Department; Pontiac, MI	1968	144	36 hours	PF	0
Hotel; Benidorm, Spain	1973	8	-	LD	38
James River, VA	1973	100	37 hours	PF	0
Odd Fellows Convention; Philadelphia, PA	1974	11	1-9 days	LD	10
American Legion Convention; Philadelphia, PA	1976	182	2-10 days	LD	16
Nottingham, England	1976-78	41	-	LD	27
Hospital; Columbus, OH	1977	15	-	LD	13
Hospital; Kingsport, TN	1977	33	-	LD	19
Hospital; Burlington, VT	1977	69	-	LD	25
Hospital, Los Angeles, Ca	1977-79	106	-	LD	26
Hotel, Bloomington, IND	1977-78	56	4-12 days	LD	12
Country Club; Atlanta, GA	1978	8	-	LD	13
New York City, NY	1978	38	-	LD	8
VFW Convention, Dallas, TX	1978	18	-	LD	6
Hospital; Memphis, TN	1978	39	2-10 days	LD	13
Hospital; Norwalk, CN	1978	28	-	LD	36

*LD = Legionnaires' Disease; PF = Pontiac Fever.

between August and September, 1978, the garment district of New York City recorded approximately 398 confirmed and presumptive cases of LD (Meyer et al., 1980).

The spordaic cases confirmed by the CDC extend over most of the United States. However, most of the cases, according to Meyer et al. (1979), have been in the northeast and middlewest with a late summer to early fall peak incidence. Patient age has ranged from 2 to 84 years with a median of 54, with males predominating (Fraser et al., 1979). However, Fisher-Hoch et al. (1981), reported an unusual case of a six-month-old female contracting the disease.

The incidence of Legionnaires' Disease has been two to four times greater in men than women (Fraser, 1981; Band and Fraser, 1981; Helms et al., 1981). A summary of the age-sex ratio for Great Britain is shown in Table 3.

Nosocomial Legionnaires' Disease

Hospital-acquired or nosocomial infections are said to develop two or more days after hospitalization and are not present or incubating on admission, or within 14 days of discharge from the hospital (Helms et al., 1983). Since the original description of the outbreak of respiratory illness due to infection by Legionella

TABLE 3

Age-Sex Ratio in Legionnaires' Disease in Great Britain*

Age (Years)	Cases Identified		
	Male	Female	Total
20 - 29	3	2	5
30 - 39	9	0	9
40 - 49	12	6	18
50 - 59	17	6	23
60 - 69	14	8	22
70 - 79	6	1	7

*Bartlett, C.L.R. (1979): a survey of sporadic Legionnaires' Disease in Great Britain between January 1, 1976 and September 30, 1978.

pneumophilia, nosocomial infections with this organism in transplant and other compromised patients has been reported both in the United States and in other countries. Gum et al. (1979), reported that nine cases with underlying chronic disease or immunosuppression were detected in the summer of 1978 and accounted for 11.6 percent of nosocomial pneumonias. In another report, Helms stated that between March and December, 1981, 24 cases of nosocomial LD were identified at the University of Iowa Hospital. Of the 24 cases, the median age of the patients was 43 years; the youngest patient was a 10 year-old girl who had a bone marrow-transplantation, whereas the oldest patient was a 75 year-old man with chronic diseases. Males outnumbered females, three to one, and most of the patients were immunocompromised, and had underlying diseases.

In the new Veterans Administration Wadsworth Medical Center, (Western Los Angeles) California, 111 hospital-acquired cases, with 17 deaths were confirmed between May, 1977 and August, 1979 (Kirby et al., 1978 and 1979; Haley et al., 1979). Haley further observed that LD accounted for 8 of 73 cases of nosocomial pneumonias, and for 4 of 14 fatal nosocomial pneumonias.

A CDC study of nosocomial pneumonia in 40 hospitals showed that LD accounted for 3.8 percent of the fatal

cases (Cohen et al., 1979). This, according to Meyer et al. (1980), suggested that 950 cases of fatal nosocomial LD occur yearly in the United States.

Sporadic Cases Outside United States

Since the 1976 Philadelphia outbreak, the disease has appeared as a sporadic respiratory illness in the United Kingdom and other countries in Europe (Macrea et al., 1977; Lawson et al., 1978; Reid et al., 1978; Bartlett et al., 1979). Nineteen cases, with two deaths, occurred in Nottingham, England between 1976 and 1978 (Meyer et al., 1980).

During a five-year period, between 1973 and 1978, tourists from Scotland and other parts of the United Kingdom have accounted for at least 10 cases of LD, with five deaths, after they stayed at the same hotel in Benidorm. Other tourists from western and northern Europe have acquired LD on Mediterranean cruises and trips to Corfu, France, Germany, Italy and Portugal (Kirby et al., 1979). Other sporadic cases have been reported in Australia, Canada, the Netherlands, Norway, Israel, Italy, Greece, Yugoslavia, South Africa, Denmark, Sweden, Austria and Switzerland (Bergman et al., 1979; Fraser and McDade, 1979; Kirby et al., 1979; Meenhorst et al., 1979; Fraser et al., 1979 and 1981; CDC, 1980

and 1981; Kaplan et al., 1980). It is interesting that cases have been discovered in almost every area in which they have been carefully sought (Fraser and McDade, 1979).

Predisposing Factors

Most outbreaks of legionellosis have occurred in July, August and September, although sporadic cases have been identified throughout the year (Fraser, 1981). Cohen and Storch et al. (1979), and Band and Fraser (1981), stressed that factors which tend to increase the risk of sporadic LD include cigarette smoking, heavy alcohol use, construction work, residing near excavation or construction sites, travel and immunosuppressive medications. Others have reported that late middle-age and elderly males are also considered high risk for developing LD (Kirby et al., 1978 and 1979; Fraser et al., 1979; Haley et al., 1979; Politi et al., 1979; Broome et al., 1979; Dondero et al., 1980). Also at risk are patients with a history of chronic lung disease, cancer, arteriosclerotic cardiovascular disease, diabetes mellitus and those who have received renal homografts (Broome et al., 1979; Haley et al., 1979; Meyer and Finegold, 1980). Predisposing factors are summarized in Table 4.

TABLE 4

Summary of Factors that Predispose to Legionellosis*

Risk Factors	Associated With Outbreaks (+ = Yes, - = No)	Associated With Sporadic Cases (+ = Yes, - = No)
Advancing age**	+	+
Male sex	+	+
Underlying chronic disease	+	+
Immunosuppressive therapy	+	+
Cigarette smoking	+	+
Heavy alcohol consumption	-	+
Residence near ongoing construction or excavation	+/-	+
Recent travel	+/-	+

*Modified from Band and Fraser, 1981.

**Late middle-age to elderly

Mode of Transmission

Investigation into the mode of LD transmission during the 1976 Philadelphia outbreak included the following general categories: person-to-person, food, tobacco, alcohol, water, animals, ice, fomites and air (Fraser et al., 1979). However, after further investigation, conclusive proof of the mode of transmission was not determined. It became clear from the studies of families of those who did not attend the convention that secondary person-to-person spread was not the mode of transmission. Also the uniform attack rate across districts militated against any possibility of person-to-person contact. Similarly, other factors do not support direct implication of the other general categories except air. Band and Fraser (1981), reported that airborne transmission of L. pneumophila has been the only documented natural mode of spread, although in most outbreaks and in sporadic cases, the mode of transmission has not been proven.

In the 1968 outbreak of Pontiac Fever (see Outbreaks and Sporadic Cases, this Chapter), it was found that only persons who visited the health department building when the air-conditioning system was operating became ill (Glick et al., 1978). To

test the airborne theory of transmission, guinea pigs were exposed to unfiltered air in the building and they developed pneumonia, whereas animals in the control group, exposed only to filtered air, were largely unaffected. Similarly, aerosols of water from the evaporative condenser in the health department building also produced pneumonia in guinea pigs (Fraser, 1981; Kaufmann et al., 1981).

In the 1976 outbreak in Philadelphia, certain observations suggested that the disease might have been spread through the air. Fraser and McDade (1979), observed that Legionnaires who became ill had spent, on the average, about 60 percent more time in the lobby of the Bellevue - Stratford Hotel than those who remained healthy. The affected Legionnaires also spent more time on the sidewalk in front of the hotel than had their unaffected fellow conventioneers. The report suggested that if the disease agent had contaminated the air in the lobby and passed through the front door to the sidewalk, it could have affected Legionnaires and other hotel visitors in just that pattern - and it did. It appeared, therefore that the most likely mode of transmission was airborne (Fraser and McDade, 1979).

In another outbreak of LD in a Memphis hospital in 1978, the case distribution closely corresponded to air

patterns from a contaminated cooling tower (Dondero et al., 1980). A significant association was noted between acquisition of LD and hospitalization in rooms that received their air supply from intake vents located near the auxillary cooling tower.

The Atlanta Country Club outbreak (Band and Fraser, 1981) in 1978 was supportive of the hypothesis of airborne transmission. The illness was associated with increased golfing activity in the two to ten days prior to onset of symptoms. This resulted from increased exposure to the exhaust from the evaporative condenser which was projected toward the practice area. Isolation of L. pneumophila from the evaporative condenser, located at the clubhouse, further strengthened the case for airborne transmission. Band and Fraser (1981), concluded that it was very likely that the infection occurred through inhalation of the bacteria as droplet nuclei.

