Comparison of Virulent and Avirulent Legionella pneumophila and Evaluation of Fish as a Potential Environmental Reservoir/Experimental Model

Sandra Reading Sommer
Virginia Commonwealth University

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Comparison of Virulent and Avirulent Legionella pneumophila and Evaluation of Fish as a Potential Environmental Reservoir/Experimental Model

A dissertation submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy at Virginia Commonwealth University

by

Sandra Reading Sommer
B A , Wartburg College, 1969
M.S , Virginia Commonwealth University, 1974

Director: Dr. Harry P. Dalton, Professor of Pathology
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Virginia Commonwealth University
Richmond, Virginia
December, 1987
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Comparison of Virulent and Avirulent *Legionella pneumophila* and Evaluation of Fish as a Potential Reservoir/Experimental Model

Abstract

A dissertation submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy at Virginia Commonwealth University

Sandra R. Sommer, Ph.D.

Medical College of Virginia - Virginia Commonwealth University, 1987

Major Director: Dr. Harry P. Dalton

*Legionella pneumophila* was first recognized as a cause of human pneumonia in 1976. Since then, much has been learned about the microbiology, pathophysiology and epidemiology of this organism. The features which permit one strain but not another to invade human lung tissue and produce disease remain incompletely understood. This study evaluated several attributes of a virulent and an avirulent strain of *L. pneumophila* in an attempt to identify characteristics which would distinguish the two.

Evaluation of a new medium, buffered egg yolk agar,
showed that virulence was maintained after 26 passages, which was the same as the buffered charcoal yeast extract agar used for comparison. However, growth appeared earlier and was heavier on the charcoal-containing medium.

Morphologically, the avirulent strain produced greater numbers of filamentous forms and was found to be encapsulated more frequently. Treatment with polymixin B produced morphologic changes similar to those previously reported but failed to alter the virulence of either strain. No plasmids were found in either strain nor were consistent differences in protein content demonstrated using sodium dodecylsulfate polyacrylamide gel electrophoresis. Both strains reacted less intensely as cultures aged with a monoclonal but not a polyclonal antibody in a direct fluorescent antibody assay. This change was more pronounced when the virulent organism was tested.

Chemotactic assays showed similar tendencies when human or guinea pig mononuclear cells were compared to two estuarine species (hogchoker and spot) and one freshwater species (golden shiner minnow) of fish. In vivo results were also similar when two of the three species of fish were tested, suggesting that either the spot or the minnow may be used in evaluation of certain
characteristics of *L. pneumophila*.

Organisms were isolated from apparently healthy fish up to 15 days after inoculation in some instances. This suggests that fish may be a possible additional environmental reservoir for *Legionella pneumophila*. It was also to become famous as the site of the first recognized epidemic of what is now known as Legionnaires' Disease (LD), since most of the cases occurred in those who had attended the convention (121, 201). Symptoms typically developed 7 days after the Legionnaire had arrived at the convention and became severe enough that 147 of the 131 patients (66%) required hospitalization. Pneumonia was the major clinical finding with death occurring in 10% a median of 7 days after the onset of illness. Those who recovered showed radiographic evidence of improvement about 10 days after onset of illness with a more rapid clinical improvement (121). The widespread publicity this epidemic received led to an intensive search for the etiologic agent. Tests for known bacteria, fungi, chlamydiae, rickettsiae and viruses were negative. Approximately 6 months after the initial outbreak, McDade and Shepard from the Centers for Disease Control (CDC) announced that a previously unrecognized bacteria had been isolated from lung
INTRODUCTION

The Bellevue-Stratford Hotel in Philadelphia was the site of the 58th annual convention of the American Legion, Department of Pennsylvania, July 21-24, 1976. It was also to become famous as the site of the first recognized epidemic of what is now known as Legionnaires' Disease (LD), since most of the cases occurred in those who had attended the convention (121, 201). Symptoms typically developed 7 days after the Legionnaire had arrived at the convention and became severe enough that 147 of the 182 patients (81%) required hospitalization. Pneumonia was the major clinical finding with death occurring in 16% a median of 7 days after the onset of illness. Those who recovered showed radiographic evidence of improvement about 10 days after onset of illness with a more rapid clinical improvement (121).

The widespread publicity this epidemic received led to an intensive search for the etiologic agent. Tests for known bacteria, fungi, chlamydiae, rickettsiae and viruses were negative. Approximately 6 months after the initial outbreak, McDade and Shepard from the Centers for Disease Control (CDC) announced that a previously unrecognized bacterium had been isolated from lung
tissue from patients from the Philadelphia epidemic using guinea pig inoculations (263). This organism was found to be an aerobic gram-negative bacillus which, on the basis of extensive biochemical testing including guanine plus cytosine content and DNA hybridization studies, was determined to belong to a new species for which the name *Legionella pneumophila* was proposed (41, 207).

Serologic testing revealed that two previous outbreaks of undiagnosed illness, one which occurred in 1965 in Washington, D.C. (290) and the other in Pontiac, Michigan in 1968 (132, 176) were in fact due to *L. pneumophila*. McDade et al. (207) also demonstrated that the organism was involved in isolated cases over a wide geographic area in the United States. Later, a rickettsia-like agent (OLDA) originally isolated in 1947 from guinea pigs inoculated with the blood of a patient with an upper respiratory tract infection was shown to be the same species (205).

Much has been learned since this organism was first described. It is unusual in its biochemical composition and is one of the few bacteria which are classified as facultative intracellular parasites. It appears to be a fastidious organism in its growth requirements in the laboratory and yet is ubiquitous in the environment. There are currently 23 species recognized with possibly 11 more to be named (38).
Two forms of disease are described (14). The first is the pneumonia originally associated with it; the second is an acute, febrile self-limited disease known as Pontiac fever. Cases are more common in the summer months (99, 190, 305) and nosocomial pneumonia, especially in the immunocompromised patient, is frequent (146, 181, 210, 214). The disease and the bacterium now have been reported from many areas of the world. Outbreaks have been associated epidemiologically with cooling towers (70, 79, 127, 183, 213), evaporative condensers (70), and potable water, especially hot water tanks (12, 34, 115, 146, 270, 281, 310, 343).

Although many questions have been answered concerning *Legionella pneumophila*, much remains to be done. There is currently no acceptable, reasonably easy way to determine which of the many strains found in the environment is most likely to cause disease. Epidemics of nosocomial LD have been reported in association with a variety of environmental isolates, only 1 or 2 of which were consistently isolated from patients. On the other hand, other institutions have isolated *legionellae* in various water sources with apparently little associated disease (72, 77, 120, 316). Controversy exists as to whether it is necessary to eradicate the organism in this situation. If a simple test were available to determine virulence of the various isolates, this decision would be easier to make.
Several factors associated with virulence in other bacteria have been found in the Legionellaceae, including endotoxin-like activity (166, 336), hemolysin (9, 125, 301), phospholipase C activity (8), and several proteases (13, 20, 82, 218, 300). Which, if any, of these is necessary for virulence is unknown. Some researchers have associated the presence of plasmid(s) with decreased virulence (29, 47) and *L. pneumophila* is known to lose its ability to cause disease after continuous passage on a variety of artificial media (33, 103, 206, 338). Others have shown a relationship with certain monoclonal antibody reaction patterns (169, 236, 306, 319).

Another question relates to the ability of the organism to cause 2 distinct diseases, LD and Pontiac fever. The isolates from an outbreak of each type were indistinguishable in experimental infections induced in guinea pigs (161). There is also one report of both diseases occurring after a common-source exposure (130).

*Legionella pneumophila* is commonly found in the environment. It has been associated with blue-green algae (303), green algae (162), several species of amoebae (1, 10, 155, 224, 258, 309) and ciliated protozoans (10, 113), all ubiquitous in water. These may account for the growth and survival of *L. pneumophila* in many instances, but not all. There has been little work reported concerning other possible
amplifiers and/or reservoirs in the environment. Survival mechanisms employed by this fastidious (in the laboratory) organism are still unclear. The seasonal occurrence of disease is also unexplained, but may be due to increased virulence in the summer months. If so, the reason for this change is unknown.

Legionnaires' Disease (LD)

This pneumonia is caused by *Legionella pneumophila*, most often serogroup 1 (145). Other species may cause a clinically indistinguishable disease (Table 1), identified by the more general term, legionellosis, which refers to all clinical syndromes produced by organisms classified within the genus *Legionella* (14). Pneumonia caused by *L. micdadei* is the only other type which has been characterized and is similar to that caused by *L. pneumophila* (216). Smaller numbers of infections by other species have been reported but there is no evidence that unique clinical manifestations are produced (14).

LD appears to be a relatively common pneumonia.
Clinical Disease

Two types of disease are associated with the Legionellaceae. Most common is the pneumonic form seen in the original 1976 epidemic and known as Legionnaires' Disease. The other has been reported less frequently and is an acute nonpneumonic illness, Pontiac fever. Other types of infections have been reported and the infection in children may cause symptoms different from those seen in adults (239).

Legionnaires' Disease (LD)

This pneumonia is caused by *Legionella pneumophila*, most often serogroup 1 (245). Other species may cause a clinically indistinguishable disease (Table 1), identified by the more general term, legionellosis, which refers to all clinical syndromes produced by organisms classified within the genus *Legionella* (14). Pneumonia caused by *L. micdadei* is the only other type which has been characterized and is similar to that caused by *L. pneumophila* (216). Smaller numbers of infections by other species have been reported but there is no evidence that unique clinical manifestations are produced (14).

LD appears to be a relatively common pneumonia
Table 1
Legionella Species Isolated From Patients With Pneumonia*

<table>
<thead>
<tr>
<th>Species</th>
<th>Source</th>
<th>Location</th>
<th>Isolated By</th>
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<td>L. pneumophila</td>
<td>human lung tissue</td>
<td>Philadelphia, PA</td>
<td>McDade and Weaver 1977</td>
<td>207</td>
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<td>L. bozemanii</td>
<td>human lung tissue</td>
<td>Key West, FL</td>
<td>Bozeman, 1957, and Hebert, 1979</td>
<td>39, 142, 297</td>
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<tr>
<td>L. micdadei</td>
<td>human blood</td>
<td>Fort Bragg, NC</td>
<td>Tatlock, 1943, Hebert, 1979</td>
<td>143, 237</td>
</tr>
<tr>
<td>L. dumoffii</td>
<td>cooling tower water</td>
<td>New York, NY</td>
<td>Gorman, 1978</td>
<td>39, 194</td>
</tr>
<tr>
<td>L. longbeachae</td>
<td>human trans-tracheal aspirate</td>
<td>Long Beach, CA</td>
<td>Porschen, 1980</td>
<td>208</td>
</tr>
<tr>
<td>L. jordanis</td>
<td>river water</td>
<td>Bloomington, IN</td>
<td>Gorman, 1978</td>
<td>38, 59</td>
</tr>
<tr>
<td>L. wadsworthii</td>
<td>human sputum</td>
<td>Los Angeles, CA</td>
<td>Edelstein, 1981</td>
<td>91</td>
</tr>
<tr>
<td>L. feeleii</td>
<td>water</td>
<td>Windsor, Canada</td>
<td>Gorman, 1981</td>
<td>150, 294</td>
</tr>
<tr>
<td>L. maceachernii</td>
<td>home evaporator-cooler water</td>
<td>Phoenix, AZ</td>
<td>Gorman, 1979</td>
<td>40, 329</td>
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<tr>
<td>L. hackeliae</td>
<td>human bronchial biopsy</td>
<td>Ann Arbor, MI</td>
<td>Hackel, 1981</td>
<td>40, 330</td>
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which may be underdiagnosed, especially in institutions where this diagnosis is not specifically and conscientiously sought. Macfarlane et al. (197) found 15% of community-acquired pneumonia to be due to *Legionellaceae*; this was the second most common cause in their prospective study conducted in England. Nearly 50% of patients admitted with pneumonia during July, August and September were found to have LD. Muder et al. (214) in a similar study, found that in a community teaching hospital in the United States where LD had never been documented, 9.5% of all pneumonias and 14.3% of nosocomial pneumonias were caused by *Legionellaceae*. Their study was conducted in February, April, and June, months in which the incidence of LD outbreaks is lower (99, 190) so these rates could be even higher if other months were evaluated. Initially, reported cases were associated with outbreaks, but a survey by England et al. (105) indicated that sporadic LD was twice as frequent, and Sanford (263) suggests that LD comprises 4-10% of all sporadic pneumonias.

The incubation period for LD appears to range from 2-11 days (121, 290) although an apparent incubation of 28 days has been reported (181). The patient then typically experiences a period of malaise, diffuse myalgias and headache followed in 12-48 h by sudden onset of high nonremittant fever and recurrent shaking chills. Gastrointestinal symptoms (nausea, vomiting,
watery diarrhea) may then occur, followed by a nonproductive cough and dyspnea (283). The disease may progress to respiratory failure requiring mechanical ventilatory assistance, hypotension and death (181, 307). Renal dysfunction and encephalopathy are also frequent (148, 283). Clinical manifestations are usually not distinctive enough to allow differentiation from other causes of pneumonia, although unexplained encephalopathy and hematuria were found to be suggestive in one study (148) while these plus diarrhea were frequent in two others (180, 272). Hyponatremia was a useful distinguishing feature in a study by Miller (211), but this was not the case in the work of Sharrar et al. (272). Macfarlane et al. (196) found no distinctive radiographic features when 49 sporadic cases of LD were compared to 147 cases of pneumococcal, mycoplasma or psittacosis pneumonia. Reported fatality rates vary from 8-25% (44, 78, 121, 145, 180, 183, 190, 290) although this may improve with more rapid diagnosis and treatment (202). Factors associated with increased risk of LD include sex (male), age (≥ 50 yrs.), smoking, underlying malignancy, immunosuppressive drug therapy, diabetes mellitus, and increased alcohol consumption (105, 190, 279). Treatment with erythromycin alone or with rifampin is effective (14, 121, 181). Tetracycline appears to be less effective and trimethoprim-sulfamethoxazole may have been effective in a few cases
Pontiac Fever

This form of legionellosis is characterized by the abrupt onset of fever, chills, headache, and myalgia (132). Cough is uncommon and the illness is similar to influenza and may be indistinguishable from a variety of respiratory syndromes induced by viruses (14). In contrast to the 0.4-4% attack rate associated with LD (181), 95% were affected in the first reported outbreak of Pontiac fever (132). Other differences include a shorter incubation period (mean, 36h), the absence of pneumonia, and it is a benign self-limited illness lasting 2-5 days (132). The Pontiac, Michigan outbreak was caused by L. pneumophila serogroup 1, but an outbreak in Windsor, Ontario, was shown to be caused by L. feeleii (150). This outbreak was clinically similar to that caused by L. pneumophila (149). There is one reported instance of simultaneous occurrence of LD and Pontiac fever in Vermont (130) but only one case of each was seen.

Other Infections

Several unusual findings have been reported in association with LD, including pyelonephritis (80), acute interstitial nephritis (268), intractable seizures (240), peritonitis (271), and pericardial effusion (271). Rarely, legionellae have been associated with infection in the absence of pneumonia. Pericarditis
(223), prosthetic valve endocarditis (204), sinusitis (265), and perirectal abcess (5), and possibly acute pancreatitis (4) have been reported. Infection of a hemodialysis fistula occurred in 2 patients, one with concurrent LD and another who had completed erythromycin therapy for LD 3 weeks earlier (173). Another patient presented initially with pericarditis and developed ocular involvement; only after more than 2 weeks did pulmonary findings develop (123). Pastoris et al. (239) have suggested that in children infection may take the form of cardiac arrhythmia or myocarditis without pneumonia. Typical LD is uncommon in children (2, 14).

Pathologic Findings

Consistent pathologic findings in fatal LD are limited to the lung. Macroscopically, the lungs are heavy with varying degrees of consolidation, usually bilateral, and no predilection for any one lobe (334). Abscesses appear to be rare, with only 2 instances noted in 42 cases reported (28, 334).

Microscopically, the histologic pattern is that of an acute fibrinopurulent pneumonia. There is extensive exudation of neutrophils (PMN), macrophages, and fibrin into the alveolar spaces. In some cases there is extensive lysis of the inflammatory cells, but this is not uniformly distributed within the lung. The underlying lung structure remains relatively uninvolved; the inflammatory process has not been noted to involve
blood vessel walls or large bronchi (27, 28, 334). Erythrocyte extravasation into the alveoli varies. Most sections have none or only a few; but areas of moderate to severe hemorrhage are occasionally present (27).

Chandler, Hicklin, and Blackmon (55) originally reported the unusual staining properties of the organism. It stains faintly or not at all with the usual tissue stains for bacteria, but is visualized using the Dieterle silver impregnation method. The organisms are seen within the pneumonic process using this method, with higher numbers associated with necrosis of the inflammatory cells (28, 334). They appear as short, pleomorphic rods 2-4 um in length and up to 1 um in diameter. Some appear to be bipolar and beaded forms may be seen. Clusters are commonly observed within macrophages as well as extracellularly (55). Alveolar damage, if it is seen, is most likely due to treatment (oxygen therapy) or is a nonspecific reaction to sepsis or shock (53, 151). Surgical specimens may show changes similar to those seen at autopsy; however, the organism may not be seen (55).

Laboratory Identification

The laboratory may aid in the evaluation of both clinical and environmental sources of legionellae. The organism or its soluble products may be detected directly in the specimen or by isolation of the bacteria
by culture methods. Serologic diagnosis also may be made in the case of clinical infection by demonstrating a four-fold change in antibody titer or by a single high titer.

Direct Examination of Specimens

Cherry and co-workers (60) first described the use of a direct immunofluorescent assay (DFA) to detect organisms in lung tissue samples. Initially, Broome et al. (43) found that of patients shown to have LD by other criteria, only 24% also had DFA positive sputum samples. They suggested that the more invasive lung biopsy might be necessary in order to demonstrate the organism by DFA. More recent work has proven the value of testing respiratory secretions using DFA (93, 246, 333) with bronchial and tracheal secretions having a positivity rate almost as high as lung specimens (246). Unfortunately, while the specificity is about 99% (87) the sensitivity of DFA testing, as compared to culture diagnosis, ranges from 25 to 70% with 60% the average (88). The lower limit of detectability in seeded sputum using DFA is about $10^4$ organisms per ml (135). DFA testing is becoming impractical with the increasing number of species and serogroups. One group (48) has evaluated an indirect fluorescent antibody system using a panvalent antibody pool and labeled goat anti-rabbit antibody with promising results. Most of these studies evaluated reactions with L. pneumophila serogroups, but
similar findings have also been reported for *L. micdadei* (71, 331). Monoclonal antibodies have been prepared by several groups (49, 133, 137, 236, 269, 318, 167) all are species-specific and most are also specific for serogroup. Studies have shown that reactivity is equivalent to the polyclonal reagents available (90, 289). Since most LD is caused by *L. pneumophila* serogroup 1 and there is evidence that these monoclonal antibodies do not cross-react as the polyvalent occasionally has been reported to do (95, 174, 233, 289) they may be more useful.

Soluble *Legionella* antigens have been detected in urine using radio- and enzyme-immunoassay techniques (19, 25, 186, 187, 188, 285, 302) and reverse passive agglutination (200, 284); staphylococcal coagglutination has also been suggested (326). Lower amounts of antigen have been detected in serum (25) and it has been suggested that antigen detection could be useful in evaluating other tissues and body fluids (111, 302). Another promising tool for rapid detection of the organism in clinical material is the recently developed nucleic acid probes (92, 106, 136, 189).

Environmental water samples have been tested using DFA as described by Morris and co-workers (213) and modified by Orrison, Cherry, and Milan (234). These specimens require concentration by filtration or centrifugation and DFA then has been shown to detect
$10^5$ organisms/ml (234). It must be remembered, however, that DFA originally was developed for clinical specimens and only *L. pneumophila* serogroups 1-4 have been extensively evaluated for cross-reactions, so caution is required in interpreting DFA data in the absence of cultures. Electron-capture gas chromatography has been suggested for detection in water specimens (129). Presently, culturing samples appears to be the most widely used technique, alone or together with DFA.

