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This is to certify that the dissertation prepared by Terrence James Ravine entitled *Legionella pneumophila*: Virulent and Avirulent Interactions with *Acanthamoeba castellanii* has been approved by his committee as satisfactory completion of the dissertation requirement for the degree of Doctor of Philosophy.

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***Legionella pneumophila*: Virulent and Avirulent Interactions with *Acanthamoeba castellanii*.**

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

By

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ABSTRACT

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

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Advisor: Harry P. Dalton, Ph.D.

Legionella pneumophila is an organism that is gaining greater attention in the medical community. Increasing evidence of *Legionella* as a major cause of community acquired pneumonia has ignited renewed interest in its close association with free-living soil and aquatic amoeba.

The current study investigated differences in the interaction of a virulent *L. pneumophila* and its agar-derived avirulent mutant with the free-living amoeba *A. castellanii*. Utilizing unique coculture conditions it was established that only the virulent phenotype undergoes intracellular multiplication in the amoeba. Replication of the avirulent form occurs only in the extracellular spaces but is enhanced by the amoeba. Numerous filamentous forms were noted in the avirulent phenotype which were attributed to defective cross-wall formation. The presence of these filaments does not interfere with replication in amoeba-free cultures. Alternately, filaments interfere with accurate spectrophotometric

adjustment of desired inoculum size. Similarly, the virulent form also replicated in amoeba-free cultures, although both forms required incubation times greater than 5 days. Passage of virulent forms in axenic amoeba cultures did not affect virulence potential in amoeba. Additionally, no changes were seen in outer membrane preparations of recovered bacteria. A previously described low molecular weight band seen in avirulent outer membranes has been reclassified at ~6.5 kDa. This band represents a highly glycosylated protein normally expressed at the the virulent form inner membrane.

INTRODUCTION

Legionella is a bacterium which appears to lead a double life. As a human pathogen it is regarded as a facultative intracellular parasite with fastidious *in vitro* growth requirements. However, it can be commonly isolated from a variety of aquatic and soil habitats and therefore is considered to be ubiquitous (124; 53). Establishing an ecological niche in an environment teaming with wildlife seems to be no small feat. When considering how far down *Legionella* is on the aquatic food chain it is remarkable how well it has adapted to its environment. Competition for available nutrients with more rapidly growing bacteria combined with growth requirements not commonly found in fresh water results in an apparent paradox regarding its occurrence in nature (48).

Since its identification in early 1977 as the causative agent of Legionnaires' disease, 32 *Legionella* species are now recognized containing 51 serogroups (23). Sixteen species have been characterized as human pathogens. The remainder are considered to be environmental isolates which have not yet been implicated in human disease (9). Historically, the *Legionella* bacterium was first isolated in 1947 with no indication that this unclassified organism could cause human disease (90). *Legionella* gained notoriety as a pathogenic bacterium during the American Legion convention held 21-24 July, 1976 in Philadelphia, Pennsylvania. Although the

disease is primarily linked to that convention it only accounted for 83% of cases meeting the clinical disease definition. Of 182 diagnosed cases, 32 patients did not attend the convention. They were most likely exposed by either entry into or being within proximity of the contaminated building. One hundred and forty seven (81%) patients required medical intervention, while 29 individuals developed severe pneumonia and died (54).

The Disease: Legionnaires' disease and Pontiac fever

Early attempts to isolate the causative agent of Legionnaires' disease proved unfruitful. Traditional culture methods for routinely recovered microbiologic agents of pneumonia did not yield a common organism. The agent finally was isolated using rickettiologic techniques. Intraperitoneal injection of fresh autopsy tissue from fatal cases into guinea pigs resulted in a pneumonia similar to Legionnaires' disease. Recovered guinea pig liver, spleen and lungs were then inoculated into embryonated hen's eggs. Use of the Gimenez stain allowed visualization of rod shaped bacteria in recovered yolk sacs. Serological evidence for Legionnaires' disease was accomplished by indirect fluorescent antibody testing of convalescent patient serum (60; 98). Once fluorescent antibody testing became available retrospective studies were conducted which involved incidents of respiratory illness that demonstrated a similar pattern to legionellosis. One such study linked a fatal outbreak of respiratory illness in 1965 at St. Elizabeth's Hospital (Washington, D.C.) to *Legionella*. In this episode, 81 patients displayed various clinical symptoms of legionellosis, including several cases

of pneumonia. Subsequently, fourteen patients (17%) died and no etiologic agent was determined. *Legionella* titers were performed on paired sera from 26 cases stored previously at Center for Disease Control (CDC, Atlanta, Ga.). Nineteen (85%) patients demonstrated titers with serologic evidence of legionellosis (141). A separate epidemic of febrile illness occurred in July - August of 1968 at the county health department building in Pontiac, MI. Ninety five percent of the employees working in this structure were affected. This outbreak of nonpneumonic illness was desiganated as Pontiac fever (61). In 1973 two episodes of Pontiac fever were documented. The first occurred when nine men were engaged to clean a steam turbine condenser situated on the James River in Virginia. Within 43 hours all nine individuals had developed signs consistent with Pontiac fever. No episodes of pneumonia were recorded in any of these workers. Sera from these individuals were tested for *Legionella* by CDC with five displaying significant titers against *L. pneumophila* (55). During that same month, 3 out of 189 British tourists returning from vacationing in Brenidorm, Spain died of pneumonia. Again, CDC provided serologic evidence of legionellosis (123). Fallon and Rowbotham investigated an outbreak of suspected Pontiac fever, which occurred at a leisure complex in Lochgoelhead, Scotland (42). Initially, all direct environmental cultures were negative, but use of a permissive amoeba host, *Acanthamoeba polyphaga*, allowed for the isolation of *L. micdadei* from whirlpool water. Convalescent sera from infected individuals demonstrated anti-*L. micdadei* antibodies. In September 1974, a similar outbreak occurred at an Odd Fellows convention held ironically, at the same hotel which would later host the 1976

Legionnaires' convention. Of 20 patients meeting case definition of Legionnaires' disease 2 (10%) died. Once again, serologic evidence provided for a definitive diagnosis of legionellosis and strongly implicated that both Philadelphia episodes were caused by the same agent. (141).

Legionella infections manifest two distinct forms. Legionnaires' disease refers to *Legionella* pneumonia, whereas non-pneumonic legionellosis also is known as Pontiac fever (54; 61). Legionnaires' disease appears to affect men two to three times more frequently than women. Other risk factors include smoking, alcoholism, diabetes and the chronically ill who received corticosteroids or immunosuppressive therapy. Seasonal variation is noted, with most cases occurring in the summer or autumn. To date epidemiologic studies indicate that inhalation remains to be the primary route of disease transmission. No evidence has been presented which would suggest person-to-person transmission (17).

The disease presents itself as do most lobar pneumonias and exhibits no unique clinical features. Laboratory findings are generally unremarkable except for mild elevations of white blood cell (WBC) counts, slightly abnormal liver enzymes and sodium electrolyte disturbances. Usually, an incubation period of 2-10 days is seen followed by an abrupt onset of high fever, malaise, headache and myalgia. Chest pain was of a pleuritic nature and noted in approximately one third of the Legionnaires attending the 1976 Philadelphia convention. Also dyspnea, abdominal pain and diarrhea were seen to occur in many infected individuals. Chest radiographs were abnormal in over 90%, early on demonstrating patchy interstitial infiltrates or patchy consolidation progressing to a

more widespread consolidation. Acute respiratory failure was the most important immediate complication. Lung infiltrates remained unilateral in over 50% of the cases and effusion, when present, was minimal. Cavitation was not seen. Coughs frequently became productive but were rarely purulent (54). Histologic examination revealed legionellae in areas of pneumonia most often within necrotic debris in alveoli and respiratory brochioles. Rarely were they located in bronchi, larger brochioles or alveolar septa (23). Macroscopic pathology was best described by Winn, *et al.* in fatal cases of Legionnaires' disease in Burlington, VT (155). Gross pathologic examination revealed extensive consolidation of air spaces, usually multilobar. Cut surfaces were moist and airless becoming granular and friable after formalin fixation. Other notable findings included fibrinous pleuritis, emphysema, singular macroscopic abcesses and pulmonary emboli, listed in decreasing order of occurrence.

The disease remains difficult to diagnose, with testing protocol having changed little since 1984. Serologic evidence of *Legionella* infection is heavily relied upon in making a diagnosis. However, culture recovery remains the "gold standard" and is considered to be very sensitive if performed early in the disease process (37).

Patients with Pontiac fever present early complaints similar to those seen in Legionnaires' disease. A shorter incubation period averaging 36 h is seen. Symptoms including fever, chills, headache and myalgia lasting two to five days were reported by Glick, *et al.* during the 1968 outbreak in Pontiac, MI (61). The illness is usually characterized as self-limiting with no secondary cases being

detected. The incidence of community acquired pneumonia (CAP) attributed to *Legionella species* is currently believed to range from 1 to 5%. Preliminary data from a large regional study of CAP requiring hospitalization suggests that the incidence of *Legionella*-associated pneumonia may be severely underestimated. Reasons given for such an underestimation include: a large proportion of the patients not being hospitalized; not all infected individuals present with pneumonia; and diagnostic techniques such as antigen detection and culture are not 100% sensitive (17).

The Bacterium

In 1979, Brenner, *et al.* proposed the establishment of the family *Legionellaceae* containing only one genus, *Legionella* (18). Although various legionellae have been isolated from patient sources, *L. pneumophila* remains as the major pathogen and is clinically the most frequently encountered organism in the group. Legionellae are aerobic, nonsporeforming, gram-negative rods. Phenotypically, individual bacterial cells vary in size depending on the source of origin. Isolates from tissue specimens appear as short, tapering rods with nonparallel sides and tapering ends. These measure 0.5 μm in width by 1 to 2 μm in length. However, *in vitro* cultivation yields organisms with wide variation in size. Reports indicate while widths are similar to specimens obtained from tissue, 0.3 to 7 μm, lengths can vary from the typical 2 to 3 μm to lengths of greater than 20 μm. Most legionellae are motile by either polar or subpolar flagella, which generally occur singularly but sometimes can be found in pairs. Pili also can be found. Biochemically, with the exception of *L. worsleiensis*, all species

demonstrate weak catalase and peroxidase activity. Excluding *L. micdadei, L. feeleii* and *L. nautarum*, all species liquify gelatin and most produce a soluble brown pigment in the presence of tyrosine. Neither nitrate reduction nor urea hydrolysis is seen (124; 125). Glucose and other carbohydrates are not generally utilized as a primary energy source, although *L. pneumophila* can metabolize glucose as a carbon source (101). Specific nutrient requirements including the amino acids arginine, isoleucine, methionine, serine, threonine, valine, and leucine. Except for *L. oakridgensis* a definitive requirement for L-cysteine exists. Depending on the medium used, incorporation of α-ketogluturate exhibits a stimulatory effect on legionellae growth. A source of iron appears to be essential for good growth of *L. pneumophila*. However, exclusion of iron from some liquid complex or defined medium may not preclude its growth, but tends to extend the length of the lag phase (151). Growth enhancement also occurs with the addition of trace metals such as calcium, cobalt, copper, magnesium, manganese, nickel, vandium and zinc (125).

Following the initial *in vivo* isolation procedure developed by McDade, *et al.* various artificial media have been developed to support legionellae growth (98). These media facilitate ease in culturing the organism in both research and clinical settings. Early attempts to grow *Legionella pneumophila* on 17 different bacterial agars were unsuccessful. Weaver and Feeley used Mueller-Hinton agar supplemented with 1% hemoglobin and 1% IsoVitalex® (152). Further studies by these individuals led to the development of Feeley-Gorman (F-G) agar, in which soluble ferric pyrophosphate or ferric nitrate is substituted for hemoglobin and

IsoVitaleX® is replaced by L-cysteine-HCl, using a Mueller-Hinton agar base. However although supportive, growth did not yield adequate numbers of organisms required for testing. Consequently, charcoal yeast extract (CYE) agar was generated by modifying F-G agar to enhance recovery of *L. pneumophila* from tissue specimens. Yeast extract replaced peptone constituents, while activated charcoal was substituted for starch. The use of yeast extract, not only enhanced growth but lowered levels of inhibitory NaCl. Addition of starch or charcoal is thought to interfere with the inhibitory effects of free fatty acids such as oleic acid and may serve to scavenge toxic oxygen radicals (101; 125). Later, CYE agar was improved by the addition of ACES buffer (BCYE), (116). This formulation with the addition of α-ketoglutarate facilitates good recovery of legionellae from tissue sources. It can be found readily as a primary isolation medium in clinical laboratories. Corresponding liquid media also were developed in which the agar base was deleted. Also, several chemically defined media were generated by various investigators (151; 119; 133; 82).

In vitro growth temperature appears to be optimal at 35°C although *L. pneumophila* will also grow between 25-42°C, albeit at a slower rate (152). A very narrow pH range of 6.85-6.95, medium dependent, generally is required for consistent *in vitro* growth (44). This is in contrast to a much broader pH range of 5.4 to 8.1 as noted by Fliermans, *et al.* during a survey of aquatic habitats throughout the United States (53). *L. pneumophila* displays a great capacity for acid tolerance. Wong, *et al.* demonstrated that it could survive exposure in hydrochloric acid, pH 2.0, for 30 min (156). The organism grows best aerobically

with 2.5% CO_2. Decreased growth occurs with reduced oxygen tension and no growth under anaerobic conditions. The lack of anaerobic growth suggests that *L. pneumophila* has no fermentative capability and therefore derives its energy from strictly a respiratory metabolism (125). Several *Legionella* species synthesize compounds which fluoresce after exposure to long-wave UV light. Little is known about the significance of these fluorescent constituents but they may be involved in transport of compounds across the cell membrane. Legionellae are very susceptible to drying as compared to other bacteria commonly encountered in tap water (ie. pseudomonads). A four log drop in viability can be seen within the first 30 sec whereas no viable organisms can be recovered after 90 min drying (101).

Legionellae demonstrate little structural evidence for possession of a peptidoglycan layer. Still, they demonstrate key components of peptidoglycan and lipopolysaccharide, namely diaminopimelic acid and 2-keto-3-deoxyoctonic acid. An unusual finding was the high percentage of branch-chain fatty acids (81 to 90%) present in *L. pneumophila*. This abundance of fatty acids is typically associated with a few thermophilic gram-negative organisms. Other *Legionella* species demonstrate similar, unique profiles of branched-chain fatty acids (104; 105). *L. pneumophila* also possess cytoplasmic β-hydroxybutyric acid granules which have been characterized as a common storage polymer in bacteria (152).

Pathogenicity of legionellae in human phagocytic cells

Although a great deal of information about Legionnaires's disease can be found in medical literature, very little is understood about *L. pneumophila*'s

pathogenicity mechanisms. As previously indicated, legionellae are facultative intracellular parasites which can multiply in both monocytes and alveolar macrophages (71; 92). As with most intracellular infections, specific cell mediated immune response plays a paramount role in clearing infectious bacteria. Initial host phagocytic cells, predominantly polymorphonuclear leukocytes (PMN), results in inefficient killing of ingested *L. pneumophila* (153). The ability of *L. pneumophila* to circumvent elimination by PMNs combined with intracellular growth in monocytes later in primary infection can result in overwhelming pneumonia. This process usually occurs prior to development of specific immunity (34).

In examining proposed *Legionella* virulence factors it is important to recognize that conclusions are generally based on work accomplished with more than one *Legionella* species. Additional species such as *L. micdadei* and others also have received attention due to their isolation in roughly 15% of all cases of legionellosis. Individual virulence factors described in one species may not necessarily be present in another species. Additionally, there may be not only relevant biological differences between species but also in host predilection. Whereas, *L. pneumophila* can infect a wide variety of individuals, *L. micdadei* appears to infect only immunosuppresed patients. Consequently, virulence factors as well as their interactions with host defense mechanisms may differ between these two organisms. This condition in conjunction with the use of a single *Legionella* strain when elucidating virulence factors makes it difficult to attribute their relevance to legionellae as a group (34).

A key to understanding pathogenicity of *Legionella* infections at a cellular level may involve the use of *in vitro* studies of professional phagocytes which interact to clear invading organisms. Cell types extensively studied include PMNs, alveolar macrophages and peripheral blood monocytes-macrophages.