There have been, however, other outbreaks of LD that have occurred at sites without air-conditioning or heat-rejection systems, but the evidence supports the theory of airborne transmission. In the 1965 St. Elizabeth's Hospital outbreak, the illness was significantly associated with exposure to the hospital grounds or sleeping by open windows (Band and Fraser, 1981). During that summer, extensive excavation had been

undertaken on the hospital grounds for installing a new lawn-sprinkler system. It was postulated that L. pneumophila became airborne and was disseminated during excavation or back filling, thus contaminating the air. CDC investigators found that it was primarily those patients whose beds were near windows in buildings close to excavation sites who became ill (Fraser and McDade, 1979). Fraser and McDade (1979), further observed that cases were clustered in time, with each cluster coming five or six days after an excavation site had been filled. The data suggest that contaminated dust raised in the process was spread through the air and infected the patients. The 1973 outbreak in Spain was also shown to be unrelated to air-conditioning or heat-rejection systems (cooling tower, evaporative condenser, steam-turbine condenser); all of which have been epidemiologically implicated as vehicles of transmission (Band and Fraser, 1981).

Diagnosis of Legionellosis

Clinical diagnosis of Legionnaires' Disease may be difficult because it can resemble other common bacterial pneumonias or infections caused by other organisms, most notably Mycoplasma pneumoniae, Chlamydia psittaci (psittacosis), Coxiella burnetti (Q Fever), Francisella

tularensis (tularemia), influenza, Histoplasma capsulatum, Coccidioides immitis and Taxoplasma gondii (Band and Fraser, 1981). However, there are epidemiological features, or clues, that can assist in the diagnosis of LD. They include: absence of evident person-to-person spread or notable contact with animals, occurrence predominantly in middle-aged and elderly men, incubation period of two to ten days, history of heavy cigarette smoking or alcohol use, exposure to construction or excavation sites, or a recent history of travel (Storch et al., 1979; Band and Fraser, 1981). In addition to the above, failure of an apparently bacterial pneumonia to respond to agents such as penicillins, cephalosporins, or aminoglycosides should raise the possibility of LD. Conversely, an impressive response to erythromycin suggests this diagnosis if other causes have been excluded (Meyer and Finegold, 1981).

Definitive diagnosis is made by recovery of L. pneumophila from clinical specimens, demonstration of the organism or its antigens in tissue or body fluids and/or documentation of a rising titer (≥fourfold rise) of specific antibodies. L. pneumophila has been recovered from respiratory secretions, pleural fluids, blood, sputum, placenta, and lung tissue by using special culture media such as CYE (Macrae et al., 1981; Fraser,

1981; Band and Fraser, 1981). Direct fluorescent antibody (DFA) technique (CDC, 1981, Broome et al., 1979) has been used to demonstrate the presence of, as well as, identification of the isolates of L. pneumophila. The DFA procedure may be applied to specimens of pleural fluid, sputum, or tissue. Alexander et al. (1979), using the DFA staining technique to evaluate respiratory secretions did not observe any false positives among his negative controls which indicated that the DFA technique was highly specific for L. pneumophila. Early in the course of the infection, the use of the DFA technique can make diagnosis possible within a few hours. Fraser and McDade (1979), reported that as many as 70 percent of patient specimens show positive fluorescent staining, and false positive results seem to be infrequent.

Recent studies, according to Gump et al. (1979), suggested that positive direct immunofluorescent smears can be found in as many as 71 percent of the cases, if multiple samples of sputum are obtained from the same patient. However, Band and Fraser (1981), cautioned that Mycobacteria, and an occasional strain of Pseudomonas fluorescens, may also stain brightly (false positive). In a similar note, Broome et al. (1979), found that DFA sensitivity was only 24 percent when applied to multiple sputum smears from patients with LD

diagnosed by serologic, cultural, or histologic procedures. Due to the rapidity with which the test can be conducted, direct immunofluorescent staining may be the most practical rapid diagnostic test now available for LD (Fraser, 1981). DFA also has proven to be extremely specific when it is applied to the appropriate clinical specimens (Orrison et al., 1982).

Other diagnostic tests that are commonly used include Dieterle silver stain, the Gimenez stain (Chandler et al., 1977; Winn et al., 1978; Alexander, et al., 1979) and serologic measurements of antibodies specific to L. pneumophila. These serologic measurements include indirect immunofluorescent (IFA) staining, hemagglutination, hemagglutination inhibition, microagglutination, and enzyme-linked immunosorbent assay (ELISA). ELISA involves the demonstration of the L. pneumophila antigen in the urine of patients with LD (Berdal et al., 1979; Tilton et al., 1979; Band and Fraser, 1981). If the ELISA test proves sensitive and specific, it too will also greatly enhance the rapidity of diagnosis of LD.

The diagnosis of Pontiac Fever, a second but milder form of legionellosis is made on clinical, epidemiologic, serologic bases, and has thus far been recognized only in epidemic form (Band and Fraser, 1981).

Treatment of Legionellosis

L. pneumophila is susceptible to a wide variety of antimicrobial agents. Using in vitro tests to determine minimal inhibitory concentrations, the Legionella isolates were found to be susceptible to erythromycin, rifampin, the aminoglycosides, the penicillins, cofoxitin, chloramphenicol, trimethoprim-sulfamethoxazole, and doxycycline (Band and Fraser, 1981). However, it has been reported that only erythromycin and rifampin were found to be markedly effective in prophylaxis as well as in treatment of infected embryonated hens' eggs and guinea pigs (Thornsberry, 1978; Fraser, 1979; McDade, 1979; Tsai et al., 1979; Band and Fraser, 1981). In reviewing the protocol used in treating patients in the 1976 Philadelphia, 1977 Vermont, and 1977 Los Angeles outbreaks, it was noted that the case-fatality ratio was lowest for those patients treated with erythromycin. For example, in the Vermont outbreak, the case-fatality ratio for patients receiving erythromycin was five percent, which was significantly lower than the ratio of 17 percent for patients who were not treated with erythromycin (Broome et al., 1979).

The dose, route, and duration of the most effective therapy is not known. Erythromycin has been administered to seriously ill patients and patients who cannot tolerate

oral therapy by injecting 500 to 1000 mg intravenously every six hours (15 mg/kg every six hours for children other than neonates) for at least 14 days (Fraser, 1981; Band and Fraser, 1981). It is recommended that rifampin be reserved for combined therapy with erythromycin for those patients not responding to high-dose intravenous erythromycin therapy. Rifampin has also been recommended for those patients with documented lung abscesses (Band and Fraser, 1981). Apparent relapses have been reported in a few patients for whom therapy was terminated prior to the 14th day of treatment. If a relapse is noted, erythromycin therapy should be resumed (Fraser, 1981).

Meyer and Finegold (1980), reported that ampicillin, penicillin, carbenicillin, oxacillin, cephalothin, cephalexin, cefoxitin, cefazolin, amikacin, gentamicin, and clindamycin have been used without effectiveness in patients.

In addition to antibiotic therapy, other supportive therapeutic measures are of great importance in the treatment of patients with respiratory failure, shock, or acute renal failure (Band and Fraser, 1981). Respiratory insufficiency may necessitate supplemental oxygen therapy and assisted ventilation. Renal failure requires the management of fluid and electrolyte balance, while dialysis may be needed on a temporary basis. Vasoactive

drugs may be of assistance in the management of shock (Fraser, 1981).

The prognosis of Legionnaires' Disease is greatly affected by both the patient's underlying disease and therapy being administered (Meyer and Finegold, 1980). Immunosuppressed patients at Wadsworth Hospital who received erythromycin had a case-fatality rate of only 24 percent as opposed to 80 percent for those who did not receive erythromycin. However, in non-immunosuppressed patients, the case-fatality rate was 7 percent with erythromycin therapy and a 25 percent fatality rate was observed in those patients that did not receive erythromycin (Kirby et al., 1979; Kugler et al., 1983).

Preventive measures and control of legionellosis are not fully known. Band and Fraser (1981), reported that environmental reservoirs and distribution in nature of L. pneumophila have not been defined nor has the mode of spread in sporadic cases and most outbreaks of the disease. However, in settings in which spread has been demonstrated to be due to aerosolized droplet nuclei from heat-rejection devices, stopping the production of the aerosols and eliminating the organism from the contaminated system may prevent further cases (Glick et al., 1978; Dondero et al., 1980; Band and Fraser, 1981).

Isolation of Legionella pneumophila

L. pneumophila is a fastidious, slow-growing organism which is rather difficult to isolate, expecially when associated with faster-growing, less fastidious contaminating organisms (Fitzgeorge et al., 1982). L. pneumophila has frequently been isolated from patients with LD (Bopp et al., 1982) as well as infected animals such as guinea pigs (Fraser et al., 1977). It has also been isolated from environmental sites associated with epidemic outbreaks (Morris et al., 1979 and 1980; Brenner et al., 1980; Cordes et al., 1980), as well as from lakes and streams in areas where cases of legionellosis have not been documented (Fliermans et al., 1979 and 1981; Tison et al., 1981).

The successful isolation of L. pneumophila was first accomplished by means of a method generally used for isolating non-bacterial microorganisms such as rickettsias (Fraser and McDade, 1979). This procedure is currently used to isolate L. pneumophila and other Legionella species from environmental samples. The procedure is rather complex and time-consuming. It involves intraperitoneal injection of a concentrated sample into guinea pigs which is then followed by inoculating embryonated eggs with necropsied splenic tissue resected from the guinea pig (Fraser and McDade,

1979; Morris et al., 1979). L. pneumophila can be isolated either from the tissue of the guinea pig or the egg yolk sac then plated on charcoal-yeast extract (CYE) agar and incubated at 35°C in a humidified incubator with an atmosphere of about 2.5 percent carbon dioxide for 2-4 days. Initially, however, L. pneumophila was isolated on an enriched Mueller-Hinton agar (McDade et al., 1977), showing that the organism was a bacterium and not a rickettsia.