**Isolation Methods**

McDade and co-workers (207) initially isolated *L. pneumophila* in guinea pigs inoculated intraperitoneally with tissue suspensions from fatal cases occurring during the 1976 Philadelphia outbreak. Tissues from infected guinea pigs were lethal for 6-7 day old embryonated hens' eggs. The organism did not grow on trypticase soy or blood agar or in thioglycollate broth. It was cultured successfully on Mueller-Hinton (MH) agar containing 1% hemoglobin and 1% IsoVitaleX (Incubated in a 5% CO$_2$ atmosphere) from both infected material obtained in laboratory experiments as well as from the original human tissues. It also has been shown to grow on commercially enriched chocolate agar (84).

Analysis of this modified MH agar revealed that L-cysteine HCl could substitute for the IsoVitaleX and ferric pyrophosphate for the hemoglobin (110). This medium, Feeley-Gorman agar, was further modified by the
addition of yeast extract as a protein source and activated charcoal to remove inhibitors produced during preparation (109, 154). This charcoal yeast extract agar (CYE), further modified by the addition of a buffer, N-(2-acetamido)-2-aminoethane sulfonic acid (ACES), and α-ketoglutarate (BCYE), has resulted in enhanced growth of legionellae (85, 237). The pH of the medium is important, and should be adjusted to 6.9 using KOH, as NaOH has been shown to be inhibitory (109). BCYE is satisfactory for specimens which normally are expected to be sterile (177) but the slow-growing legionellae often are inhibited or masked when contaminated specimens such as sputum or environmental samples are cultured. Glycine has been shown to be an effective selective agent by Wadowsky and Yee (315). Antibiotics have been added in various combinations (30, 85, 94) as well to improve the ability to isolate legionellae from contaminated specimens. Comparative studies have demonstrated the effectiveness of these media for clinical specimens (50, 85, 97) although strains of *L. micdadei*, *L. gormanii*, *L. dumoffii*, and possibly other species may be inhibited by some components (315).

Pretreatment of contaminated specimens using heat (97), low pH (30, 50), or negative enrichment (299), has been suggested with acid pre-treatment resulting in greater enhancement with clinical specimens, especially
when used with a semi-selective medium (50, 97). When compared with DFA, culture is more sensitive for *L. pneumophila* by a factor of 1.2 to 4 (50, 87, 88). Results with environmental samples are mixed (30, 52, 76, 85, 86, 98). Direct plating methods are generally agreed to be superior to the original guinea pig inoculation method (76, 98) but it appears that pretreatment, concentration, or use of selective media may all reduce the number of organisms recovered and/or the species isolated (52, 85, 314). Thus, no single selective medium or pretreatment method can be used exclusively, and most environmental samples will probably require a combination of several techniques for maximal recovery of legionellae.

**Identification of Isolates**

Presumptive identification at the genus level* can be made if after 3 to 5 days of incubation, small (1-2 mm in diameter) colonies of pleomorphic gram-negative bacilli appear on BCYE but not on media without L-cysteine (e.g., trypticase soy blood agar or BCYE made without it). After several days of growth, the colonies

*Three genera in the family *Legionellaceae* have been proposed: *Legionella*, *Tatlockia*, and *Fluoribacter* (45, 128). The issue remains controversial (38). This work refers to the organisms using the one genus (*Legionella*), classification system as described by Brenner et al. (40, 41).
become larger, more convex, and finally flatten out (87). Two non-Legionella organisms which might be misidentified by this growth test are a thermophilic Bacillus sp. (291) and Francisella tularensis (198). These can be distinguished by the ability to grow at 55C (Bacillus) or by colonial morphology, growth rate and carbohydrate utilization (F. tularensis).

Speciation of the legionellae is more difficult (Table 2). All species fail to produce acid from glucose, reduce nitrate, or produce urease. It has been said that all species produce catalase (38); however, Pine and co-workers (242) have shown that L. pneumophila does not actually produce catalase. Instead, this species produces only a peroxidase with weak catalase-like activity. Pine et al. (242) described a whole-cell peroxidase test which can distinguish L. pneumophila from the other species. L. pneumophila is also the only species which hydrolyzes sodium hippurate (140) although some strains of L. feeleii and L. spiritensis also may be weakly positive (40). L. micdadei has been shown to be acid-fast in tissue (221, 256) although it may lose this characteristic when grown on BCYE (152).

Autofluorescence and browning on media containing tyrosine (but not charcoal) also have been used to identify species (312). Vickers, Brown, and Garrity (311) have developed a dye-containing differential
Table 2
Phenotypic Characteristics of the 23 Known Species of Legionella

<table>
<thead>
<tr>
<th>Species</th>
<th>Catalase</th>
<th>Browning</th>
<th>Gelatin liquefaction</th>
<th>Hipurate hydrolysis</th>
<th>Motility</th>
<th>Oxidase</th>
<th>Beta lactamase</th>
<th>Autoflour-</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. pneumophila</td>
<td>2,3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+ or +/-</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>L. feeleii</td>
<td>+</td>
<td>(w)</td>
<td>-</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L. spiritensis</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>L. longbeachae</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
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<tr>
<td>L. jordanis</td>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>L. oakridgensis</td>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>L. wadsworthii</td>
<td>+</td>
<td>+</td>
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<td>-</td>
<td>+</td>
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<td>L. saintchelensi</td>
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<td>L. hackelise</td>
<td>+</td>
<td>(w)</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>L. maceachernii</td>
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<td>+</td>
<td>-</td>
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<td>-</td>
<td>+</td>
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<td>+</td>
<td>-</td>
</tr>
<tr>
<td>L. micdadei</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>L. israeliensis</td>
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<td>(w)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>L. bozemanii</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>(bluish-white)</td>
</tr>
<tr>
<td>L. dumoffii</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(bluish-white)</td>
</tr>
<tr>
<td>L. gormanii</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(bluish-white)</td>
</tr>
<tr>
<td>L. anisa</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(bluish-white)</td>
</tr>
<tr>
<td>L. cherrii</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(bluish-white)</td>
</tr>
<tr>
<td>L. steigerwaitii</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(bluish-white)</td>
</tr>
<tr>
<td>L. parisiensis</td>
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<td>+</td>
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<td>+</td>
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<td>-</td>
</tr>
<tr>
<td>L. rubrtiucens</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>L. erythra</td>
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<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>(red)</td>
</tr>
</tbody>
</table>

* Data from refs 18 and 40

It must be noted that in some cases reactions are based on one or only a few strains and may be modified in the future.

1 Browning produced on charcoal-treated yeast extract agar containing tyrosine.

2 + = positive; - = negative; +(w) = weak reaction; +/- = weakly or not always positive.

3 L. pneumophila produces a peroxidase with weak catalase-like activity (see text).
medium which is probably very useful in their institution where both \textit{L. pneumophila} and \textit{L. micdadei} are endemic (216). It is not likely to be cost effective in other settings. It must be noted that biochemical reactions in some cases are based on a single strain or only a very few strains, and these reactions may be modified as more are isolated (38).

Clinically, serogrouping is the most useful way to distinguish species (87). This may be done by DFA (60, 71, 288), agglutination (292, 293, 295, 325) or possibly counterimmunoelectrophoresis (275). There is some cross-reactivity among species and, rarely, other genera (16, 24, 95, 165). An isolate which fails to serotype still has a high likelihood of being \textit{Legionella} (either a new serogroup or species) (87). DNA hybridization studies, impractical for the clinical laboratory, may be necessary for definitive identification of species (38).

\textbf{Seroologic Diagnosis}

Serologic testing by McDade et al. (207) during the 1976 Philadelphia outbreak determined that a 4-fold increase in titer to a minimum of 1:64 or a single titer > 1:128 using an indirect fluorescence assay (IFA) could be associated with disease. Studies of titers in apparently healthy populations (147, 264, 280) led the CDC to modify these values to a 4-fold increase to at least 1:128 or a single titer of at least 1:256 for \textit{L. pneumophila} serogroup 1 antibodies (324). The incidence
of antibodies to other species is less well-defined although recent studies suggest that prevalence may be higher for at least some species than for *L. pneumophila* (32, 321, 328).

Titers described above were obtained using a heat-killed organism as the antigen in the IFA. Several other methods have been described for preparing the antigen (15, 230, 238, 287) which have been shown to result in lower titers but, if the titer which is used to determine infection is also lowered, these methods are equivalent in sensitivity (107, 323). In order to interpret titers accurately, the type of antigen must be known. Another problem in interpretation results from the fact that some patients develop species-specific antibodies (45% in one study [328]) while others appear to react with a common antigen (55% [328]) so that no single antigen could be used to detect all *Legionella* infections.

Cross-reactivity of *Legionella* with other organisms appears to be uncommon. Early concerns about *Mycoplasma pneumoniae* cross-reactivity (134) appear to have been discounted (247, 322). Rarely, sera have been shown to cross-react with certain strains of *Bacteroides fragilis* (95), *Hemophilus influenzae* and *Staphylococcus aureus* (172) and *Chlamydia psittaci* (232). Patients with antibodies to *L. bozemanii* have been shown to have similar serological reactivity with *Rickettsia typhi* and
Proteus vulgaris OX19 (276).

Other methods, which are either easier to use or can be automated have been described and may overtake the IFA in popularity. A microagglutination assay was described in 1978 by Farshy, Klein and Feeley (108) which has been shown to be useful (68, 139, 185, 286). A limitation of this procedure is the cross-reactivity of agglutinating antibodies with species of Pseudomonas (69, 184). Collins and associates (69) reported that cystic fibrosis patients often had elevated titers to Legionella antigens (41.3% of the 128 patient samples tested had titers > 1:64 to one or more as compared to 9.7% of 103 normal controls). These titers correlated directly with those to P. aeruginosa, a common cause of infection in these patients. In another study (66), this same group used crossed immunoelectrophoresis to demonstrate that L. pneumophila possessed an antigen related to the "common antigen" of P. aeruginosa. This cross-reactivity is found in several species of Legionella, but is highest with L. bozemanii (69).

Enzyme-linked immunosorbent assay has also been used for antibody detection (100, 108, 262) and a solid-phase immunofluorescence assay has recently been reported (26, 298). Indirect hemagglutination (193, 341), and immunodiffusion (277) have also been evaluated and found to be useful. Rodgers and Laverick (253) have tested a passive hemagglutination assay using flagellar
antigen-coated turkey erythrocytes which may be useful in detecting antibodies to those Legionella species which are flagellated (the majority). Sampson, Plikaytis, and Wilkinson (261) recently have shown that a 58 kilodalton (Kdal) protein common to all 7 species and 8 serogroups tested reacted with sera from 100% of patients with culture-confirmed LD (L. pneumophila serogroup 1) and 78% of those which were diagnosed serologically. This may lead to a test which is able to detect most species associated with human disease without the occasional cross-reactivity seen in assays currently in use.

Pathogenesis

Investigations of the ability of legionellae to cause disease were initially hampered by the lack of an appropriate animal model. McDade et al. (207) originally isolated the organism by intraperitoneal inoculation of guinea pigs but the pathologic lesions did not resemble those seen in human LD (56, 332). Since it seems likely that humans are infected by the respiratory route, various experimental models have been developed using this means to induce infection. Intranasal and intratracheal instillation are analogous to aspiration of oropharyngeal contents which may be an important mechanism in the development of nosocomial LD in the compromised host. Guinea pigs and rats have been
infected by these methods but there are drawbacks (332).

Exposure to concentrated bacterial aerosols is most similar to the ways humans may acquire the disease in the community. Whole body aerosols have been used with guinea pigs (22, 176, 209, 217) but many organisms wind up in body fur as well as in the lungs (332). Several investigators have used snout-only aerosol exposure to produce infection in several animal species, including guinea pigs, rats, mice, marmosets, and monkeys. Guinea pigs are moderately susceptible to disease but rats and monkeys are more resistant and mice appear to be very resistant to pneumonia when inoculated this way. Lesions produced in this manner appear to be similar to and mimic human disease rather well (332).

Once an animal model had been developed, it became possible to evaluate a variety of potential virulence mechanisms in vivo as well as in vitro. Virulence may be a result of physical attributes, the production of soluble toxic factors, or the ability to avoid or affect host defense mechanisms. *Legionella* appears to possess capabilities in all these areas, but a most important factor in producing disease well may be its ability to multiply intracellularly within monocytes and macrophages. Each of these aspects will be discussed in more detail below.

**Ultrastructure**

The legionellae have been studied using human lung
tissue, and organisms grown on various types of artificial media and in the yolk sac of fertile hens' eggs. The organism is ultrastructurally similar in all preparations, with exceptions as noted below.

In an early report, Rodgers, Macrae, and Lewis (254) described the organism in tissue as a short coccobacillus (0.6 µm wide, 1-2 µm long) with parallel sides, tapered ends and a smooth surface. Vacuole-like bodies were evident and occasional organisms had a loose undulating outer membrane. Most organisms were seen intracellularly in the cytoplasm of degenerating cells; none were found in the nuclei.

These findings were confirmed and expanded in subsequent studies (54, 178, 250). Long forms and filaments 10-20 µm long were rarely seen in tissue but were seen in preparations from cultures on artificial media (54, 250). In 1984, Bornstein, Nowicki, and Fleurette (33) correlated the average lengths of organisms grown on various media with the LD$_{50}$ of the same form for embryonated hens' eggs. They found that after 10 subcultures, the shortest forms, grown on embryonated egg yolk sacs, also had the lowest LD$_{50}$ (10$^{1.36}$) while the longest forms grown on BCYE, had the highest LD$_{50}$ (10$^{4.79}$). They suggest that increased numbers of filamentous forms may be associated with decreased virulence. In another study, Chan (Ph.D. thesis, Virginia Commonwealth University, 1982)
associated morphological changes produced by polymixin B treatment with increased virulence for guinea pigs. He also noted increased vacuolation associated with virulence.

Glavin et al. (131) observed an electron-lucent gap surrounding organisms in sections of human lung tissue and suggested that this might represent a microcapsule. Rodgers, using ruthenium red to detect the polyanions associated with capsules, detected none (250, 251). However, Hebert et al. (141) did observe rare capsules in *L. pneumophila* using ruthenium red. This discrepancy may be due to slight differences in technique, strain differences, or different growth conditions (255).

Another area in which discrepancies were found in early studies involved the presence or absence of an electron-dense peptidoglycan layer, found in gram-negative bacterial cell walls (54, 117, 178, 250). Keel and co-workers (178) saw no peptidoglycan structure on electron-microscopy, but did find typical components biochemically, with the exception of the cross-linking material, diaminopimelic acid. Chandler et al. (54) did report its presence in their study. The importance of this feature is related to the endotoxic properties of the typical gram-negative cell wall. Endotoxin activity associated with legionellae is discussed more fully in the next section.

Early studies found no evidence of flagella (54,
More gentle preparation techniques did lead to the observation of flagella and pili by several groups (57, 141, 252, 296). Most often organisms had a single polar or subpolar flagellum up to 8 um long. Occasionally pairs of polar flagella were seen. Many were straight, but gently curved as well as very curly forms were noted. The fact that not all strains had flagella or pili would suggest that this is not important for virulence in Legionella spp. Elliott and Johnson (102) did report flagella on organisms known to be virulent while none were found on those nonvirulent forms grown on artificial media. Later work by these same authors (103) showed that lack of flagella, while associated with growth on certain types of media, was not associated with virulence. Organisms grown on BCYE did not decrease in virulence and retained flagella after 12 passages. Organisms grown in yeast extract broth failed to produce flagella, but remained virulent, at least for guinea pigs injected intraperitoneally.

**Virulence Factors**

As mentioned above, gram-negative bacterial cell walls are associated with a heat-stable lipopolysaccharide (LPS) toxin (36). Wong and co-workers (216) first demonstrated that L. pneumophila does exhibit "endotoxicity" but it differs from the classic activity associated with gram-negative bacteria. It was weaker in a variety of endotoxicity tests and
reacted biochemically in an atypical fashion. L. pneumophila LPS has been shown to contain 2-keto-3-deoxyoctonate (62, 126, 153, 336) as other gram-negative LPSs do, but does not contain heptose (235) or large amounts of hydroxy fatty acids. In fact, the legionellae possess primarily unique branched-chain fatty acids which are predominately unsaturated (114, 203, 336).

Outer membrane proteins also are important in bacterial virulence (274). Several groups have isolated a major outer membrane protein (MOMP) which appears to be species-specific (51, 153, 226). This MOMP has a molecular weight in the range of 24-30 Kdal (51, 126, 153). Hindahl and Iglewski (153) characterized their 29 Kdal MOMP as similar to that of the Chlamydiae. They suggested a possible role for this MOMP in the inhibition of lysosome-phagosome fusion (see below) which L. pneumophila and C. psittici are both known to do. The fact that monoclonal antibodies apparently detect surface antigens which differ according to virulence (169, 236, 306, 319) also supports the involvement of membrane components in causing disease.

Transmissible plasmids are known to confer antibiotic resistance and have been associated with toxin production in other bacteria (36). Plasmids in varying numbers have been found in the legionellae. Most plasmids have been found primarily in environmental
isolates while the majority of clinical isolates have been plasmidless (46, 47, 96, 199, 228), although a recent study conducted at the Wadsworth Veterans' Administration Hospital in California found plasmids in 95% of their clinical isolates (96). One group (29) has reported increased virulence with the plasmidless form associated with almost all the nosocomial LD cases seen in their institution. Both types tested were environmental isolates from the potable water system; the plasmidless organism had a lower LD$_{50}$ as determined by intraperitoneal inoculation of guinea pigs as well as a shorter time to death. While plasmid transfer into Legionella species has been reported (58, 179) as well as among Legionella species (81), it was difficult and probably is not a major virulence factor.

Extracellular products capable of producing tissue damage have been described. A hemolytic cytotoxin has been reported for several species (9, 125, 301). Thorpe and Miller (301) found no difference in activity in environmental, human, animal-passaged, and laboratory-adapted strains, suggesting that this hemolysin is not solely responsible for conversion to avirulence. Baine (8) has demonstrated phospholipase C activity (associated in several bacterial species with hemolysis) which may explain at least partially the ability to damage erythorocyte membranes. Baine also suggests that this activity may inhibit lysosome-phagosome fusion. A
variety of aminopeptidases, esterases and proteases also has been reported (13, 82, 218, 219, 229, 300) including one protease which causes pulmonary damage in guinea pigs when administered intranasally or intratracheally (13). Another protease has been shown to have chymotrypsin-like activity (20). These proteases may produce pulmonary damage although major tissue-destructive proteases other than the one described above (13) have not been detected with certainty. Another toxin has been shown to affect the metabolism of human neutrophils with no apparent effect on viability or phagocytosis (124). This may play a role in the ability of legionellae to survive intracellularly. Muller (220) has developed a system to test for inducible enzymes which may correlate with virulence.

The fact that LD symptoms indicate multisystem involvement (renal, gastrointestinal, neurological) with the organism itself found only rarely outside the lung suggests that a toxin is important in causing disease. Legionellae have been isolated from the blood so the presence of bacteria in low numbers in other organs is possible as well (248).

One problem in interpreting many of these studies relating to extracellular products is the omission of passage history or determination of virulence of the organism(s) used. L. pneumophila has been shown to lose virulence when passaged on artificial media (206)
including BCYE agar (33, 163, 313). Virulence is restored upon passage in cultured human embryonic lung fibroblasts (338) or in guinea pigs or embryonated eggs (103). Chan (Ph.D. thesis) recently has evaluated a buffered egg yolk medium which appears to maintain virulence longer than BCYE. If this is so, virulence studies would be simplified by the omission of periodic passage through living material in order to maintain virulence.

Interactions with Phagocytic Cells

Intracellular organisms seen in histologic sections from lungs of patients with LD suggested that the ability to multiply within the cells found in lung was an important process in the ability of *L. pneumophila* to cause disease. Horwitz and Silverstein demonstrated in 1980 (157) that the organism did indeed multiply intracellularly in human monocytes but not in PMNs or lymphocytes. This also has been found to be true for human alveolar macrophages obtained by bronchoalveolar lavage (222). Tissue cultures of various cell lines including human embryonic lung fibroblasts also support intracellular growth (73, 231, 337) and are more convenient and more easily obtained than the human monocyte/macrophage. Various animal models, most often the guinea pig, also have been used to demonstrate similar findings in vitro. The ability to multiply well intracellularly has been associated with only the
virulent strains tested. The organisms lose this ability, which correlates with loss of in vivo virulence, when cultured on artificial media (101, 164, 182). None of these studies found that *L. pneumophila* could grow extracellularly under experimental conditions. The cells do not produce a soluble growth factor; the organism must find its way inside the cell.