Ingestion of *L. pneumophila* by PMNs requires specific antibody to fix the third component of complement (C_3) to bacterial cell surfaces. Alternately, phagocytosis of virulent *L. micdadei* requires only complement as an opsonin (153). As previously stated, PMN killing of ingested virulent *L. pneumophila* was inefficient when compared to a serum resistant bacteria. For example, Horwitz & Silverstein established that neutrophils could effectively diminish the numbers of an encapsulated, serum resistant strain of *E. coli* by 2.5 logs (71). However, only a 0.5 log reduction in numbers could be achieved with virulent *L. pneumophila* under the same conditions. The importance of a primary neutrophil response can be demonstrated by removing this component and subsequent bacterial challenge. Fitzgeorge, *et al.* studied the effects of neutrophil depletion on *Legionella* infection after administering anti-polymorph serum to guinea pigs (152). This treatment essentially lowers the infective dose of *L. pneumophila* needed to produce pneumonia, increases lung bacterial burdens and raises mortality rates. Pulmonary lesions remain basically unchanged except for an absolute decrease seen in neutrophil numbers. Such a result suggests that *L. pneumophila* is responsible for resulting pulmonary damage seen in legionellosis, not the action of neutrophil enzymes. Experiments conducted by Donowitz, *et al.* have shown that *L. micdadei* affects a variety of neutrophilic functions (33). Once ingested *L. micadadei* can

significantly inhibit bactericidal activity, chemotaxis, phagocytosis and stimulated toxic oxygen metabolites. It is of particular interest to note that viable bacteria are not required for inhibition of these processes. Nevertheless, viable bacteria have been shown to cause a greater degree of inhibition of these functions. This observation suggests that initial uptake of *Legionella* results in intracellular killing but further ingestion of bacteria causes impairment of normal neutrophil activity.

Considerable attention has been given to mononuclear phagocytes and their role in resolving *Legionella* infections. *In vitro* studies conducted by Kishimoto, *et al.* showed that approximately 5% of cynomolgus monkey alveolar macrophages contained intracellular *L. pneumophila* after 3 h of infection (89). By 24 h many macrophages demonstrated distended vacuoles filled with bacteria. This process eventually results in host cell destruction due to the rapidity of bacterial multiplication and subsequent cytoplasmic disruption. Experiments performed by Jacobs, *et al.* compared survival of virulent and avirulent *L. pneumophila* after phagocytosis by pigtail monkey alveolar macrophages. Assays utilized methods that distinguished between viable and killed bacteria after macrophage ingestion. Results indicate that intracellular killing of *Legionella* is primarily mediated by hydroxyl radical (OH^-) formation. Virulent legionellae that survived this process went on to increase by more than two logs following four days of incubation. Alternately, avirulent forms which also survived the initial oxidative burst replicated at a diminished rate during the same time period. Similarly, legionellae can also multiply rapidly in human alveolar macrophages obtained by bronchoalveolar lavage and cultured *in vitro*. Even though specific antibody and

complement enhances phagocytosis, less than 10% of the inoculum was killed. Over a three day period *L. pneumophila* numbers increased from 2.5 to 5 logs with destruction of macrophage layer, coinciding with peak growth (108). Comparable results were seen when using alveolar macrophages from rats and guinea pigs by bronchoalveolar lavage. In this case phagocytosis could be prevented by macrophage pretreatment with cytochalasin D resulting in a lack of extracellular growth (41). Similarly, pretreatment of macrophages with cytocholasin B or incubation at 4°C reduced phagocytosis of *L. micdadei* by guinea pig alveolar macrophages. Together, these studies suggest that uptake of legionellae by alveolar macrophages requires a functioning macrophage microfilament system and most likely occurs by a normal phagocytic process (92).

As seen with alveolar macrophages, *in vitro* studies conducted by Horwitz & Silverstein demonstrated that *L. pneumophila* could infect and multiply in peripheral blood monocytes (70). After several logs increase in intracellular *L. pneumophila* growth infected monocyte layers were destroyed. Both specific antibody and complement were required for any killing of virulent legionellae cells. However, only a 0.25 log reduction could be achieved even when both opsinins were present. Surviving bacteria went on to increase several logs over four days of incubation (71). Studies examining monocyte oxidative metabolism yielded similar attenuation of function as seen with neutrophils. Monocytes which previously had ingested heat-killed *L. micdadei* demonstrated decreased activity upon subsequent challenge with live *L. micdadei*. However, unlike neutrophils subsequent challenge with *S. aureus* did not result in inhibition of phagocytosis and

only minimally reduced *S. aureus* killing (32).

Albeit, *L. pneumophila* appears to suppress monocyte function in an effective manner there are methods to increase the killing capability of monocytes. Horwitz & Silverstein showed that partially activated mononuclear phagocytes could be generated by soluble stimulated lymphocyte products (71). Additionally, Con A-treated mononuclear cells exhibited decreased phagocytosis of ingested *L. pneumophila* and were able to prevent intracellular multiplication yet unable to kill the bacteria. Monocyte activation with recombinant human interferon can result in inhibition of legionellae multiplication but requires both complement and specific antibody. Again, *L. pneumophila* killing was slight as only a 0.5 log decrease was noted from the initial inoculum (12).

Cytokine induction can result in inhibition of *L. pneumophila* growth in peripheral blood monocytes. Monocytes obtained from a recovered patient of Legionnaires' disease were coincubated with formalin-killed *L. pneumophila*. Freshly explanted monocytes subjected to cytokine product of the formalin-killed bacteria were refractory to legionellae growth (73).

A great deal of attention has been focused on *Legionella*'s relationship with free-living amoeba. This can be attributed primarily to the similarities between legionellae uptake by human phagocytic cells and amoeba. Although it is appropriate to mention this relationship now it will be examined at greater length later in this review.

The ability of *Legionella* to cause human disease is thought to be due to its capability to multiply in phagocytic cells. To date, three lines of evidence support

this premise (25). First, Davis, *et al.* found that aerosol infection in guinea pigs with legionellae resulted in a rapid increase in bacteria recovered by pulmonary lavage at 24 h (30). In the first 16 h, 86% of viable bacteria were associated with recovered cells. After 24 h and until 48 h, a rapid influx of neutrophils was seen in recovered cell pellets. Fractions containing alveolar macrophages demonstrated the greatest number of recoverable bacteria whereas neutrophil fractions contained noticeably less. When examining morphology of internalized bacteria, it was noted that macrophages contained morphologically intact legionella. Alternately, legionellae in neutrophils were described as structurally damaged but still intact. Jepras, *et al.* also used aerosol infection in guinea pigs but to examine the effects of virulent or avirulent legionellae strains on lavage obtained phagocytes (81). While the virulent strain increased rapidly to a peak of 10^{11} bacteria per lung, avirulent legionellae did not replicate and were totally cleared by 21 days after infection. Alveolar macrophages contained approximately 10 times more viable bacteria than neutrophils. However, recovery of avirulent legionellae from either cell type was about equal. The location of virulent legionellae was predominantly intracellular, while avirulent bacteria were noted as extracellular. These results imply that alveolar macrophages are the primary permissive host cells in early *Legionella* infections. It also was concluded that neutrophils demonstrate some degree of *in vivo* legionellae killing, but the process is relatively inefficient and does not eliminate the bacteria (81).

The second line of evidence involves an apparent correlation between susceptibility of an animal species to *Legionella* infection and the permissiveness of

intracellular multiplication in a given species' macrophages. Although correlation was generally good in most cases, a few exceptions were noted (158; 160).

The final line of evidence pertains to *L. pneumophila* mutants which are impaired in intracellular growth and have reduced virulence in animals (76; 81; 118).

Concerning phagocytosis, differences have been found in various *Legionella* strains in regards to both their mode of uptake and ultimate intracellular fate. Horwitz described a unique method of *L. pneumophila* uptake in human monocytes, alveolar macrophages and neutrophils referred to as coiling phagocytosis (75; 77). This process entails the development of long pseudopod which coils around the object bacterium. Eventually the bacterium becomes centralized in the coiled pseudopod and forms an abnormal ribosome-studded phagosome (74). Surprisingly enough, this type of phagocytic mechanism has only been seen to occur with *L. pneumophila* Philadelphia-1 strain. Live, glutaraldehyde-killed and heat-killed Philadelphia-1 strain cells are all internalized by coiling phagocytosis (75). Both virulent and avirulent *L. pneumophila* Philadelphia 1 strain are seen to enter human monocytes by phagocytosis. Once internalized within the phagosome their interaction with the host cell begins to differ. Virulent *L. pneumophila* interacts with various cell components such as smooth versicles, mitochondria and ribosomes to inhibit lysosomal fusion. Alternately, avirulent *L. pneumophila* does not exhibit similar organelle interactions and does allow lysosomal fusion (78).

The ability of *L. pneumophila* to escape phagosome-lysosome fusion may lie

in its mode of cellular uptake. By utilizing receptor-mediated endocytosis a parasite-versicle may form which usually would not be destined to undergo lysosomal fusion. Endosomal formation demonstrating singular bacterial cells can be seen in close association with smooth or rough endoplasmic reticulum. Fusion of the bacterium with the endoplasmic reticulum would provide an ideal bacterial multiplication site while precluding lysosome fusion (49).

Several investigators have shown that other legionellae belonging to serogroup 1 and *L. micdadei* are engulfed by phagocytic cells using conventional methods and that phagolysosomal fusion does occur. The typical phagocytosis method involves pseudopod extension around the object bacterium with fusion of tips after pseudopod contact at the distal side (153; 113; 124; 40; 25; 122). As a result of coiling phagocytosis, abnormal phagosome formation and inhibition of phagolysosomal fusion are probably not required legionellae virulence factors for intracellular survival. Nevertheless, these characteristics may account for differences noted in virulence of the Philadelphia-1 strain and other *L. pneumophila* strains or other *Legionella* species. Such evidence points towards possible multifactorial interaction between *Legionella* and phagocytes in a strain or species specific manner. Inhibition of phagosome-lysosome fusion, phagosomal acidification and survival of subsequent oxidative killing mechanisms in all probability contributes to each strain's or species virulence potential (34). King, *et al.* examined the affects of cytochalsin D and methylamine on phagocytosis of legionellae by an amoeba and human monocyte-like cell line (88). Treatment of *Hartmannella veriformis* with cytocholasin D, an inhibitor of microfilament-

dependent phagocytosis, did not inhibit intracellular legionellae growth. Alternately, methylamine, an inhibitor of pinocytosis, inhibited amoeba-supported legionellae growth in a dose dependent fashion. Both cytocholasin D and methylamine inhibited legionellae growth in U937 cells. Further examination into similarities and differences in strain/species specific virulence determinants will provide valuable insight into *Legionella* infections of human phagocytes.

Potential *Legionella* virulence factors which result in alterations of normal phagocyte function are the object of current studies. Legionellae appear to interfere with early events in phagocyte cell activation. Consequently, undesired affects are seen with a phagocytic cells capability to clear an infection. Initial cell activation is usually accomplished by the well studied process of signal transduction. Blockage of the signal transduction mechanism has been demonstrated with a variety of bacterial toxins, most notably pertussis toxin (7). This toxin mode of action has been well characterized and is seen to inhibit neutrophil function. Since legionellae affect neutrophil function in a similar manner, cytotoxic products were sought which could explain the inhibitory process. As a result, two peptide factors have been reported which inhibit phagocyte activation (34). The first factor was obtained from both culture supernatants and sonicates of *L. pneumophila* and *L. micdadei*. It exhibits not only cytotoxic activity but inhibits oxidative metabolism in neutrophils and is lethal in animals (56; 57; 62; 93). Unfortunately, this moiety has only been partially purified from either source. It was first demonstrated by Freidman, *et al.* that culture filtrates of *L. pnemophila* exhibited cytotoxic activity for Chinese Hamster Ovary (CHO) cells

(56). Characteristics of the cytotoxin include, heat stability, sensitivity to papain and pronase but not trypsin and a molecular weight cut off of 1,000 via dialysis determination. Taken together, these properties suggest a small polypeptide. Treatment of neutrophils with toxin can depress hexose monophosphate shunt (HMPS) activity, block membrane depolarization and inhibit chemiluminescent response (93; 62). The second proposed toxin was isolated from *L. micdadei* by Dowling, *et al.* and partially purified by chromatographic methods (34). Treatment of neutrophils with this toxin significantly inhibited superoxide anion (O_2^-) generation in response to the chemotactic peptide N-formly-methionyl-leucyl-phenylalanine (fMLP). Toxin activity could not be destroyed by heating for 60 min at 100°C. The mechanism of toxin action remains unknown but is most likely to involve early events in the neutrophil activation process. It is likely that the toxin exhibits its effects prior to activation of phospholipase C (34).

The importance of the *Legionella* toxin as a molecular level virulence factor requires further study but holds much promise in explaining neutrophil inhibition. Several lines of evidence support its contribution to diminishing neutrophil activity. First, toxin has been recovered from all examined *Legionella* species. Second, toxin secreted into medium in which legionellae are grown blocks neutrophil activation. Third, since toxin is stable at a pH of 3.5, which is an expected level in phagosomes, it would not necessarily be inactivated by acidification of the phagosome. Fourth, toxin can produce a refractoriness to stimulation subsequent to ingestion of legionellae. Fifth, heat-killed legionellae can inhibit neutrophil function due to a heat stable toxin. Finally, protection of mice to lethal *Legionella*

challenge can be achieved by prior administration of toxin material (62; 34).

The contributions of extracellular *Legionella* enzymes as virulence factors also has been examined. The presence of legionellae proteases and hemolysins could explain pulmonary and extrapulmonary presentations seen in legionellosis (34). Production and extracellular release of these products may offer insight into extrapulmonary manifestations of *Legionella* infections such as diarrhea, liver dysfunction, hematuria, obtundation and azotemia (5).

Acid phosphatases have been detected in various *L. pneumophila* strains and *L. micdadei* (143; 110). Two separate acid phosphatases (ACP) described by Saha, *et al.* were recovered from supernatants of whole cell *L. micdadei* suspensions and purified by chromatography (129). These enzymes were designated ACP_1 and ACP_2. Pretreatment of human neutrophils with ACP_2 inhibited O_2^- production after fMLP stimulation. Alternately, ACP_1 did not demonstrate a similar effect even at concentrations a hundred fold greater than ACP_2. The mechanism of ACP_2 action appears to involve dephosphorylation of secondary intracellular messengers such as inositol phosphates or diacylglycerol. Phosphatidylinositol-4-5-biphosphate (PIP_2) and myo-inositol-1-4-5-triphosphate (IP_3) proved to be excellent *in vitro* substrates for *Legionella* phosphatase. Although originally classified as an acid phosphatase, ACP_2 now is referred to as a phosphatase since PIP_2 is optimally hydrolyzed at pH 7.0 (131). In all likelihood, it is not phosphatase activity alone which causes neutrophil refractoriness to fMLP stimulation. Most likely, it acts in conjunction with *Legionella* toxin to affect multiple activation sites following ingestion of viable bacteria (34).

All tested *Legionella* species with the current exception of *L. micdadei* possess phospholipase C activity. Consequently, extracellular phospholipase C activity is a proposed factor contributing to formation of typical pulmonary lesions seen in *Legionella* pneumonia. These lesions are characterized by considerable cellular debris in recovered necrotic material containing remnants of both mononuclear cells and PMNs. Such a histologic picture suggests that legionellae derived phospholipase C may be involved in the immense cytolysis of primary inflammatory mediator cells seen in legionellosis (2). Baine, *et al.* also showed that cultures of *L. pneumophila* lyse several species' erythrocytes including horses, sheep, rabbits, guinea pigs and humans (2). Furthermore, *Legionella* strains which lysed these erythrocytes also produced opacity on egg yolk agar indicating lecithinase activity. This result further supported the concept that most legionellae produce an appreciable quantity of phospholipase C and secrete it into growth medium (3). The importance of phospholipase C in legionellosis can be seen in three possible areas. First, is the impaired gas exchange due to hydrolysis of phosphatidylcholine, a major pulmonary surfactant. Phospholipase C enzymatically breaks down phosphatidylcholine into phosphocholine and diacylglycerol. Second, degradation of phosphatidylcholine present in eukaryotic membranes may result in cytolysis or damage of both lung tissue and responding inflammatory cells. Third, phagolysosomal fusion may be disrupted by hydrolysis of phospholipid membranes (4). Additionally, inhibition of neutrophil function by *L. pneumophila* phospholipase has been demonstrated in a dose dependent fashion (132).