L. pneumophila's fastidiousness is such that it does not grow on any conventional or standard bacteriological medium (Chandler et al., 1977), unless the medium contains a high concentration of the amino acid cysteine and iron salts such as ferric pyrophosphate. Fraser and McDade (1979), noted that a double nutritional requirement, such as this is rarely observed in bacteria. However, Weaver (Feeley et al., 1978), in an attempt to adapt the organism to conventional bacteriological media obtained suspensions of Legionnaires' Disease bacterium-infected embryonated yolk sacs and inoculated portions onto 17 different types of media, including Bordet-Gengou, cysteine-glucose-blood agar, heart infusion (HI), HI with five percent rabbit bloo, Muller-Hinton (MH), MH with five percent Fildes enrichment, MH with five percent fetal calf serum, and MH with one percent hemoglobin and one percent IsoVitalex

used for the isolation of L. pneumophila from environmental samples cannot completely replace the guinea pig/egg inoculation method, since cultural techniques allow the receovery of approximately 45 percent of the L. pneumophila isolated from known positive water samples.

CHAPTER III

MATERIALS AND METHODS

Collection of Samples

Environmental water samples used in this study were taken from three different sources:

1. Raw water, collected from:
   a. a farm pond
   b. a river
   c. three creeks in the City of Morehead
   d. two lakes
2. Potable "drinking" water, collected from the City of Morehead Water System:
   a. shower heads
   b. faucets
   c. swimming pool
   d. hot water tanks
3. Water from air-conditioning systems:
   a. cooling towers
   b. air-conditioner condensate

Raw Water Collection: Two-liter samples were collected from various aquatic habitats in sterile wide-mouth screw-cap plastic bottles (BEL-ART Products, Calvert Industrial Supplies, Calvert, Kentucky) and simultaneously measured for temperature, dissolved oxygen (Model 54 ARC; Yellow Springs Instrument Co., Yellow

Springs, Ohio), pH (Fisher Accumet Model 610A pH meter, Fisher Scientific Co., Pittsburg, Pennsylvania), and conductivity (Markson Portable Conductivity Meter, Model 10, Markson Science, Del Mar, California).

Except for Cave Run Lake and the Licking River samples collected during the first week of November, all samples were collected during the months of July, August, and September, 1983. Each sample group was transported to the laboratory in insulated containers (Freeze Safe, Polyfoam Parker Corp., Wheeling, Illinois) with ice packs (Stanbel, Springfield, Massachusetts) to prevent exposure to extreme heat. Samples not processed immediately were refrigerated at 4°C for no more than 48 hours.

Potable Water Collection: Faucet and shower samples were collected in one-liter wide-mouth, screw-cap plastic bottles and transported to the laboratory as described above. However, the shower head samples were collected after the removal of shower head aerators were effected and sterile swab applicators were inserted into shower head outlets and rotated firmly against the interior surface to dislodge residue. Swabs were placed immediately into sterile swab containers (Falcon Swab Tube Application, Fisher Scientific, Louisville, Kentucky). After swabbing, and without flushing, the one liter samples were collected.

The swimming pool sample was collected at about 2m below the surface when the swimming pool was not in use. Samples from hot water tanks were collected from drainage pipes at the bottom of each tank.

Air-conditioning Systems: In air-conditioning systems, water samples were collected:

1. Directly from cooling towers
2. From evaporative condenser tanks
3. From closed system cooling tower condensates with no direct contact between the air and air-conditioning system

Processing of Samples

Filtration: All samples were processed as illustrated in Figure I. Each sample was concentrated by filtration, using a sterile 47 mm filter assembly (Millipore Corporation, Bedford, Massachusetts) containing a 2.0 μm porosity type polycarbonate filter (Nuclepore Corporation, Pleasanton, California). Some visibly turbid samples had to be filtered through a 0.45 μm porosity type HA millipore filter (Millipore Corporation, Bedford, Massachusetts) in order to facilitate filtration of samples.

After each sample was filtered, the membrane filter was aseptically removed from the holder. The membrane was placed soiled side down into a 150 ml wide-mouth

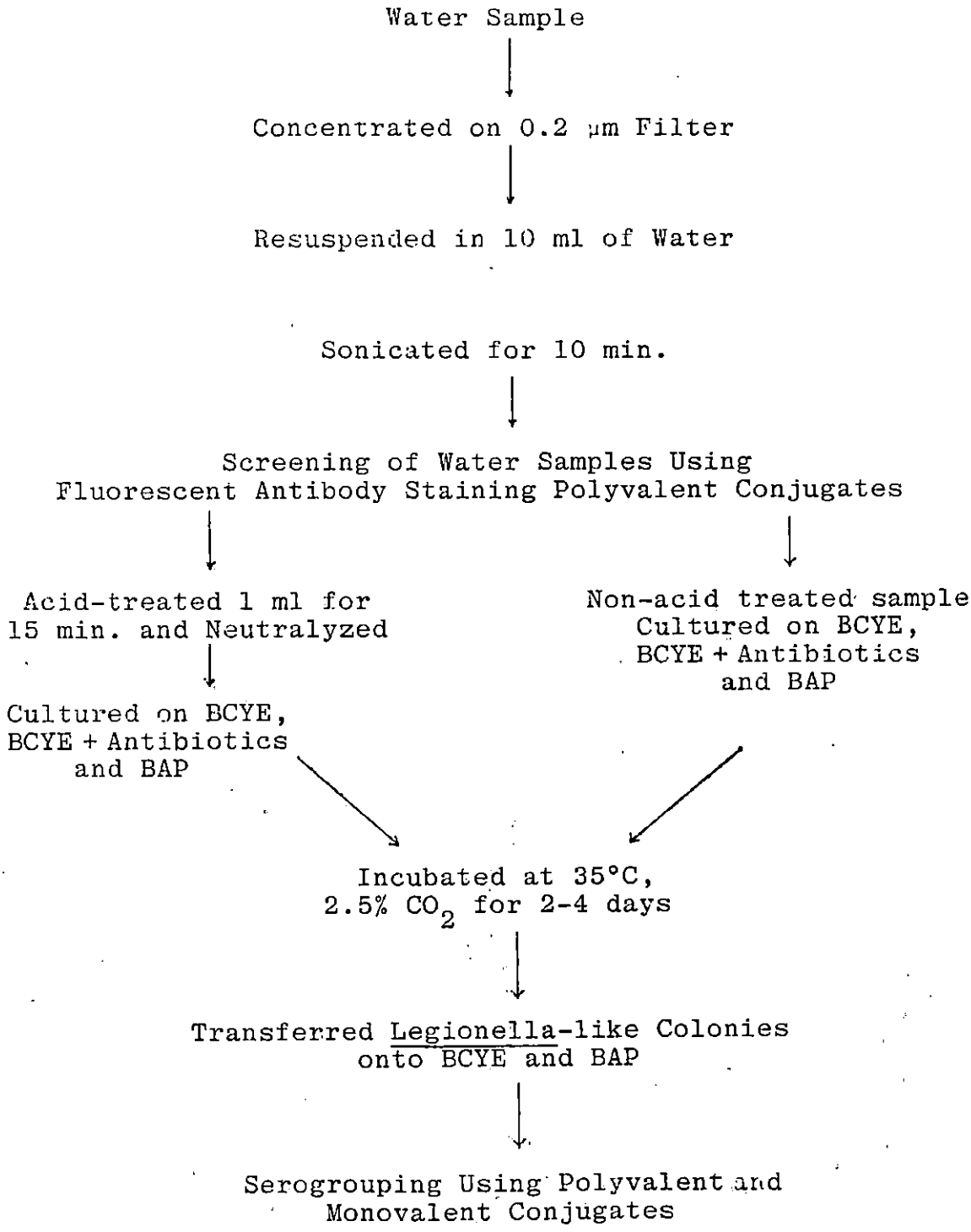

Figure I. Procedure for Screening Water Samples Using DFA and BCYE.

round jar (Kerrs Glass Manufacturing Corporation, Lancaster, Pennsylvania) containing 10 ml sterile distilled water. The filter membranes from raw water samples were resuspended in 20 ml of sample water because of the amount of inert materials accumulated in the filters.

Sonication: The concentrated samples were then placed in a sonicating bath (Sonicor Instrument Corp., Copaigue, New York) for 10 minutes to resuspend cells trapped on the filter membrane. When the sample bottles were placed into the bath, the caps were screwed on lightly to allow heat to dissepate.

Fluorescence Antibody (FA) Staining: Fluorescent antibody conjugates for 10 serogroups of Legionella species (CDC, Biological Products Division, Atlanta, Georgia) were used to screen all water samples before plating was conducted. Samples exhibiting greater numbers of fluorescing cells were considered more likely to have Legionella and were subjected to serospecific testing.

Acid Wash Treatment: Part of each sample was exposed to an acid wash treatment. Wang et al. (1979), reported that L. pneumophila is relatively resistant to a pH of 2.2 on short period exposure. Therefore, an

aliquot of each suspension was treated with acid to reduce the number of non-legionellae organisms present and to enhance the isolation of Legionella species (Bopp et al., 1981). A 0.5 ml sample aliquot was added to 4.5 ml diluent containing a 0.2 M KCl - HCl solution (pH of 2.2) to achieve a 1:10 dilution (Bopp et al., 1981; Buesching et al., 1983). This was mixed with a vortex mixer until a homogenous suspension was effected. The suspension stood at room temperature to allow a total contact time of 15 minutes with the acid solution (Gorman and Feeley, 1982). The suspension was neutralized with 0.1 N KOH so that the final pH was 6.9 (Gorman and Feeley, 1982).