Phagocytosis of legionellae is characterized by several unusual findings. Horwitz (156) found that long phagocyte pseudopods coil around the organism as it is internalized; however, this was not seen by Oldham and Rodgers (231) or Elliott and Winn (104). All agree that once internalized a novel phagolysosome is produced. Shortly after entry, most vacuoles are surrounded by smooth vesicles and mitochondria. This is quickly followed by ribosomes and rough vesicles. By 8 h after entry, all vacuoles are studded with ribosomes (157). Evidence suggests that the bacteria avoid the bacteriocidal effects of intracellular enzymes by preventing fusion of the phagosome with the enzyme-carrying lysosomes (156).

The fact that bacteria are inside the phagocytes probably protects them from the bacteriocidal effects of human serum in vivo. They have been shown to be susceptible in vitro (3, 31, 243). Interestingly, while coating with specific antibody and complement increases uptake, it does not significantly affect the ability of
L. pneumophila to multiply intracellularly (159, 182, 313, 339). Humoral immunity is thus not the major means by which the organism is cleared.

Several studies have shown that activated macrophages are able to inhibit bacterial multiplication (160, 222, 339). These studies used the supernatents from mitogen-stimulated cells to activate the phagocytes. Bhardwaj et al. (23) have shown that human recombinant \( \gamma \)-interferon at relatively high concentrations will also inhibit multiplication. They also noted that the phagosome in the activated cell was structurally similar to that observed in the nonactivated phagocyte. Most studies have demonstrated little effect on the ability of the cell to kill the organism. A study using athymic nude mice (83) suggests that cell-mediated immunity is important in host defense in vivo as well.

PMNs do not support the intracellular growth of L. pneumophila but neither do they kill the bacteria efficiently (157, 158). Lochner and co-workers (124, 195) have shown that a toxin produced by the organism leads to impaired activation of the superoxide-generating complex which may explain the reduced killing ability, since both virulent and avirulent strains are sensitive to the effects of oxygen metabolites generated in vitro.

The role of erythromycin and rifampin in therapy
seems to be to inhibit multiplication rather than to kill. These antibiotics are able to penetrate the cell, but intracellular bacteria appear to be protected. Extracellular organisms are killed at antibiotic levels near the minimal inhibitory concentrations, but intracellular organisms are resistant to killing at higher concentrations equal to or greater than peak serum levels in humans. Failure to treat for an adequate length of time may lead to relapses as once the antibiotic was removed the bacteria began to multiply again (156).

Transmission

The large number of branched-chain fatty acids found in *Legionella* species are common in thermophilic bacteria (118). This association led Fliermans and colleagues to look for legionellae in a variety of aquatic habitats. Theirs was the first report of nonepidemic-related isolation of *L. pneumophila* in natural lakes in the Southeast (120). This study was expanded and they subsequently found legionellae to be a common inhabitant of freshwater lakes and rivers over a wider geographic area (119). The organism also was isolated from a thermally altered lake. They found it to be able to survive extreme ranges of environmental conditions (i.e., isolated from waters ranging in temperature from 5.7 to 63°C and pH from 5.5 to 8.1).
also has been detected in mud (213), rainwater (308), and in oxidation ponds and fish ponds (17). In most instances, even in those specimens with the highest numbers, *L. pneumophila* comprised less than 1% of the total bacterial population (119). Legionella-like organisms have been observed in the Caribbean Ocean but in areas tested in the United States neither isolates nor DFA positive samples were obtained once salinity levels increased to saltwater concentrations (118). Skaliy and McEachern (273) showed that 4 strains of *L. pneumophila* were able to survive 69-139 days in distilled water and 364-369 days in tap water in seeded samples.

Once legionellae were known to survive and multiply in water, the question of amplifiers and disseminators arose. Early outbreaks were associated with cooling towers and evaporative condensers (70, 79, 138, 183, 213). These heat rejection systems were suspected to be important in dissemination of disease. This mechanism could not account for all outbreaks (e.g., St. Elizabeth's Hospital used the "open window" air conditioning system [290]) nor for all cases in some (215). Airborne spread as a result of soil excavations was postulated to be the cause in the St. Elizabeth's outbreak.

Reports of isolation of legionellae from potable water and its association with LD then began to appear
(12, 34, 115, 146, 270, 281, 310, 343). As Muder, Yu, and Woo point out in their review (215), virtually all outbreaks occurring after this time cite the water distribution system as a culprit, usually with no stronger evidence than earlier studies incriminating heat rejection systems.

Airborne transmission with inhalation of contaminated aerosols is undoubtedly an important means of infection; however, other ways may be possible. Human pneumonias may develop following aspiration, usually of organisms in the oropharynx (215). The generally low attack rates associated with LD as well as the high incidence of nosocomial LD in patients with conditions predisposing to subclinical aspiration (e.g. general anesthesia with endotracheal intubation) might support this theory. A weakness is the lack of evidence that *L. pneumophila* colonizes the oropharynx. Bridge and Edelstein (42) found only 3 of 186 (1.6%) throat specimens positive by DFA using CDC's criterion, and no organisms were isolated. Their study group included 40 patients housed in a building which had been associated with endemic LD; the conditions of the patients were not described. Aspiration of contaminated drinking water is possible. *L. pneumophila* has been isolated from ice machines which may be the primary source of a patient's drinking water (215).

Ingestion as a means of infection has received less
attention. This is interesting, as the only parameter that could be statistically linked to acquisition of disease in the 1976 outbreak was ingestion of water (121). Katz and Matus (175) have shown that guinea pigs develop a mild pneumonitis and fever when given infected water to drink. A study by Jones et al. in Connecticut (170) found a significant association of nosocomial disease with antacids. The role of antacids is unclear but they postulate that this neutralizes gastric pH, thus facilitating infection by ingestion, possibly followed by aspiration and pneumonia.

Heat Rejection Systems

Cooling towers and evaporative condensers are widely used to dissipate unwanted heat into the atmosphere. All depend on contact between circulating water and ambient air. As the heated water is passed through an airstream, evaporation occurs and the remaining water is cooled. This cooled water collects at the bottom of the unit and is discharged intentionally at intervals to control the build-up of sediment. This water may be "bled" into a storm sewer, sanitary sewer, or even a nearby stream or lake. In addition, the airstream leaving the unit is saturated and may contain a small portion of the circulating water in the form of droplets known as drift. Airborne contaminants near the unit may be absorbed and organisms in the circulating water may be released into the
environment (212).

The air conditioning system was implicated in the 1968 outbreak of Pontiac fever (176). Sentinel guinea pigs exposed to the incriminated building developed disease. Laboratory studies using whole body aerosol exposure to evaporative condenser water collected at the site also led to disease in the guinea pigs. L. pneumophila was isolated from cooling tower water during an outbreak of LD in Memphis, Tennessee (79). This tower was thought to be a source for the outbreak but from evidence gathered at the time, other reservoirs were probable and the possibility of diagnostic bias in identifying cases of LD could not be excluded. Stronger evidence implicating an evaporative condenser at a Georgia country club was reported by CDC researchers investigating an outbreak of LD (70). L. pneumophila serogroup 1 was isolated only from condenser water and tracer studies suggested that exhaust was directed toward certain tees on the golf course. The degree of golfing activity during the likely exposure period was a risk factor for illness.

A more recent study in Rhode Island (127) also incriminates cooling towers as a source. L. pneumophila was isolated from potable water but the decline in cases of LD following treatment of only the cooling towers led the investigators to conclude that this was the primary disseminator. The follow-up period was brief (10
months) and did not include the months when most cases initially occurred. This conclusion may be premature given the seasonality of LD and the possibility of less diligent searches for LD after treatment of the towers. **Potable Water**

Tobin and colleagues were the first to report the isolation of *L. pneumophila* from potable water taken from a shower unit in the area in which patients with LD were housed (11). Since that time, hospital potable water has been found to contain *L. pneumophila* in many sites within an institution as well as in institutions all over the world (12, 34, 72, 115, 146, 209, 270, 281, 310, 316, 343). These isolates have been associated with nosocomial LD outbreaks in some instances (12, 34, 115, 146, 270, 281, 310, 343), but not in others (72, 77, 120, 316). An interesting case is the epidemic at Wadsworth Veterans' Administration Hospital. Originally thought to be the result of contaminated cooling towers, the number of cases declined dramatically only after the potable water supply was treated. No isolates of *L. pneumophila* and no cases of LD have been found in over 2 years after treatment (270). The fact that many LD patients do not shower in the hospital nor come into contact with aerosols generated by hot water faucets suggests other methods of exposure. Cases of LD have been reported when various aerosol-producing devices are filled or rinsed with tap water, including a mechanical
humidifier (171) a portable room humidifier and jet nebulizers used in respiratory therapy (6). Non-hospital associated epidemics in which potable water has been suggested also have been seen (11, 12, 335). Legionellae have been isolated in residential potable water without apparent associated illness (7, 64). Organisms have not been isolated from treated water entering the distribution network (64, 304), although in one case (304) low numbers were seen using DFA. This would suggest that public water supplies are not the source or at most would seed only small numbers into the distribution system.

Conditions in hot water sources may be conducive to survival and multiplication. Water temperatures lower than 60°C have been associated with isolates (7, 244, 340) as well as stagnation which occurs during periods when tanks are not in use (61, 115). Faucet obstructions (e.g. aerators, backflow preventors) have been shown to support the survival of _L. pneumophila_ (61). Another material implicated in increasing the ability of the organism to survive is the rubber washer (115, 267). A study by Niedenveld and associates (225) found 9 of 14 rubber compounds and 15 of 30 constituents would support the growth of _L. pneumophila_ in naturally contaminated hot water.

Other bacteria may provide nutrients for the legionellae. Stout et al. (282) found that the presence
of environmental bacteria enhanced the survival of *L. pneumophila*. Schofield and Loeci (266) showed that in the absence of other organisms, *L. pneumophila* failed to colonize various washer fittings in laboratory experiments. Both groups suggest that sediments and stagnant warm water allow increased multiplication of other flora which may in turn provide nutrients for the growth or survival of legionellae.

### Role of Protozoa and Algae

In addition to the bacteria found in association with legionellae, there is evidence to suggest interactions with other organisms occur, which are favorable to the legionellae. Tison et al. (303) reported in 1980 that *L. pneumophila* would grow in association with the blue-green algae (cyanobacteria). They isolated *L. pneumophila* from a thermophilic algal mat community growing at 45°C in a man-made thermal effluent. Their experiments suggested that the *L. pneumophila* were using algal extracellular products as carbon and energy sources and that growth was dependent on active photosynthesis. They theorized that this may be a mutualistic association, with the algae providing oxygen for the bacteria while the algae used the carbon dioxide produced by the bacteria for photosynthesis.

Since then, species of green algae (chlorophyta) have also been shown to enhance growth of *L. pneumophila* (162). Since algae are common contaminants in aerosol-
generating water systems it is possible that inhalation of a single algal cell carrying *L. pneumophila* would be an infective dose.

In the same year (1980), T. J. Rowbotham reported that *L. pneumophila* was pathogenic for free-living, ubiquitous freshwater and soil amoebae of the genera *Acanthamoeba* and *Naegleria* (258). He suggested that a fecal vacuole or amoeba full of legionellae could be the infective particle for humans. Other researchers have confirmed this preliminary work with a variety of amoebal species (1, 224, 309). Fields and associates (113) have demonstrated a similar relationship with the ciliated protozoan *Tetrahymena pyriformis*.

There appears to be a limited range of infectivity, with some species of amoebae supporting growth of only certain strains of certain serogroups of *L. pneumophila* (258). The legionellae have been shown to grow intracellularly and viable amoebae are required (1, 113, 155, 224). Newsome et al. (224) have shown that the intracellular vacuoles produced in the amoebae are similar to those seen in human macrophages. There is an alignment of mitochondria and ribosome-like structures along the vacuole membrane.

Barbaree and co-workers (10) were the first to look for protozoa as well as legionellae in investigating an outbreak of legionellosis. They found amoebae as well as two species of ciliates in cooling tower water
containing \textit{L. pneumophila}. Two of the 4 cooling towers investigated were implicated in the spread of disease and both had amoebae and/or a ciliate as well as the \textit{L. pneumophila}. One tower which was not implicated had bacteria and amoebae; the other tower had only legionellae. They suggested that the protozoa may be reservoirs for supporting survival and multiplication of virulent legionellae. Rowbotham (259) suggests that \textit{L. pneumophila} may be protected from biocides used in various water treatment programs by infecting amoebae which then encyst. The resulting cysts are resistant, especially to the chlorine commonly used in the water supply industry. A study by Tyndall and Dominque (309), using only one strain of \textit{L. pneumophila} isolated from the environment, suggests that neither the infected amoebae nor the \textit{L. pneumophila} has increased virulence or pathogenic potential as a result of their relationship. This may not be true for other strains of bacteria or for interactions with other species of protozoa.

In addition to their roles as reservoirs and amplifiers, amoebae have been postulated to play a role in human disease. Rowbotham (260) suggests that Pontiac fever is a result of hypersensitivity pneumonitis to the vesicles of acanthamoebae coupled with a mild infection by \textit{L. pneumophila}. When the vesicles and intact \textit{Legionella}-containing amoebae are inhaled by someone
with increased risk, LD results. Dual infection, which has been reported rarely, also could be explained on this basis as well, since a single amoeba has been shown to be infected by more than one type of Legionella (260). Since the intracellular changes in amoebae and human leukocytes are similar, it is likely that humans are only incidental hosts for the legionellae, whose natural habitat is the aquatic environment. As pointed out by Spriggs (278), "It's humbling to consider that Legionella's shift from amoebae to humans is less a leap than a lateral shuffle."

The possibility of other animal reservoirs has received little attention. Serological studies have shown that a surprisingly high number of horses (31.4% in one study [67]) have elevated titers to L. pneumophila when values found in humans are used to determine infection. Direct challenge has not resulted in disease, but seroconversions were noted (65). Various species of primates as well as small numbers of cattle, swine, sheep, antelope, water buffalo, and dogs have had elevated titers (63, 65, 67). Aquatic birds have been shown to have titers which paralleled those seen in the normal human population, and a few birds (e.g. pelicans) had values much higher, suggesting an unusual prevalence in their environment or a unique susceptibility to infection (65). The possibility of
cross-reactions, discussed previously, in the serological studies cannot be eliminated. Only one study has reported attempts to isolate legionellae from animals. Cohen et al. (63) reported that 17% of 139 bovine cadaver lungs and lungs from 4% of 29 slaughtered calves were positive by DFA; however, only 2 strains of \textit{L. pneumophila} serogroup 1 were isolated from 2 cadavers.

**Objectives of the Study**

1. To evaluate a buffered egg yolk medium for its ability to support the growth and maintain virulence of \textit{L. pneumophila}.

2. To confirm the ability of polymixin B to alter morphology and virulence of another strain of \textit{L. pneumophila}.

3. To compare virulent and avirulent \textit{L. pneumophila} for differences in morphology, plasmid content, proteins as detected by sodium dodecylsulfate-polyacrylamide gel electrophoresis, and reactivity with monoclonal and polyclonal antibodies.

4. To evaluate three species of fish both as a model for infection and as a potential environmental reservoir for \textit{L. pneumophila}.
MATERIALS AND METHODS

Organisms and Media

Virulent and avirulent *Legionella pneumophila*, serogroup 1, were kindly provided by Dr. Washington C. Winn, Jr. (Department of Pathology, University of Vermont, College of Medicine, Burlington, VT 05405). This strain, Burlington 1, originally was isolated from a patient with Legionnaires’ Disease. The original isolate was propagated on modified Mueller-Hinton agar to produce the avirulent form.

Virulence was confirmed by inoculating guinea pigs with growth from a 72 h culture of each organism as described below. Aliquots of the liver and spleen suspensions (see virulence studies) from infected animals were frozen at -70°C for future use. In addition, organisms isolated from these tissues as well as those from the original culture were used to make heavy suspensions in 1 ml aliquots of freezer broth (50% glycerol, 50% trypticase soy broth) and frozen at -70°C. For each experiment requiring bacteria, an aliquot of either tissue or culture suspension was thawed, plated on buffered charcoal yeast extract agar and incubated at 35°C in an atmosphere of 5% CO₂ for 72 h.

Buffered charcoal yeast extract agar containing α-
ketoglutarate (BCYE) prepared as shown in Appendix A was routinely used to culture the organism. When material suspected of contamination was cultured, it was necessary to use a semi-selective medium (BMPA α), prepared as shown in Appendix A. Also in this Appendix are the formulas for the test medium, egg yolk buffered agar (EYB), and yeast extract buffered broth (YEVB).

**Animals**

Male Hartley strain guinea pigs weighing 250 to 300 g were housed individually in plastic cages and allowed free access to standard guinea pig food and water. During and after inoculation the animals were handled and housed within a biologic safety cabinet.

Hogchoker (Trinecte maculatus) 7-12 cm in length (see Figure 1) were captured by trawl net from the Ware and Nansemond Rivers on several occasions. Salinities and water temperature were roughly equivalent in the two river systems. Fish for the chemotactic study were held in tanks of flowing seawater at ambient temperature until use and fed Zeigler's trout chow daily. Fish for the virulence studies were housed at room temperature in ten gallon aquaria (approximately 20 fish per tank) containing Instant Ocean® (Aquarium Systems, Mentor, Ohio 44060) prepared so that the specific gravity fell between 1.020 and 1.023. Approximately 30% of the water in each aquarium was replaced with fresh Instant Ocean every other day. Fish were fed frozen brine shrimp.
every other day. The aquaria were kept in a biological safety hood during all experiments.

Spot (Leiostomus xanthurus) 9-13 cm in length (see figure 2) were captured and handled as described above for the hogchoker.

Golder shiner minnows (Notemigonus crysoleucas) 7-13 cm in length (see Figure 3) were purchased from

![Image of fish]

Figure 1

Ware or Nasemond River Hogchoker (Trinecte maculatus)
every other day. The aquaria were kept in a biological safety hood during all experiments.

Spot (*Leiostomus xanthurus*) 9-13 cm in length (see Figure 2) were captured and handled as described above for the hogchoker.

Goldie shiner minnows (*Notemigonus crysoleucas*) 7-13 cm in length (see Figure 3) were purchased from Paul's Bait Shop, 3400 Mechanicsville Turnpike, Richmond, Virginia 23223. These fish were housed at room temperature in ten gallon aquaria (approximately 20 fish per tank) containing tap water treated with Start Right® (Jungle Laboratories Corporation, Cibolo, Texas 78108). Approximately 30% of the water in each aquarium was replaced with fresh treated tap water every other day. Fish were fed frozen brine shrimp daily. The aquaria were kept in a biological safety hood during all experiments.

**Virulence Studies**

A. Guinea pigs

1. Determination of virulence

Organisms initially were evaluated to confirm virulence or avirulence. Cultures of each isolate grown on BCYE for 72 h at 37°C in a 5% CO₂ atmosphere were suspended in 9 ml of YEBB and adjusted to match approximately the opacity of a McFarland No. 3 standard. Serial 100-fold dilutions were made in YEBB; 0.1 ml aliquots of each dilution were plated in duplicate on
Figure 2

Ware or Nansemond River Spot (*Leiostomus xanthurus*)
Figure 3

Golden Shiner Minnow (Notemigonus crysoleucas)
BCYE; streaked, and incubated to determine the colony count. One ml aliquots of the original suspension were injected intraperitoneally into the guinea pigs. Animals were observed daily for signs of infection for 7 days. Moribund animals were killed with an overdose of CO₂ gas and livers and spleens removed aseptically. Portions of these tissues were emulsified in saline and cultured on BCYE and EYB. The remaining suspensions and tissues were aliquoted and frozen at -70°C. Animals which remained healthy for 7 days were sacrificed as above.