Early studies focusing on legionellae protease activity have resulted in the discovery of various serum proteolytic enzymes and a variety of aminopeptidases (106; 107). Among the several serum proteins tested, Muller found that α_1-antitrypsin was actively degraded (106). This observation may help to explain the emphysema-like symptoms seen in legionellosis. Additionally, proline-specific endopeptidase activity was detected by Berdal, *et al.* (11). An earlier study by Berdal, *et al.* demonstrated chymotrypsin-like activity in four *Legionella* species including *L. pneumophila* but not *L. micdadei* (10). Whole cell lysates exhibited little chymotrypsin-like activity indicating that legionellae secrete the majority of this protease. Whereas Muller speculated that proteases play a major role in pathogenicity of legionellosis (106). Conlan, *et al.* demonstrated formation of pulmonary lesions when administering purified *L. pneumophila* protease to guinea pigs. Six discrete proteases were isolated, one causing lung damage similar to that seen in legionellosis. The same enzyme demonstrated *in vitro* activity against collagen, casein & gelatin. Intradermal injection of 100-fold concentrate of *L. pneumophila* culture supernatant elicited skin lesions with pale central necrosis reaching maximal size by 2 h. Purification of casein active supernatant by ion exchange chromatography yielded bands of 41, 42 and 19.8 kDa. During the same year Dreyfus and Iglewski characterized what appeared to be the same protease which Colan, *et al.* had used in their pulmonary lesion studies (35; 27). Two antigenically indistinguishable proteins of 38 and 40 kDa molecular weights were recovered by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). However, gel electrophoresis yielded only a single 40 kDa protein which

suggested that the 38 kDa moiety was most likely derived during purification form autoproteolysis of the 40 kDa protein. In a further study, Conlan, *et al.* demonstrated the presence of tissue destructive protease in lung tissue recovered after intranasal inoculation of guinea pigs (28). Using immunocytochemical staining, Williams et al., demonstrated the presence of tissue destructive protease in pulmonary samples of experimentally induced *L. pneumophila* lesions. Investigators have also described a 38-42 kDa zinc metalloprotease which possesses cytotoxic activity (85; 121). Initially, Keen and Hoffman demonstrated that selected *Legionella* exhibiting cytotoxic activity also possessed hemolytic activity whether of virulent or avirulent origins (85). *Legionella* which did not express cytotoxicity such as *L. micdadei* and *L. feeleii* were nonhemolytic. When derived from culture supernatants both hemolytic and proteolytic moieties were found to purify as a single protein moiety. Quinn, *et al.* accomplished genetic sequencing of the 38 kDa protease showing that a 1.2 kilobase (kb) DNA sequence was responsible for enzyme encoding (121). This DNA fragment designated *pro* is responsible for proteolytic, hemolytic and cytotoxic activities of *L. pneumophila* serogroup 1. These same individuals produced a mutant strain (PRT8) deficient in metalloprotease expression when compared to parental wild-type organism. The PRT8 strain, although serologically and biochemically identical to its parental strain lacked both hemolytic and cytotoxic capabilities. Using the Philadelphia-1 strain, a probe was constructed to the metalloprotease sequence and tested against various *L. pneumophila* strains and other *Legionella* species. Hybridization was seen with all tested *Legionella pneumophila* serogroups

but not with *L. dumoffi*, *L. micdadei*, *L. feeleii* or *L. jordanis* strains. Cloned protease reacted with convalescent serum from a *L. pneumophila* infected patient but not with patient sera infected with other *Legionella* species (121). Despite similarities in proteolytic activity, including cytotoxic and hemolytic activity, ion requirements for iron or zinc, ethylenediaminetetra acetic acid (EDTA), temperature and pH requirements, there are still distinctive cytotoxic, genetic and immunological differences in proteolytic activities between *Legionella* species. The same group sequenced the structural gene encoding for the extracellular metalloprotease and found a single large open reading frame (ORF) 1.629 nucleotides in length (13). The proposed polypeptide possessed 543 amino acid residues with a molecular weight of 60.7 kDa. This protein was vastly larger than the observed 38 kDa of previously determined native and recombinant proteins. Homology analysis revealed extensive amino acid identity to *Pseudomonas aeruginosa* elastase. Similarly, *P. aeruginosa* ORF was found to be substantially larger than the mature elastase protein. Extensive homology was seen between *L. pneumophila* protease at the enzymatically active site of *P. aeruginosa* elastase. Additionally, elastase inhibitors and related thermolysins were shown to inhibit *L. pneumophila* protease activity. Therefore, *L. pneumophila* protease and *P. aeruginosa* elastase appear to share similar proteolytic mechanisms, at least at a molecular level (13). Szeto & Shuman examined possible regulation of the zinc metalloprotease using transposon mutagenesis (140). The structural gene for the major secretory protein (Msp) was isolated from a genomic *L. pneumophila* library and a plasmid vector was constructed. Vector mediated mutagenesis of an *E. coli*

host yielded mutants lacking protease activity or mutants with increased proteolytic ability, suggesting that the *msp* gene's expression may be regulated. One mutant (Tn9) failed to demonstrate protease production but like the wild type was still able to infect, multiply and kill human macrophages. Guinea pigs challenged with aerosolized Msp^+ and Msp^- *L. pneumophila* showed similar results at both 50 and 100% lethal doses. Both the Msp^+ parent and Msp^- mutant increased in a comparable fashion, of 4 logs growth by 72 h, compared with organisms recovered from lung tissue of infected guinea pigs. Resulting pulmonary lesions demonstrated similar pathology between both organisms. Furthermore, *in vivo* passage of a Msp^- mutant did not result in reversion to parental wild-type (14). Consequently, the cytotoxic extracellular protease does not appear to be a requirement for infection but may serve to enhance virulence via macrophage destruction (85). Alternately, inactivation of protease inhibitors such as α_1-antitrypsin or impairment of phagocytic may prove relevant to the pathogenesis of legionellosis (29; 132).

Protective immunity can be induced by aerosol exposure of guinea pigs to live avirulent *L. pneumophila* mutant. Both a strong cell-mediated and humoral response was elicited in mutant immunized but not control animals. Subsequent challenge with lethal aerosol exposure resulted in 40-83% survival of mutant immunized guinea pigs compared to 0% in the control group. Mutant immunization provided protection comparable to wild-type immunization resulting in 57% survival after challenge with wild-type organism (14). Further studies by Blander and colleagues examined the ability of *L. pneumophila* membranes to

induce protective immunity in guinea pigs (15). Aerosol challenge with *L. pneumophila* after aerosol or subcutaneous inoculation resulted in an 80% survival rate. Aerosol inoculation with formalin-killed *L. pneumophila* did not result in protective immunity upon subsequent lethal challenge. Since purified membrane did not contain detectable Msp or induce anti-Msp antibody production it is likely that other distinctive immunoprotective molecules than Msp are present in these membrane preparations (14).

Defining the role of protein kinases in *Legionella* infections is an area which has attracted much interest. Protein kinases function to catalyze the phosphorylation of specific serine, threonine or tyrosine residues expressed by protein substrates. Phosphorylation and dephosphorylation of critical proteins may result in regulation of host phagocytic cell function. Saha, *et al.* demonstrated that *L. micdadei* possesses two protein kinases (PK) designated PK I and PK II (130). Column purified sonicate supernatants revealed PKs with maximum activity seen between pH 6.8-7.0. Neutrophil derived cytosol and soluble membrane fractions were incubated with purified *L. micdadei* PK to determine if suitable substrates were possessed by phagocytic cells. Numerous proteins present in either fraction were phosphorylated in the presence of bacterial PK. Further experimentation by the same group indicated that *L. micdadei* PK catalyzes phosphorylation of both tubulin and phosphatidylinositol (PI) at the expense of adenosine triphosphate (ATP). Phosphorylation of both PI and tubulin was seen *in vitro* whereas phosphorylation of only PI was seen in intact neutrophils. Phosphorylation of tubulin monomers inhibits microtubule

assembly. Therefore, it could adversely affect phagocytic activation or function. Ultimately, *L. micdadei* PK may affect various tubulin-mediated phagocytic activities including motility, phagocytosis and phagolysosomal fusion (130).

Bender et al., provided evidence for the hemolytic protein legiolysin *(lly)* exhibiting gene conservation in legionellae with the highest degree of homology seen in *L. pneumophila* (8).

In examining the cell envelope *of L. pnemophila* Knoxville-1 strain, Hindal and Iglewski noted two forms of cytoplasmic membrane (CM-1, CM-2) an intermediate form (IM) and two outer membrane forms (OM-1, OM-2), (64). The CM-1 fraction being purely a cytoplasmic form whereas CM-2 contained small amounts of peptidoglycan. Phosphotidyl choline and phosphotidyethanolamine, which are seldom observed in bacterial cells, were determined to be major phospholipid components of the membrane fraction (51). Interestingly enough, the carbohydrate and 2-keto-3-deoxyoctonic acid (KDO) content of the outer membrane fractions was not appreciably greater than the cytoplasmic fraction (64). However, this was not the case as reported by Gabay and Horwitz who localized KDO to the outer membrane fraction (59).

Outer membranes isolated from various *L. pneumophila* serogroups and other *Legionella* species demonstrate a single exposed, major surface protein of 29 kDa. This protein is tightly associated with LPS and is equally distributed in IM, OM-1 and OM-2 fractions. Alternately, the cytoplasmic membrane fraction contained a single major protein of 65 kDa. Isolated LPS from *L. pneumophila* was described as atypical v. other gram-negative bacilli when visualized by silver

staining of SDS-PAGE gels (59). It was further shown that *Legionella*-derived LPS does not exhibit classical gram-negative LPS endotoxicity but only induces a weak pyrogenic response in rabbits and low toxicity in mice (156).

Early outer membrane work by Ehert and Ruckdeschel demonstrated the 29 kDa MOMP in all ten *L. pneumophila* serogroups examined (38). It is generally accepted that all *Legionella* species except for *L. bozemanii* serogroup 1 possess the disulfide-cross-linked 29 kDa MOMP (19). Work performed by Gabay, *et al.* resulted in classifying the MOMP as a cation selective porin (58). Porins are a type of bacterial protein which are capable of bacterial and/or host cell membrane insertion creating channels allowing ion passage. It has been proposed that the 95 kDa complex reported by Butler, *et al.* consists of four disulfide-cross-linked subunits of MOMP resulting in a peptidoglycan bound porin (19). Butler and Hoffman determined that *L. pneumophila* MOMP is anchored via a 31 kDa protein to peptidoglycan in a covalent manner (20). This anchor protein appears to be covalently bound to both the 28 kDa and 31 kDa subunit proteins by interchain disulfide bonds. The total molecular weight of the MOMP complex appears to be 100 kDa when using SDS-PAGE under nondenaturing conditions, which upon reduction results in 31 and 28 kDa monomers in a ratio of 1:2 or 1:3. Further characterization by this group confirmed previous suspicions that the 31 kDa fragment is actually a 28 kDa monomer bound to a fragment of peptidoglycan (20). Furthermore, the 28 kDa monomers are cross-linked to each other via interchain disulfide bonds. Continuing their investigation of MOMP, Hoffman, *et al.* cloned the structural gene, encoding for both the 28 and 31 kDa

fragments (67). Nucleotide sequencing analysis determined an ORF of 891 base pairs (bp) which encodes a polypeptide of 297 amino acids in length. The mature protein contains 276 amino acids with a leader sequence comprised of the remaining 21 amino acids. The DNA sequence appears to be highly conserved among *L. pneumophila* serogroup 1 and may be environmentally regulated. The contribution of MOMP to *L. pneumophila* virulence remains unclear since MOMP is expressed by both virulent and avirulent forms (19). However, transformation of *E. coli* with a 750 bp DNA fragment from virulent *L. pneumophila* results in expression of *Legionella* MOMP and increased virulence in chicken embryos compared to parental *E. coli*. A modification or decreased expression of *L. pneumophila* MOMP may result in decreased phagocytosis of avirulent strains when compared to their virulent counterparts (34).

Pearlman, *et al.* investigated a distinctive 24 kDa protein, which in addition to the MOMP elicits strong antigenic activity in rabbits inoculated with killed *L. pneumophila* (117). Cianciotto, *et al.* mutated the gene encoding the 24 kDa surface protein resulting in a loss of its expression (24). When compared to its isogeneic parent strain, mutant infectivity of U937 cells, a transformed human macrophage-like line, was significantly impaired. An 80-fold greater inoculum size of mutant was required to produce a comparable infection to the isogeneic parent strain. Additionally, reintroduction of the 24 kDa gene imparted full infectivity potential, indicating a possible role for Mip (macrophage infectivity potentiator) in increasing virulence capabilities of infective legionellae. A Mip$^-$ mutant inoculated intratracheally into guinea pigs killed fewer animals and resulted in an extended

time period prior to development of illness than the parental wild-type. Once again, reintroduction of the *mip* gene resulted in an infection similar to the isogeneic parent strain. Differences in proteolytic, phosphatase activity, complement fixation, serum resistance or LPS structure could not be detected between the Mip$^-$ mutant or wild-type parent (24). Engleberg, *et al.* sequenced the *mip* gene and determined that a long ORF of 699 bp was present with a resulting molecular weight of 29.4 kDa (41). *L. pneumophila* appears to be the only *Legionella* species expressing the Mip protein although other *Legionella* species may exhibit Mip-related proteins (26).

All examined *Legionella* species and serogroups have been found to express a 58 to 60 kDa protein produced upon heat shock. This protein (htb) contains both genus-specific epitopes as well as other epitopes cross-reactive with numerous species of gram-negative bacteria. Nucleotide sequencing accomplished via *E. coli* shows that htbB shares considerable homology with other bacterial heat shock proteins. It is highly immunogenic and is seen to be the predominant protein reactive with convalescent phase patient serum obtained from patients with confirmed legionellosis (68; 134).

Avirulent *Legionella* mutants are considered to be valuable tools in analyzing virulence determinants. The analysis of avirulent bacteria which are unable to multiply in permissive host cells allows for determination of those factors possessed by the parent strain which are lacking in the mutant. Production of avirulent mutants follows two basic lines. First, passage on suboptimal artificial media can result in spontaneous conversion of an organism normally virulent in

guinea pigs to an avirulent form (22; 114; 112; 16; 99). Catrenich and Johnson used supplemented Mueller-Hinton (SMH) agar in order to evaluate growth differences between virulent and avirulent populations of *L. pneumophila* (22). This agar primarily interfered with the growth of virulent cells but not with avirulent organisms. Analysis of SMH constituents indicated that the casein acid hydrolysate contained the inhibitory agent, identified as sodium chloride. Ormsbee, *et al.* reported a loss of virulence in *L. pneumophila* after prolonged cultivation on SMH indicated by decreased plaque formation in chicken embryo yolk sacs and lethality in guinea pigs (114). Organisms propagated on modified Mueller-Hinton agar demonstrated vastly increased 50% infective doses (ID_{50}) of 10^5 organisms vs. 10^1 for those recovered from egg yolk sacs. Loss of virulence is also associated with a conversion of *L. pneumophila* cells to long filamentous rods upon subculture on agar medium (16). By using predialysis of yeast extract incorporated into a modified charcoal yeast extract Nowicki, *et al.* were able to maintain short non filamentous forms of *L. pneumophila* which were seen to be more virulent than those grown on conventional media (112). Again, there was a positive correlation between the mean length of the bacilli and the 50% lethal dose (LD_{50}) in guinea pigs inoculated either intraperitoneally or by inhalation of aerosols.

The second mutant type is derived via genetic engineering producing an attenuated organism (25). This process may possess a distinct advantage since a fully virulent wild-type parent strain is readily available for comparison purposes . A further advantage in using this type of mutant is that studies utilizing legionellae

derived from propagation on substandard medium may not have a well defined passage history (34).

Kishimoto, *et al.* showed that an avirulent mutant derived from Philadelphia 1 could not survive in guinea pig derived peritoneal macrophages, whereas virulent strains of *L. pneumophila* serogroup 1 could survive as well as to multiply intracellularly (89). As previously described, Jacobs, *et al.* demonstrated that both virulent and agar-derived avirulent organisms were susceptible to early phagocyte killing (80). Later in infection, virulent numbers were seen to increase in number by at least 2 logs during 96 h post-infection whereas avirulent bacteria multiplied in a less rapid fashion over the same period. Horwitz produced 44 mutants by passage of wild-type *L. pneumophila* on SMH agar (76). All 44 mutant clones were phagocytosed but were avirulent for human monocytes. They did not multiply whereas wild-type bacteria increased in numbers by 2.5-4.5 logs. Even though, mutant bacteria were not killed by the monocytes, avirulence was attributed to an inability to undergo intracellular multiplication. The mutant bacteria also failed to demonstrate the distinctive ribosome-lined phagosome characteristic of the isogeneic parent strain (76). Using a semidefined medium, Mintz, *et al.* isolated and characterized tryptophan and thymidine auxotrophs of the Philadelphia 1 strain (102). When inoculated into peripheral blood monocytes the thymidine auxotroph was incapable of extracellular survival. Alternately, the tryptophan auxotroph grew well under the same conditions. Pearlman, *et al.* found that U937 cells could sustain intracellular growth of *L. pneumophila* with resulting cytopathic effect (CPE), (118). Virulent strain LD_{50} in guinea pigs, was

determined to be 10^6 organisms and produced noticeable CPE. In contrast, inoculating 10^7 avirulent organisms did not result in guinea pig death or CPE. Four pairs of virulent/avirulent strains of *L. pneumophila* were utilized by Summergill, *et al.* in characterizing differences in adherence/uptake and activation of human peripheral blood monocytes (139). Oxidative response was assessed by both flow cytometry and superoxide generation inhibition. Avirulent strains within the pairs of isolates stimulated oxidative response in monocytes to a greater degree than their virulent counterparts. No significant difference was noted in the uptake of any virulent/avirulent pair. In a similar study virulent and avirulent *L. pneumophila* were examined for their effect on PMN oxidative metabolism. A reduced oxidative burst was once again seen with the virulent organism (139). Dreyfus examined infectivity of *L. pneumophila* using HeLa cells (36). Here virulent and avirulent isolates were inoculated into HeLa cell monolayers. The virulent isolate efficiently entered HeLa cells yet the avirulent derivative did so at nearly a 1,000-fold decrease. Decreased avirulent invasion was not attributed to a lack of bacterial association with the cell monolayer.