Preparation of BCYE Media: The preparation of a buffered charcoal yeast-extract (BCYE) agar base (Remel, Regional Medical Laboratories, Lenexa, Kansas), used to isolate Legionella from all samples, was carried out under the Germ-Free Bioflow Hood (Germ-Free Laboratories, Miami, Florida), using strict aseptic techniques. The media composition is shown in Table 5. The BCYE media (40.0 grams) was placed into one liter of deionized water and boiled until the media was in solution. The media was neutralized by adding 40 ml of 1 N KOH prior to autoclaving at 121°C for 15 minutes. It was then placed into a 50°C water bath prior to the addition of the antibiotics and

TABLE 5

Composition of Buffered Charcoal Yeast-extract Medium

Type of Constituents	Amount/Liter
Yeast-extract	10.0 g
Activated Charcoal (Norit SG)	2.0 g
L-cysteine HCl.2 H_2O	0.4 g
Ferric Pyrophosphate Soluble	0.25 g
Agar (Difco)	17.0 g
ACES Buffer (n-2 acetamido-2-aminoethane sulfonic acid)	10.0 g
Polymixin B (selective)	40,000 U
Vancomycin (selective)	0.5 mg
Anisomycin (selective)	80.0 mg

nutritional additives. L-cysteine, ferric pyrophosphate and triple antibiotics [polymyxin-B, anisomycin and vancomycin (PAV)] were added to the cooled BCYE media. Using 1 N KOH, the final pH of the media was adjusted to 6.91. The cooled media was dispensed (20 ml/plate) into sterile 15 x 100 mm petri dishes (1029 Petri Dish, American Scientific Products, Cleveland, Ohio). The contents of the flask was periodically swirled to maintain a suspension of the charcoal particles.

Plating and Incubation: Each bottle of concentrated sample was swirled to mix the suspension. Both acid treated and non-acid treated samples were plated by transferring, a 0.1 ml aliquot of the neutralized suspension with a sterile pasteur pipet, onto one plate each of BCYE, BCYE + antibiotics (PAV), and five percent sheet blood agar (G.S. Media and Reagents, Lexington, Kentucky). The plating procedure was carried out under the Germ-Free Bioflow Hood. A sterile smooth glass-rod was used to spread the inoculum evenly over each plate.

Inoculated plates were incubated at 35°C in candle extinction jars under an atmosphere of approximately 37 percent humidity and about 2.5 percent carbon dioxide (Feeley et al., 1978; Grace et al., 1981; Gorman and Feeley, 1982; Remel, 1983). Three uninoculated plates

were also incubated after being momentarily open to check possible contamination.

Quality Assurance: The performance of the media can only be ascertained by the growth characteristics of the Legionella bacterium (Gorman and Feeley, 1982; Remel, 1983). This was demonstrated by the growth of L. pneumophila Serogroup I (ATCC 33152) used as a quality assurance control to evaluate protocol. L. pneumophila (American Type Culture, Parklawn Drive, Rockcastle, Maryland) was obtained in a freeze-dried preparation. The freeze-dried culture was rehydrated and transferred into half- strength BCYE broth (5 ml BCYE agar + 5 ml deionized sterile water). From this broth, a sample was taken and processed the same way as water samples and inoculated onto one place each of BCYE, BCYE + antibiotics, and 5 percent sheep blood agar.

Interpretation of Plates: All cultures were examined after 48 hours and every 24 hours thereafter for 7 to 10 days. BCYE plates were observed microscopically with the aid of disecting microscope (American Optical, Buffalo, New York) for the presence of bacterial colonies resembling Legionella. Colonies with morphological characteristics of Legionella species were further examined with long wave UV light (Ultra Violet Products,

Models UVSL - 25 and C-5, San Gabriel, Calfornia) for blue-white-fluorescence. Suspect colonies were aseptically transferred onto (a) BCYE agar and (b) sheep blood agar. Inoculated portions of the plates were streaked with a sterile loop to provide areas of heavy growth then incubated for 24 hours. Plates demonstrating growth on BCYE agar only were presumptive Legionella species and were examined further with DFA specific conjugates and Gram stain. Positive and negative controls were included in each test. Slides were view independently by two observers (Evans and Washington, 1981).

Fluorescent Antibody Techniques: Direct fluorescent antibody (DFA) staining is an immunofluorescence procedure developed to detect the presence of Legionella bacteria in human lung tissue, respiratory tract fluids and cultures. DFA has also been used to screen environmental samples for Legionella prior to inoculating guinea pigs or BCYE with polyvalent conjugates prepared against the Legionella serogroups (Cherry et al., 1978; McKinney et al., 1979).

Materials and methods employed in this study were those recommended by the U.S. Department of Health and Human Services, Public Health Service, CDC (1983). They

included:

1. Slides with two wells each, 10 mm in diameter (Fluoroslides, Erie Scientific, Sybron Corporation, Ohio)

2. Legionella pneumophila Polyvalent Pool A, Legionella Polyvalent Pool B and Legionella Polyvalent Pool C (Appendix A)

3. Fluorescein isothiocynate (FITC) - labeled normal rabbit control globulin (Appendix A)

4. Legionella Grouping FITC - labeled globulins (Appendix A)

5. Phosphate buffered saline (PBS), pH 7.6 (0.01 M buffer; 0.85% NaCl)

   a. Concentrated stock solution was prepared as follows:

      Na_2HPO_4 (anhydrous reagent grade). . 12.36 g

      NaH_2PO_4 (reagent grade). . . . . 1.80 g

      NaCl (reagent grade) . . . . . 85.00 g

      Distilled water to make . . . . . 1,000 ml

   b. Working solution, pH 7.6 (0.01) buffer, NaCl) was prepared as follows:

      Concentrated stock solution . . . . 100 ml

      Distilled water to make . . . . . 1,000 ml

      pH was adjusted to 7.6

6. Glycerol mounting medium

   Buffer, pH 9.0, was prepared first by adding 4.4 ml of 0.5 M $NaCO_3$ (5.3 g in 100 ml distilled water) to 100 ml of 0.5 M $NaHCO_3$

   One part of this buffer was added to nine parts of glycerol (glycerin) neutral

7. Cover slips No. 1, 24 x 60 mm (Microscope Cover Glass, Fisher Scientific Co., Louisville, Kentucky)

8. Coplin jars for PBS soak

9. Incubation chamber - large petri dish (1048 Petri Dish, Falcon, Oxnard, California), with a moistened filter paper (or a comparable moisture-containing chamber)

Direct Fluorescent Antibody Staining Procedure:

Using a microliter pipette dropper (Cooke Laboratory Products, Dynatech Laboratories, Alexandria, Virginia) the concentrated sample (0.05 ml) was spotted onto a two-welled slide (Fluoroslides, Erie Scientific, Sybron Corporation, Ohio), air dried, gently heat fixed, then stained with 1-2 drops of polyvalent fluorescent antibody conjugates to Legionella pneumophila Serogroups 1, 2, 3, 4, and 6; L. bozemanii Serogroup 1; L. gormanii Serogroup 1; L. micdadei Serogroup 1; L. dumoffii Serogroup 1; and L. longbeachae Serogroup 2. Pre-immune serum conjugate with fluorescein isothiocyanate (FITC) was used as a negative control. Each conjugate was quality assurance tested against its specific antigen as a positive control (Cherry, et al., 1978). DFA conjugates were kindly supplied by the Biological Products Division, CDC, Atlanta, Georgia. A water sample containing L. pneumophila Serogroup 1 (ATCC 33152 American Type Culture Collections, Rockville, Maryland) was included as an additional positive control in each test. The fluorescent antibody staining procedure is presented in Figure II.

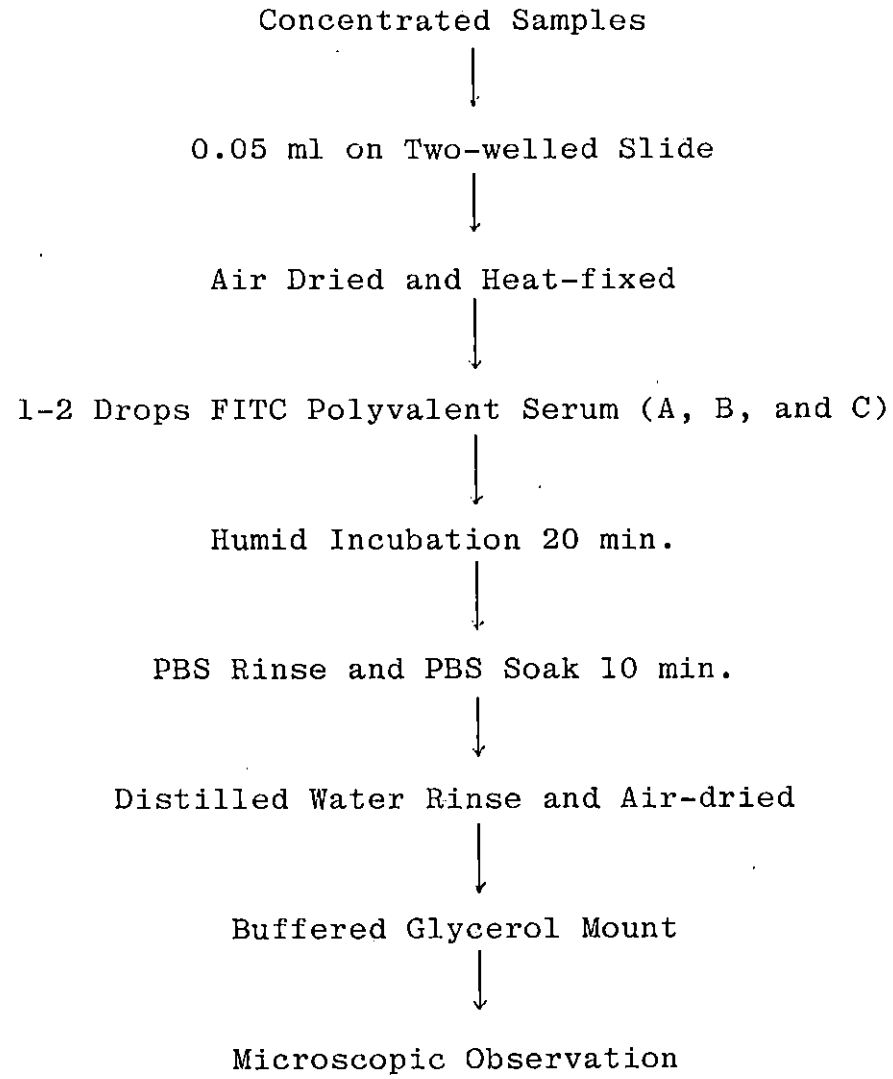

Figure II. Direct Fluorescent Antibody Staining Procedure Flow Chart.