2. Media evaluation

EYB and BCYE were tested for their ability to maintain virulence. Organisms isolated from spleen or liver suspensions of moribund animals were transferred every 7 days to the same type of medium (BCYE or EYB) and incubated in a 5% CO₂ atmosphere. Animals were inoculated as described above using organisms grown for 72 h at the following intervals: after 15, 20, 25, and 26 transfers. The 25th passage also was tested by injecting 1 ml of a 10-fold dilution of the original suspension of organisms grown on BCYE or EYB.

3. Polymixin B studies

Polymixin B (Pfizer, Inc., New York, New York 10017) was added to bacterial suspensions to evaluate its effect on the virulence of the organisms. One ml of a 10 X solution of polymixin B was added to 9 ml of
bacteria suspended in YEBB to yield a final antibiotic concentration of 160 µg/ml. One ml of saline was added to another suspension to serve as a control. All suspensions were incubated at 37°C in a 5% CO₂ atmosphere for 5 h. Following the incubation period, aliquots were removed for colony counts and one ml was injected intraperitoneally into each guinea pig. Two guinea pigs were inoculated per suspension. Bacterial suspensions were prepared from organisms grown on BCYE for 72 h as described above. The avirulent organism was tested using only the initial suspension with a turbidity approximating that of the McFarland No. 3 standard. The virulent strain was tested using the initial suspension and ten-fold and one-hundred-fold dilutions of this preparation treated with both.

B. Fish

1. Hogchoker

This species of fish was chosen for the preliminary studies because it was thought to be the hardiest of the three and was available in larger numbers than the spot. It was necessary to establish guidelines for inoculum size and volume since little relevant data were found. Initially, bacterial suspensions were prepared as described for the guinea pig studies, substituting sterile teleost saline (0.7% NaCl) for the YEBB. This number of organisms (0.3 ml injected) had no effect on the fish, so subsequent
experiments used bacterial suspensions prepared as follows: each strain (virulent and avirulent) was streaked for confluent growth on one BCYE plate and incubated at 35°C in a 5% CO₂ atmosphere for 72 h. The organisms from each of these plates were suspended in 10 ml of sterile 0.7% NaCl. Aliquots of these suspensions were removed for injection and further dilution for either injection or colony counts.

Each fish received up to 0.5 ml of a bacterial suspension inoculated intraperitoneally using a 25 gauge, 5/8 inch needle (Becton-Dickinson, Rutherford, New Jersey 07070) for each. Fish were marked by removing a portion of one fin prior to injection. Uninoculated, unmarked fish were added to the aquaria to serve as controls. Tanks were checked at least twice daily and any dead fish removed. The peritoneal cavity of each dead fish was entered aseptically and cultured on BMPAα medium. Surviving fish in some experiments were killed by severing the spinal cord adjacent to the skull and the peritoneal cavity cultured as above.

Aquaria in some experiments were tested for the presence of Legionella by culturing aliquots of the water on BMPAα medium 24 h after injecting the fish and by culturing the charcoal filter at the end of the experiment. All cultures were incubated a minimum of 10 days before being discarded.
Isolates with colonial morphology typical of \textit{L. pneumophila} were stained using Gram's method and subcultured to a sheep blood agar plate and a BCYE plate. Isolates with typical gram reaction and morphology which grew on the BCYE but not the blood agar plate after 7 days were presumptively identified as \textit{L. pneumophila}. Questionable isolates were tested using a direct fluorescent antibody reaction (Litton Bionetics, Inc., Charleston, South Carolina 29405).

2. Spot

Virulence was determined for this species as described for the hogchoker. Early studies suggested that the spot were more susceptible than the hogchoker. To test this hypothesis, spot and hogchoker were injected intraperitoneally with 0.5 ml each of the same suspension of either virulent or avirulent \textit{L. pneumophila} prepared as described above. The fish were housed in the same tank with uninoculated fish of each species added to serve as controls.

In another experiment, smears of the peritoneal cavity of each spot were made, stained with Wright's stain and then stained with Giemsa stain. These smears were examined for the presence of various cell types.

This species was also used to evaluate the effects of continuous transfer of the virulent \textit{L. pneumophila} on artificial media. Suspensions of virulent \textit{L. pneumophila} transferred twice and 27 times on BCYE were
prepared as described above in teleost saline. Fish received either 0.5 ml or 0.4 ml of these suspensions via intraperitoneal inoculation.

3. Minnows

Since *L. pneumophila* is known to survive in fresh water for prolonged periods of time, an experiment was done to determine the effects of adding known numbers of organisms to water similar to that to be used to house the fish. Ninety-nine ml of tap water was inoculated with one ml of a suspension containing either a $3.6 \times 10^4$ virulent or $4.7 \times 10^4$ avirulent organisms. These bottles were incubated at room temperature with the tops kept loose in a biologic safety cabinet. Aliquots were removed daily for 7 days and plated or diluted and plated onto BMPA\(\alpha\) agar. These plates were incubated and examined for the presence of *L. pneumophila*. In addition, water in the aquaria was cultured daily for the presence of *L. pneumophila* by plating 0.01 ml in duplicate on BMPA\(\alpha\) medium. The charcoal filter was also cultured at the end of each experiment, using BMPA\(\alpha\) medium.

Virulence of *L. pneumophila* for this species was determined as described for the hogchoker.

**Chemotactic Assays**

A. **Human**

Fourteen ml of heparinized blood was collected from each of 4 volunteers. Mononuclear cells were isolated
using a ficoll-hypaque gradient (LSM solution, Litton Bionetics, Inc.). The cells were washed 3 times in
Eagle Minimum Essential medium (Hazleton Research
Products, Inc., Denver, Pennsylvania 17517) containing
10% fetal calf serum (Eagle MEM). Viability and cell
concentration were determined by trypan blue exclusion
and the cells suspended to a concentration of $6 \times 10^6$
viable cells/ml in Eagle MEM.

Bacterial suspensions were prepared using virulent
and avirulent L. pneumophila grown on BCYE for 72 h at
35°C in a 5% CO$_2$ atmosphere as well as E. coli cultured
on sheep blood agar for 24 h using the same incubation
conditions. Each organism was suspended in 9 ml of
saline and adjusted to match approximately the opacity
of a McFarland No. 3 standard. Colony counts were done
and the suspensions centrifuged for 20 min at 3,000 rpm
(2,000 x g) using a Sorvall RC-3 automatic refrigerated
centrifuge with an HG-4L head (DuPont Instruments,
Wilmington, Delaware 19898) to sediment the cells.
Cells were killed by resuspending them in a 10% formalin-saline solution. Prior to use in the
chemotactic assay, organisms were washed once and
resuspended in Eagle MEM to a concentration of $5 \times 10^8$
cells/ml.

The assay was carried out using a Boyden chamber
(35) and testing each organism as well as a negative
control in duplicate. The bottom well of the chamber
(Ahlco Corporation, Meriden, Connecticut) contained 0.2 ml of the bacterial suspension of medium alone. A 5.0 μm membrane filter (Wallabs, Fairfax, California 94930) was placed over this well, the upper chamber attached, and 0.2 ml of the mononuclear cell suspension added. Chambers were incubated at 37°C in a 10% CO₂ atmosphere for 90 min. After incubation, the filters were removed and stained with Wright's stain. Dried filters were coverslipped and examined using 45X magnification. Mononuclear cells were differentially counted on the upper and lower surfaces of the filter.

Results were expressed as a chemotactic index, or the ratio of cells on the lower surface of the filter to the total number of cells counted (500). Chemotactic activity was considered to be positive when the number of cells migrating through the filter equaled or exceeded 2.5 times the negative control.

B. Guinea pig

Peritoneal macrophages were collected using a modification of the technique described by Rocklin (249). Briefly, 10 ml of light mineral oil was injected intraperitoneally into male Hartley strain guinea pigs about 72 h prior to performing the chemotactic assay. Animals were anesthetized with ether prior to injection. Animals were sacrificed using an overdose of ether. A 6 to 7 cm skin incision down to the peritoneum was made and 50 ml of cold Eagle MEM injected intraperitoneally.
A 15 gauge needle attached to a plasma transfer set (Fenwal Laboratories, Division of Travenol Laboratories, Inc., Deerfield, Illinois 60015) was inserted into the abdomen. The oil and wash solution was collected via this tubing into sterile plastic 50 ml conical centrifuge tubes. Tubes were centrifuged for 15 min at 5C and 1500 rpm in the Sorvall RC-3 centrifuge. Oil and supernatent were removed and the cells transferred to sterile 12 ml centrifuge tubes and washed twice more (5 min at 5C and 1000 rpm) in Eagle MEM. Cell viability and concentration were determined by trypan blue exclusion and the cells suspended to a concentration of 5 x 10^6 viable cells/ml in Eagle MEM.

Chemotactic assays were performed using guinea pig peritoneal macrophages as described for the human mononuclear cells above.

C. Fish

Kidney macrophages were isolated from all three species using the same technique. Fish were killed by severing the spinal cord adjacent to the skull with a scalpel. Kidneys were aseptically removed, washed in teleost buffered saline and transferred to Minimal Essential Medium (MEM) (Gibco Diagnostics, Lawrence, Massachusetts 01843) containing 10% fetal calf serum. Kidney tissue was homogenized and clumps of cells allowed to settle in a 10 ml plastic test tube. Macrophages were separated from the cell suspension
using the Percoll density gradient technique of Braun-Nesje et al. (37) as modified by Weeks and Warinner (320). The macrophage fractions were removed from gradients and washed once in teleost buffered saline. Trypan blue exclusion was used to determine viability and cell concentration. Cells were suspended to a concentration of $5 \times 10^5$ viable cells/ml in MEM.

Chemotactic activity was quantified by a modification of the Boyden technique (320) using a double chamber apparatus (Nucleopore Corporation, Bethesda, Maryland). The lower chamber contained 0.2 ml formalin killed \textit{L. pneumophila} (virulent or avirulent) or \textit{E. coli} prepared as in the human chemotactic assay technique except that the cells were washed and resuspended in teleost buffered saline with 10% v/v human serum. Medium alone was added to three chambers to serve as a negative control. The upper chamber of each contained 0.2 ml of the macrophage suspension. Chambers were separated by a 8.0 µm membrane filter (Millipore SSWPO 1,300 membrane). Chambers were incubated in triplicate at 15°C, membranes removed at 30, 60, and 90 min intervals, fixed with methanol, and stained with Wright's stain. Macrophages were differentially counted on the upper and lower surfaces of the filter. Results were expressed as a chemotactic index, or the ratio of macrophages on the lower surface of the filter to the total number of cells counted (at
plasmid Analysis

Virulent and avirulent *L. pneumophila* were analyzed for the presence of plasmids using the procedure described below. A loopful of organisms grown on BCYE for 72 h as previously outlined was suspended in 100 µl of Schlae's TE buffer (see Appendix B) and mixed vigorously using a vortex mixer. Next, 200 µl lysing buffer (see Appendix B) was added and mixed gently by inversion 12 times. Following a 20 min incubation at room temperature, 600 µl of phenol·chloroform, 1:1 (v/v), was added and this solution inverted gently. Samples were centrifuged 6 min at 15,600 x g in an Eppendorf Micro Centrifuge Model 5414 (Brinkmann Instruments Company, Westbury, New York 11590). The aqueous supernatent (lysate) was aspirated and 20 µl of lysate added to 5 µl of sample buffer (see Appendix B).

This lysate-sample buffer mixture (20 µl) was applied to a well made in a 0.6% agarose gel. This gel was placed in a horizontal gel electrophoresis apparatus (Bethesda Research Laboratories, Gaithersburg, Maryland 20877) containing 1X E buffer (see Appendix B), power turned to 100 volts for 5 min and then to 50 volts until the blue tracking dye reached the edge of the gel (2-3 h). Power was turned off and the gel stained with ethidium bromide (50 µl of a 1 mg/ml ethidium bromide solution added to 50 ml of tap water in a metal pan) for
30 min followed by destaining for 30 min in tap water. Gels were analyzed using long wave ultraviolet light and photographed with a Polaroid camera.

**Electron Microscopy**

Virulent and avirulent *L. pneumophila* were grown on BCYE for 72 h at 37°C in a 5% CO₂ atmosphere. Bacteria were suspended in 9 ml of YEBB and adjusted to match approximately the opacity of a No. 3 McFarland standard. One ml of a 10X solution of polymixin B was added to one suspension of virulent organisms to yield a final concentration of 160 μg/ml. One ml of 0.85% NaCl was added to another suspension to serve as a negative control. The avirulent organisms were treated similarly. All suspensions were incubated for 5 h at 37°C in a 5% CO₂ atmosphere. Following this incubation, 2 ml of 2.5% (v/v) glutaraldehyde was added to each tube and the tubes incubated at 4°C overnight. The bacterial cells were then centrifuged for 10 min and washed 3 times (10 min each) with sodium cacodylate buffer (pH 7.2) containing 3mM CaCl₂. The cells were post-fixed with 2% osmium tetraoxide (OsO₄) for 2 h. The cells were washed once in sodium cacodylate buffer as above and then embedded in 2% Noble agar following post-fixation. These blocks were dehydrated in a graded alcohol series and then placed in propylene oxide. The blocks were left overnight in a solution of 50% propylene oxide and 50% Epon 812. This propylene
oxide/Epon mixture was replaced by 100% Epon the next morning for 3 h. The blocks were then embedded in plastic capsules filled with fresh Epon and incubated at 60°C for 3 days. The Epon blocks were cut using an ultramicrotome equipped with a diamond knife. Sections with gray translucence were picked up on a 200 mesh copper grid and stained for 30 sec using saturated aqueous uranyl acetate followed by lead citrate for 1 min. The ultrathin sections were examined at 20 KV in a Philips EM 400 electron microscope.

Other bacterial suspensions were analyzed for the presence of capsules using ruthenium red. Cells were prepared as described above with the exception of the post-fixation step. The 2% OsO₄ solution was modified by the addition of 1.5 mg ruthenium red per ml of OsO₄. The rest of the procedure remained the same.

Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The procedure used was a modification of the technique of Laemmli (191); all reagents and chemicals were supplied by Bio-Rad Laboratories (Rockville Center, New York 11571) unless otherwise noted.

A. Specimen preparation

Each bacterial strain was streaked for confluent growth on 3 BYCE plates (1 plate for the gradient gel) and incubated for 72 h at 37°C in a 5% CO₂ atmosphere. All visible growth was collected and suspended in 10 ml
of sterile saline using cotton-tipped swabs. Cells were sedimented by centrifugation for 15 min at 23 C at 3,000 rpm using the Sorvall RC-3 centrifuge. The pellet was resuspended in 5 ml of a solution of phenol, glacial acetic acid, and water in a ratio of 2:1:0.5. The pellet was vigorously mixed by using a vortex mixer for 2 min and this mixture incubated at 37C for 1 h.

Following this incubation, the mixture was centrifuged for 10 min at 10,000 rpm (15,000 x g) at 4C using a Sorvall RC-5B refrigerated superspeed centrifuge equipped with a SA 600 head (DuPont Instruments, Wilmington, Delaware 19898). The supernatent was collected into a 13 x 100 mm test tube and the soluble proteins precipitated by the addition of cold acetone to fill the tube. The tube was incubated at 4C a minimum of 15 min and the proteins pelleted by centrifuging at 3,000 rpm for 15 min. The residual phenol and acetic acid were removed by washing the pellet with 1 ml cold acetone 3 times. The pellet was then allowed to dry.

The pellet was suspended in 300 µl (400 µl for the gradient gel) of sample buffer (see Appendix B) and the proteins disrupted using a pasteur pipette. This mixture was transferred to a 400 µl polyethylene microcentrifuge tube. The molecular weight standard was prepared by adding 10 µl of the standard to 190 µl of the sample buffer in a microcentrifuge tube. This standard was composed of the following: lysozyme
(14,400 daltons) soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), bovine serum albumin (66,200) and phosphorylase B (92,500).

All samples were heated in a boiling water bath for 5 min and then centrifuged for 15 min at 10,000 rpm. The resulting supernatent was placed in the wells using a Hamilton microliter syringe (Hamilton Company, Reno, Nevada 89520).

B. Slab gel electrophoresis

Gels were prepared using a Hoefer molded vertical slab electrophoresis unit with casting stand (Hoefer Scientific Instruments, San Francisco, California 94107). Dimensions of the gels were as follows: 14 cm wide, 16 cm long, 1.5 cm thick. Formulas for all solutions may be found in Appendix B.

The lower, resolving gel contained 12% acrylamide and was prepared by combining 16.25 ml of lower gel buffer with 26.00 ml acrylamide solution and 22.75 ml distilled water. This was mixed by swirling and deaerated by applying a vacuum for 15 min. Fresh 10% ammonium persulfate was prepared and 110 5 µl added to the monomer solution as well as 16 9 µl of N,N,N',N'-tetramethylethylenediamine (TEMED) to catalyze polymerization. This solution was swirled gently to mix and placed between 2 glass "sandwiches" in the casting stand. A 50 ml syringe was used to aspirate the solution and deliver it to a height about 4.0 cm from
the top of each of two "sandwiches." A layer of water was added in the same way and the gels allowed to polymerize overnight.

The next morning the gel surface was rinsed once with water and the 5% stacking gel prepared. Distilled water (14.30 ml) was mixed with 6.25 ml stacking gel buffer and 4.29 ml acrylamide solution and deaerated as above. The separating combs were placed at an angle between the glass plates in each "sandwich." Polymerization was initiated by adding 75.00 µl of ammonium persulfate and 25.00 µl TEMED to the stacking gel solution. This was then delivered using a 50 ml syringe to a level about 2 cm below the top of each "sandwich." The combs were then slowly pushed into the solution and centered. The level of monomer solution was adjusted to fill the "sandwich". After polymerization was complete (60-90 min), the combs were removed and the wells rinsed once with distilled water.

The upper buffer chamber was attached to the top of the glass plate assemblies in the casting stand and filled with running buffer to cover the central ridge (approximately 600 ml). The lower buffer chamber was filled with about 1200 ml of running buffer, the heat exchanger placed inside, and cool tap water flow initiated. A stir bar was added to the lower buffer chamber, the unit placed on a magnetic stirrer, and the glass plate assemblies placed inside the lower buffer
Samples were added to the wells as follows: wells 1 and 10, blank; wells 2 and 9, 25 µl standard; and wells 3 through 8, 5, 10, 15, 20, 25 and 30 µl of either the virulent or avirulent preparation. The electrode leads were attached and the power supply (Model 500, E-C Apparatus Corporation, St. Petersburg, Florida 33709) set to provide a constant current of 30 ma (15 ma/gel) until the tracking dye reached the bottom of the stacking gel and then 60 ma (30 ma/gel) until the dye reached the bottom of the resolving gel. Total running time was between 3 and 4 h. When the run was complete the power supply was turned off, the leads disconnected, and each gel placed in a plastic dish containing 400 ml of 40% methanol-10% acetic acid (v/v) fixative solution. The containers were covered and gels stored until stained (no longer than 48 h).

C. Gradient Gel Electrophoresis

Precast gradient (3-27%) SDS-polyacrylamide gels (Sepragel®) were purchased from Integrated Separation Systems (Newton, Massachusetts 02164). Gels were placed in the Hoefer casting stand using the alignment spacers and slide clamps as directed by the package instructions. A sample applicator (Sepracomb®) was placed on top of each gel, the upper buffer chamber attached and filled as above, and any bubbles inside each sample well removed according to the instructions.
The gels were placed inside the lower buffer chamber prepared as above. Samples were added to each well as follows: wells 1, 2, 12, 13 - blank; wells 4, 7, 10-25 µl standard; wells 3, 6, 9-20, 25, and 30 µl of the virulent sample; and wells 5, 8, 11-20, 25 and 30 µl of the avirulent sample. The power supply was turned on and the current adjusted to 60 ma (30 ma/gel) until the tracking dye reached the bottom of the gel (about 3 hours). Gels were removed and placed in the fixative as described above.