Although the majority of avirulent *L. pneumophila* have been shown to be incapable of intracellular multiplication both cellular and molecular mechanisms responsible for this inability remain unknown. Whether decreased phagocytosis, decreased complement fixation, inability to survive in a intracellular environment or possibly a combination of each situation results in this defect requires further study (34). Furthermore, interpretation of results using passage derived avirulent mutants may be complicated by the presence of more than one mutation (24).

Amoebae

Small free-living amoeba such as *Acanthamoeba* and *Naegleria* are distributed worldwide and can be isolated from a variety of habitats (31). *Naegleria* spp. have been isolated from both soil and water environments including fresh water ponds and lakes, water supplies, power plant thermal effulents sewage, soil, swimming pools, whirlpools, aquaria and from the nasal passages of healthy children. Similarly, *Acanthamoeba* can be isolated from numerous environmental sources including fresh water, sea water, industrial cooling water, swimming pools, dental units, dialysis units and contact lens cases. Additionally, it can be recovered from vegetables, mushrooms, fish, reptiles, birds and a variety of human body fluids and most notably from corneas (31; 147)). Of the six known species of *Naegleria* only *N. folweri* is thought to cause human disease. It produces an acute form of hemorrhagic necrotizing meningoencephalitis referred to as primary amoebic meningoencephalitis (PAM), which usually results in death 7 to 10 days after infection (97). In PAM, *Naegleria* gain access to nasal passage after inhalation of dust or aspiration of contaminated water containing trophozoites. Some individuals demonstrate respiratory symptoms which may be the result of hypersensitivity reaction or subclinical amoeba infection (95). Alternately, *Acanthamoeba* are known to cause a subacute or chronic infection, primarily in the disabled or immunosuppressed, known as granulomatous amebic encephalitis (GAE) or a vision-threatening keratitis (147). The route of entry for *Acanthamoeba* into the central nervous system (CNS) appears to be of hematogenous, originating at a primary lesion in the skin or lung (95). The

ability*Acanthamoeba* to cause keratitis appears to be related to its preference for lower temperatures, as those seen in the eye compared to the rest of the human two-fold. First, due to low environmental temperatures or lack of infectivitybody (31).

The life cycles of *Naegleria* and *Acanthamoeba* are similar due to the presence of both trophozoite and cyst stages. However, *Naegleria* can also exhibit a third flagellate stage. Both amoeba encyst when unfavorable conditions are present (95).

Since members of the genus *Acanthamoeba* are being isolated from clinical specimens at an increased frequency, Visvesvara suggests that they be identified to at least genus level. Determination of amoeba species based soley on morphology would prove difficult (148).

The ability of *Acanthamoeba* to encyst may contribute to the survival of internalized legionellae during chlorine exposure or exposure to other biocides. Cysts from infected trophozoites were seen to provide protection to at least 50 mg/l free chlorine (86). In a similar study, *Tetrahymena pryiformis* provided for a >50-fold resistance of several intracellular bacterial pathogens, including *L. gormanii*, to free chlorine (87). *Acanthamoeba* have also been used to recover otherwise undetectable legionellae from both clinical specimens and environmental sources (127; 135; 159).

Pathogenicity of legionellae in amoebae

The relationship between *Legionella* and free-living amoeba appears to be

legionellae can serve as a nutrient source for the amoeba (1; 126; 45; 136). Second, the amoeba may become a victim of what is usually a rapid and lethal episode of intracellular legionellae multiplication (126). Because of the numerous parallels in the interaction of legionellae and amoeba, several investigators pursued amoeba models in hopes of uncovering basic mechanisms responsible for legionellosis. Consequently, numerous coculture models have been established to examine this host-parasite relationship. As of the current year five genera of amoeba and one ciliated protozoan have been shown to support intracellular multiplication of *L. pneumophila.* Of all the *Legionella* species tested only *L. pneumophila* demonstrated the ability to replicate in all six test hosts (50).

In 1980, Rowbotham published a preliminary report indicating that *L. pneumophila* was a pathogen for the free-living amoebae *Acanthamoeba* and *Naegleria* (126). In this study *A. castelanii, A. polyphaga, A.* spp (unknown), *N. gruberi & N. jadini* were inoculated with *L. pneumophila* representing six serogroups. Findings indicated that the resulting intracellular legionellae growth was both serogroup and amoeba species dependent. Using the Knoxville or Los Angeles serotypes, Tyndall and Domingue examined the association of legionellae with *A. royreba* and *N. lovaiensis* (145). At 24 h 99.9% of inoculated legionellae were killed, but after several weeks in coculture a chronic infection of the amoeba could be established. Inoculation of guinea pigs with amoeba-associated legionellae from chronically infected cultures showed no increase in virulence when compared to bacteria grown in yeast extract broth (145).

Anad, *et al.* examined the effects of incubation temperature on the

interaction of *L. pneumophila* with *A. palestinensis* (1). Cocultivation of organisms at 35°C resulted in intracellular legionellae multiplication with an increase in numbers by 3 logs by day 5. In contrast, legionellae were initially digested at 20°C by day 2 but still could be recovered in low numbers by day 6. In order to stimulate a more natural aquatic environment Fields, *et al.* chose to examine *L. pneumophila* interaction with the ciliate *T. pyriformis* in tap water culture (45). Previous investigators had utilized either solid agar with killed nutrient bacterial lawns or liquid amoeba supportive growth medium. Within a 5 day period the *L. pneumophila* population increased by approximately 2 logs in a temperature dependent fashion. Suspensions of lysed protozoa, filtered culture supernatant or sterile tap water were not seen to support legionellae growth. Holden, *et al.* looked for the presence of amoeba derived products which could support extracellular growth of *L. pneumophila* (69). Previously, Tison, *et al.* had demonstrated extracellular growth of legionellae in association with cyanobacteria (blue-green algae), (144). Experiments conducted by Holden and colleagues employed the use of parabiotic chambers in which a 0.4μm filter was used to separate *A. castellanii* from the Philadelphia 1 strain (69). Bacterial growth was not seen indicating that a growth supportive, diffusible, extracellular factor is not produced by the amoeba. When cultivated together, *L. pneumophila* numbers increased by three to four orders of magnitude after 48 to 72 of incubation. This would suggest that direct amoebae-legionellae contact is a prerequisite for bacterial growth (69). However, Wadowsky, *et al.* recently reported extracellular multiplication of avirulent *L. pnemophila* in coculture with the amoeba *H.*

veriformis. Furthermore, virulent *L. pneumpohila* multiplication was noted in *H. veriformis* growth-supportive medium in the absence of amoeba (150).

Using electron microscopy Newsome, *et al.* documented events associated with *N. fowleri* infection by internalized *L. pneumophila* (109). Photomicrographic evidence suggests that intracellular legionellae replication takes place in a similar manner to that seen in human monocyte infections. As noted in previous cocultures, *Legionella* numbers also increased by approximately 2 logs after 6 days incubation (109).

Henke and Seidel investigated the association of amoeba and *L. pneumophila* recovered from groundwater, drinking water and whirlpools (63). Amoeba could be isolated in these water sources up to a temperature of 57°C. Membrane concentration technique yielded *L. pneumophila* with amoeba in 55% of samples tested. Environmental isolates of *Acanthamoeba* sp. supported growth of *L. pneumophila* serogroup 4 in both artificial medium and autoclaved tap water. At the site of a legionellosis outbreak Barbaree, *et al.* isolated amoeba and two ciliates, *Tetrahymena sp.* and *Cyclidium sp.* from cooling tower water containing legionellae (6). Cocultivation studies revealed that isolated amoeba and the *Tetrahymena* sp. support intracellular multiplication of *L pneumophila*. In a similar investigation of Pontiac Fever Fields, *et al.* probed the ability of *L. anisa* to replicate in *Hartmannella veriformis* (47). Both *L. anisa* and *H. veriformis* were isolated from an indoor fountain suspected of being a legionellae reservoir. Although the bacteria multiplied in the presence of *H. veriformis*, they failed to do so when inoculated into cultures containing *T. pyriformis* or human mononuclear

cells. Additionally, it failed to infect guinea pigs. The failure of *L. anisa* to multiply in human phagocytes may explain the difference in exposed individuals contracting Pontiac Fever instead of pneumonic legionellosis (47). Taking a somewhat different view, Rowbotham suggests that Pontiac Fever may be the result of a hypersensitivity pneumonitis to *Acanthamoeba* or their fecal vesicles containing legionellae (128).

Vandenesch, *et al.* compared virulence of coculture-derived legionellae to legionellae grown on CDYE agar (146). Aerosol infection studies in guinea pigs yielded similar LD_{50} using either organism source. The ability of *H. veriformis* to support growth of various legionellae was accomplished by Wadowsky, *et al.* (149). Multiplication, in replicate cultures, was seen with *L. bozemanii* (WIGA strain), *L. dumoffii* (NY-23 & TX-KL strains), *L. micdadei* (two environmental strains) and *L pneumophila* (six environmental strains and one clinical isolate). Conversely, *L. anisa* (one strain), *L. bozemanii* (MI-15 strain), *L. micdadei* (clinical isolate), *L. longbeachae* (one strain) and *L. pneumophila* (Philadelphia strain) could not multiply in any culture setup. Additionally, *L. gormanii* and one *L. pneumophila* environmental isolate replicated in only one of three cocultivations (149).

Merkurov, *et al.* showed that an avirulent *L. pneumophila* strain could not replicate in *T. pyriformis*, whereas its virulent counterpart increased by 1,000-fold. A loss of hemolytic activity in the avirulent mutant correlated with a loss of infective capacity (*100*).

After a suitable incubation period with *A.* castellanii, Moffat and Thompkins used gentamicin to kill extracellular legionellae and study the kinetics

of intracellular legionellae growth (103). Cocultivation was carried out under amoeba growth-limiting conditions. Here various *Legionella* strains increased in number at 37°C while avirulent organisms did not. Furthermore, none of the tested legionellae, virulent or avirulent multiplied at room temperature (103).

Fields, *et al.* examined comparative virulence of seventeen *Legionella* strains (11 species) by intraperitoneal injection into guinea pigs and inoculation into *T. pyriformis* cultures (46). Data analysis suggests four distinct categories of legionellae virulence. First, organisms that infect and kill guinea pigs and multiply in *T. pyriformis*. Second, organisms that infect but do not kill guinea pigs and multiply in *T. pyriformis*. Third, organisms that do not infect guinea pigs but can kill at high doses and multiply in *T. pyriformis*. Fourth, organisms that neither infect nor kill guinea pigs and can not multiply in *T. pyriformis* (46). Additionally, in a later study, Fields, *et al.* indicated that certain *Legionella* species, particularly *L. anisa* demonstrated varying results when virulence is compared in guinea pigs, protozoan and human cell cultures (47). This study included both a *H. veriformis* and *L. anisa* recovered form an indoor fountain associated with an outbreak of Pontiac fever. In this case, *L. anisa* multiplied in two *H. veriformis* strains but could not infect *T. pyriformis* cultures. Furthermore, the same strain failed to infect both guinea pigs and human macrophage cells. The authors suggest that the inability of this *L. anisa* strain to infect human monocytes may result in a febrile illness in lieu of Legionnaires' disease (47).

Recently, Wadowsky, *et al.* described the multiplication of 4 agar derived avirulent *L. pneumophila* mutants in coculture with H. veriformis (150).

Alternately, the Philadelphia-1 strain was similarly tested and could multiply whereas the avirulent derivative could not. Here again, avirulent multiplication progressed at a much slower rate than their virulent counterparts. The virulent parent strains outpaced their corresponding avirulent mutants by approx. 3 to 4 logs growth at 4 days coincubation. However, by day 7 the avirulent strains had substantially increased with at least one strain demonstrating 3.5 logs growth The corresponding virulent organism was recovered at 4.4 logs growth. The remaining avirulent organisms narrowed the gap seen at day five by approx. 2 logs growth. None of the tested strains multiplied in amoeba-free cultures (150).

Ott, *et al.* investigated the effect of temperature variation on *L. pneumophila* replication in *A. castellanii*, the same species used in the current study (45). This study utilized the Philadelphia-1 strain and two environmental strains recovered from a hot water tank, serogroups 1 and 6 (U1S1 and U21S6 respectively). Cocultures received a 1 h pre-gentamicin treatment prior to coincubation for 24 h. Here, both the wild-type Philadelphia-1 and U1S1 strains grew in a 37°C coculture while the avirulent mutant and U21S6 strains did not. As with the present study, PYG medium was used in all coculture experiments. However, when the incubation temperature was lowered to 30°C the avirulent Philadelphia-1 exhibited appreciable growth to within 1.5 logs of the wild-type parent strain and within 1 log of U1S1. Again, U21S6 did not exhibit growth potential at the lower temperature. Growth of the wild-type Philadelphia-1 strain at both temperatures correlated well with virulence in guinea pigs, by IP injection, and multiplication in U-937 cells. A similar result was seen with U1S1.

Conversely, the U21S6 isolate did not exhibit multiplication in U-937 cells, infect guinea pigs or grow in amoeba cocultures at either temperature. The avirulent Philadelphia-1 strain was similar in its activity to that of U21S6 except that multiplication was noted in 30°C amoeba cocultures (45). Additional a study by States, *et al.* examined temperature dependent survival of *L. pneumophila* when coincubated with *H. veriformis* (137). Coculture temperatures varied form 10 to 44°C with 5°C increments until 40°C and then 1°C thereafter. A very low MOI of 1:50 (*Legionella*:amoeba) was used. Here, peak bacterial growth were seen at 37°C of 4.92 logs with growth at 30°C seen to be not far behind at 4.45 logs (137).

Overall, the differences in the ability of *Legionella* strains to multiply in an amoeba host may be affected by differences in legionellae passage history, type of coculture medium, species of protozoan or temperature of incubation. Further studies using similar cocultivation conditions are needed to establish a direct correlation and/or differences between amoeba and human phagocytes in infectious legionellosis. As a result the current study was initiated to:

1. Establish a growth-supportive amoeba coculture system to study the differences in the interaction of virulent and avirulent *L. pneumophila* with the amoeba *A. castellanii*.

2. Determine if either virulent or avirulent *L. pneumophila* forms undergoes intracellular replication in *A. castellanii*.

3. Determine the effects of long-term *L. pneumophila* passage into axenic amoeba cultures on amoeba virulence potential.

4. Examine phenotypic differences in avirulent and virulent *L.*

pneumophila which may contribute to organism virulence in amoeba and guinea pigs.

5. Further characterization of a unique low molecular weight band seen in avirulent *L. pneumophila* outer membrane profiles.

MATERIALS AND METHODS

Organisms

Virulent and avirulent forms of *Legionella pneumophila* serogroup 1 were acquired from Dr. Sandra R. Sommer (Department of Medical Technology, Virginia Commonwealth University, Richmond, VA). These organisms were originally obtained by Dr. Sommer from Dr. Washington C. Winn Jr.. (Department of Pathology, University of Vermont, College of Medicine, Burlington, VT). The virulent strain (VLp), designated Burlington 1 was initially isolated from an individual with Legionnaires' disease. The avirulent form (ALp) was derived from multiple passage of parental wild-type on modified Mueller-Hinton agar. Additionally, some studies utilized a patient isolate recovered from open lung biopsy material. This CDC confirmed *Legionella pnemophila* serogroup 1 isolate was designated as EJ1.

A culture of *Acanthamoeba castellanii* was kindly provided by Dr. Francine Marciano-Cabral (Department of Microbiology & Immunology, Virginia Commonwealth University, Richmond, VA). Stock Ac cultures were maintained by weekly passage of amoeba in peptone yeast glucose medium (PYG), also known as ATCC 712, (see appendix A) with contamination checks accomplished at time of passage by plating an aliquot of organism suspension on both 5% sheep

blood agar and buffered charcoal yeast extract supplemented with α-ketoglutarate (α-BCYE) plates (see appendix B) and examining both plates for growth at 72 h incubation.

Virulence potential was established by inoculating guinea pigs with either VLp or ALp retrieved from 96 h α-BCYE agar plates incubated at 35°C with 5% CO_2. Suspicious *Legionella pneumophila* colonies were confirmed with direct fluorescent antibody staining using a fluorescein-labelled, monoclonal anti-*Legionella pneumophila* antibody (Genetic Systems Corp., Seattle, WA.). Necropsied tissue from infected guinea pigs was frozen at -70°C for future use. Confirmed *Legionella* isolates from these tissues in addition to those used for guinea pig inoculation were suspended in freezer broth (50% glycerol, 50% trypticase soy broth) and frozen at -70°C. Aliquots of these suspensions were thawed, distributed to α-BCYE agar plates and incubated at 35°C with 5% CO_2 for 72 h prior to testing. This protocol was subsequently used to recover *Legionella pneumophila* for all testing procedures. Contamination checks of previously frozen *Legionella pneumophila* aliquot were routinely accomplished by inoculating 5% sheep blood agar plates and examining for growth after 72 h incubation at 35°C with 5% CO_2.