Slides were incubated in a moist chamber at room temperature for 20 minutes. Excess conjugate was removed by holding each slide level but perpendicular and tapping it against a tissue tower. Quickly and gently the smears were rinsed with a stream of phosphate buffered saline (PBS) from a wash bottle while slides were held horizontally with the long edge tipped downward. Specific conjugate was prevented from coming into contact, even momentarily, with the control smear. Slides were immersed in PBS in separate coplin jars for 10 minutes. After rinsing with distilled water, the slides were air dried and mounted with alkaline glycerol and covered with a Corning No. 1 cover slip, and examined with a fluorescent microscope. Each sample preparation showing specific staining was tested with the negative (control) conjugate to ensure that the observed reaction was serologically specific. The same protocol was employed in the preparation of samples for serospecific (monovalent) staining.

In the DFA procedure an antigen is fixed on a slide and is then overlaid with FITC - labeled globulin directed against the antigen. The antigen binds the labeled immune globulins and the resulting antigen-antibody complexes are rendered visible upon excitation of the FITC by ultraviolet light. When exposed to ultraviolet light,

(500 nm), FITC emits long wavelengths of light in the yellow-green (500 nm) portion of the color spectrum and the Legionella cells are observed as brilliantly fluorescent yellow-green rods.

Examination of Slides

Slides were examined on a binocular fluorescent microscope (Vertical Fluorescence, American Optical Corporation, Scientific Instrument Division, Buffalo, New York) using a 50 watt mercury vapor lamp (to provide a rich source of excitation energy), collector lens system and field diaphragm to effectively control the beam. Exciter filters (BG12 + KV418) transmit selected wavelengths (500 nm). The barrier filters (OG515) delete extraneous wavelengths.

Smears were examined first under the 10X plain-acromatic objective. In strongly positive preparations, such as the control and some of the positive samples, the bacteria were visible as uniform dots. The objective was switched to 20X and 40X for rapid screening and to confirm and select the area where organisms were present. At 100X or oil immersion, the bacterial cells were visible, as single short to long rods (0.3 µm to 0.9 µm by 2 µm and up to 20 µm) that fluoresced yellow-green around the entire periphery, thus confirming the observation. A

sample was considered positive if a smear contained brightly fluorescing bacilli that were compatible morphologically with legionellae (Evans and Washington, 1981).

CHAPTER IV

RESULTS AND DISCUSSIONS

Forty-four water samples were collected and processed in an attempt to demonstrate the presence of L. pneumophila. The direct immunofluorescent antibody (DFA) method and a direct plating method using BCYE medium were employed. Those establishments which were used as sites for the collection of samples were kept confidential.

Raw Water

Eight of the 44 samples were collected from raw water sources. Using polyvalent sera and the DFA technique, these samples were screened for Legionella-like cells. Six of eight samples displayed fluorescent antibody-positive cells (see Table 6).

On initial examination with polyvalent pools A, B, and C (Appendix A), the Cave Run Lake sample was positive for both polyvalent A and C conjugates. Only L. pneumophila Serogroups 1 and 3 (Knoxville 1 and Bloomington 2, respectively), as well as L. gormanii Serogroup 1, were identified using serospecific conjugates. The serospecific reagent for L. longbeachae was not available. When the Cave Run Lake sample was plated on BCYE and blood agar, some colonies grew on both plates.

TABLE 6

Isolation of Legionnaires' Disease Bacteria from Raw Water
Using Direct Immunofluorescence and BCYE Media

Sample	Date	Source	Direct Analysis of Samples		
			DFA*	BCYE*	DFA Group-specific
WS 600	11/03/83	Cave Run Lake	+	+	+
WS 601	8/08/83	Eagle Lake	+	+	+
WS 602a	11/02/83	Farm Pond	-	-	-
WS 602b	11/02/83	Pond Mud	-	-	-
WS 603	11/03/83	Licking River	+	-	**
WS 604	7/29/83	Triplett Creek	+	-	***
WS 605	8/08/83	Evans Branch	+	+	+
WS 606	8/08/83	Oxley Branch	+	-	+

*DFA = Direct immunofluorescent antibody; BCYE = buffered charcoal yeast-extract.

**No serospecific conjugate was available for L. longbeachae; ***not performed.

(+) = Presence of Legionella-like colonies; (-) = absence of Legionella-like colonies.

However, only those colonies appearing on BCYE agar at, or after, 48 hours, and showing similar morphological characteristics to L. pneumophila, as well as bluish-white fluorescence under ultraviolet (UV) light (Ultra Violet Products, Models UVSL-25 and C-5, San Gabriel, California), were presumptively identified as Legionella isolates. Attempts to subculture these Legionella-like colonies onto fresh BCYE agar plates were not successful due to the presence of contaminants which may have inhibited the growth of, or overgrew, the small Legionella colonies. However, when some colonies were resuspended in 5 ml of sterile water and stained with serospecific conjugates, only L. pneumophila Bloomington 2 was confirmed. Either the other two serogroups did not grow or their colonies were not successfully recovered. It may be that cross-reactivity occurred between the Knoxville 1 and Bloomington 2 serogroups.

The Eagle Lake sample (Table 6) was similarly examined and found to be positive for polyvalent reagents A, B, and C. There were more fluorescing cells per field in polyvalents A and B than in polyvalent C. However, only L. pneumophila (Knoxville 1) was identified when serospecific conjugates were used. Further examination of colonies on BCYE revealed several small colonies which fluoresced under UV light, but were apparently too small

to be successfully subcultured, because a heavy inoculum was required for Legionella to survive the transfer process (Cherry et al., 1978; Finegold and Martin, 1982). Due to the small size of the colonies after two to three days of incubation the plates were incubated for an additional three days. After five days of incubation, contaminants overgrew the culture plates, thus, it was impossible to make a positive identification of Legionella-like colonies. However, when a suspension was prepared from these non-pure colonies, L. pneumophila Knoxville 1 was detected by serospecific staining.

The Licking River sample was DFA positive for polyvalent C conjugate, however, no fluorescing cells were observed using L. gormanii and L. micdadei serosepcific reagents (Appendix A). Legionella-like colonies were not detected on BCYE medium (Table 6).

Samples from three creeks (Triplett Creek, Oxley Branch and Evans Branch) in the City of Morehead exhibited fluorescent antibody positive cells (Table 6). On further analysis using serospecific reagents, Evans Branch was found to have L. pneumophila Knoxville 1 and Bloomington 2, and one other species, L. gormanii Serogroup 1. This sample was also found culture positive on BCYE agar. Both the Triplett Creek and Oxley Branch samples were negative on BCYE agar. The DFA analysis of Evans Branch sample with

serospecific reagents detected L. pneumophila Knoxville 1 and Bloomington 2 as well as L. gormanii, but the Triplett Creek and Oxley Branch samples were not analyzed serospecifically.

Three of the raw water samples (Licking River, Triplett Creek and Oxley Branch) were found positive for DFA, but culture negative. This may be explained by the fact that some of the fluorescing bacteria may belong to other sepcies that are serologically and morphologically similar to Legionella species, such as Pseudomonas fluorescens (Cherry et al., 1978), Mycobacterium tuberculosis (Cherry et al., 1979), and Bacteriodes fragilis (Edelstein et al., 1980). These organisms indicate false-positive DFA tests. It is also possible that, because of the number of manipulations involved, Legionella species may lose viability (a) by impaction on the filter membrane or because of the toxicity of the membrane itself, (b) by trauma of the blending operation, or (c) by resuspension and dilution before plating (Orrison et al., 1981). The media used may also be suboptimum for cultural recovery (Cherry et al., 1979).

It should be noted that both samples from a farm pond lacked fluorescing cells, and did not produce Legionella-like colonies on BCYE agar. The negative results could be due to either sampling error or that the Legionella

bacteria were present in undetectable numbers.

Analysis of raw water samples revealed that 75 percent of the samples exhibited fluorescing cells, while Legionella-like colonies were isolated on BCYE media from only 38 percent of the samples. Morris et al. (1979) and Fliermans et al. (1979), reported 75 percent of creek samples and 90 percent of lake samples were DFA positive. In both studies, Legionella were recovered through animal inoculation with DFA positive samples. However, Tison et al. (1983), in a recent survey of lakes and rivers in the southern United States, isolated Legionella from only 15 percent (47 of 318) of DFA positive samples using culture media. Bopp et al. (1981), reported a recovery rate of up to 45 percent for Legionella when an acid procedure in combination with CCVC selective medium and BCYE medium were employed.