D. Staining the Gels

Gels were stained using the Bio-Rad Silver stain kit according to the directions for > 1.0 mm gels. Gels were gently rotated using a rocker platform (Bellco Biotechnology, Vineland, New Jersey 08360) throughout the entire staining process. Briefly, following 12-48 h of room temperature fixation, gels were immersed in two changes (400 ml each) of 10% ethanol-5% acetic acid (v/v) for 30 min each. Following the second period, gels were each immersed in 200 ml oxidizer for 10 min followed by 3 washes in 400 ml deionized water for 10 min each. Gels were then immersed in 200 ml silver reagent for 30 min and washed for 2 min in 400 ml deionized water. Developer (200 ml) was then added and allowed to remain until the solution turned yellow or a brown "smokey" precipitate appeared (about 30 sec). This developer was discarded and 200 ml of fresh
developer added, and incubated 5 min. This was followed by another 5 min incubation in 200 ml of fresh developer. The reaction was stopped using 400 ml of 5% acetic acid (v/v) for 5 min. Gels were stored in about 10 ml of distilled water in Ziploc® bags (Dow Chemical company, Indianapolis, Indiana 46268) until photographed with a Polaroid camera.

Serological Studies

A. Monoclonal antibody analysis

Organisms were tested using the monoclonal antibody in the direct immunofluorescent antibody kit of Genetic Systems (Seattle, Washington 98121) according to package directions. Both virulent and avirulent *L. pneumophila* were grown on BCYE at 37°C in a 5% CO₂ atmosphere and tested at the following times: 25 h, 48 h, 72 h, 8 days, and 17 days after inoculation. A culture of *E. coli* incubated as above and tested at the same intervals was used as a negative control. A separate slide was used for each test. Slides were examined using an American Optical vertical fluorescence microscope, model 2071 (Reichert Scientific Instruments, Buffalo, New York 14215) equipped with a filter system for fluorescein isothiocyanate.

B. Polyclonal antibody analysis

Organisms were tested using the direct immunofluorescent polyclonal antibody kit produced by Litton Bionetics, Inc (Charleston, South Carolina
according to package directions. Virulent and avirulent *L. pneumophila* and *E. coli* were tested as described above.

**RESULTS**

Virulence of the organisms was confirmed in the initial guinea pig studies. The animal which received the suspension of virulent *L. pneumophila* (5.2 x 10⁸ colony forming units [CFU]) exhibited signs of infection including ruffled fur, eye discharge, and lethargy within 48 h and became moribund approximately 91 h after injection. This guinea pig (GP 41) was sacrificed and the spleen and liver cultured. After 5 days, heavy growth of what appeared to be *L. pneumophila* was observed. Gram stain of the isolate (Figure 4) showed typical morphology and a direct fluorescent antibody stain confirmed the finding. This isolate was injected (2 x 10⁸ CFU) into another animal which succumbed in a manner similar to the first. Cultures of this animal's spleen and liver (GP 42) also yielded a heavy growth of *L. pneumophila*. Isolates from both animals were passed once a week on BCYE and EBYB agars.

The avirulent *L. pneumophila* failed to affect a guinea pig when 9.0 x 10⁸ CFU were injected. This animal remained apparently healthy until sacrificed 7 days after inoculation. Cultures of tissues from this animal yielded 30 colonies from the spleen homogenate.
RESULTS

Virulence of the organisms was confirmed in the initial guinea pig studies. The animal which received the suspension of virulent *L. pneumophila* \((5.2 \times 10^8\) colony forming units [CFU]) exhibited signs of infection including ruffled fur, eye discharge, and listlessness within 48 h and became moribund approximately 51 h after injection. This guinea pig (GP #1) was sacrificed and the spleen and liver cultured. After 5 days, heavy growth of what appeared to be *L. pneumophila* was observed. Gram stain of the isolate (Figure 4) showed typical morphology and a direct fluorescent antibody stain confirmed the finding. This isolate was injected \((2 \times 10^8\) CFU) into another animal which succumbed in a manner similar to the first. Cultures of this animal's spleen and liver (GP #2) also yielded a heavy growth of *L. pneumophila*. Isolates from both animals were passed once a week on BCYE and EYB agars.

The avirulent *L. pneumophila* failed to affect a guinea pig when \(9.0 \times 10^8\) CFU were injected. This animal remained apparently healthy until sacrificed 7 days after inoculation. Cultures of tissues from this animal yielded 30 colonies from the spleen homogenate and 2 from the liver which were identified as *L.*
Results of the media evaluation study are shown in Table 3. Since the animal which received the isolate from GF 41 passed 20 times on the medium remained healthy while the 26th passage on this medium killed within 48 hours, the 25th passage of the isolate from GF 42 was tested. The results of this experiment are shown in Table 4.

![Figure 4](image)

**Figure 4**

Typical Gram Reaction of Virulent or Avirulent *Legionella pneumophila* Isolated on BCYE Agar
Results of the media evaluation study are shown in Table 3. Since the animal which received the isolate from GP #1 passed 20 times on CYE remained healthy while the 26th passage on this medium killed within 48 hours, the 25th passage of the isolate from GP #2 was tested. The results of this experiment are shown in Table 4.

Table 5 shows the results of polymixin B treatment of the virulent *L. pneumophila*. Of the two guinea pigs inoculated with the initial suspension, one died within 48 h, the other appeared to be unaffected throughout the observation period. All 4 animals inoculated with the 10-fold dilutions died within 5 days. Two animals receiving the 100-fold dilutions (one received the saline-treated suspension, the other, the polymixin B treated organisms) exhibited signs of illness including lethargy, eye discharge, and labored breathing. The other animal in each pair remained apparently well throughout the 10 days.

Treatment of the avirulent *L. pneumophila* with polymixin B resulted in no deaths and no apparent illness in either of the animals which received the polymixin B-treated or the saline treated organisms (Table 6). Since an occasional animal appeared to be unaffected by usually lethal amounts of the virulent bacteria (Tables 3, 4, and 5) blood was drawn by cardiac puncture from these animals one day prior to injection.
<table>
<thead>
<tr>
<th>Passages</th>
<th>Organisms Injected (CFU)*</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>8.7 x 10^8</td>
<td>moribund at 2 days</td>
</tr>
<tr>
<td>CYE</td>
<td>4.8 x 10^8</td>
<td>moribund at 2 days</td>
</tr>
<tr>
<td>EYB</td>
<td>7.0 x 10^8</td>
<td>alive and well at 7 days</td>
</tr>
<tr>
<td>20</td>
<td>3.6 x 10^8</td>
<td>dead at 4 days</td>
</tr>
<tr>
<td>CYE</td>
<td>7.0 x 10^8</td>
<td>alive and well at 7 days</td>
</tr>
<tr>
<td>EYB</td>
<td>3.6 x 10^8</td>
<td>dead at 4 days</td>
</tr>
<tr>
<td>26</td>
<td>4.7 x 10^8</td>
<td>dead at 2 days</td>
</tr>
<tr>
<td>CYE</td>
<td>6.1 x 10^8</td>
<td>dead at 5 days</td>
</tr>
</tbody>
</table>

*bacteria originally isolated from GP #1.
Table 4

Ability of Virulent *L. pneumophila* to Affect Guinea Pigs Following 25 Passages on BCYE or EYB Agar

<table>
<thead>
<tr>
<th>Medium</th>
<th>Organisms Injected (CFU)</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYE</td>
<td>3.5 x 10^8</td>
<td>dead at 2 days</td>
</tr>
<tr>
<td></td>
<td>3.5 x 10^7</td>
<td>dead at 3 days</td>
</tr>
<tr>
<td>EYB</td>
<td>1.4 x 10^8</td>
<td>dead at 3 days</td>
</tr>
<tr>
<td></td>
<td>1.4 x 10^7</td>
<td>alive and well at 7 days</td>
</tr>
</tbody>
</table>

*bacteria originally isolated from GP #2*
Table 5

The Effect of Polymixin B Treatment of Known Virulent *Legionella pneumophila* on Guinea Pig Morbidity and Mortality

<table>
<thead>
<tr>
<th>Suspension</th>
<th>Colony Count (CFU)</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-treatment</td>
<td>Post-treatment</td>
<td></td>
</tr>
<tr>
<td>undiluted</td>
<td>5.5 x 10^8</td>
<td>3.2 x 10^8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 dead at 2 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 alive and well at 10 days</td>
</tr>
<tr>
<td>10-fold dilution polimixin B treated</td>
<td>ND*</td>
<td>3.5 x 10^7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 dead at 4 days</td>
</tr>
<tr>
<td>10-fold dilution saline treated</td>
<td>ND</td>
<td>2.6 x 10^7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 dead at 4 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 dead at 5 days</td>
</tr>
<tr>
<td>100-fold dilution polimixin B treated</td>
<td>ND</td>
<td>2.5 x 10^6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 sick days 3-6; recovered</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 alive and well at 10 days</td>
</tr>
<tr>
<td>100-fold dilution saline treated</td>
<td>ND</td>
<td>2.4 x 10^6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 sick days 2-6; recovered</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 alive and well at 10 days</td>
</tr>
</tbody>
</table>

*ND = not done*
The sera were tested for the presence of antibodies specific for *L. pneumophila* using an indirect fluorescent antibody technique. One animal in each treatment group had a titer less than 1:16 while the other had a weak reaction at 1:16 only.

**Table 6**

Effect of Polymixin B Treatment of Known Avirulent * Legionella pneumophila on Guinea Pig Morbidity and Mortality

<table>
<thead>
<tr>
<th>Suspension</th>
<th>Colony Count (CFU)</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-treatment</td>
<td>Post-treatment</td>
<td></td>
</tr>
<tr>
<td>Polymixin B treated</td>
<td>1.3 x 10⁹</td>
<td>5.9 x 10⁸ 2/2 alive and well at 7 days</td>
</tr>
<tr>
<td>Saline treated</td>
<td>1.3 x 10⁹</td>
<td>9.1 x 10⁸ 2/2 alive and well at 7 days</td>
</tr>
</tbody>
</table>

CFU/ml was injected into the fish. Normal saline was injected into the hogchoker to test virulence used organisms prepared so that 0.3 ml of a suspension containing approximately 10⁸ CFU/ml was injected into the fish so inoculated were infected. Subsequent experiments used bacteria which remained on the surface of the suspension as well as three serial 10-fold dilutions. The effects of injecting 0.3 ml of this suspension into the hogchoker are shown in Table 7. Those fish injected with the virulent *L. pneumophila* were housed separately from those receiving the avirulent strain due to the large numbers of fish involved. The fish began to die in both tanks in an apparently random manner after 6 days. The peritoneal cavity of each was cultured as soon as the death was observed. Culture results are shown in Tables 8 and 9.

Virulent *L. pneumophila* was recovered from 9 of the 12 fish inoculated with this strain. One fish was not cultured; 82% of those in which cultures were obtained had at least a few viable *L. pneumophila* and 4 fish had large numbers. The two in which the organisms was not
The sera were tested for the presence of antibodies specific for *L. pneumophila* using an indirect fluorescent antibody technique. One animal in each treatment group had a titer less than 1:16 while the other had a weak reaction at 1:16 only.

Early experiments with the hogchoker (*Trinecte maculatus*) to test virulence used organisms prepared so that 0.3 ml of a suspension containing approximately $10^8$ CFU/ml was injected per fish. None of the fish so inoculated were infected. Subsequent experiments used all bacteria which could be removed from one BCYE plate streaked for confluent growth suspended in 10 ml of 0.7% saline. The effects of injecting 0.3 ml of this suspension as well as three serial 10-fold dilutions into the hogchoker are shown in Table 7. Those fish injected with the virulent *L. pneumophila* were housed separately from those receiving the avirulent strain due to the large numbers of fish involved. The fish began to die in both tanks in an apparently random manner after 6 days. The peritoneal cavity of each was cultured as soon as the death was observed. Culture results are shown in Tables 8 and 9.

Virulent *L. pneumophila* was recovered from 9 of the 12 fish inoculated with this strain. One fish was not cultured; 82% of those in which cultures were obtained had at least a few viable *L. pneumophila* and 4 fish had large numbers. The two in which the organism was not
Table 7

Mortality of Hogchoker (*Trinecte maculatus*) Infected with Virulent or Avirulent *Legionella pneumophila*

<table>
<thead>
<tr>
<th>Number of Organisms Injected (CFU)**</th>
<th>Organisms Injected (CFU)**</th>
<th>Deaths at 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5 x 10^7</td>
<td><em>L. pneumophila</em></td>
<td>0/3</td>
</tr>
<tr>
<td>7.5 x 10^6</td>
<td><em>L. pneumophila</em></td>
<td>0/3</td>
</tr>
<tr>
<td>7.5 x 10^5</td>
<td><em>L. pneumophila</em></td>
<td>1/3</td>
</tr>
<tr>
<td>3.3 x 10^9</td>
<td><em>L. pneumophila</em></td>
<td>0/3</td>
</tr>
<tr>
<td>3.3 x 10^8</td>
<td><em>L. pneumophila</em></td>
<td>0/3</td>
</tr>
<tr>
<td>3.3 x 10^7</td>
<td><em>L. pneumophila</em></td>
<td>0/3</td>
</tr>
<tr>
<td>3.3 x 10^6</td>
<td><em>L. pneumophila</em></td>
<td>0/3</td>
</tr>
</tbody>
</table>

* fish injected with each strain were kept in separate tanks

** total volume injected was 0.3 ml per fish in each case
### Table 8

Recovery of Virulent *Legionella pneumophila* from the Peritoneal Cavities of Hogchoker Inoculated with Varying Numbers of Organisms

<table>
<thead>
<tr>
<th>Number of Organisms Injected (CFU)</th>
<th>Time of Death (days)</th>
<th>Culture Results*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>7.5 x 10⁸</strong></td>
<td>7</td>
<td>3 + <em>L. pneumophila</em></td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>1 colony (not legionella)</td>
</tr>
<tr>
<td></td>
<td>(sacrificed)</td>
<td>2 + <em>L. pneumophila</em></td>
</tr>
<tr>
<td><strong>3.3 x 10⁸</strong></td>
<td>7</td>
<td>1 + <em>L. pneumophila</em></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1 + <em>L. pneumophila</em></td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>no growth</td>
</tr>
<tr>
<td><strong>7.5 x 10⁷</strong></td>
<td>9</td>
<td>2 + <em>L. pneumophila</em></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>3 + <em>L. pneumophila</em></td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>4 colonies (not legionella) and 1 + <em>L. pneumophila</em></td>
</tr>
<tr>
<td><strong>7.5 x 10⁶</strong></td>
<td>5</td>
<td>not done</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1 + mixed flora</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>2 + mixed flora</td>
</tr>
<tr>
<td><strong>Uninoculated</strong></td>
<td>8</td>
<td>2 colonies (not legionellae)</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>2 + mixed flora</td>
</tr>
</tbody>
</table>

*1+ = colonies in primary streak only
2+ = colonies in primary and secondary streaks only
3+ = colonies in all three streaked areas
4+ = nearly confluent growth in all streaked areas
Table 9

Recovery of Avirulent *Legionella pneumophila* from the Peritoneal Cavitie of Hogchoker Inoculated with Varying Numbers of Organisms

<table>
<thead>
<tr>
<th>Number of Organisms Injected (CFU)</th>
<th>Time of Death (days)</th>
<th>Culture Results*</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3 x 10⁹</td>
<td>9</td>
<td>4 + <em>L. pneumophila</em></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>4 + <em>L. pneumophila</em></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>3 + <em>L. pneumophila</em></td>
</tr>
<tr>
<td>3.3 x 10⁸</td>
<td>7</td>
<td>2 + mixed flora</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>3 + mixed flora</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>2 + <em>L. pneumophila</em> (sacrificed)</td>
</tr>
<tr>
<td>3.3 x 10⁷</td>
<td>7</td>
<td>3 + <em>L. pneumophila</em></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>3 + <em>L. pneumophila</em></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>3 + <em>L. pneumophila</em></td>
</tr>
<tr>
<td>3.3 x 10⁶</td>
<td>7</td>
<td>2 + <em>L. pneumophila</em></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>2 colonies (not legionella) and 1 + <em>L. pneumophila</em></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>2 + <em>L. pneumophila</em></td>
</tr>
<tr>
<td>Uninoculated</td>
<td>8</td>
<td>4 colonies (not legionella)</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>no growth</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>3 + mixed flora</td>
</tr>
</tbody>
</table>

* 1+ = colonies in primary streak only
2+ = colonies in primary and secondary streaks only
3+ = colonies in all three streaked areas
4+ = nearly confluent growth in all streaked areas

*No growth* indicates that no *L. pneumophila* were isolated. The number of fish in each group was 10. Only the hogchoker received the avirulent strain, these two fish in which the avirulent *L. pneumophila* were not isolated received an intermediate number of 3 x 10⁵. No infections occurred in the un inoculated controls that received the undiluted original stock of *L. pneumophila*.
recovered both received the lowest number initially. *Listeria pneumophila* was not isolated in any of the uninoculated fish housed in the same tank. This was also the case with the uninoculated controls in the tank containing those fish which received the avirulent form. *Listeria pneumophila* was reisolated from 10 of the 12 fish (83%) inoculated with the avirulent form. These fish had generally higher numbers of *Listeria pneumophila* than did those which received the virulent strain. The two fish in which the avirulent *Listeria pneumophila* was not isolated received an intermediate number ($3.3 \times 10^8$) initially.

Both hogchoker and spot (*Leiostomus xanthurus*) became readily available, so the next experiments involved simultaneous testing of both species. Suspensions of virulent *Listeria pneumophila* were prepared as above and 0.3 ml of each as well as 0.3 ml of two serial ten-fold dilutions were injected into both species. Only the hogchoker received the avirulent strain; these fish were housed in another aquarium. The results of this experiment are shown in Table 10. Since only one death occurred in 7 days, on day 8 two fish each from the groups that received the undiluted original suspensions were sacrificed and the peritoneal cavities cultured. The culture results are shown in Table 11. Remaining fish survived until the experiment was terminated on day 10.

Table 12 shows the results of this experiment
<table>
<thead>
<tr>
<th>Organisms Injected (CFU)**</th>
<th>Deaths in 7 Days</th>
<th>Hog Choker</th>
<th>Spot</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virulent L. pneumophila</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$1.2 \times 10^{10}$</td>
<td>0/3</td>
<td>0/3</td>
<td></td>
</tr>
<tr>
<td>$1.2 \times 10^9$</td>
<td>0/3</td>
<td>0/3</td>
<td></td>
</tr>
<tr>
<td>$1.2 \times 10^8$</td>
<td>0/3</td>
<td>1/3</td>
<td></td>
</tr>
<tr>
<td>Uninoculated</td>
<td>0/4</td>
<td>0/3</td>
<td></td>
</tr>
<tr>
<td>Avirulent L. pneumophila</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$5.2 \times 10^8$</td>
<td>0/3</td>
<td></td>
<td>ND^</td>
</tr>
<tr>
<td>$5.2 \times 10^7$</td>
<td>0/3</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>$5.2 \times 10^6$</td>
<td>0/3</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Uninoculated</td>
<td>0/3</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

* fish injected with each strain were housed in separate tanks
** total volume injected was 0.3 ml per fish in each case
^ not done
Table 11

Recovery of Virulent or Avirulent *Legionella pneumophila* from the Peritoneal Cavities of Hogchoker and Spot 7 Days After Inoculation with Known Numbers of Organisms

<table>
<thead>
<tr>
<th>Oragnisms Injected (CFU)</th>
<th>Culture results *</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Virulent</strong> <em>L. pneumophila</em></td>
<td>1.2 x 10^10</td>
</tr>
<tr>
<td>Hogchoker #1</td>
<td>3 + <em>L. pneumophila</em></td>
</tr>
<tr>
<td>#2</td>
<td>3 + <em>L. pneumophila</em></td>
</tr>
<tr>
<td><strong>Avirulent</strong> <em>L. pneumophila</em></td>
<td>5.2 x 10^8</td>
</tr>
<tr>
<td>Hogchoker #1</td>
<td>1 + <em>L. pneumophila</em></td>
</tr>
<tr>
<td>#2</td>
<td>2 + <em>L. pneumophila</em></td>
</tr>
</tbody>
</table>

*total volume injected was 0.3 ml per fish in each case

* 1+ = colonies in primary streak only
2+ = colonies in primary and secondary streaks only
3+ = colonies in all three streaked areas
4+ = nearly confluent growth in all streaked areas
Table 12

Mortality of Hogchoker and Spot Infected with Virulent or Avirulent Legionella pneumophila (0.3 ml Dose) and Housed in the Same Tank

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Injected (CFU)*</th>
<th>Deaths in 7 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virulent L. pneumophila</td>
<td>1.1 x 10^{10}</td>
<td>0/3</td>
</tr>
<tr>
<td>Avirulent L. pneumophila</td>
<td>1.4 x 10^{10}</td>
<td>0/3</td>
</tr>
<tr>
<td>Uninoculated Controls</td>
<td>0</td>
<td>0/2</td>
</tr>
</tbody>
</table>

*total volume injected was 0.3 ml per fish in each case
repeated with these modifications: all fish were housed in the same tank, both species received identical inoculations, and 0.3 ml of only the original, undiluted suspensions was injected into each. Mortality was similar for the hogchoker; however, there was one death within 48 h in each spot group. The volume of each inoculum was next increased to 0.5 ml. This resulted in only one hogchoker death but 8 of the 9 injected spot died within 48 h (Table 13).