Virulence Confirmation

Organisms were grown on α-BCYE agar at 35°C for 72. with 5% CO_2 prior to evaluation of virulence potential. Each form was suspended in 0.89% saline to approximate a No. 3 McFarland standard. Intraperitoneal injection of a 1

ml suspension was performed on male Hartley guinea pigs weighing 250-300 grams using a 26 ga, 3/8" needle. Determination of original inoculum size was accomplished by performing serial 100-fold dilutions in 0.89% saline and plating 0.1 ml of each dilution on α-BCYE agar plates in duplicate. Colony counts were performed after 4 days incubation at 35°C with 5% CO_2. Guinea pigs were examined every other hour for eight hours on the day of inoculation and twice daily thereafter. Moribund animals were sacrificed by a carbon dioxide inhalation overdose and subjected to necropsy. Surviving animals were sacrificed by the same method 14 days after inoculation. Spleens, livers and lungs were aseptically removed from each animal and emulsified tissue samples were inoculated onto both α-BCYE and α-BCYE with PAC (see appendix C) agar and frozen at -20°C as previously described.

Cocultures

Establishment of Coculture Model

Evaluation of *Legionella pneumophila* virulence potential in *Acanthamoeba castellanii* was accomplished as follows. Three days prior to coculture experiments 8-10, 25 cm^2 Falcon 3013 tissue culture flasks (TCF), (Becton Dickinson, Oxnard, CA.) containing 9 ml PYG were seeded with 1 ml of amoeba stock culture. Frozen aliquot of VLp and ALp were thawed and inoculated onto a α-BCYE plate and a 5% sheep blood agar plate as previously described. On the day of coculture setup, coculture flask caps were tightened securely, sealed with

Parafilm™ (American National Can, Greenwich, CT) and placed in an ice bath for 15 min. Culture flasks were then removed and blotted for residual water. Amoeba were gently dislodged from the bottom and sides of each TCF using a sterile Falcon 3085 cell scrapper (Becton Dickinson). Suspensions were aseptically transferred to Falcon 2070, 50 ml polypropylene tubes (Becton Dickinson) and subjected to centrifugation for 10 min at 200xg using a Sorvall RC-3 floor model centrifuge (Dupont Instruments, Newton, CN.) at room temperature (RT). Supernatants were discarded and amoeba pellets resuspended in 1 ml fresh PYG. Amoeba viability and numbers were determined by using trypan blue (0.4% solution in 0.85% saline, ICN Biomedicals, CA) exclusion staining. Viable amoeba were counted with an improved Neubaeur hemocytometer (AO/Spencer, Buffalo, NY). *A. castellanii* numbers were subsequently adjusted to approximately $2.0x10^6$ Ac/ml in fresh PYG.

Isolated colonies from either VLp or ALp plates were transferred to a sterile screw top tube containing 5 ml of fresh PYG broth. A Sequoia-Turner model 340 spectrophotometer (Mountainview, CA) was set to 625 nm and adjusted to an optical density (O.D.) of 0 with 5 ml fresh PYG broth. *L. pneumophila* were then adjusted to an O.D. reading of 0.2 for VLp and 0.1 for ALp. Preliminary O.D. studies indicated that these readings would result in approximately $1x10^8$ CFU/ml for each *Legionella* form. A 1:100 dilution was performed on each *Legionella pneumophila* suspension to result in approximately $1x10^6$ CFU/ml ALP and VLp. Plate counts were accomplished by performing serial 10-fold dilutions in 0.89% saline and transferring 0.1 ml each to triplicate

BCYE plates. *Legionella pneumophila* numbers were determined after 4 days incubation at 35°C with 5% CO_2.

Cocultures and corresponding control flasks were set up in the following manner. Twenty five cm^2 TCF were filled with 8 ml fresh PYG broth. Added to one ml of approximately $1.0x10^6$ CFU/ml ALp or VLp suspended in PYG and 1 ml of approximately $2.0x10^6$ Ac/ml suspension was added to the medium. Control flasks consisted of 9 ml fresh PYG inoculated with either approximately 1 ml of ALp or VLp adjusted to $1.0x10^6$ CFU/ml or 1 ml of approximately $2.0x10^6$ Ac/ml. All flasks were incubated at 30°C in ambient air without rotation.

Each experimental flask was sampled at 24 h intervals for a period of 5 days. Sampling was accomplished by placing coculture and control TCF in an ice bath for 15 min followed by gentle dislodgement of organisms using sterile cell scrapers on each flask. The resulting suspension was transferred to sterile screw top glass tubes and thoroughly mixed using a vortex (Vortex-Genie™, Scientific Industries, Bohemia, NY) setting #3. One ml aliquot were transferred to clean tubes and amoeba numbers determined. The remaining suspensions were centrifuged for 10 min at 200 x g at room temperature. Five ml of resulting supernatant were transferred to sterile screw top tubes and plate counts were accomplished on BCYE agar as previously described.

Effect of Multiplicity of Infection (MOI) on Cocultures

Legionella pneumophila were recovered from frozen aliquot as previously stated. Additionally, amoeba were processed as described in the previous section

to yield approximately $2.0x10^6$ Ac/ml in PYG. Isolated VLp or ALp were suspended in fresh PYG to approximate an O.D. reading of 0.2 and 0.1, respectively, to yield approximately $1x10^8$ Lp/ml. A 1:100 dilution was accomplished for each form to yield approximately $1x10^6$ Lp/ml. Plate counts were performed on both the initial inoculum and their corresponding 1:100 dilutions. Experimental flasks contained 8 ml PYG, 1 ml of approximately $2.0x10^6$ Ac/ml and 1 ml of either $1x10^8$ or $1x10^6$ Lp/ml. The correspondingcontrol flasks consisted of 9 ml PYG plus 1 ml of either the undiluted or 1:100 dilution of VLp or ALp. Cocultures were incubated at 30°C in ambient air, without rotation. Sampling of each coculture condition occurred on days 1,3 and 5 according to previously specified protocol. Amoeba numbers were also determined on coculture harvesting days.

Effect of Filtration on Virulence Potential of ALp

Using previously stated protocol, both *Legionella pneumophila* and *Acanthamoeba castellanii* were recovered and adjusted to approximately $1.0x10^6$ Lp/ml and $2.0x10^6$ Ac/ml respectively. In addition, the entire first zone of the same ALp plate was suspended in 10 ml PYG. This suspension was prefiltered, under vacuum, using a sterilized Nalgene filter unit (Nalge Co, Rochester, NY) fitted with a MSI 42 mm, 20μm nylon membrane filter (Micron Separations Inc., Westboro, MA). The resulting filtrate was transferred to a second Nalgene filter unit (Rochester, NY) fitted with a MSI 42 mm, 5μm filter (Westboro, MA), filtered and subsequently transferred to a sterile screw-top test tube. The OD of

the filtered ALp was adjusted to 0.1 to 0.2 at 625 nm and plate counts performed as with the unfiltered ALp and VLp suspensions. A 1 ml suspension of VLp, ALp and filtered ALp were inoculated into 8 ml PYG and 1 ml of adjusted Ac suspension. Bacterial length of the ALp filtrate was determined by examining at least 10 high power oil immersion fields (1,000X) of 100 bacteria using a calibrated micrometer (AO/ Spenser, Buffalo, NY.)

Passage and Time Studies

Coculture organisms were adjusted and incubated as previously described except for the following modifications. Three or four conditions were directly monitored during the entirety of these studies which included: total *L. pneumophila* ; internalized *L. pneumophila*; external *L. pneumophila*; and associated *L. pneumophila*. Total *L. pneumophila* was comprised of the number of bacteria recovered in external, internalized and amoeba associated fractions. It was measured by harvesting the complete coculture suspension as previously described. Sampling was accomplished by placing a well-mixed aliquot in a sterile scew-top tube which contained an equal volume (v/v) of 0.45- 0.52mm acid-washed glass beads (Thomas Scientific, Swedesboro, NJ). This sample was then subjected to vortexing using a SMI model 2600 multi- tube vortxer (Scientific Manufacturing Industries, Emeryville, CA) at a setting of 6 for 3 min duration. These conditions were shown to be sufficient in lysing nearly 100% of intact *Ac* (>95%) without decreasing viability of associated *L. pneumophila*. Resulting lysates were plated onto α-BCYE and *L. pneumophila* numbers enumerated.

Internalized bacteria were measured by subjecting the coculture to a gentamicin pre-treatment prior to sampling flask contents. The PYG medium was removed and replaced with 10 ml of Ac buffer (see appendix D). This buffer was also removed and replaced by 10 ml of 30°C Ac buffer containing 100μg/ml gentamicin sulfate (Sigma) which had been previously demonstrated to kill all non-internalized *Legionella pneumophila*. Cocultures were then placed into a 35°C ambient air incubator for 2 h. The buffer-gentamicin solution was then removed and replaced with 10 ml 35°C Ac buffer. Wash buffer was then removed and plated onto α-BCYE to ensure completeness of gentamicin action. An additional 10 ml Ac buffer was added to coculture flasks and adherent amoeba were harvested as previously described. These suspensions were subjected to the same vortex procedure as total bacterial samples and enumerated by plate counts.

External bacteria were measured by harvesting the entire coculture suspension, centrifuging samples at 200xg for 10 min, An aliquot of the resulting supernatant was removed and plated for colony counts.

In contrast, associated bacteria were measured by removing the coculture medium and replacing it with 10 ml of 30°C Ac buffer. This wash procedure was repeated twice more. The third wash was replaced by 10 ml Ac buffer after aspiration and the suspensions harvested. Samples from the first and third washes were centrifuged for 10 min at 200 x g and plated for bacterial plate counts to ensure a significant (>95%) decrease in *L. pneumophila* numbers between the first and third washes. Resulting suspensions were subjected to the glass bead vortexing procedure and subsequently plated for bacterial count determination.

At 5 days of incubation an additional coculture flask was subjected to the associated *L. pneumophila* procedure except that 1 ml of the vortexed suspension was used to inoculate a new set of cocultures to be incubated and subsequently tested. This 1 ml *L. pneumophila* suspension was inoculated into 8 ml PYG medium containing approximately $2.0x10^5$ Ac/ml. Cocultures were then processed at 5 day intervals with the last set being sampled at day 30 of incubation. Amoeba numbers were determined for each condition. Furthermore, recovered *L. pneumophila* were placed on α-BCYE plates for OMC processing and aliquot were frozen at -70°C in freezer broth.

Outer Membrane Components Isolation

Outer membrane components (OMC) were isolated using a slight modification of the sodium N-lauryl-sarconate (Sarkosyl) procedure of Ehret and Ruckdeschel (38). Previously frozen (-70°C) VLp and ALp were grown for 3 days on α-BCYE agar at 35°C with 5% CO_2. Organisms were removed from a confluent plate using sterile cotton-tipped swabs and suspended in 15 ml conical polystyrene tubes (Baxter Health Corp, McGaw Park, IL) containing 5 ml of Dulbecco's modified phosphate buffered saline (0.05M sodium phosphate, pH 7.4, 0.1M sodium chloride; JRH Biosciences, Lenexa, KS). Bacterial suspensions were then centrifugated in a Sorvall RC-3 (Newton, CN) at 1,700xg for 20 min at RT. Supernatants were decanted and 5 ml of 10mM N-hydroxyethyepipperazine-N'-2-ethane sulfonic acid (HEPES) containing protease inhibitors (see appendix E) was added to bacterial pellets and mixed. Suspensions were processed

using a Bronwill "Biosonik" sonicator (Bronwill Scientific, Rochester, NY) at 4x30 sec with 1 min intervals between sonications while suspended in an ice bath. A probe intensity of 80 was used. Rough cell debris was removed by centrifugation at 1,700xg for 20 min. Resulting supernatant were transferred to methanol cleaned Beckman 14x89 mm polyallomer ultracentrifugation tubes (Becton Dickinson Inc, Palo Alto, CA) and filled to within 1 mm of the top with 10mM HEPES buffer. Sample tubes were transferred to a Beckman SW40 Ti rotor (Palo Alto, CA) and centrifugated at 100,000xg for 60 min at 4°C in a Beckman L8-70M ultracentrifuge (Palo Alto, CA). The resulting gel-like pellet was considered to be the total membrane preparation. Supernatant were discarded and pellets resuspended in 5 ml HEPES buffer containing 1% Sarkosyl. Pellets were gently broken up, tubes corked and wrapped in Parafilm™ , placed on a Dade rotator V (American Dade, Miami, FL) and vigorously agitated for approximately 30 min at RT or until particles were completely resuspended. Each tube was filled to within 1 mm of the top with HEPES with 1% Sarkosyl. Tubes were subjected to ultracentrifugation at 100,000 x g for 60 min a 4°C. Supernatant were discarded and resulting pellets resuspended in 200μl of double distilled water (ddH_2O). Protein concentrations were determined using the Lowry micro-protein assay (Sigma, Protein Assay Kit P 5656) according to manufacturers instructions. Outer membrane protein samples were adjusted to 2μg/μl and frozen at -20°C prior to electrophoresis.

Proteinase-K Digestion

Proteinase-K (PK) digestion of *Legionella pneumophila* was accomplished employing a modified procedure of Hitchcock & Brown in which previously frozen (-20°C) OMC samples were substituted for the normal whole-cell lysates (66). In this procedure 20μl of 2μg/μl adjusted bacterial OMC were placed in 1.5 ml Eppendorf microtubes (Cole-Palmer, Niles, IL) to which 10μl of 2X sample buffer was added. Samples were then boiled at 100°C for 10 min. After allowing samples to cool for 5 min, 10μl of a 2.5 mg/ml solution of PK (Sigma) in sample buffer was added to one set of tubes. Alternately, 10μl sample buffer without PK was added to the other set of tubes to function as a control set. Tubes were then placed into a 60°C water bath for 1 h VLp and ALp controls were accomplished by adding an equal amount of 2X sample buffer to each sample. A 0.5 mg/ml *Salmonella minnesota* LPS (Sigma) control was also prepared and diluted with an equal volume of 2X sample buffer. All samples were boiled at 100°C for 5 min and allowed to cool for 5 min before loading SDS-PAGE gels.

Electrophoresis

Electrophoresis of Sarkosyl insoluble outer membrane components was accomplished under a variety of conditions. Unless otherwise noted all reagents were procured from Bio-Rad Laboratories.

Sodium Dodecylsulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Discontinuous, denaturing SDS-PAGE was performed using a modification of the Laemmli (1970) method as described by Ehret and Ruckdeschel (1985).

An EC 500 power supply (E-C Apparatus Corp., St Petersburg, FL) was attached to either a SE 600 Hoefer (Hoefer Scientific Instruments, San Francisco, CA), Bio-Rad Protean xII (Bio-Rad Laboratories,CA) or a Bio-Rad Mini-protean vertical slab gel electrophoresis units yielding 1.5 mm thick gels of 14x16 cm and 16x18 cm respectively. Compositions of polyacrylamide and buffer solutions can also be found in Appendix F. Gel casting was accomplished in a similar fashion using either manufacturers equipment. Glass plates were pre-soaked for at least 30 min in Bio-Rad cleaning concentrate, rinsed 2 times in tap water followed by 2 rinses in ddH_2O and allowed to air dry. Plates were cleaned with absolute ethanol, inspected for imperfections and once determined to free of such were assembled into glass sandwiches using 1.5 mm spacers. Sandwiches were then transferred to their corresponding casting stand and gel solutions mixed in the following manner. Separating gels requiring a final polyacrylamide concentration of 12 to 15% were prepared as indicated in Appendix F and degassed under vacuum in a side-arm Erlenmeyer flask for 10-15 min. To the degassed solution both 250 μl of 10% ammonium persulfate (APS) and 50 μl of N,N,N',N'-tetramethylethylenediamine (TEMED) were added. The solution was swirled vigorously to mix components and distributed into the glass sandwich until a level was achieved which would result in a stacking gel with 2 cm from the bottom of the sample well to the separating gel interface. Gels were overlaid with water-saturated isobutanol and allowed to polymerize for at least 45 min. Once polymerized the isobutanol solution was removed, the gel interface was twice washed with ddH_2O and then layered with ddH_20 until the stacking gel was

poured. A stacking gel of a 4% concentration was prepared as indicated in appendix F and degassed for 10-15 min. To this mixture 125 μl of 10% APS and 25 μl TEMED were added. Prior to adding the APS and TEMED solutions, the water overlay was removed from the separating gel interface and the area blotted to remove excess water. At this point, 10-15 well teflon combs of 1.5 mm thickness were inserted into the glass sandwich after being moistened with ddH_2O and excess water removed. Combs were placed at an angle to preclude bubble formation during pouring. The well mixed stacking gel solution was distributed into the glass sandwich while the combs were gently pushed down into their final position. Stacking gels were allowed to polymerize at least 45 min. After polymerization had occurred, sample wells were rinsed with ddH_2O to remove unpolymerized polyacrylamide and filled with running buffer until samples were loaded.