The concentration of fluorescing cells per liter of raw water samples in this study ranged from 10^4 to 10^5, which were comparable with the 10^3 to 10^6 cells per liter of sample reported by Morris et al. (1981), and Fliermans et al. (1981).

Selected physical and chemical characteristics of the habitats under study (Table 7), were comparable to those observed in a survey of aquatic habitats by Fliermans et al. (1981). Both studies demonstrate that a

TABLE 7

Summary of Habitat Characteristics of *Legionella* Species from Non-epidemic Sources

Sample	Habitat	Temperature (°C)	Conductivity (μmho/cm)	Dissolved Oxygen (mg/ℓ)	pH
WS 600	Cave Run Lake	22.2°	12	8.4	5.5
WS 601	Eagle Lake	21.8°	14	7.8	5.8
WS 602	Farm Pond	22.6°	85	7.8	5.0
WS 603	Licking River	21.5°	12	7.6	6.0
WS 604	Triplett Creek	28.2°	65	7.0	6.4
WS 605	Evans Branch	24.2°	76	7.4	6.8
WS 606	Oxley Branch	23.8°	74	6.8	6.6

wide range of habitats are capable of harboring these bacteria. These organisms can survive in temperatures of 5.7 to 63°C, pH values of 5.8 to 8.1, dissolved oxygen of 0.3 to 9.6 parts per million (ppm), and conductivity of 18 to 106 μmho/cm (Fliermans et al., 1981).

Cooling Towers

Seven samples were collected from cooling towers, four of which WS 508, 511, 512, and 513 were positive by direct immunofluorescent polyvalent conjugates (A, B, and C) and serospecific conjugates (Table 8). However, only two of the seven samples (WS 511 and 512) were culture positive. Samples WS 500, 509, and 510 were negative for all tests. Of these three negative samples (43 percent), one was collected from a cooling tower (WS 500) which was not treated with biocidal agents. The remaining two and the rest of the four positive samples were collected from cooling towers which received regular treatment. The biocidal agents used were called DEARSIDES 713, 717, and 904. The active ingredients of the Dearcides are quaternary ammonium compounds and bis (tri-n-butyltin) oxide.

Since only 50 percent of the DFA positive samples produced colonies on BCYE agar, the fluorescing cells observed in the rest of the DFA positive samples were

TABLE 8

Isolation of Legionnaires' Disease Bacteria from Cooling Towers
Using Direct Immunofluorescence and BCYE Media

Sample	Date	Source	Direct Analysis of Samples		
			DFA*	BCYE*	DFA Group-specific
WS 500	9/08/83	Cooling Tower	-	-	-
WS 508	9/09/83	Cooling Tower	+	-	+
WS 509	9/09/83	Cooling Tower	-	-	-
WS 510	9/09/83	Cooling Tower	-	-	-
WS 511	9/09/83	Cooling Tower	+	+	+
WS 512	9/09/83	Cooling Tower	+	+	+
WS 513	9/09/83	Cooling Tower	+	-	+

*DFA = Direct immunofluorescent antibody; BCYE = buffered charcoal yeast-extract.

(+) = Presence of Legionella-like colonies; (-) = absence of Legionella-like colonies.

apparently dead as a result of the biocides in water treatment. The non-viability could also be attricutable to a number of other reasons that were previously discussed under the section concerning raw water.

A report by Orrison et al. (1981), showed that water cooling towers and evaporative condensers are proven sources for the dissemination of LD bacteria. Similarly both epidemiological and environmental studies have implicated air-conditioning systems in many outbreaks of LD (Fraser et al., 1978; CDC, 1978; Glick et al., 1978; Terranova et al., 1978; Macrae et al., 1979; Cordes et al., 1980; Dondero et al., 1980; Band et al., 1981; Fisher-Hoch et al., 1981). The fact that some samples were both DFA positive and culture positive, in spite of the daily treatment with biocidal agents, underscores the need for the continued monitoring and treatment of these systems in order to prevent potential outbreaks of legionellosis. The situation becomes even more critical when these organisms are found in the air-conditioning systems of health institutions, hotels and other public corporations where populations of high risk individuals may be exposed to contaminated air.

Air-conditioning Condensates

Three of seven samples (43 percent) collected from condensates of air conditioners were DFA positive, but

only one sample (14 percent) was considered positive on BCYE agar (Table 10). Of the three DFA positive samples (WS 501, 502, and 506), two samples, WS 502 and 506 reacted with group-specific conjugates. On further analysis, L. pneumophila Bloomington 2 and Chicago 2, and L. gormanii Serogroup 1 were identified using monovalent sera.

In one study, Morris et al. (1979), reported negative results in all eight condensate samples collected from air exchange units of the cooling towers analyzed. The positive findings in this work were probably related to the fact that some of the condensates were trapped in outlet pipes, thus providing an opportunity for Legionella to colonize and proliferate within the system. The culture positive sample was a mixture of condensate and air-conditioner reservoir water. The water present in the base of the reservoir floor was exposed to the air, thus providing an opportunity for Legionella to colonize the water and to utilize the organic debris present in the stagnating water.

Potable Water Samples

Potable water samples from hot water tanks, faucets, showers, and a swimming pool (Table 9) were screened for Legionella. These samples constituted 50 percent (22 of 44) of all samples assayed in this study.

TABLE 9

Isolation of Legionnaires' Disease Bacteria from Air-conditioning Systems Condensates Using Direct Immunofluorescence and BCYE Media

Sample	Date	Source	Direct Analysis of Samples		
			DFA*	BCYE*	DFA Group-specific
WS 501	9/08/83	Recycled Water	+	-	-
WS 502	9/08/83	A/C Reservoir	+	+	+
WS 503	9/08/83	Condensate	-	-	-
WS 504	9/08/83	Condensate	-	-	-
WS 505	9/08/83	Condensate	+	-	+
WS 506	9/08/83	Condensate	-	-	-
WS 507	9/08/83	Recycled Condensate	-	-	-

*DFA = Direct immunofluorescent antibody; BCYE = buffered charcoal yeast-extract.

(+) = Presence of Legionella-like colonies; (-) = absence of Legionella-like colonies.

Analysis of these samples using polyvalent conjugates showed that 77 percent (17 of 22) were DFA positive. However, on BCYE agar, only 18 percent were positive (samples collected from a faucet, two shower heads and a swimming pool). Swab cultures collected from shower heads (WS 204 and 205) gave similar results.

The DFA negative samples included those collected from a hot water tank, a faucet and three shower heads (Table 10). These negative results may be indicative of portions of an uncontaminated water system, or that Legionella-like cells were present in undectable numbers.

The number of DFA positive cells in these samples were high. However, since the DFA technique stains both viable and non-viable cells, one plausible explanation for the large number is that the treatment procedures employed successfully inactivated the Legionella bacteria. The detection of fluorescing cells, dead or alive, raised several important points: a) that Legionella are present in the city potable water system, b) that the treatment rendered to potable water prior to consumption appears to be effective, and c) that the potential does exist for Legionella to colonize, proliferate, and become a potential health hazard should there be a breakdown in the treatment process (Tobin et al., 1980). Publish data show that Legionella are known to survive in the water

TABLE 10

Isolation of Legionnaires' Disease Bacteria from Potable Water
Using Direct Immunofluorescence and BCYE Media

Sample	Date	Source	Direct Analysis of Samples	
			DFA*	BCYE*
WS 400	9/07/83	Hot Water Tank	+	-
WS 401	9/07/83	Hot Water Tank	+	-
WS 402	9/07/83	Hot Water Tank	+	-
WS 403	9/07/83	Hot Water Tank	-	-
WS 404	9/08/83	Hot Water Tank	+	-
WS 300	9/06/83	Faucet	+	-
WS 301	9/06/83	Faucet	+	-
WS 302	9/06/83	Faucet	+	-
WS 303	9/06/83	Faucet	+	-
WS 304	9/07/83	Faucet	+	-
WS 305	9/08/83	Faucet	+	-
WS 306	9/08/83	Faucet	+	-
WS 307	9/07/83	Faucet	+	+
WS 308	9/06/83	Faucet	-	-
WS 200	9/06/83	Shower	-	-
WS 201	9/06/83	Shower	-	-
WS 202	9/06/83	Shower	+	-
WS 203	9/06/83	Shower	-	-
WS 204	7/26/83	**Shower Head	+	+
WS 205	9/08/83	**Shower Head	+	+
WS 206	9/08/83	Shower	+	-
WS 207	7/26/83	Swimming Pool	+	+

*DFA = Direct immunofluorescent antibody; BCYE = buffered charcoal yeast-extract.

**Similar results were observed for water samples and swab cultures taken from the same shower heads.

(+) = Presence of Legionella-like colonies; (-) = absence of Legionella-like colonies.

distribution lines, especially those that are not frequently used or where low flow rates prevail (Tison et al., 1980; Tobin et al., 1981).

Stout et al. (1980), observed that sediments in the plumbing system, including faucets, shower heads, hot water tanks and storage tanks do serve as concentration mechanisms whereby Legionella proliferate, especially in the hot water system, and are disseminated to other areas through peripheral outlets where they reside in the sediments (Helms et al., 1983). Stagnant deposits in the hot water tanks are reported to have higher titers of Legionella (Fisher-Hock et al., 1981), and non-sterile tap water were reported to support the growth of these organisms (Yee and Wadowsky, 1982).