Table 14 shows the mortality of spot only injected with 0.5 or 0.4 ml of either the virulent or avirulent L. pneumophila. Since any deaths which appeared to be related to the inoculation occurred within 48 h, this period was used to determine mortality in this experiment. Although both the virulent and avirulent groups experienced significant numbers of deaths, there appeared to be some time relationship. Table 15 shows the number of deaths as they occurred at 24, 36, and 48 h (spot only) in both this and the previous experiment. Smears of peritoneal material were stained with Wright's stain and then Giemsa's stain at the time of death in this experiment. Control fish and those still alive which received the avirulent L. pneumophila were sacrificed and smears made. Figure 5 shows a typical field seen in peritoneal smears of the sacrificed control fish. Cells were few in number and primarily red blood cells. Occasional leukocytes were seen; most
### Table 13

Mortality of Hogchoker and Spot Infected with Virulent or Avirulent *Legionella pneumophila* (0.5 ml Dose) and Housed in the Same Tank

<table>
<thead>
<tr>
<th>Organisms Injected (CFU)*</th>
<th>Deaths in 7 Days</th>
<th>Hogchoker</th>
<th>Spot</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virulent <em>L. pneumophila</em> 1.0 x 10^{10}</td>
<td>0/3</td>
<td>5/5</td>
<td></td>
</tr>
<tr>
<td>Avirulent <em>L. pneumophila</em> 1.5 x 10^{10}</td>
<td>1/3</td>
<td>3/4</td>
<td></td>
</tr>
<tr>
<td>Uninoculated Controls</td>
<td>0/3</td>
<td>0/4</td>
<td></td>
</tr>
</tbody>
</table>

*total volume injected was 0.5 ml per fish in each case*
Table 14

Mortality of Spot (Leiostomus xanthurus) Infected with Two Different Volumes of Virulent or Avirulent Legionella pneumophila and Housed in the Same Tank

<table>
<thead>
<tr>
<th>Volume Injected (ml)</th>
<th>Organisms Injected (CFU)</th>
<th>Deaths in 48 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Virulent strain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>$1.3 \times 10^{10}$</td>
<td>4/4</td>
</tr>
<tr>
<td>0.4</td>
<td>$1.0 \times 10^{10}$</td>
<td>4/4</td>
</tr>
<tr>
<td><strong>Avirulent strain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>$1.8 \times 10^{10}$</td>
<td>3/4</td>
</tr>
<tr>
<td>0.4</td>
<td>$1.5 \times 10^{10}$</td>
<td>1/4</td>
</tr>
<tr>
<td><strong>Uninoculated Controls</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0/5</td>
</tr>
</tbody>
</table>
### TABLE 15
Spot Mortality Due to Virulent and Avirulent

*Legionella pneumophila*, Serogroup I

<table>
<thead>
<tr>
<th>L. pneumophila strain type</th>
<th>Organisms injected ($\times 10^3$)</th>
<th>Number of fish injected</th>
<th>Time of Death (hours)</th>
<th>Total Dead</th>
<th>Total Living 48 hours After Injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>24</td>
<td>36</td>
<td>48</td>
</tr>
<tr>
<td>Virulent</td>
<td>1.0</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Avirulent</td>
<td>1.5</td>
<td>4</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Virulent</td>
<td>1.3</td>
<td>4</td>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Virulent</td>
<td>1.0</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Avirulent</td>
<td>1.8</td>
<td>4</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Avirulent</td>
<td>1.5</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Wright-Giemsa Stained Smear of Peritoneal Cavity of Uninoculated Spot (original magnification, 400X)
appeared to be macrophages (Figure 6). The fish injected with the avirulent *L. pneumophila* showed a more cellular infiltrate. Cells seen in fish which were found dead exhibited degenerative changes and were difficult to identify. The one fish alive at 48 h which received 0.5 ml was sacrificed; representative fields from this fish's peritoneal smear are seen in Figures 7, 8, and 9. Erythrocytes were seen as in the control, but more leukocytes were apparent. A variety of types were seen, predominately macrophages and lymphocytes, although a few granulocytes were also observed. Clumps of mononuclear cells (Figures 10 and 11) were found as well. All fish inoculated with the virulent strain were found dead. Similar degenerative changes were seen and cell types were very difficult to differentiate. Representative fields are shown in Figures 12 and 13. No erythrocytes were identified, but a few mononuclear cells were seen. Occasional sheets of cells with indistinct cytoplasmic borders (Figures 14, 15 and 16) were observed as well.

The spot were also used to determine the effects of serial passages on BCYE on the virulence of the bacteria. All fish (including controls) were found dead at 24 h (Table 16).

Tank water cultured 24 h after fish were inoculated and filters cultured at the end of each experiment yielded no detectable *L. pneumophila* at any time.
Figure 6

Wright-Giemsa Stained Smear of Peritoneal Cavity of Uninoculated Spot
(original magnification 1,000 X)
Figure 7

Wright-Giemsa Stained Smear of Peritoneal Cavity of Spot Sacrificed 48 Hours After Inoculation with 1.8x10^{10} Avirulent *Legionella pneumophila* (original magnification 400 X)
Figure 8

Wright-Giemsa Stained Smear of Peritoneal Cavity of Spot Sacrificed 48 Hours After Inoculation with 1.8X10^{10} CFU Avirulent Legionella pneumophila Demonstrating a Mononuclear Cell (original magnification 1,000 X)
Figure 9

Wright-Giemsa Stained Smear of Peritoneal Cavity of Spot Sacrificed 48 Hours After Inoculation with 1 \(8\times10^{10}\) CFU Avirulent Legionella pneumophila Demonstrating Two Mononuclear Cells (original magnification 1000 X)
Figure 10

Wright-Giemsa Stained Smear of Peritoneal Cavity of Spot Sacrificed 48 Hours After Inoculation with 1.8X10^{10} CFU Avirulent Legionella pneumophila Showing Clumps of Mononuclear Cells (original magnification 400 X)
Figure 11

Wright-Giemsa Stained Smear of Peritoneal Cavity of Spot Sacrificed 48 Hours After Inoculation with $1.8 \times 10^{10}$ CFU Avirulent \textit{Legionella pneumophila} Showing Clumps of Mononuclear Cells (original magnification 1,000 X)
Figure 12

Wright-Giemsa Stained Smear of Peritoneal Cavity of Spot Dead 48 Hours After Inoculation with $1.3 \times 10^{10}$ CFU Virulent Legionella pneumophila-Representative Field (original magnification 400 X)
Figure 13

Wright-Giemsa Stained Smear of Peritoneal Cavity of S. pneumoniae Dead 48 Hours After Inoculation with 1.3x10^6 CFU Virulent Legionella pneumophila - Another Representative Field (original magnification 400 X)
Figure 14

Wright-Giemsa Stained Smear of Peritoneal Cavity of Spot Dead 48 Hours After Inoculation with $1.3 \times 10^{10}$ CFU Virulent Legionella pneumophila Demonstrating Sheets of Cells (original magnification 100X)
Figure 15

Wright-Giemsa Stained Smear of Peritoneal Cavity of Spot Dead 48 Hours After Inoculation with 1.3X10^{10} CFU Virulent Legionella pneumophila Demonstrating Sheets of Cells (original magnification 400 X)
Figure 16

Wright-Giemsa Stained Smear of Peritoneal Cavity of Spot Dead 48 Hours After Inoculation with 1.3X10^10 CFU Virulent Legionella pneumophila Demonstrating Another Sheet of Cells (original magnification 400 X)
Legionellae are known to survive in fresh water, so before virulence studies were begun using the fresh water fish, it was necessary to determine whether the

Table 16

Ability of Virulent *Legionella pneumophila* to Kill Spot After 2 and 27 Transfers on BCYE Agar

<table>
<thead>
<tr>
<th>Volume Injected (ml)</th>
<th>Organisms Injected (CFU)</th>
<th>Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Transfers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>$1.7 \times 10^{10}$</td>
<td>5/5</td>
</tr>
<tr>
<td>0.4</td>
<td>$1.4 \times 10^{10}$</td>
<td>5/5</td>
</tr>
<tr>
<td>27 Transfers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>$1 \times 10^{10}$</td>
<td>5/5</td>
</tr>
<tr>
<td>0.4</td>
<td>$8 \times 10^9$</td>
<td>5/5</td>
</tr>
<tr>
<td>Uninoculated Controls</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>6/6</td>
</tr>
</tbody>
</table>

Uninoculated controls were initially injected with 0.5 ml and 0.3 ml of bacterial suspensions prepared as in the hogchoker and spot studies. All inoculated fish died within 4 days (Table 16). Peritoneal cultures of fish which died 24 hours or more after inoculation are shown in Table 19 as well as the three living control fish which were sacrificed on day 4. No *L. pneumophila* was isolated from the uninoculated controls but was recovered from 11 of 13 (84.6%) of the inoculated fish. Minnows were then injected with 0.1 ml of the bacterial suspension. Since the fish still died rapidly (Table 20), the next experiment used 0.1 ml of a 10-fold dilution of the original suspension as well. Results are shown in Table 21. None of the fish receiving the avirulent strain died within 10 days. Two of the fish receiving the undiluted suspension of virulent bacteria died within 24 h, the third was dead within 4 days, and the fourth
Legionellaceae are known to survive in fresh water, so before virulence studies were begun using the fresh water fish, it was necessary to determine whether the bacteria would multiply under the test conditions. Addition of $10^6$ CFU of virulent or avirulent \textit{L. pneumophila} to 99 ml of tap water (final bacterial concentration: $10^4$/ml) resulted in the reisolation of no detectable \textit{L. pneumophila} of either strain, even after 7 days of incubation at room temperature (Table 17).

The golden shiner minnows (\textit{Notemigonus crysoleucas}) were initially injected with 0.5 ml and 0.3 ml of bacterial suspensions prepared as in the hogchoker and spot studies. All inoculated fish died within 4 days (Table 18). Peritoneal cultures of fish which died 24 hours or more after inoculation are shown in Table 19 as well as the three living control fish which were sacrificed on day 4. No \textit{L. pneumophila} was isolated from the uninoculated controls but was recovered from 11 of 13 (84.6%) of the inoculated fish. Minnows were then injected with 0.1 ml of the bacterial suspension. Since the fish still died rapidly (Table 20), the next experiment used 0.1 ml of a 10-fold dilution of the original suspension as well. Results are shown in Table 21. None of the fish receiving the avirulent strain died within 10 days. Two of the fish receiving the undiluted suspension of virulent bacteria died within 24 h, the third was dead within 4 days, and the fourth
Table 17

Recovery of Virulent and Avirulent Legionella pneumophilia After Inoculation in Carbon-Filtered Richmond City Tap Water Followed by Incubation at Room Temperature

<table>
<thead>
<tr>
<th>Day</th>
<th>Virulent**</th>
<th>Avirulent**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Organisms Isolated (CFU x 10^4)</td>
<td>L.pneumophilia/Total*</td>
</tr>
<tr>
<td>0 (prior to inoculation)</td>
<td>0/1.6</td>
<td>0/1.6</td>
</tr>
<tr>
<td>1</td>
<td>0/51</td>
<td>0/41</td>
</tr>
<tr>
<td>2</td>
<td>0/107</td>
<td>0/88</td>
</tr>
<tr>
<td>3</td>
<td>0/43</td>
<td>0/36</td>
</tr>
<tr>
<td>4</td>
<td>0/58</td>
<td>0/36</td>
</tr>
<tr>
<td>5</td>
<td>0/16</td>
<td>0/16</td>
</tr>
<tr>
<td>6</td>
<td>0/34</td>
<td>0/35</td>
</tr>
<tr>
<td>7</td>
<td>0/50</td>
<td>0/25</td>
</tr>
</tbody>
</table>

* Average of duplicate cultures of 10^-2, 10^-4, 10^-6 dilutions of each sample

** 3.6 x 10^6 CFU Virulent bacteria suspended in 1 ml of 0.7% saline added to 99 ml water

4.7 x 10^6 CFU Avirulent bacteria suspended in 1 ml of 0.7% saline added to 99 ml water
Table 18

Mortality of Golden Shiner Minnows (Notemigonus crysoleucas) inoculated with 0.5 ml or 0.3 ml Doses of Virulent or Avirulent Legionella pneumophila and Housed in the Same Tank

<table>
<thead>
<tr>
<th>Volume Injected (ml)</th>
<th>Organisms Injected (CFU)</th>
<th>Deaths After 4 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Virulent strain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>$1.5 \times 10^{10}$</td>
<td>4/4</td>
</tr>
<tr>
<td>0.3</td>
<td>$9.3 \times 10^9$</td>
<td>4/4</td>
</tr>
<tr>
<td><strong>Avirulent strain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>$1.0 \times 10^{10}$</td>
<td>4/4</td>
</tr>
<tr>
<td>0.3</td>
<td>$6.3 \times 10^9$</td>
<td>4/4</td>
</tr>
<tr>
<td><strong>Uninoculated Controls</strong></td>
<td>0</td>
<td>1/4</td>
</tr>
</tbody>
</table>

*1+ = colonies in primary streak only
2+ = colonies in primary and secondary streaks only
3+ = colonies in all three streaked areas
4+ = nearly confluent growth in all streaked areas

** = isolates appeared typical of *L. pneumophila*; however, the organisms failed to grow on subculture
Table 19

Recovery of Legionella pneumophila from the Peritoneal Cavities of Minnows Inoculated with Varying Numbers of Organisms Which Died 24 or More Hours After Injection

<table>
<thead>
<tr>
<th>Number of Organisms Injected (CFU)</th>
<th>Time of Death (days)</th>
<th>Culture Results*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Virulent strain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$1.5 \times 10^{10}$</td>
<td>1</td>
<td>3+ <em>L. pneumophila</em>; 1+ mixed flora</td>
</tr>
<tr>
<td>*$9.3 \times 10^9$</td>
<td>2</td>
<td>2+ <em>L. pneumophila</em>; 1+ mixed flora</td>
</tr>
<tr>
<td><strong>Avirulent strain</strong></td>
<td>1</td>
<td>3+ <em>L. pneumophila</em></td>
</tr>
<tr>
<td>$1.0 \times 10^{10}$</td>
<td>2</td>
<td>2+ mixed flora</td>
</tr>
<tr>
<td>*$6.3 \times 10^9$</td>
<td>3</td>
<td>3+ mixed flora</td>
</tr>
<tr>
<td><em>Uninoculated Controls</em></td>
<td>4</td>
<td>3+ mixed flora</td>
</tr>
<tr>
<td>(sacrificed)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*All deaths occurred with the first 36 hours after inoculation, and the fish were sacrificed at 7 days.

*1+ = colonies in primary streak only
2+ = colonies in primary and secondary streaks only
3+ = colonies in all three streaked areas
4+ = nearly confluent growth in all streaked areas

** = isolate appeared typical of *L. pneumophila*; however, the organisms failed to grow on subculture
Table 20

Mortality of Golden Shiner Minnows Inoculated With 0.1 ml Doses of Virulent or Avirulent *Legionella pneumophila* and Housed in the Same Tank

<table>
<thead>
<tr>
<th>Organisms Injected (CFU)</th>
<th>Deaths After 7 Days*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virulent strain</td>
<td>4.6 x 10^9</td>
</tr>
<tr>
<td>Avirulent strain</td>
<td>5.8 x 10^9</td>
</tr>
<tr>
<td>Uninoculated Controls</td>
<td>0</td>
</tr>
</tbody>
</table>

*All deaths occurred with the first 36 h; remaining fish were sacrificed at 7 days.*
Table 21

Mortality of Minnows Inoculated With 0.1 ml Doses of Undiluted or a 1:10 Dilution of Virulent or Avirulent Legionella pneumophila and Housed in the Same Tank

<table>
<thead>
<tr>
<th>Organisms Injected (CFU)</th>
<th>Deaths After 10 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Virulent strain</strong></td>
<td></td>
</tr>
<tr>
<td>4.4 x 10^9</td>
<td>3/4 (8h, 24h, and day 4)</td>
</tr>
<tr>
<td>4.4 x 10^8</td>
<td>1/4 (day 7)</td>
</tr>
<tr>
<td><strong>Avirulent strain</strong></td>
<td></td>
</tr>
<tr>
<td>5.3 x 10^9</td>
<td>0/4</td>
</tr>
<tr>
<td>5.3 x 10^8</td>
<td>0/4</td>
</tr>
<tr>
<td><strong>Uninoculated Controls</strong></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1/3 (day 2)</td>
</tr>
</tbody>
</table>

Organisms varied from 4 to 45 colonies.
did not succumb within the 10 days. Only 1 of the 4 which received the 10-fold dilution died in the 10 days; this death occurred on day 7. The peritoneal cavity of one surviving fish in each group was cultured on day 10. *L. pneumophila* was reisolated from all except the one which received the 10-fold dilution of the avirulent strain (numbers varied from 4 to 45 colonies).

There appeared to be some changes in the ability of the virulent *L. pneumophila* to kill consistently when similar numbers of bacteria were injected. The age of the BCYE agar was suspected to be the culprit. To test this hypothesis, the virulent strain only was cultured on media stored for 2 months at 4 C and on plates prepared within 72 h. The results of this experiment are shown in Table 22.

Tank water was cultured daily in all experiments except the last. *Legionellaceae* were isolated sporadically from the water and only in the first two days following inoculation (Table 23). Filter cultures resulted in heavy growth, but no *L. pneumophila* was isolated, even from subcultures of the original plates.

Chemotactic activity of human mononuclear cells directed toward both strains was more than the negative control, but still less than that with the *E. coli* (Table 24). Using 2.5 times the control mean (12.3) as the minimum for a positive result, both the *E. coli* and the avirulent *L. pneumophila* would be considered to be
Table 22

Mortality of Golden Shiner Minnows Injected with 0.1 ml Doses of Virulent *Legionella pneumophila* Grown on Fresh or Two-Month-Old BCYE Agar and Housed in the Same Tank

<table>
<thead>
<tr>
<th>Organisms Injected (CFU)</th>
<th>Deaths After 7 Days*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh BCYE</td>
<td>1.3 x 10^9</td>
</tr>
<tr>
<td>Old BCYE</td>
<td>1.6 x 10^9</td>
</tr>
<tr>
<td>Uninoculated Controls</td>
<td>0</td>
</tr>
</tbody>
</table>

* All deaths occurred within the first 24 hours.

* Water was tested at two dilutions (10^-2 and 10^-3) in duplicate using BMPA- medium and the results averaged except in the first tank where only the 10^-2 dilution was cultured. Results are rounded to the nearest 500.