Sample buffer was prepared as indicated in appendix F. and both samples and molecular weight standards processed as follows. Each outer membrane sample was diluted 1:2 with 2X sample buffer resulting in 1 μg/μl samples. Protein molecular weight standards consisted of Diversified Biotech, low range markers (Newton Centre, MA), hereafter referred to as low molecular weight (LMW) marker, and Bio-Rad SDS-PAGE or SDS-PAGE silver low range markers, hereafter referred to as high molecular weight (HMW) marker and/or Bio-Rad SDS-PAGE broad range (BMW). The LMW covers a range from 2.5 kilodaltons (kDa) to 20.4 kDa, whereas HMW spans from 14.4 kDa to 97.4 kDa and BMW 6.5 kDa to 200 kDa. Appropriate dilutions were made of each marker

as indicated for the staining procedure. Both samples and markers were placed in a boiling water bath for 5 min, removed and allowed to cool to RT. Insoluble particulate matter was removed by a 1-2 sec spin-down at 10,000 x g in an Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY). Sufficient running buffer was added to the lower buffer chamber and the upper buffer chamber was assembled in accordance with manufacturer's instructions. Sample wells were filled with running buffer and subsequently loaded in the following manner. Sample supernatant and molecular weight markers were loaded with a small volume (0-100μl) Eppendorf pipette equipped with Costar microtips (Cambridge, MA) at 2 to 30 μl per sample well. Molecular weight markers were loaded at 3-5 μl for LMW, 10-15 μl for HMW and 5μl for BMW. Electrophoresis for the Hoeffer and Bio-Rad Protean xII units was conducted using constant current and applying 70V until tracking dye had entered the separating gel. Current was increased to 60 mA and maintained until the dye front had reached 1 cm from the bottom of the gel sandwich. The Bio-Rad mini-protean unit was placed in an ice bath and adjusted to 150-200 volts, constant voltage, until the dye front had reached the desired area. At this time electrophoresis was discontinued and gels placed in a 50% methanol (Corco Chemical Co., Fairless, PA) and 10% glacial acetic acid (HAc) (Columbia Diagnostic Inc, Springfield, VA) solution and slowly rotated overnight.

Staining of SDS-PAGE Gels

Coomassie Blue Stain

Gels requiring Coomassie blue staining were accomplished as listed in Appendix G. Briefly, a priorly fixed gel was placed in a 50% methanol, 10% acetic acid and 0.05% Coomassie brilliant blue R solution (Sigma) and slowly rotated for 1-3 h. Gels were then briefly washed with a small amount of destaining solution composed of 5% methanol and 7% acetic acid, which was replaced by a sufficient amount of destain solution to completely cover the gel. After 1 h the destain solution was discarded and replaced with fresh destain and slowly rotated overnight. The next morning, gels were checked for completeness of destaining and fresh destain solution added if required to completely remove any residual background stain. Destain was replaced with ddH_2O when complete clearing of the background was noted.

Silver Stain

Gels requiring silver staining were processed by utilizing the Bio-Rad silver stain kit in accordance with the suggested protocol. Briefly, gels were removed from the overnight fixative and placed in a clean glass container containing 40% methanol and 10% acetic acid for 60 min. This fixative was aspirated and replaced by a 10% ethanol (EtOH) (Pharmco Products Inc., Bayonne, NJ) and 5% acetic acid fixative for 30 min. This fixation step was repeated once more for an additional 30 min. Oxidizer then replaced the fixative for 10 min. This step was followed by 3-5, 10 min washes with ddH_2O until the oxidizer was completely removed. Water was replaced by silver reagent for 30 min followed by a 2 min rinse with ddH_2O. Developer replaced the water rinse and was aspirated at the

first sign of brown "smokey" precipitate. Fresh developer was added to the gel for approximately 5 min. At this point developer was replaced with fresh developer and development progress until the desired band intensity was reached. The developer was replaced with a 5% acetic acid (Columbia Diagnostics) stop solution and rotated for approximately 5 min. The stop solution was subsequently replaced with ddH_2O.

An alternate periodic acid oxidation step was used to highlight carbohydrates in proteinase-K digest gels. The staining procedure remained essentially the same except for the substitution of a periodate oxidation step in lieu of the normal perchromate-based oxidizer solution provided with the silver stain kit. Additionally, a decreased concentration of acetic acid was utilized during the fixative step. Briefly, after an overnight fixation in 40% EtOH/5%HAc, gels were placed in an oxidizer solution comprised of 40% EtOH, 5% HAc & 0.7% periodate for 5 min. The remainder of the staining procedure was performed as listed above.

Both Coomassie blue and silver stained gels were photographed using a 35mm single lens reflex camera with Kodak Ektachrome 100HC film while backlit on a Hall fluorescent light box (Hall Industries, San LuisObiso, CA).

Periodic Acid Schiff (PAS) stain

Gels requiring demonstration of glycoproteins were subjected to a modified PAS stain procedure. Gels were immersed in 12.5% trichloracetic acid for 30 min. They were subsequently transferred to 200 ml of ddH_2O for 2 min with slow

rotation. The water was replaced by 100 ml of a 1% periodic acid/3% HAc oxidizing solution for 10 min with slow rotation.. At this time, gels were washed for 10 min in 4 washes of 200 ml dds_2O using slow rotation. They were then immersed in a volume of Schiff's reagent (Sigma) sufficient to cover the gel for 30 min in the dark. Stain was removed and replaced by 100 ml of a 0.5% sodium metabisulfite solution for 10 min followed by two additional metabisulfite washes of 10 min each. Gels were then immersed in 200 ml dds_2O under gentle agitation and water was replaced until excess stain was removed.

Giminez Staining of Ac

Cocultures were harvested by placing flasks on a wet ice bath for 15 min followed by gentle dislodgement of adherent amoeba with a sterile cell scraper Briefly, after an overnight fixation in 40% EtOH/5%HAc, gels were placed in an oxidizer solution comprised of 40% EtOH, 5% HAc & 0.7% periodate for 5 min. (Falcon). Cell suspensions were transferred to sterile screw-top tubes and amoeba pelleted by centrifugation at 200 x g for 10 min. Resulting pellets were resuspended in 10 ml Page's amoebal saline (PAS) or Ac buffer, and subjected to an additional 10 min centrifugation at 200 x g. Supernatants were discarded and pellets resuspended in 1 ml PAS or Ac buffer. Using a sterile transfer pipette, 1 to 2 drops of the amoeba suspension was placed on a clean 50 x 24 mm coverslip and Ac allowed to attach at least 30 min or until the drop was almost dry. Three drops of 80°C, 10% (v/v) formalin (Baxter Health Care) were added to each coverslip for 2 min. Coverslips were then transferred to a RT, 10% (v/v) formalin

bath for an additional 2 min. Next, coverslips were rinsed briefly in tap water and allowed to air dry. Coverslips were suspended in a Falcon 1058 large petri dish (Becton Dickinson, NJ) on wooden applicator sticks. Staining was accomplished by filtering 50°C buffered carbol fuschin stain through a Whatman #1 filter paper and allowing 3-5 drops of stain to cover the area with adherent amoeba. The cover was placed on the petri dish and allowed to remain in place for 10 min. Coverslips were then washed thoroughly with tap water and allowed to air dry. Each coverslip was then mounted onto a glass slide using Accu-Mount™ mounting medium (Baxter Health Care, McGaw Park, IL) and examined for stain quality. Brightfield photomicroscopy was accomplished using a Leitz Didex 22 light microscope (Wetzlar, W. Germany) equipped with a 35 mm camera loaded with Kodak Ektachrome 64T (Eastman Kodak, Rochester, NY)

Fluorescent Antibody (FAb) Stain

Recovery and subsequent processing of *L. pneumophila* or *A. castellanii* was accomplished as described in the Gimenez stain procedure except for the following modifications. Dual well fluorescent microscopy slides (Genetic Systems, Seattle WA.) were inoculated with 2-3 drops of viable amoeba suspensions and allowed to attach for approximately 30 min or until the drops were almost dry. At this time, 2-3 drops of cold (4-6°C) acetone (Sigma) was placed onto the well for 2 min duration. Slides were transferred to RT, 10% (v/v) formalin for an additional 2 min, briefly washed in tap water and allowed to air dry. A commercially available *Legionella* immunofluorescent antibody test kit (Genetics Systems) was

utilized to locate bacteria associated with amoeba. A control slide was prepared using the provided *L. pneumophila* positive control suspension in one well and *Escherichia coli* in the other well acting as the negative control. All prepared amoeba slides were rapidly heat-fixed and slides were treated with 1-3 drops of anti-*Legionella*, FITC-labelled monoclonal antibody. *L. pneumophila* slides were prepared by removing a suspect colony and suspending it in a small amount of 1% formalin in saline to approximate a McFarland No. 1 turbidity standard. Two to three drops of each suspension were placed onto the provided slides and excess suspension removed with a clean Pastuer pipette. Smears were allowed to air dry and were then heat-fixed. All slides were transferred to a moisture chamber and incubated for 30 min at 35°C. After incubation, slides were placed at a 90° angle and excess staining reagent blotted. Slides were briefly dipped in deionized water followed by optional counterstaining of amoeba in 8 drops of Kallestad quantafluor Evan's blue stain (Chaska, MN) in 200 ml PBS. Slides were then transferred to deionized water for 2 min, air dried and mounted with the provided mounting medium. Fluorescent photomicroscopy was accomplished using a Zeiss epifluorescent microscope (Thornwood, N.Y.) equipped with a 35 mm camera using Kodak Ektachrome 400HC film (Eastman Kodak, Rochester, NY).

Scanning Electron Microscopy (SEM)

Scanning electron micrographs were performed on fixed specimens of VLp and ALp. Each organism was recovered from previously frozen stocks and plated onto BCYE medium. Cultures were incubated for 4 days at 35°C in 5% CO_2.

Fixation of *L. pneumophila* was performed using the following procedure. Fifteen to twenty-five well isolated Lp colonies were suspended in 5 ml PBS (JRH Biosciences, Lenexa, KS), well mixed and subjected to centrifugation in a Sorval RC-3 centrifuge for 20 min at 1,700 x g. The supernatant was discarded and bacterial pellets resuspended in 5 ml, 2% glutaraldehyde solution (Polysciences, Warrington, PA) for 30 min. Glutaraldehyde was removed by pelleting bacteria at 1,700 xg for 20 min, discarding the supernatant and resuspending bacterial pellets in 5 ml PBS. Again, *L. pneumophila* were pelleted, supernatant was discarded and bacteria resuspended in phosphate buffer. Specimen processing continued in the following manner. Four pieces of 15 cm Whatman #1 filter paper (W & R Balston, England) were placed in a small petri dish to which specimen holders were positioned onto the top of the filter papers. Filter paper was then wet thoroughly to result in a moisture chamber. One drop of poly (lysine) hydrobromide, 0.1% aqueous, M.W. 60-120, (Polysciences) was placed onto the central portion of the specimen holder and allowed to remain there for 15 min. Specimen holders were then briefly dipped in water and excess water blotted. A drop of either *L. pneumophila* suspension was placed onto the poly (lysine) covered area and allowed to remain there for 15 min. Next, specimens were processed in an alcohol dehydration series of 70% ethanol for 5 min, 95% ethanol for 5 min and 100% ethanol for 5 min. The series was followed by immersing specimens in 50% ethanol/50% freon 113 (Polyscience) and processing samples with a Polaron critical point dryer (Polaron Instruments, Waterford, England). Critical point dried samples were then gold/palladium coated using a

Polaron SEM Coating Unit E5100 (Polaron Instruments) in accordance with manufacturers instructions. Samples were then scanned and photomicrographs were taken using a Phillips EM400 STEM scanning/transmission electron microscope (Eindhoven, Netherlands) operating at 20kV.

Transmission Electron Microscopy (TEM)

Transmission electron micrographs were made on *A. castellanii* or *L. pneumophila* recovered from cocultures using a modification of Fields, *et al.* in a manner similar to that used during FAb and Gimenez staining (46).

A. castellanii suspensions were harvested from coculture as previously described. Flask contents were transferred to a 15 ml conical polystyrene screw-top tube (Baxter) and amoeba pelleted for 10 min by centrifugation at 200 x g. The supernatant was discarded, amoeba pellets resuspended in 10 ml Ac buffer and subjected to pelleting as described above. The supernatant was once again discarded and amoeba pellets resuspended in 5 ml phosphate buffered 2% glutaraldehyde (Polysciences). Tubes were then tightly capped wrapped with Parafilm™ and rotated slowly for 45 min to 1 h. After rotation each tube was subjected to two washes using phosphate buffer and final amoeba pellets resuspended in residual buffer.

L. pneumophila were processed for fixation by flooding 72 h culture plates with 5 ml PBS (JRH Biosciences) and dislodging colonies with a bent Pastuer pipette. Resulting organism slurries were transferred to 15 ml conical tubes as above and bacteria pelleted by centrifugation at 2,600 x g for 20 min. Bacterial

supernatants were discarded and pellets were resuspended in 5 ml PBS and repelleted as before. Supernatants were again discarded and bacterial pellets resuspended in 5 ml phosphate buffered 2% glutaraldehyde and tubes rotated for 45 min to 1 h as with amoeba samples. Bacteria were then pelleted as before and washed two times in phosphate buffer. Supernatants were discarded and bacterial pellets resuspended in residual buffer.

At this point both amoeba and bacteria were processed in the same manner. A 4% agarose (Sigma) solution was made, placed in a 80°C water bath and allowed to dissolve. Once removed, the solution was allowed to cool to 45°C and was then transferred to a 1.5 ml Eppendorf microcentrifuge tube (Cole-Palmer). At this time the tubes were placed into the Eppendorf microcentrifuge (Brinkman Industries, Westbury, N.Y.) and sample added to the top of the liquid agarose. The tubes were capped and centrifugated at approximately 10,00xg for 10 secs. Tubes were removed and agarose allowed to solidify with organism pellets at the bottom of each tube.

A. castellanii or *L. pneumophila* samples were removed from their corresponding tubes by cutting each tube to release the agarose pellet. Pellets were cut into small blocks and transferred to 5 ml phosphate buffer for 5 to 10 min. They were then post-fixed using an osmium-tetroxide solution (Polysciences) for 1 h and rinsed with phosphate buffer. Each specimen was put through a alcohol dehydration series of 50, 70, 95 and (X3) 100% ethanol using a Linx tissue processor (Leica, Deerfield, IL). Specimen blocks were transferred to a 50/50 mix of polypropylene/Poly-Bed (Polysciences, Warrenton, PA) and then into Poly-Bed

embedding medium. Embedded sections were made using a Diatome diamond knife (Ft. Washington, PA) on a LKB ultromicrotome (Stockholm, Sweden). Thin sections were stained with uranyl acetate/lead citrate using a LKB ultostainer (Stockholm, Sweden). Electron micrographs of both organisms were accomplished using a Phillips EM400 STEM scanning transmission electron microscope operating at 60 kV.

RESULTS

Guinea Pigs

Guinea pigs in the VLp group received 1 ml intraperitoneal injections of 2.50 x 10^8 CFU/ml suspended in 0.89% saline. All three guinea pigs demonstrated signs of distress, such as ruffled neck fur, and both nasal and eye secretions by 24 h post-inoculation. By 48 h two guinea pigs had died and the third was moribund, exhibiting no signs of movement. In a similar fashion, the second group of guinea pigs received an injection containing 2.03 x 10^9 CFU/ml of ALp. This group was monitored daily for fourteen days and did not exhibit any distress related symptoms. Subsequently, these animals were sacrificed. Organisms confirmed by FAb staining as *Legionella pneumophila* were recovered from the lungs, spleens and livers of all guineas in the VLp group. In contrast, the lung specimen of one ALp guinea pig and the spleen of another each demonstrated one colony which when Gram stained revealed long, filamentous gram-negative rods At this time, these organisms were thought to be plate contaminants and were not followed by FAb staining.