Recent epidemiologic investigations continue to implicate water distribution systems as a source of nosocomial LD (Tobin et al., 1980; Cordes et al., 1981; Fisher-Hoch et al., 1981; Stout et al., 1982; Meenhorst et al., 1983). One environmental Protection Agency (EPA) official reported that Legionella had recently been isolated from distribution lines at elvels ranging between one to four CFU/ℓ, and that the organisms may colonize gaskets and other fixtures (Geldreich, 1983). Thus, it appears that Legionella are able to utilize substrates in hot water tanks, faucets, and shower heads which allow the

bacteria to increase in water and pose a potential health hazard to high risk groups.

Although the infectious dose of Legionella bacteria is not known, experimental work with guinea pigs has demonstrated that approximately 10^3 to 10^4 L. pneumophila caused fatal infection when administered as an aerosol (Fitzgeorge et al., 1980). Berendt et al. (1980), reported a median infectious dose of $\leq$ 121 organisms when using a laboratory-adopted strain of L. pneumophila. However, association between exposure to contaminated potable water and the risk of developing Legionella pneumonia in patients has yet to be established. Host susceptibility is undoubtedly a critical factor for the development of the infection (Stout et al., 1982).

Classification of Legionella Isolates

Serospecific fluorescent antibody conjugates were used to identify the various serogroups of Legionella species in samples previously determined positive by DFA using polyvalent reagents (Table 11).

Four serogroups of L. pneumophila were identified: a) Knoxville 1, b) Bloomington 2, c) Los Angeles 1, and d) Chicago 2. Knoxville 1 and Bloomington 2 were the most frequently detected isolates; five of eleven and seven of eleven samples respectively. They demonstrated a very strong positive reaction in most samples, whereas Chicago 2

TABLE 11

Classification of Isolates of L. pneumophila Using Direct Immunofluorescence*

Sample	Source	Serogroups of L. Pneumophila				
		Knoxville 1	Togus 1	Bloomington 2	Los Angeles 1	Chicago 2
WS 502	A/C Reservoir	-	-	4+	-	3+
WS 503	Condensate	-	-	-	2+	-
WS 505	Condensate	-	-	2+	-	-
WS 508	Cooling Tower	-	-	3+	-	-
WS 511	Cooling Tower	4+	-	4+	-	-
WS 512	Cooling Tower	-	-	-	-	-
WS 513	Cooling Tower	4+	-	3+	-	-
WS 600	Cave Run Lake	3+	-	4+	-	-
WS 601	Eagle Lake	**	**	**	**	**
WS 602	Farm Pond	-	-	-	-	-
WS 603	Licking River	4+	-	-	-	-
WS 604	Triplett Creek	**	**	**	**	**
WS 605	Evans Branch	4+	-	4+	-	-

*DFA = Direct immunofluorescent antibody.

**Not performed

(+) = Presence of Legionella-like cells; (-) = absence of Legionella-like cells.

and Los Angeles 1 were positive in one sample each. Togus 1 was not detected in any sample screened. In addition to the serogroups of L. pneumophila, two additional species of Legionella were identified: they were L. gormanii and L. micdadei. Both were detected in four and five samples respectively (Table 12). One other species, L. bozemanii was not observed in any of the 11 samples examined. The monovalent sera for L. longbeachae were not available. Criteria for reporting DFA results are presented in Appendix B.

Legionella Population Density

Selected positive samples were quantitated microscopically using DFA examination (Table 13). The population density of three of the four serogroups of L. pneumophila, varied as follows: a) Knoxville 1 ranged from 3.2×10^4 to 5.0×10^5 cells/ℓ; b) Bloomington 2 was 4.0×10^4 to 5.2×10^5 cells/ℓ; and c) Chicago 2, identified in only one sample, had a high count of 2.8×10^5 cells/ℓ. Other species quantitated were L. gormanii 1 with a density of 2.0×10^4 to 5.0×10^5 and the number of fluorescing cells for L. micdadei ranged from 4.0×10^4 to 3.0×10^5 cells/ℓ. These numbers are comparable to those reported by Tison et al. (1983), in potable drinking water supplies; they are also consistent with those observed by Fliermans et al., 1981. L. pneumophila type strain

TABLE 12

Classification of Isolates of Other *Legionella* Species
Using Direct Immunofluorescence*

Sample	Source	*L. bozemanii* 1	*L. gormanii* 1	*L. micdadei* 1
WS 502	A/C Reservoir	-	4+	-
WS 503	Condensate	-	-	3+
WS 505	Condensate	-	-	-
WS 508	Cooling Tower	-	3+	-
WS 511	Cooling Tower	-	-	4+
WS 512	Cooling Tower	-	-	4+
WS 513	Cooling Tower	-	-	3+
WS 600	Cave Run Lake	-	3+	-
WS 601	Eagle Lake	**	**	**
WS 602	Farm Pond	-	-	-
WS 603	Licking River	-	-	3+
WS 604	Triplett Creek	**	**	**
WS 605	Evans Branch	-	4+	-

*Staining intensity measured on a scale of 1+ to 4+, 4+ = very strong reaction; (-) = no reaction. Conjugates for *L. longbeachae* were not available.

**Not performed.

(+) = Presence of *Legionella*-like colonies; (-) = absence of *Legionella*-like colonies.

TABLE 13

Legionella Population Densities of Selected Habitats Determined by Using Direct Immunofluorescence

Sample	Source	*L. pneumophila* Serogroups			*Legionella* Species	
		Knoxville 1	Bloomington 2	Chicago 2	Gormanii 1	Micdadei 1
WS 503	A/C Reservoir	-	5.2×10^5	2.8×10^5	3.1×10^5	-
WS 508	Cooling Tower	-	6.0×10^4	-	1.0×10^5	-
WS 511	Cooling Tower	5.0×10^5	4.8×10^5	-	-	3.0×10^5
WS 512	Cooling Tower	-	-	-	-	3.2×10^5
WS 513	Cooling Tower	4×10^4	2.0×10^5	-	-	2.4×10^5
WS 600	Cave Run Lake	3.2×10^4	4.0×10^4	-	2.0×10^4	-
WS 601	Eagle Lake	3.2×10^4	-	-	-	-
WS 603	Licking River	1.0×10^5	-	-	-	4.0×10^4
WS 605	Evans Branch	4.0×10^5	3.0×10^5	-	5.0×10^5	-

Counts are based on microscopic observation of strongly fluorescing *Legionella*-like bacteria in 0.05 ml of sample concentrate and are reported as the number of fluorescing cells/liter.

Togus 1 and L. bozemanii were not observed; serospecific conjugate for L. longbeachae was not available.

Heterotrophic Plate Counts

Heterotrophic plate counts of representative environmental samples are summarized in Table 14. A standard plate count medium (Difco) was used, and the plate count data varied considerably. Potable water samples had the lowest count of bacterial flora; these samples ranged from 1.4×10^4 colony forming units (CFU) per liter in the faucet samples to 1.3×10^5 CFU/ℓ and 4.4×10^5 CFU/ℓ in hot water tank and shower head samples respectively. The highest counts were obtained in samples from the Cave Run Lake (1.8×10^8 CFU/ℓ), and Eagle Lake (8.0×10^6 CFU/ℓ).

The heterotrophic plate counts were high, thus acid treatment of samples was undertaken to reduce bacterial flora and enhance the isolation of Legionella. Acid-treated samples showed relatively lower bacterial counts per liter than non-acid-treated samples. The range was 1.9×10^3 to 2.0×10^4 CFU/ℓ for acid-treated samples and 1.3×10^6 to 1.8×10^8 CFU/ℓ for non-acid-treated samples (Table 15).

TABLE 14

Heterotrophic Plate Counts of Selected Environmental Samples
Using Standard Plate Count Medium

Sample	Source	CFU/ℓ*
WS 207	Shower	4.4×10^5
WS 306	Faucet	1.4×10^4
WS 405	Hot Water Tank	1.3×10^5
WS 504	Condensate	1.6×10^6
WS 508	Cooling Tower	1.3×10^6
WS 603	Licking River	7.6×10^6
WS 601	Eagle Lake	8.0×10^6
WS 600	Cave Run Lake	1.8×10^8

*CFU/ℓ = Colony forming units/liter.

TABLE 15

Heterotrophic Plate Counts of Non-acid Wash and Acid Wash Samples Using Standard Plate Count Medium

Sample	Source	CFU/ℓ*	
		Non-acid Wash	Acid Wash**
WS 501	Recycled Water	4.8×10^6	2.4×10^3
WS 502	A/C Reservoir	6.8×10^6	2.0×10^4
WS 508	Cooling Tower	1.3×10^6	4.6×10^3
WS 511	Cooling Tower	7.2×10^6	2.5×10^3
WS 512	Cooling Tower	4.3×10^6	1.9×10^3
WS 600	Cave Run Lake	1.8×10^8	2.8×10^4
WS 601	Eagle Lake	8.0×10^6	3.8×10^3
WS 603	Licking River	7.6×10^6	6.0×10^3

*CFU / ℓ = Colony forming units/liters.

**Each sample was left in acid buffer (pH 2.2) for a total contact time of 15 minutes.

CHAPTER V

CONCLUSION

This study has demonstrated that Legionnaires' Disease bacteria can be isolated from environmental sites. These findings are significant because Kentucky is one of the states in which sporadic cases of legionellosis have been reported; however, legionellosis has not been documented for Rowan County. Since LD remains a fatal pneumonia-like syndrome and L. pneumophila is one of the frequent causes of nosocomial infections, there is a need for preventive steps to eliminate LD bacteria from potential dissemination sources. It would be advisable, therefore, for hospitals, nursing homes, hotels, schools and universities, and other public institutions to regularly monitor their water distribution systems for Legionella and, if contaminated, to treat them appropriately.