** Filters were swabbed and plated directly onto BMPA- medium in duplicate. In all instances there was nearly confluent growth and no *L. pneumophila* could be isolated on subcultures.
Table 23
Recovery of *Legionella pneumophila* from Water and Charcoal Filters in Tanks Used to House Golden Shiner Minnows

<table>
<thead>
<tr>
<th>Number of Fish</th>
<th>Volume Injected (ml)</th>
<th>Organisms Isolated (CFU x 10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>L. pneumophila/Total</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tank 1</td>
</tr>
<tr>
<td>Water, Day*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0/160</td>
<td>0/47.5</td>
</tr>
<tr>
<td>1</td>
<td>&gt;100/TNTC</td>
<td>4.5/50.0</td>
</tr>
<tr>
<td>2</td>
<td>2.5/TNTC</td>
<td>0/55.0</td>
</tr>
<tr>
<td>3</td>
<td>0/TNTC</td>
<td>0/45.0</td>
</tr>
<tr>
<td>4</td>
<td>0/10.5</td>
<td>0/75.0</td>
</tr>
<tr>
<td>5</td>
<td>ND</td>
<td>0/62.5</td>
</tr>
<tr>
<td>6</td>
<td>ND</td>
<td>0/27.5</td>
</tr>
<tr>
<td><strong>Filter</strong></td>
<td></td>
<td>0/TNTC</td>
</tr>
</tbody>
</table>

* Water was tested at two dilutions (10^{-2} and 10^{-4}) in duplicate using BMPAα medium and the results averaged except in the first tank where only the 10^{-2} dilution was cultured. Results are rounded to the nearest 500.

** Filters were swabbed and plated directly onto BMPAα medium in duplicate. In all instances there was nearly confluent growth and no *L. pneumophila* could be isolated on subcultures.
Chemotactic Index of Human Mononuclear Cells Toward Two Strains of _Legionella pneumophila_

**Chemotactic Index**

<table>
<thead>
<tr>
<th>Virulent Strain</th>
<th>Avirulent Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>8.1</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>14.9</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>12.7</td>
</tr>
<tr>
<td>Experiment 4</td>
<td>8.2</td>
</tr>
</tbody>
</table>

Mean of all Experiments 11 14.4

Values are expressed as the ratio of mononuclear cells on the lower surface of the filter to the total number of mononuclear cells counted. Each value represents the mean of duplicate chambers. Negative control mean was 4.9 and the E. coli mean was 19.2.
chemotactic, while the virulent strain would be borderline, but negative.

Guinea pig peritoneal macrophages were attracted to all three organisms tested (Table 25). The virulent L. pneumophila strain, however, would not be considered positive in relation to the negative control (2.5 times the control mean = 11.0). Both the E. coli and the avirulent L. pneumophila would be considered to be positive with the E. coli slightly less chemotactic.

The hogchoker macrophages reacted positively at 90 min with the E. coli and the virulent L. pneumophila, but not the avirulent L. pneumophila (2.5 times the negative control mean = 20.8), as seen in Table 26. The E. coli chemotactic index was nearly twice that of the virulent strain, which only minimally exceeded the control value standard. The reverse was the case with the spot macrophages (Table 27). The avirulent strain was the most chemotactic of the three at 90 min, which were all positive using a value of 20 to determine chemotactic activity. None of the bacteria tested produced chemotactic activity which exceeded that of the controls when the golden shiner minnow macrophages were used (Table 28). Table 29 compares the chemotactic responses of all species tested.

Results of the plasmid analysis are seen in Figure 17. No plasmids were detected in either the virulent or the avirulent L. pneumophila.
Table 25

Chemotactic Index of Guinea Pig Peritoneal Macrophages Toward Two Strains of * Legionella pneumophila *

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Virulent Strain</th>
<th>Avirulent Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.2</td>
<td>10.6</td>
</tr>
<tr>
<td>2</td>
<td>9.9</td>
<td>20.3</td>
</tr>
<tr>
<td>3</td>
<td>5.0</td>
<td>10.0</td>
</tr>
<tr>
<td>4</td>
<td>13.1</td>
<td>16.7</td>
</tr>
</tbody>
</table>

Mean of all Experiments 9.3 14.4

Values are expressed as the ratio of macrophages on the lower surface of the filter to the total number of macrophages counted. Each value represents the mean of duplicate chambers. Negative control mean was 4.4 and the * E. coli * mean was 13.3.
Table 26

Chemotactic Index of Hogchoker (Trinecte maculatus) Macrophages Toward Two Strains of *Legionella pneumophila*

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>Virulent Strain</th>
<th>Avirulent Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 minutes</td>
<td>20.3</td>
<td>5.5</td>
</tr>
<tr>
<td>60 minutes</td>
<td>35.3</td>
<td>10.8</td>
</tr>
<tr>
<td>90 minutes</td>
<td>21.8</td>
<td>14.3</td>
</tr>
</tbody>
</table>

Values are expressed as the ratio of macrophages on the lower surface of the filter to the total number of macrophages counted. Each value represents the mean of duplicate chambers from three experiments using 12-25 fish per experiment. Negative control mean was 8.3 and the *E.coli* mean was 38 at 90 minutes' incubation time.
### Table 27

Chemotactic Index of Spot (Leiostomus xanthurus) Macrophages Toward Two Strains of *Legionella pneumophila*

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>Virulent Strain</th>
<th>Avirulent Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 minutes</td>
<td>10.5</td>
<td>25.7</td>
</tr>
<tr>
<td>60 minutes</td>
<td>22.3</td>
<td>40.5</td>
</tr>
<tr>
<td>90 minutes</td>
<td>24.3</td>
<td>60.5</td>
</tr>
</tbody>
</table>

Values are expressed as the ratio of macrophages on the lower surface of the filter to the total number of macrophages counted. Each value represents the mean of duplicate chambers from three experiments using 12-25 fish per experiment. Negative Control mean was 8 and the *E. coli* mean was 38 at 90 minutes' incubation time.
Table 28

Chemotactic Index of Golden Shiner Minnow (Notemigonus crysoleucas) Macrophages Toward Two Strains of Legionella pneumophila

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>Virulent Strain</th>
<th>Avirulent Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 minutes</td>
<td>7.1</td>
<td>5.1</td>
</tr>
<tr>
<td>60 minutes</td>
<td>9.9</td>
<td>6.6</td>
</tr>
<tr>
<td>90 minutes</td>
<td>6.5</td>
<td>5.9</td>
</tr>
</tbody>
</table>

Values are expressed as the ratio of macrophages on the lower surface of the filter to the total number of macrophages counted. Each value represents the mean of duplicate chambers from four experiments using 12-25 fish per experiment. Negative control mean was 11.3 and the E.coli mean was 3 at 90 minutes' incubation time.
Table 29

Comparison of Chemotactic Activity of Human Mononuclear Cells, Guinea Pig Macrophages, and Macrophages from Three Species of Fish Using Virulent or Avirulent \textit{L. pneumophila} as the Stimulus

<table>
<thead>
<tr>
<th></th>
<th>Virulent Strain</th>
<th>Avirulent Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Mononuclear Cells</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Guinea Pig Peritoneal Macrophages</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Fish Macrophages</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hog Choker</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Spot</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Minnow</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Chemotaxis was considered positive (+) when test values exceeded 2.5 times the control value. Chemotaxis was negative (-) if the value was less. Borderline values (+) were those which were +1.5 that of the negative control value times 2.5.
Figure 17

Analysis for the Presence of Plasmids in Virulent and Avirulent *Legionella pneumophila*

Lane 2  Avirulent *Legionella pneumophila*

Lane 3  Virulent *Legionella pneumophila*

Lane 8  E. coli with multiple plasmids

Lanes 1, 4, 5, 6, and 7 contain environmental *L. pneumophila* isolates with known plasmid content
Both virulent and avirulent forms were similar morphologically. The virulent form (Figure 18) demonstrated the typical trilaminar outer membranes with evenly distributed nuclear material; large vacuoles were seen inside many of the cells. Only three cells were found to have any suggestion of a capsule (Figure 19) after prolonged searching. The avirulent \textit{L. pneumophila} had a similar appearance with 2 differences. The intracellular vacuoles were smaller and usually less distinct (Figure 20) and apparently encapsulated organisms (Figure 21) were easily found. Polymixin B treatment of both strains also produced similar changes in morphology (Figures 22 and 23). Bleb formation was noted to be evenly distributed over the outer surfaces of the bacteria and the nuclear material was distributed in a patchwork pattern.

Electrophoresis using a 12\% sodium dodecylsulfate polyacrylamide gel (SDS-PAGE) to separate soluble proteins suggested the presence of several bands in the avirulent form that were not seen in the virulent \textit{L. pneumophila} (see Figures 24 and 25). One additional band appeared to be present between the 31 and 45 kd standards, two between the 21.5 and 31 kd standards, and one at the same point as the 21.5 kd standard. An attempt was then made to clarify these bands as well as to separate possible additional bands using the more sensitive gradient SDS-PAGE. Results of electrophoresis
Figure 18

Ultrastructure of Virulent *Legionella pneumophila* (70,000 X)
Possible Encapsulated Virulent Legionella pneumophila (70,000 X)
Figure 20

Ultrastructure of Avirulent Legionella pneumophila (90,000 X)
Figure 21

Encapsulated Avirulent *Legionella pneumophila* (90,000 X)
Figure 22

Appearance of Virulent *L. pneumophila* Following Treatment with 160 µg/ml Polymixin B (70,000 X)
Figure 23

Appearance of Avirulent *L. pneumophila* Following Treatment with 160 μg/ml Polymixin B (70,000 X)
Figure 24

Pattern of the Soluble Proteins of Virulent *Legionella pneumophila* After 12% Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis
Figure 25

Pattern of the Soluble Proteins of Avirulent Legionella pneumophila After 12% Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis
using a 3-27% gradient gel are shown in Figure 26. This time there were no apparent differences in the patterns seen between the virulent and avirulent *L. pneumophila*.

Testing the organisms with a direct fluorescent antibody reaction using a monoclonal antibody resulted in a drop in the number of intensely stained bacteria as the cultures grew older (Table 30). The virulent strain demonstrated only 25% which were as brightly stained as the positive control by the eighth day of culture, whereas the 8 day culture of the avirulent *L. pneumophila* showed 50% of the bacteria fluorescing this intensely. When a polyclonal antibody was used in the same reaction, no reduction in intensity was noted for either the virulent or the avirulent form 2, 3, 8, or 17 days after the BCYE plates were inoculated. The organisms were not tested at 24 h as originally planned because there was little visible growth on the plate inoculated with the virulent *L. pneumophila*. 

*Figure 26*

Patterns of the Soluble Proteins of Virulent and Avirulent *L. pneumophila* After Gradient (3-27%) Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis

V = virulent

Av = avirulent
Figure 26

Patterns of the Soluble Proteins of Virulent and Avirulent L. pneumophila After Gradient (3-27%) Sodium Dodecylsulfate Polycrylamide Gel Electrophoresis

V = virulent  Av = avirulent
Table 30

Comparison of Direct Immunofluorescent Reactions of Virulent and Avirulent *Legionella pneumophila* Using Monoclonal or Polyclonal Antibody

<table>
<thead>
<tr>
<th>Age of Culture (Days)</th>
<th>Virulent Strain</th>
<th>Avirulent Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Monoclonal (°)</td>
<td>Polyclonal (°)</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>17</td>
<td>25</td>
<td>100</td>
</tr>
</tbody>
</table>

Bacterial cells in all tests were fluorescent; results are expressed as the approximate percentage which were as bright as the positive control supplied with each kit.
DISCUSSION

Ability of the two strains of *L. pneumophila* serogroup 1 to cause disease was repeatedly confirmed throughout this study. The virulent form was able to kill most guinea pigs at concentrations as low as $2.6 \times 10^7$ CFU/ml and produce observable signs of infection in half the animals at $2.5 \times 10^6$ CFU/ml (Tables 3, 4, and 5). These values are similar to those reported by others (29, 33, 112, 116, 122, 342). It is difficult to make direct comparisons owing to the variety of strains used as well as differences in culture conditions and criteria for infection. Early works also often lack data concerning passage history of the isolate(s) used, a factor now known to be important in assessing virulence. Intraperitoneal inoculation of the animals is also a less sensitive indicator of virulence. Infection via aerosol inhalation results in consistently lower LD$_{50}$ values (22, 75) and is probably the method of choice when comparison with human disease is desired. This requires more equipment and technical expertise than intraperitoneal inoculation and may not be feasible except in large research facilities where many animals must be infected.

Twelve guinea pigs received at least $1 \times 10^8$ CFU of
the virulent strain. Two animals never developed visible signs of infection. The possibility that some animals may have been exposed to legionellae prior to injection was considered. Four animals were tested for antibodies using an indirect fluorescent assay; significant titers were not found. This apparently random resistance remains unexplained, but is unlikely to be due to prior exposure. Animals were outbred so variations in innate susceptibility to infection would seem to be the best explanation. Variability in virulence of the organism can be ruled out since the suspension which failed to infect one of the animals was lethal for 4 of 4 animals which received a 1:10 dilution of this same suspension (Table 5).

Comparison Studies of EYB and BCYE Media

Both strains of L. pneumophila were able to grow on BCYE and EYB agars. The avirulent, agar-adapted form consistently showed visible growth earlier than the virulent form, especially when the virulent strain was to be isolated from a tissue suspension. Both strains showed browning and a clear zone in the agar around colonies on EYB. This is in agreement with Chan's (Ph.D. thesis) results; however, the colonies were cream-color in this study as opposed to the sunshine yellow, deepening as the colonies aged, seen by him. The most obvious explanation is strain differences since
Chan used the Knoxville 1 strain rather than Burlington 1. The Burlington 1 strains also grew better on BCYE than the EYB. In one instance, no visible growth was seen on EYB at 72 h, but colonies were seen on BCYE. More colonies were seen on BCYE in most instances as well. This is in agreement with Keathley and Winn (177) who suggested that media without charcoal do not support the growth of legionellae as well as those which contain it. The charcoal probably removes inhibitors produced when yeast-extract containing media are autoclaved (154).

Virulence was maintained for 26 passages on either agar (Tables 3 and 4). Chan found that virulence was lost after 25 passages on BCYE but not on EYB. Again, strain differences may account for this discrepancy as well as the small numbers of animals used in both studies. Other researchers have found that virulence is lost after as few as 5 transfers on modified Mueller-Hinton agar (103) or 10 on BCYE (33). In contrast, others have found no loss of virulence after 12 passages on BCYE (103, 338) and some have suggested that 30 transfers or more may be necessary to produce an avirulent variant (163, 257, 313).

Ability to cause disease may be related to variables other than media components. In this study, apparently random events seemed to result in changes in the ability of the virulent organism to affect guinea
pigs. After 20 passages on BCYE, the animal receiving $7 \times 10^8$ CFU failed to develop disease, yet 6 additional transfers resulted in death of another guinea pig 2 days after receiving $4.7 \times 10^8$ CFU. Age of the medium was suspected, but this was not confirmed when fish were used as the experimental model (Table 22). Edelstein (89) has suggested incubation temperature may affect virulence. All cultures were incubated at 37°C in this study and Chan did not find temperature to affect virulence, either. Legionellae require very humid conditions for survival and growth (21, 74). Danielson and Cooper (74) have suggested that evaporation and syneresis associated with agar plates may affect the ability of legionellae to grow on laboratory media. Their solution is to use a biphasic slant or to incubate the inverted petri dishes with sterile water in the lids. This may explain the results seen here or other undefined changes which occur in preparation and/or storage of media may be responsible. Pine et al. (241) suggests the age of either the inoculum or medium or both could limit subsequent growth. Solid media may select for auxotrophs which require either amino acids or purine for growth. These organisms may also be able to grow in the presence of compounds inhibitory for the more virulent forms.

Effects of Polymixin B Pre-treatment
Polymixin B treatment also failed to alter virulence. Morphological changes seen on electron microscopy were similar to those reported by Chan. Blebs were formed in both virulent and avirulent strains after exposure to polymixin B (Figures 22 and 23). These were evenly distributed over the outer surfaces of the bacteria. Size was proportional to antibiotic concentration (data not shown). The nuclear material was distributed in a patchwork pattern. There was no apparent change, however, in the numbers of virulent organisms required to produce disease and/or death as a result of pre-treatment (Table 5). Treatment of the avirulent strain failed to produce visible signs of infection as well (Table 6). The failure to confirm Chan's findings regarding increased virulence with polymixin B treatment may also be explained by strain differences and/or the small numbers of animals used in each study. Wong, et al. (336) suggested that polymixin B treatment results in decreased toxicity through binding of the lipid A component of endotoxin. Toxicity was not significantly reduced in their experiments using mice; this work using guinea pigs would support their finding as well.

Detection of Virulence Factors

Morphologically, there was little difference between the two strains. The avirulent strain showed
higher numbers of very long filamentous forms in a Gram stained smear. As the cultures aged, the virulent strain produced increased numbers of filamentous forms but not to the level seen in the agar-passaged strain. This is compatible with the findings of Bornstein, Nowicki, and Fleurette (33). They studied filament length in various culture conditions and related this to virulence. Bacteria with the longest average length also had the highest LD$_{50}$. In vivo findings also would support this theory. Short, cocco-bacillary forms are most often seen in tissue sections while greatly elongated forms are rare (54, 250).

Electron microscopy of isolates showed them to be similar as well (Figures 18 and 20). No flagella or pili were noted. This is not unusual; early reports failed to note appendages in the several strains examined (54, 250). Later studies did reveal the presence of flagella (57, 252, 296). The Burlington 1 strain has been shown to produce streamers and a curly polar flagellum when stained using a simplified Leifson method (296). Several groups (102, 252, 296) note that passage on artificial media may result in loss of the ability to produce flagella and that very gentle handling during processing is required to maintain attachment to the bacteria. Hebert and associates (141) noted that only a small number of cells had flagella in their analysis of the OLDA strain of _L. pneumophila._
Elliott and Johnson (103) demonstrated that loss of the ability to produce flagella does not alter the ability to produce disease in guinea pigs via intraperitoneal injection. This is also the case in the present study. In one instance, an aliquot was injected into a guinea pig while another portion of the suspension was processed for electron microscopy. It is possible that flagella were either not being produced or were lost in processing. In either case, the virulent strain was able to cause disease.

One difference in the strains was the size of the intracellular vacuoles. These vacuoles were smaller and usually less distinct in the avirulent form. Chan also noted increased vacuolation in forms thought to be more virulent. Vacuoles also are easily found in organisms seen in human lung tissue (54, 178, 250). It is possible that these vacuoles contain products which increase the virulence of the bacteria. They are more likely, however, to be storage granules produced in times of decreased availability of nutrients in the environment. As discussed above, the avirulent form is able to grow better on artificial media and is probably able to utilize more components more efficiently than the virulent strain. Chan noted increased vacuolation in organisms grown on EYB but not on BCYE. This is consistent with this interpretation as in the present study organisms did not grow as well on EYB, which would
lead to increased vacuolation if this theory is correct. Intracellular organisms in vivo would also deplete nutrients as they multiply within the macrophages. These granules stain with Sudan black and appear to be poly-B-hydroxybutyrate-like (251, 317). This would also support the idea that these are storage granules rather than enzymes or other factors which would result in increased ability to cause disease.

More interesting is the fact that capsules were found without much difficulty in the avirulent strain (Figure 21). Only 3 cells of the virulent strain were noted to have a possible capsule (Figure 19). Encapsulation remains a controversial question. Glavin and associates (131) suggested that the clear space surrounding bacteria they saw in human lung tissue might represent a microcapsule or slime layer in some strains. Hebert et al. (141) used ruthenium red to look specifically for capsules. Capsules were readily found in L. micadei, L. bozemanii, and L. dumoffii, but were rare in the strain of L. pneumophila examined. Rogers and Davey (251) did not observe capsules using ruthenium red in either organisms in lung cell vacuoles or in intact cells grown in the laboratory. They suggest that this discrepancy may be due to slight differences in technique, strain differences, or the fact that capsules occur rarely, if at all, in L. pneumophila (255). The present study found capsules, but most often in the
avirulent form.

Capsules are known to increase virulence by increasing resistance to phagocytosis (36). Since L. pneumophila multiplies only intracellularly, it may be that the presence of a capsule actually hinders its ability to multiply in the animal host and would thus be associated with decreased virulence. The ability to survive and multiply within macrophages is associated with virulence. Avirulent strains are either killed (182) or multiply more slowly and to a lower magnitude (313). Jepres et al. (164) found that guinea pigs infected with an avirulent strain had mostly extracellular organisms on lung lavage. The virulent strain was found mostly intracellularly in the same situation. They suggest that avirulent strains resist phagocytosis or are more effectively killed by the macrophage following ingestion. The presence of capsules would support the first mechanism.

Plasmids were not seen in either strain (Figure 17). This finding is consistent with other work. Both plasmid-containing (96) and plasmidless (29, 46, 47) isolates have been associated with outbreaks of LD. Plasmid-containing isolates retain them on transfers so these are probably most useful as an epidemiologic tool (228). The presence or absence of plasmids has no apparent relationship to virulence.