Characteristics of Organisms

VLp and its medium derived avirulent counterpart, ALp, exhibit several differences when examined closely. For example, the colonial morphology of VLp

at 72 h incubation on α-BCYE agar (Fig. 1) usually demonstrates colonies ranging from pin-point, or barely discernable, to those reaching 1.5-2.0 mm in diameter. Conversely, ALp colonies at the same incubation time appear to be uniform in size at approximately 2 mm in diameter with little variation noted. Colonies of VLp can be lifted readily from the plate using a standard bacteriologic inoculating loop. However, ALp colonies cannot be lifted readily off the same type of agar plates and appear to be "sticky" when this process is attempted. Gram stain smears of VLp (Fig. 2) are typically seen as weakly stained gram-negative rods of 1-2 μm in length occurring singularly or in pairs. Occasionally, small filaments of 5-10μm in length can be seen. The amount of filaments detected can vary. Alternately, on 100% of the stains examined ALp (Fig. 3) present themselves as filaments of varying length. Some filaments appear to be only 10-20μm in length, whereas others may span 2-3 oil immersion fields (1,000X). The overall impression of the smear is best described as the organism resembling "angel hair" pasta. Filaments are usually seen as a highly entangled mass which can also be detected near the periphery of the smear. Additionally, single rods are seen interspersed between the filaments, closely resembling their virulent parent. Each coculture utilized previously frozen ALp and VLp organisms derived from a single stock pool. Variation was noted in the amount of filaments seen between recoveries. Furthermore, closer examination of previously stained VLp coculture inoculum smears revealed varying amounts of filaments and lengths ranging from 10μm to those seen in ALp smears. EJ1 colony characteristics and organism morphology closely resemble that seen with VLp except for a much weaker Gram

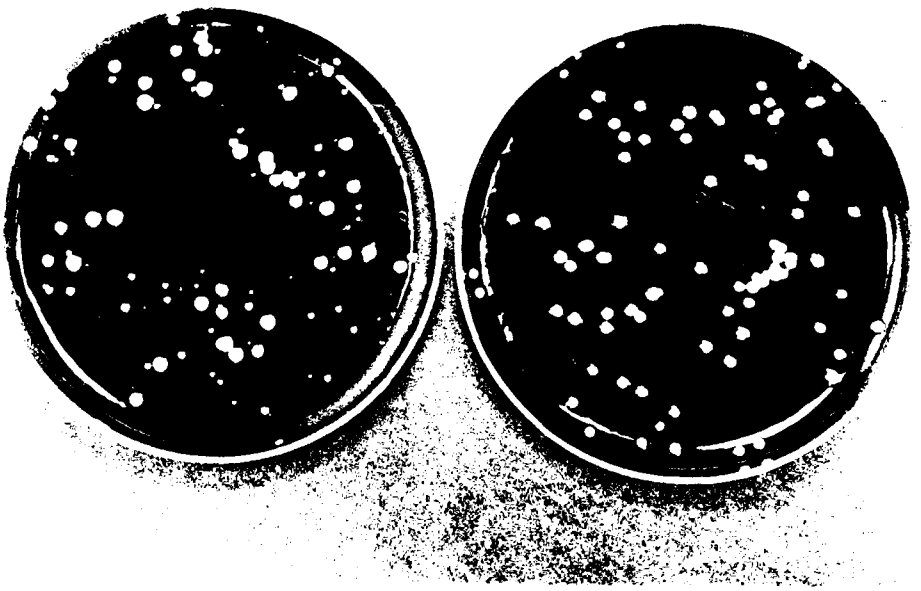


Figure 1. Colony size difference between VLp (left) and ALp at 72 h on α-BCYE agar.

Figure 2. Typical Gram stain reaction of VLp at 72 h incubation on α-BCYE agar (1,000X).

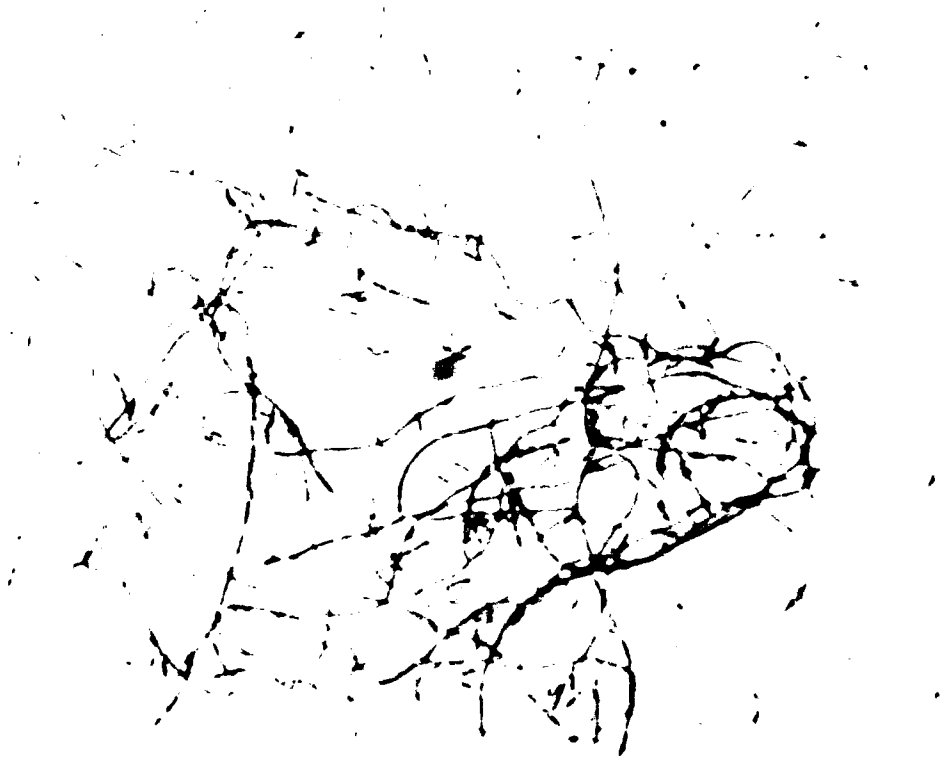


Figure 3. Typical Gram stain of ALp at 72 h incubation on α-BCYE agar (1,000x).

stain reaction.

Scanning electron micrographs of VLp (Fig. 4) reveal short, uniform sized rod-shaped bacilli with lenghts of 1 to 2 μm. Conversely, ALp forms (Fig. 5) vary from short bacilli similar to those seen with VLp to rod-shaped forms which appear to span nearly the entire photomicrograph. These filaments (Fig. 6) appear to form during bacterial division as a result of an elongation due to an inability to complete normal cross-wall division. No apparent differences in bacterial cell surfaces was noted in either organism.

VLp and ALp transmission electron micrographs of 72 h α-BCYE cultures reveal several basic differences in each bacterium. VLp (Fig. 7) appears to be comprised of numerous uniformly sized, darkly stained granules with vacuoles seen in the cytosol. The vacuoles can be seen singularly or in large numbers. Well defined cell membranes are readily apparent. In contrast, the ALp form (Fig. 8) appears less densely packed with granules similar to those seen in VLp and can exhibit multiple defects in cell wall synthesis. Occasionally, forms may be seen which are similar in appearance to that of VLp. In general, the cell walls of ALp are less defined than those seen in VLp. When internalized at 24 h, VLp morphology (Fig. 9) appears to alter slightly. Bacteria l cells appear less than their counterparts which have not been placed in coculture with amoeba. However, the overall impression of the density remains greater than that seen with ALp grown on α-BCYE agar. Additionally, the well-circumscribed cell membranes seen with VLp grown on α-BCYE agar are not seen. Instead, a ruggated or wavy outer membrane structure is noted which conforms to the

Figure 4. Scanning electron micrograph of VLp at 72 h incubation on α-BCYE agar (18,750X).

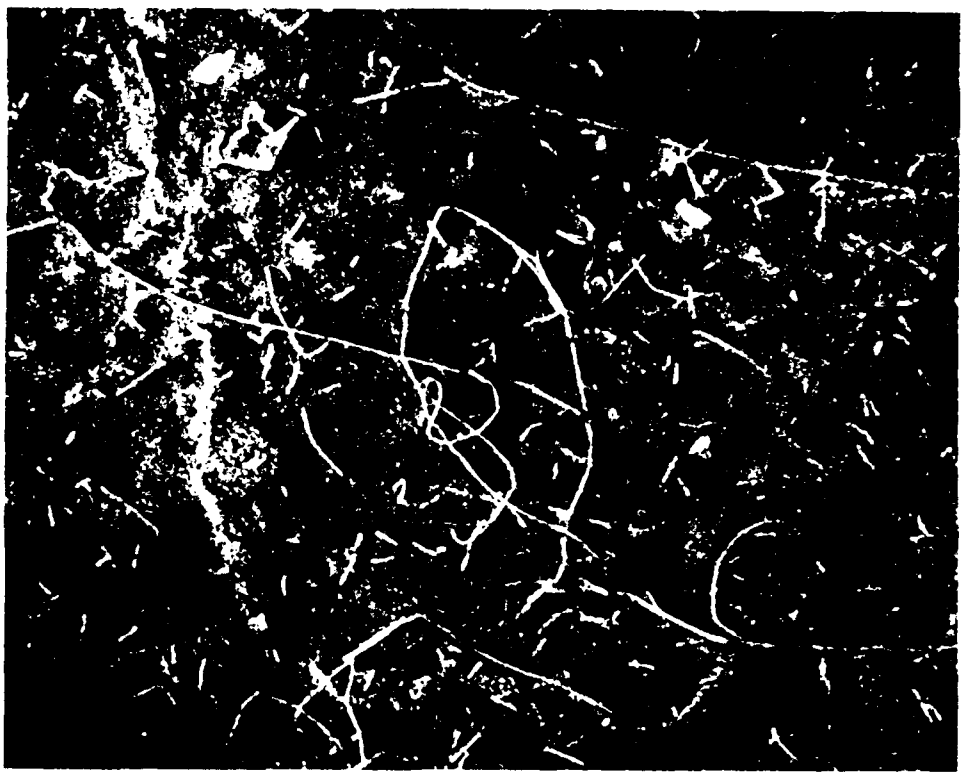


Figure 5. Scanning electron micrograph of ALp filament formation at 72 h incubation on α-BCYE agar (1,500X).

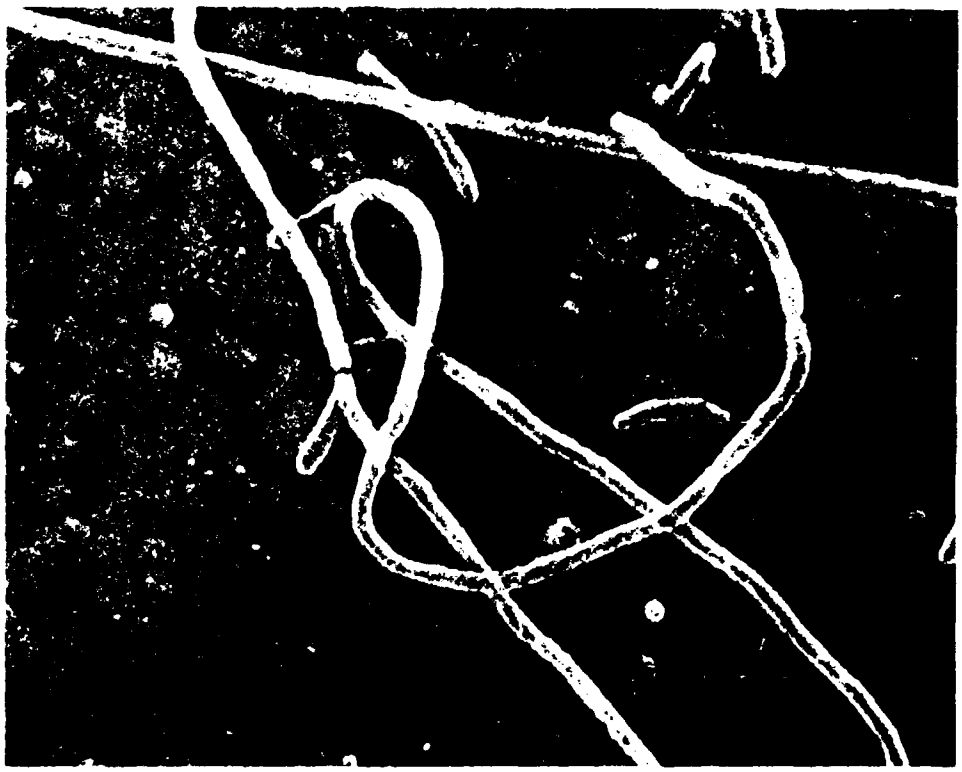


Figure 6. . Scanning electron micrograph of ALp at 72 h incubation on α-BCYE agar. Note the pinched area which appears to be an attempt at cross wall formation (9,600X).

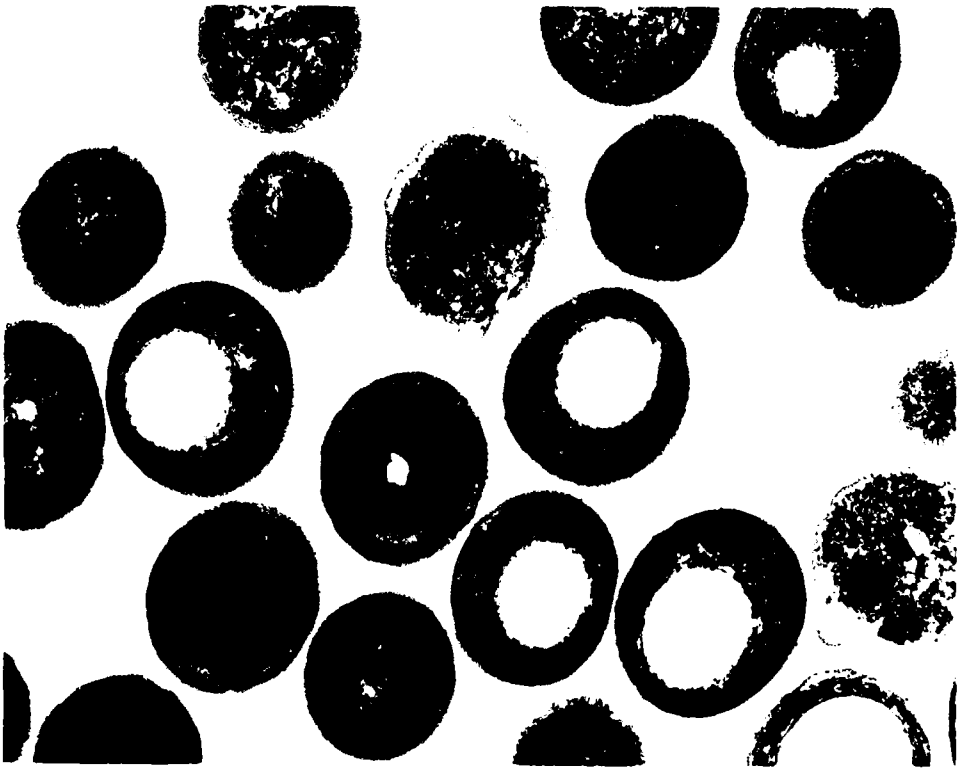


Figure 7. Transmission electron micrograph of VLp at 72 h incubation on α-BCYE agar. Note both the dense granular material and large vacuoles (36,000X).

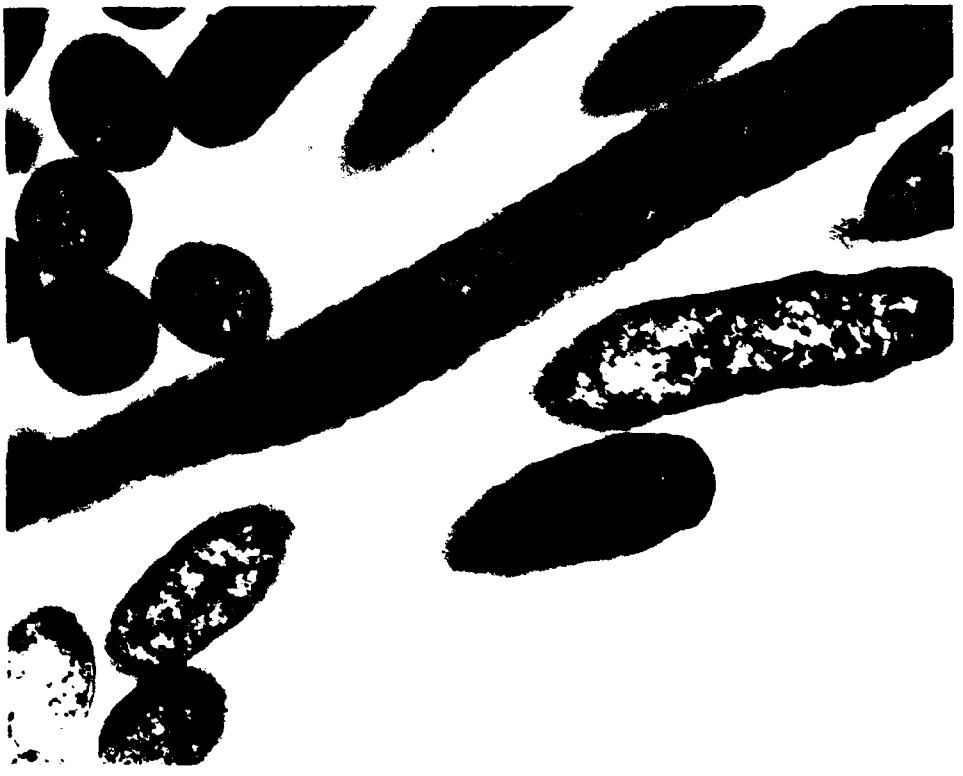


Figure 8. Transmission electron micrograph of ALp at 72 h incubation on α-BCYE agar. Note area which appears to be defective cell wall formation (22,000X).