Cooling towers of air-conditioning systems have been implicated in many LD outbreaks. These dissemination sources should be treated with effective cooling tower biocides such as chlorine, tri-n-butyltin, 2,2-dibromonitrilopropionamide and quarnary ammonium compounds. Combinations of these biocides, such as Dearcides, have been shown to be effective against

L. pneumophila (Orrison et al., 1981; England III et al., 1982). Chlorination alone has been used in cooling tower decontamination in the United Kingdom (Fisher-Hock et al., 1981).

Legionella pneumophila has also been recovered from cold water outlets (Orrison et al., 1981). Continuous chlorination at 1-2 ppm of free chlorine has been reported effective in eliminating the organism from cold water (Fisher-Hock et al., 1981; Tobin et al., 1980). Storage tanks can be decontaminated using 50 ppm free chlorine and allowing the water to stand for three hours before draining (Tobin et al., 1980).

Hot water tanks have been known to harbor Legionella, especially when the temperature is warm enough (45°C) to drive off free chlorine, yet cool enough to support L. pneumophila. Decontamination of hot water tanks can be effected by raising the temperature to 55°C to 60°C (Fisher-Hock et al., 1981). It should be noted that hospital plumbing systems in this country may be an especially favorable environment for Legionella since the Joint Commission on Accreditation of Hospitals has required that hospitals maintain hot water in tanks and showers at lower temperatures, thus permitting the possible multiplication of Legionella in plumbing fixtures, tap water, and water and sediment at the bottoms of the

tanks (Wadowsky et al., 1982).

In addition to decontamination treatments (Dearcides, chlorination, and raising of water temperature), other preventive measures have been proposed. Dr. Gary C. duMoulin of Harvard Medical School (1983), said that purging or flushing water lines prior to admission of a patient into a room that has not been recently used will reduce the concentration of L. pneumophila. He pointed out that when a shower is not in use, bacterial flora, including Legionella, may concentrate at the shower head and constitute a potential health hazard. Tobin et al. (1980), also reported that any interrupted water supply may be a source of L. pneumophila. They suggested that hospital shower bath design should be considered with a view to reducing unnecessary backwaters.

Aerosol dissemination of L. pneumophila has been documented in several legionellosis outbreaks (Dondero et al., 1980; Cordes et al., 1980; Glick et al., 1980; Helms et al., 1983). If water systems are purged of LD bacteria through chlorination or high temperature, the chances of LD will be greatly reduced or eliminated, and aerosols generated from showers, faucets and nebulizers will no longer be considered a potential health hazard for elderly and immunocompromised patients and other high

risk individuals.

Summary

One objective of this study was to establish whether Legionella pneumophila, the etiological gent of Legionnaires' Disease, was present in Rowan County environmental sites. Another was to become familiar with the ecology of the bacteria. Additionally, positive isolation would provide the impetus for making area physicians cognizant of their presence and for considering the bacteria in the diagnosis of pneumonitis patients. Also, it was hoped that this study would initiate a periodic monitoring of selected sites for the presence of LD bacteria to possibly prevent any potential outbreak of legionellosis.

During the summer and fall of 1983, 44 water samples were collected from natural waters, air-conditioning systems, and potable water sources in Rowan County. These samples were processed and assayed for Legionella species, using both cultural methods and direct immunofluorescent antibody (DFA) staining techniques. Upon DFA examination, using Legionella polyvalent conjugates, it was found that 75 percent of all samples exhibited fluorescing Legionella-like cells with morphological characteristics of Legionella

pneumophila. Potable water had the highest DFA positive cells (77 percent), followed by raw water samples (75 percent), air-conditioning cooling tower samples (57 percent), and lastly, air-conditioning condensates (43 percent). However, using BCYE medium, raw water samples had the highest culture-positive Legionella colonies (38 percent), whereas air-conditioning cooling tower samples, potable water samples, and air-conditioning condensates were 29 percent, 18 percent, and 14 percent culture-positive, respectively.

Using serospecific fluorescent sera on the DFA positive samples and some of the colonies, it was possible to identify three different Legionella species: a). L. pneumophila, b). L. gormanii and c). L. micdadei. It was also possible to identify four common serogroups of L. pneumophila: a). Knoxville 1, b). Bloomington 2, c). Los Angeles 1 and d). Chicago 2. Of the three species, L. pneumophila was the most common. Similarly, of the different serogroups, Knoxville 1 and Bloomington 2 were identified in more samples (45 percent and 64 percent, respectively) than were Chicago 2 and Los Angeles 1, both of which were observed in 9 percent of the samples.

The heterotrophic plate counts of some samples were considerably high. Potable water samples had the lowest counts of 10^4 to 10^5 CFU/ℓ, whereas the raw water samples

had the highest counts (10^6 to 10^8 CFU/ℓ, while air-conditioning samples were 10^6 CFU/ℓ. However, the number of cells reacting with Legionella-specific fluorescent antibody conjugates in air-conditioning systems and raw water samples were generally reduced 10- to 100-fold in air-conditioning samples and 10-100-fold to 1000-fold in the raw water samples.

This study should be the beginning of a continuing epidemiologic and laboratory investigation that will yield considerable information about Legionnaires' Disease and Legionella pneumophila. Some local physicians have already indicated willingness to participate and to screen patients with pneumonitis for L. pneumophila with the objective of preventing and determining the incidence of LD in the Gateway District Health Department Area.

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APPENDICES

APPENDIX A

Direct Fluorescent Antibody Conjugates

I. Polyvalent Pool Conjugates:

1). L. pneumophila Polyvalent Pool A (Serogroups 1, 2, 3 and 4) FITC-labeled Rabbit Globulin for FA (with TMRITC-labeled NRS Diluent).

2). Legionella Polyvalent Pool B FITC-labeled Rabbit Glubulin for DFA. L. pneumophila Serogroup 5 (Dallas 1E), Serogroup 6, (Chicago 2), L. dumoffii Serogroup 1, Tex-KL), L. longbeachae Serogroup 1, (Long Beach 4) (with TMRITC-labeled NRS Diluent).

3). Legionella Polyvalent Pool C FITC-labeled Rabbit Globulin for DFA. L. micdadei Serogroup 1 (Wiga), L. gormanii Serogroup 1, (LS-13), L. longbeachae Serogroup 2, (Tucker-1) (with TMRITC-labeled NRS Diluent).

4). FITC - labeled Normal Rabbit Control Globulin for Legionella FA.

II. Serospecific Conjugates:

1). L. bozemanii Serogroup 1 (Wiga Isolate) Control Antigen for FA.

2). L. bozemanii Serogroup 1 (Wiga Isolate) FITC-labeled Rabbit Globulin for FA (with TMRITC-labeled NRS Diluent).

3). L. gormanii Serogroup 1 (LS-12 Isolate) Control Antigen for FA.

4). L. gormanii Serogroup 1 (LS-13) (Isolate) FITC-labeled Rabbit Globulin for FA (with TMRITC-labeled NRS Diluent).

5). L. micdadei Serogroup 1 (Tatlock Isolate) Control Antigen for FA.

6). L. micdadei Serogroup 1 (Tatlock Isolate) LITC-labeled Rabbit Globulin for FA (with TMRITC-labeled NRS Diluent).

7). L. pneumophila Serogroup 1 (Knoxville 1 Isolate) FITC-labeled Rabbit Globulin for FA (with TMRITC-labeled NRS Diluent).

8). L. pneumophila Serogroup 2 (Togus 1 Isolate) Control Antigen for FA.

9). L. pneumophila Serogroup 2 (Togus 1 Isolate) FITC-labeled Rabbit Globulin for FA (with TMRITC-labeled NRS Diluent).

10). L. pneumophila Serogroup 3 (Bloomington 2 Isolate) Control Antigen for FA.

11). L. pneumophila Serogroup 3 (Bloomington 2 Isolate) FITC-labeled Rabbit Globulin for FA (with TMRITC-labeled NRS Diluent).

12). L. pneumophila Serogroup 4 (Los Angeles 1 Isolate) Control Antigen for FA.

13). L. pneumophila Serogroup 4 (Los Angeles 1 Isolate) FITC-labeled Rabbit Globulin for FA (with TMRITC-labeled NRS Diluent).

14). L. pneumophila Serogroup 6 (Chicago 2 Isolate) Control Antigen for FA.

15). L. pneumophila Serogroup 6 (Chicago 2 Isolate) FITC-labeled Rabbit Globulin for FA (with TMRITC-labeled NRS Diluent).

APPENDIX B-1

Criteria for Reporting Results of DFA for Legionnaires' Disease Bacterium in Lung Tissue and Fluids*

Lung Tissue and Fluids	
Results	Report
> 50 strongly fluorescing bacteria/ field	**IF + (many)
2-50 strongly fluorescing bacteria/ field	IF + (moderate)
> 25 strongly fluorescing bacteria/ smear but	
> 1 strongly fluorescing bacterium/field	IF + (few)
< 25 strongly fluorescing bacteria/smear	Report numbers only
0 strongly fluorescing bacteria / smear	IF -
> 5 strongly fluorescing bacteria/ smear	IF + (number)

*Cherry et al., 1978.

**IF = Immunofluorescence

APPENDIX B-2

Criteria for Reporting Results of DFA for Legionnaires' Disease Bacterium in Lung Tissue and Fluids

*In Other Specimens	
Results	Report
≥25 strongly fluorescing bacteria/smear	FA+
<25 strongly fluorescing bacteria/smear	Numbers only
0 strongly fluorescing bacteria/smear	Fa-

*CDC (Cherry and McKinney, 1979.