Polyacrylamide gel electrophoresis (PAGE) results
were puzzling. Initial work using a 12% resolving gel suggested the presence of bands in the avirulent strain not seen in the virulent organisms (Figures 24 and 25). This is similar to the work of Reardon (M.S. thesis, Virginia Commonwealth University, 1985). He found two major bands present in the avirulent strain in addition to three bands prominent in both strains. Differences in technique (Reardon used an 8% resolving gel) may account for the small differences in relative mobilities of the major soluble proteins seen in this and Reardon's study. Gradient PAGE failed to confirm the differences between strains (Figure 26). This method was more sensitive; many more bands were discernable. They appeared to be identical in relative mobility and staining properties in the virulent and avirulent organisms. Lema and Brown (192) have compared electrophoretic patterns of the soluble proteins from 5 species as well as 21 strains of 6 serogroups of *L. pneumophila*. They found only minor differences within a species and suggested that patterns were distinctive enough to allow species identification with SDS-PAGE. The *L. pneumophila* serogroup 1 strains analyzed (13) were similar although some demonstrated bands of lighter staining intensity suggesting lower production of certain proteins. Lema and Brown thought this may correlate with the presence of a plasmid, which may promote increased synthesis of a peptide. It is
difficult to compare their band patterns with those seen in this work since different methods of preparing the specimens and gels were used. Generally, many bands could be seen in both, with an apparently heavier concentration of low molecular weight proteins. There may be minor strain differences, but these are probably not related to virulence. The additional bands seen in the avirulent strain when the 12% separating gel was used could be due to the relative insensitivity of this procedure. The virulent organism may produce these proteins, but at levels detectable only with the gradient gel. Variations in media (see earlier discussion) may have led to altered protein production which affected the virulent strain to a greater extent than the agar-adapted avirulent strain in the 12% gel experiments.

Results of direct fluorescent antibody staining were interesting. Both strains stained uniformly and with the same intensity as the positive control when the polyclonal reagent was used. The number of cells which stained as intensely as the positive control decreased at 72h of growth when the monoclonal antibody test system was used. This remained constant at about 50% for the avirulent form but declined to 25% at 8 and 17 days for the virulent strain (Table 30). All cells at all times were fluorescent, but those which did not stain as brightly as the positive control diminished in
staining intensity as the cultures aged so that at day 17, 50% of the avirulent cells and 75% of the virulent cells were only weakly positive (l+). Cells which were brightly stained were primarily the filamentous forms which increased in number as the cultured aged. This variation in fluorescence intensity with the age of the culture is noted in the product information supplied with the Genetic Systems reagents. However, they describe a lack of uniform staining over the cell surface rather than cell-to-cell differences in intensity.

These results support the findings of a study by Petitjean et al. (Abstr. Ann. Meet. ICAAC, 1986, 1072, p. 293). They found a change in the affinity constant and the number of low affinity binding sites as a virulent organism was subcultured to produce an avirulent derivate. Loss of reactivity with their monoclonal antibody was related to loss of virulence. In the present study the virulent form lost reactivity. These discrepancies are easily explained since different monoclonal antibodies were used. Petitjean and co-workers used an antibody identical to CDC Mab 2, which reacts only with subgroups of \textit{L. pneumophila} serogroup 1 (168). The Genetic Systems monoclonal antibody is species-specific and reacts with all strains from the 9 serogroups tested (133). It is probable that a change in the epitope itself or in accessibility to the
antigenic determinant occurs as the culture ages. Increased "stickiness" is seen on aging. This may be the result of the production of a slime layer which would obscure the antigenic determinant to a greater degree in the virulent form. Vigorous mixing did not change the reactions, suggesting an alteration in individual cells rather than changes as a result of increased clumping. The polyclonal reagent contains a mixture of antibodies with different specificities which may explain the fact that all cells at all ages reacted with the same intensity. Certain antibodies in the mixture may react differently, but this is not detected in microscopic analysis.

Other investigators have shown that strains of L. pneumophila which give certain patterns when tested with a panel of monoclonal antibodies are more likely to be associated with clinical illness than other strains with different reactivities using the same panel (236, 306, 319). It is possible that proteins which react (or fail to react) with certain monoclonal antibodies are associated with virulence. The fact that filamentous forms fluoresced to a greater extent using the Genetic Systems antibody and may be associated with decreased virulence would also tend to support this view. These areas require further investigation.

Fish as an Experimental Model
Chemotactic assays gave similar results when human mononuclear cells, guinea pig peritoneal macrophages, or spot kidney macrophages were tested (Table 29). The *E. coli* and the avirulent *L. pneumophila* were chemotactic for all 3 species. The virulent *L. pneumophila* resulted in minimal chemotactic activity in all three instances when 2.5 times the negative control mean was used to evaluate this response. Macrophages migrated through the filters in greater numbers when virulent *L. pneumophila* was tested than when only medium was used, in sufficient quantity to result in a positive interpretation only with the spot macrophages. These findings are similar to those of Horwitz and Silverstein (159) who found that phagocytosis of *L. pneumophila* in the absence of antibody and complement is very inefficient.

Data from the hogchoker and minnow chemotactic experiments are different from those discussed above. The hogchoker macrophages responded positively to the *E. coli* and the virulent *L. pneumophila* but not to the avirulent strain. Minnow macrophages were unresponsive to any of the bacteria tested. It is possible that test conditions were not optimal in the case of the minnows. A variety of systems was evaluated (data not shown) with essentially similar results. It is likely that the minnow macrophages do not respond in the same way as mammalian cells do. Little is known about the
chemotactic response of macrophages in fish and this function only recently has been studied in the hogchoker and spot (320).

In vivo experiments using the 3 species of fish were frustrating at times as well as exciting. Laboratory conditions were not ideal for long-term maintenance of healthy fish. Overcrowding in the aquaria was an important problem. Frequent partial changes of water and prompt removal of excess food and obviously sick fish allowed the survival of all 3 species in an apparently healthy condition for up to 6 weeks and possibly longer. Another drawback in the fish studies was the fact that when large numbers of fish died in a short period of time, the non-specific toxins released into the water affected other fish in the same aquarium. This meant that in a few instances, control fish were found dead as well, making results difficult to interpret (e.g., Table 16). Experiments were modified so that fewer fish were housed in each aquarium when large numbers of deaths were expected. This meant that variables might be introduced as a result of potential differences in the environments of different aquaria and/or that periodic sacrifice of infected fish must be limited.

Once these problems were overcome, the fish proved to be an acceptable experimental model. Deaths occurred within 24 to 48 h which was similar to times noted for
the guinea pigs and consistent with those reported by
other investigators using mammalian models (116, 144,
257). All species of fish were more resistant to
infection than the guinea pigs. Virulent L. pneumophila
at concentrations of \(10^7\) CFU was able to kill guinea
pigs but all species of fish required inoculum sizes
between \(10^9\) and \(10^{10}\) CFU before deaths consistently
occurred. The minnows appeared to be most susceptible;
the hogchokers the least; and the spot were intermediate
in susceptibility. In this regard, the fish are similar
to rats and mice which are also more resistant to
infection than guinea pigs when injected
intraperitoneally (83, 101, 257). There were some differences in the ability of
similar numbers of virulent organisms to cause deaths
(Tables 7, 10, 12, 13, 14, 18, 20, 21). This is most
likely to be due to the small numbers of fish used in
each experiment. Another possibility is the media
variability discussed earlier. Age of the medium was
considered but this was not confirmed (Table 22). Other
unidentified factors related to the medium may be
responsible. Analysis of the data also suggested that
the volume injected may be related in some way to the
ability to cause death. The amount of fluid alone is
unlikely to be responsible (B.A. Weeks, D.Paylor,
personal communications). It may be that a toxin is
released during the preparation of the suspension or
that cells are disrupted during the mixing process. Thus, more nonviable cell fragments and/or perhaps toxin(s) (either soluble or bound to these cells) would be injected as well as viable bacteria as volume is increased. Endotoxin alone is unlikely to be responsible, as the *Legionella* endotoxin is atypical and weak in in vivo tests (366). Rolstad and Berdal (257) suggest that in the rat, intracellular multiplication is not required to produce pathologic changes. If their animals were affected, they died rapidly (within 24 h) and they suggest that the rat is responding to a toxin or other virulence factor only, whereas the guinea pig is also susceptible to the effects of bacterial multiplication within the macrophage. The results shown here suggest that this may be important in the fish as well.

Some fish did die after injection with the avirulent strain, although not in the numbers that those which received the virulent organisms did. No cross-infection among fish is likely as legionellae were never isolated from control fish. This is compatible with a difference in amount or type of virulence factors produced. Fish which died more than 48 h after injection appeared to do so in a random manner. This also supports the theory that intracellular multiplication is not prominent in the fish as a pathogenic mechanism. Viable bacteria were isolated in
apparently healthy fish of all three species. The bacteria may survive within the fish and possibly multiply, but with no apparent ill effects.

Guinea pigs infected intraperitoneally with virulent *Legionella* often are found to have a peritoneal exudate. This did not appear to be the case with the spot. Spot injected with the avirulent strain had more leukocytes in Wright-Giemsa stained peritoneal smears than either the controls or those receiving the virulent strain. Mononuclear cells predominated with few granulocytes seen (Figures 7, 8, 9, 10, and 11). These mononuclear cells were mostly lymphocytes and macrophages with occasional plasmoid lymphocytes and plasma cells. These findings are compatible with the chemotactic studies, which suggested that the avirulent organisms were more chemotactic in vitro (Table 27). Sheets of mononuclear cells were also seen in the spot receiving the virulent bacteria (Figures 14, 15, and 16). These cells had rounder nuclei with a more open chromatin pattern and indistinct cytoplasmic borders. These are most likely mesothelial lining cells rather than macrophages. Similar sheets also occasionally were seen in controls as well as fish inoculated with the avirulent strain. These results must be considered preliminary as only a few fish were examined.

Degenerative changes were prominent in fish found dead; fish should be sacrificed at intervals and smears
examined to verify these findings.

Isolation of viable bacteria from all three species is interesting. Hogchokers cultured up to 14 days after inoculation (Tables 8, 9, 11), spot up to 7 days (Table 11), and minnows up to 10 days had positive cultures. Whether the bacteria actually multiplied or only survived in the peritoneal cavity cannot be determined from the data. In several instances, the fish were apparently healthy and were sacrificed rather than cultured after death. The minnows had fewer organisms than either the hogchoker or spot. Fish which come into contact with legionellae or amoebae containing them in the environment may become infected and serve as additional reservoirs for the bacteria during adverse conditions. Legionellae have not been isolated from salt water (118) but evidence exists that birds associated with the ocean can be infected (65). It is possible that estuarine fish such as the hogchoker or spot may protect the legionellae from the effects of salt water as well as provide a means for wider dissemination of the bacteria. Both freshwater as well as marine fish should be captured and cultured from a variety of sites to test this hypothesis.

In summary, virulence of *L. pneumophila* is most likely a result of a combination of toxin production and intracellular multiplication in susceptible cells. Comparison of the virulent and avirulent forms of the
Burlington 1 strain of *L. pneumophila* demonstrated that:

1) virulence is maintained for at least 26 passages on either EYB or BCYE media, but appears to be affected by unknown medium-related variables.

2) polymixin B treatment does not affect either strain's ability to cause disease in guinea pigs inoculated intraperitoneally.

3) morphologically, the avirulent strain produces more filamentous forms and possibly is encapsulated more frequently.

4) virulence is not related to the presence of a plasmid and is unlikely to be a result of production of proteins separable by SDS-PAGE.

5) virulent cells react less intensely with monoclonal antibody in the DFA test as they age probably as a result of concealment, alteration or loss of the antigenic determinant.

6) the avirulent strain is more generally chemotactic for macrophages although neither is potent in this respect.

7) estuarine and freshwater fish may be additional environmental reservoirs and can serve as experimental models in the evaluation of certain properties of *L. pneumophila*. 
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Buffered Charcoal Yeast Extract Agar (BCYE)

BCYE dehydrated media* 19.15 g
L-cysteine HCl • H2O 0.2 g

Suspend BCYE in 450 ml of deionized water. Adjust to pH 6.9 with 1N KOH. Add enough water to bring the total volume to 495 ml.

Autoclave at 121 °C for 15 min and cool to 50 °C.

Prepare a fresh solution of L-cysteine (0.2 g in 5 ml of water), sterilize by filtration (0.2 μm millipore filter**) and add to basal medium.

Mix well and pour into petri dishes.

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* Gibco Diagnostics, Lawrence, Massachusetts 08143
**Geiman Sciences, Inc., Ann Arbor, Michigan 48106
Buffered Charcoal Yeast Extract Agar (BCYE)

BCYE dehydrated media* 19.15 g
L-cysteine HCL \cdot H_2O 0.2 g
Agar* 17 g

Suspend BCYE in 450 ml of deionized water. Adjust to pH 6.9 with IN KOH. Add enough water to bring the total volume to 495 ml.

Autoclave at 121 C for 15 min and cool to 50 C.

Prepare a fresh solution of L-cysteine (0.2 g in 5 ml of water), sterilize by filtration (0.2 µm millipore filter**) and add to cooled basal medium.

Mix well and pour into petri dishes.

* Gibco Diagnostics, Lawrence, Massachusetts 08143
**Gelman Sciences, Inc., Ann Arbor, Michigan 48106
Egg Yolk Buffered Agar (EYB)

Yeast Extract* 10 g
ACES Buffer 10 g
Agar* 17 g
8.0 N KOH 5 ml
L-Cysteine HCl • H₂O 0.4 g
Ferric pyrophosphate 0.25 g
50% Egg yolk suspension* 10 ml

Add yeast extract, ACES buffer and agar to 965 ml of deionized water. Autoclave at 121 C for 15 min, then cool to 50 C.
Prepare fresh solutions of L-cysteine (0.4 g in 10 ml of water) and ferric pyrophosphate (0.25 g in 10 ml of water).
Filter KOH, L-cysteine, and ferric pyrophosphate solutions through a 0.2 µm filter (Gelman) and add to cooled basal medium. Add the egg yolk suspension, mix well, and pour into petri dishes.

*Difco Laboratories, Detroit, Michigan 48232
Semi-Selective Medium (BMPA α)*

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCYE dehydrated media (Gibco)</td>
<td>19.15 g</td>
</tr>
<tr>
<td>Agar (Difco)</td>
<td>5 g</td>
</tr>
<tr>
<td>L-Cysteine HCL • H₂O</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Polymixin B</td>
<td>80 µg/ml</td>
</tr>
<tr>
<td>Anisomycin</td>
<td>80 µg/ml</td>
</tr>
<tr>
<td>Cefamodole</td>
<td>4 µg/ml</td>
</tr>
</tbody>
</table>

Add BCYE agar to 450 ml deionized water. Adjust to pH 6.9 with IN KOH. Add enough water to bring the total volume to 493 ml.

Heat to boiling to dissolve; then autoclave at 121 C for 15 min and cool to 50 C.

Prepare a fresh solution of L-cysteine (0.2 g in 5 ml of water). Filter all solutions through a 0.2 µm millipore filter (Gelman) to sterilize and add to cooled basal medium.

Mix well and pour into petri dishes.

Yeast Extract Buffered Broth (YEBS)

Yeast Extract (Difco) 10 g
ACES Buffer 10 g
8.0 N KOH 5 ml
L-Cysteine HCL • H2O 0.4 g
Ferric pyrophosphate 0.25 g

Add yeast extract to ACES buffer to 975 ml of deionized water.
Autoclave at 121°C for 15 min, then cool to 50°C.
Prepare a fresh solution of L-cysteine (0.4 g in 10 ml of water) and ferric pyrophosphate (0.25 g in 10 ml of water).
Filter all solutions through a 0.2 µm millipore filter (Gelman) to sterilize and add to cooled basal medium.
Mix well and dispense into 100 ml bottles.
APPENDIX B

Solutions Used in Plasmid Analysis

1. Schine's TE buffer (0.05 M Tris HCl, 0.01 M EDTA). Combine 4 g Tris HCl with 1.86 g EDTA; bring to 500 ml with distilled water; and adjust to pH 8.0 with glacial acetic acid.

2. Lysing buffer (50 mM Tris, 5% SDS). Dissolve 1.5 g Tris acetate and 7.5 g SDS in 250 ml distilled water; adjust to pH 12.4 with 3N NaOH.

3. 20% sodium dodecyl sulfate (SDS). Dissolve 2.0 g SDS in 10 ml distilled water.

4. 10 X E Buffer.
   Add 97 g Tris acetate to 13 g EDTA; mix with 2 l of distilled water; adjust to 7.9 with glacial acetic acid. Dilute to 1:10 for 1 X E buffer (40 mM Tris acetate, 2 mM EDTA).

5. Sample buffer.
   Mix 5 ml 10 X E buffer with 5 ml glycerol, 350 µl 20% SDS and 7 mg bromophenol blue.

6. 0.6% agarose.
   Combine 1.5 g agarose with 250 ml of 1 X E buffer; bring to a boil to dissolve; then cool to 50°C.
Solutions Used in Plasmid Analysis

1. Schlae's TE buffer (0.05 M Tris HCl, 0.01 M EDTA). Combine 4 g Tris HCl with 1.86 g EDTA; bring to 500 ml with distilled water; and adjust to pH 8.0 with glacial acetic acid.

2. Lysing buffer (50 mM Tris, 3% SDS). Dissolve 1.5 g Tris acetate and 7.5 g SDS in 250 ml distilled water; adjust to pH 12.6 with 3N NaOH.

3. 20% sodium dodecyl sulfate (SDS). Dissolve 2.0 g SDS in 10 ml distilled water.

4. 10 X E Buffer. Add 97 g Tris acetate to 13 g EDTA; mix with 2 l of distilled water; adjust to 7.9 with glacial acetic acid. Dilute to 1:10 for 1 X E buffer (40 mM Tris acetate, 2 mM EDTA).

5. Sample buffer. Mix 5 ml 10 X E buffer with 5 ml glycerol, 350 µl 20% SDS and 7 mg bromophenol blue.

6. 0.6% agarose. Combine 1.5 g agarose with 250 ml of 1 X E buffer; bring to a boil to dissolve; then cool to 50 C.
Solutions Used in Polyacrylamide Gel Electrophoresis

1. Lower gel buffer (1.5 M Tris, 0.4% SDS).
   Combine 36.33 g Tris and 0.80 g sodium dodecylsulfate (SDS); bring to 200 ml with distilled water; adjust to pH 8.8 with 1N HCl; store at 4 C up to one month.

2. Stacking gel buffer (0.5 M Tris, 0.4% SDS).
   Combine 6.06 g Tris and 0.40 g SDS; bring to 100 ml with distilled water; adjust to pH 6.8 with 1N HCl; store at 4 C up to one month.

3. Acrylamide solution.
   Combine 58.4 g acrylamide, 1.6 g bis-acrylamide and 140 ml distilled water; filter using a 0.22 micron vacuum filter (Falcon Plastics, Oxnard, California 93030); store in a brown bottle at 4 C up to one month.

4. Stock running buffer (0.25 M Tris, 1.92 M glycine, 1% SDS).
   Combine 30.3 g Tris, 144.0 g glycine, and 10.0 g SDS; bring to 1 l with distilled water; store at room temperature up to one month. Dilute 1:10 with distilled water just before use.

5. Sample buffer (0.0625 M Tris, 2.3% SDS, 0.05% bromphenol blue).
   Combine 0.1892 g Tris, 0.575 g SDS, 0.0125 g bromphenol blue, 2.5 ml glycerol, and 21.91 ml distilled water; adjust to pH 6.8 with 1 N HCl; store in 1 ml aliquots at 4 C up to one month. Allow to come to room temperature and add 50 µl of 2-mercaptoethanol per ml just before use.
Publications


Sommer, S. "Hematology Case Study," The Virginian Technoscope, December, 1985, p. 10.


Abstracts


Sommer, S; Escobar, MR; Dalton, HP; and Allison, MJ. 1974. "Viral identification by immunoelectro-osmophoresis (IEOP)." Bacterial Proc. 74.