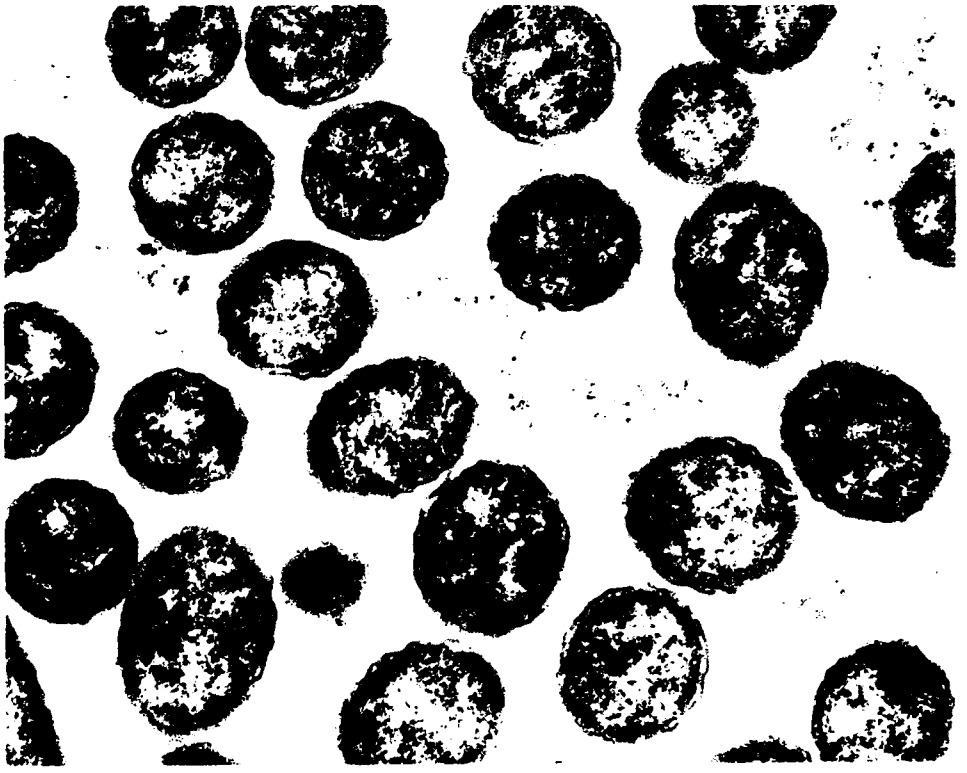


Figure 9. Transmission electron micrograph of internalized VLp after 24 h coculture incubation. Note the wavy appearance of the cell membranes (28,000X).

general cell shape. Small vacuoles can also be detected.

Uninfected *Acanthamoeba* at 24 h incubation in PYG medium (Fig. 10) present numerous cytoplasmic projections, a well-defined nucleus and evenly distributed mitochondria. Amoeba infected with ALp (Fig. 11) demonstrate a well-defined phagosome with a single bacterium by 1 h coculture incubation. Here, the bacteria appear to be in various states of degradation. By 4 h incubation, intact bacteria were not seen even upon examination of numerous bacteria-containing phagosomes. Internalized ALp could not be detected in 30 min coculture specimens. Alternately, intact VLp could be seen in amoeba after 30 min coculture incubation. They were located with relative ease by examining each field for darkly stained bacterial forms exhibiting vacuoles. This was not the case with ALp, since numerous fields, >30, had to be examined at a similar low magnification before locating internalized bacterial forms. Electron micrographs at 1 h incubation (Fig. 12) reveal the presence of rough endoplasmic reticulum (RER) which have surrounded the phagosome. Additionally, mitochondria appear to be infiltrating the phagosome area when compared with an uninoculated amoeba control. All phagosome-bound VLp forms appear to be intact and did not exhibit similar degradation forms as seen with ALp forms. Gimenez staining of amoeba (Fig. 13) reveals numerous well- defined vacuoles, some containing rod-shaped bacterial forms consistent with *Legionella*. Another interesting difference is readily apparent when attempting to adjust ALp and VLp to a similar inoculum size using a spectrophotometer set at 625 nm. An optical density twice that of ALp (Fig. 14) is needed to recover the same amount of VLp. If

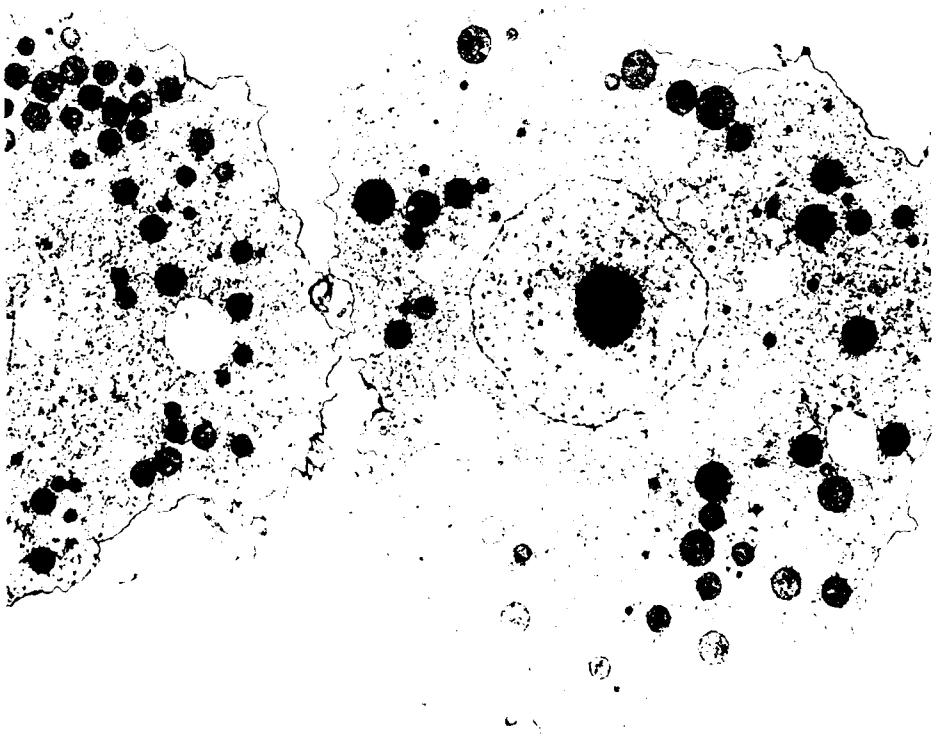


Figure 10. Transmission electron micrograph of *Acanthamoeba* in PYG medium at 24 h incubation (2,800X).

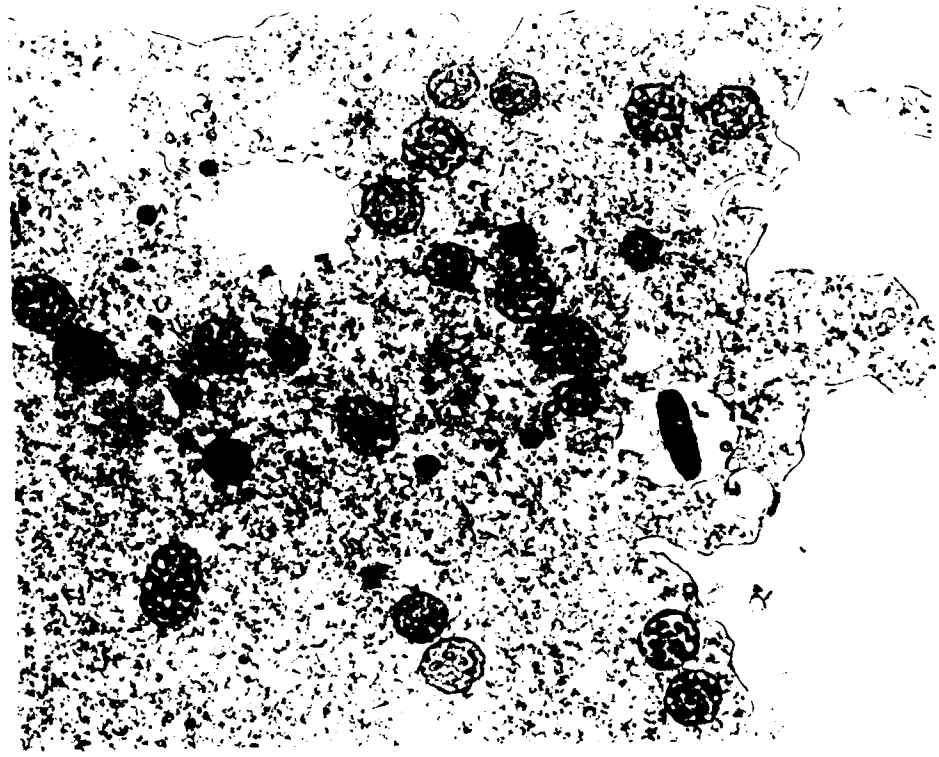


Figure 11. Transmission electron micrograph of amoeba with internalized ALp at 1 h coculture incubation.

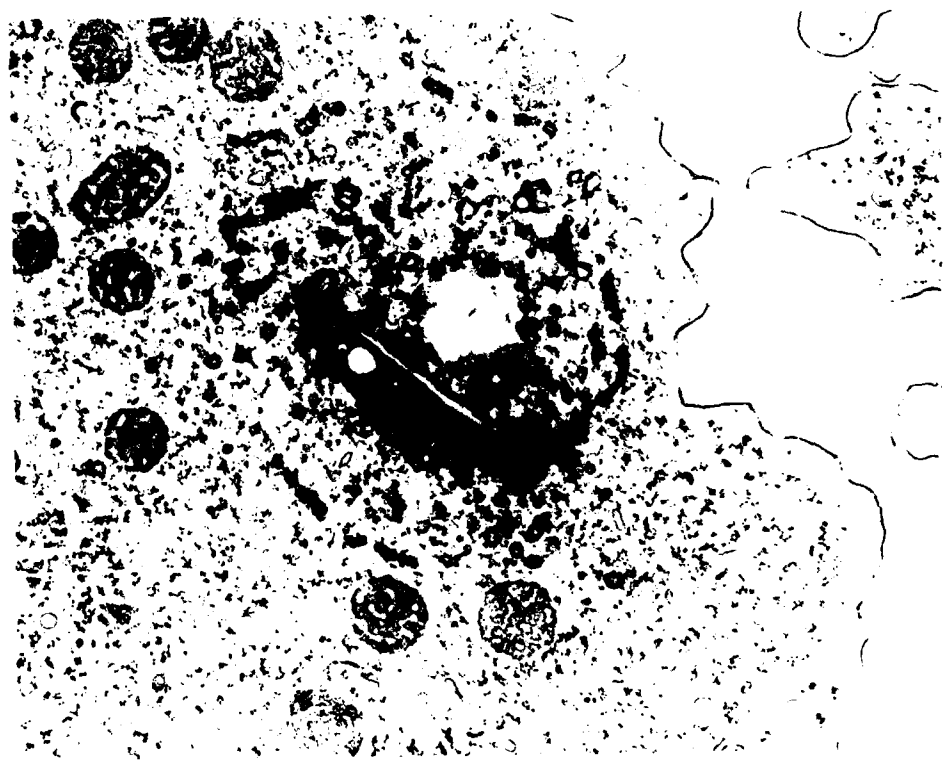


Figure 12. Transmission electron micrograph of amoeba with internalized VLp at 1 h coculture incubation. Note the localization of mitochondria and encirclement of bacterium by RER (10,000X).

Figure 13. Gimenez stain of VLp infected amoeba at 5 days incubation. Note the well defined vacuoles containing VLp organisms (1,000X).

Table 1. Difference seen when attempting to adjust inoculum size of ALp and VLp. Note that VLp requires an O.D. reading twice that of ALp in order to recover similar bacterial counts.

O.D. VLp	Count (log10)	O.D. ALp	Count (log10)
0.204	8.32	0.101	8.07
0.203	8.27	0.108	8.24
0.205	8.32	0.100	7.79
0.209	8.15	0.103	7.21
0.207	8.29	0.102	8.13
0.208	8.05	0.104	8.35
0.207	8.01	0.104	8.18
0.200	8.05	0.105	8.44
0.202	8.07	0.108	8.11
0.201	8.07	0.101	7.85
0.203	8.05	0.108	8.34
0.206	8.08	0.103	8.15
0.201	8.19	0.104	7.53
0.206	8.26	0.103	8.22
0.202	8.14	0.105	8.31
0.208	8.34	0.107	7.87
Std. Dev.	8.1663 ± 0.1168		8.0494 ± 0.3278

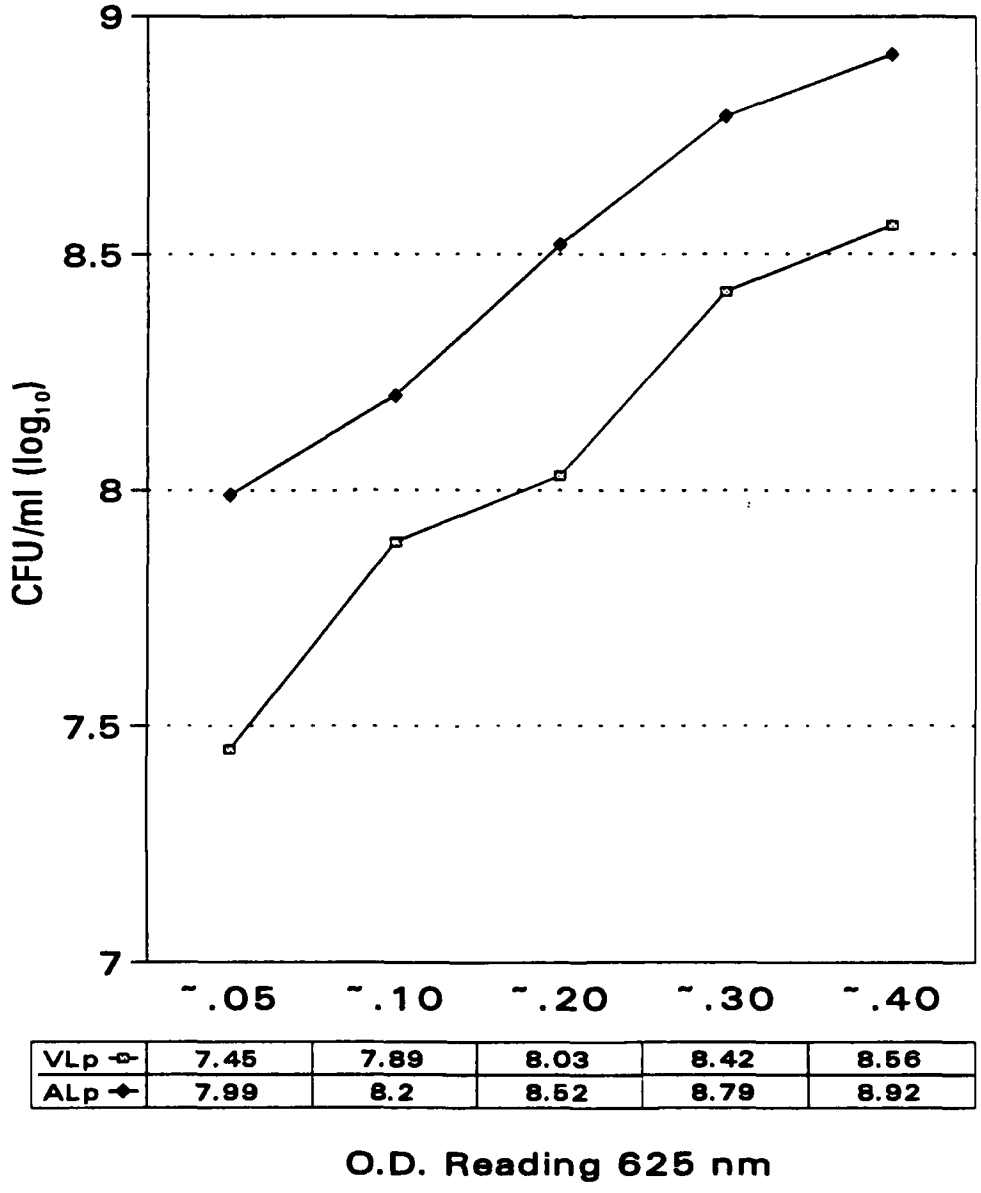


Figure 14. Difference in the amount of VLp and ALp recovery at similar optical densities (O.D.)

approximately 1 x 10^8 CFU/ml is desired, the VLp absorbance reading must be adjusted to 0.2, whereas the ALp reading must be near 0.1. This phenomenon appears to be reproducible between adjustment attempts. Additionally the variation (Table 1) in the amount of ALp recovered at similar O.D. readings exhibits a standard deviation which is three times as great as that seen with VLp.

Coculture Results

Initially, studies utilizing only VLp were conducted in which coculture flasks were sampled daily for a total of seven days. These studies indicated that VLp in association with the amoeba (Fig. 15) would initially decrease in numbers at 24 h. Between 24 and 48 h there would be no apparent change in recovered bacterial numbers. Subsequently, between 48 h and 5 days a significant increase (> 1 log) was seen. By day seven the number of VLp in coculture had increased by approx. 2 to 3 logs. Peak VLp numbers were seen between days 5 and 7. The VLp control in PYG without amoeba demonstrated either a decrease or stabilization in numbers throughout the sampling period. Later, ALp interaction was also monitored. In this case, bacteria in coculture with amoeba (Fig. 16) displayed an initial downward trend through day 6. Then, an unexpected increase of greater than 1 log growth occurred between days 6 and 7. The corresponding ALp control demonstrated a steady decrease in numbers through day 7. Alternately, the VLp with amoeba responded in the same fashion as it had in the previous studies. On the other hand, the VLp control was seen to increase dramatically, approximately 2 logs, between days 6 and 7. Monitoring of amoebal

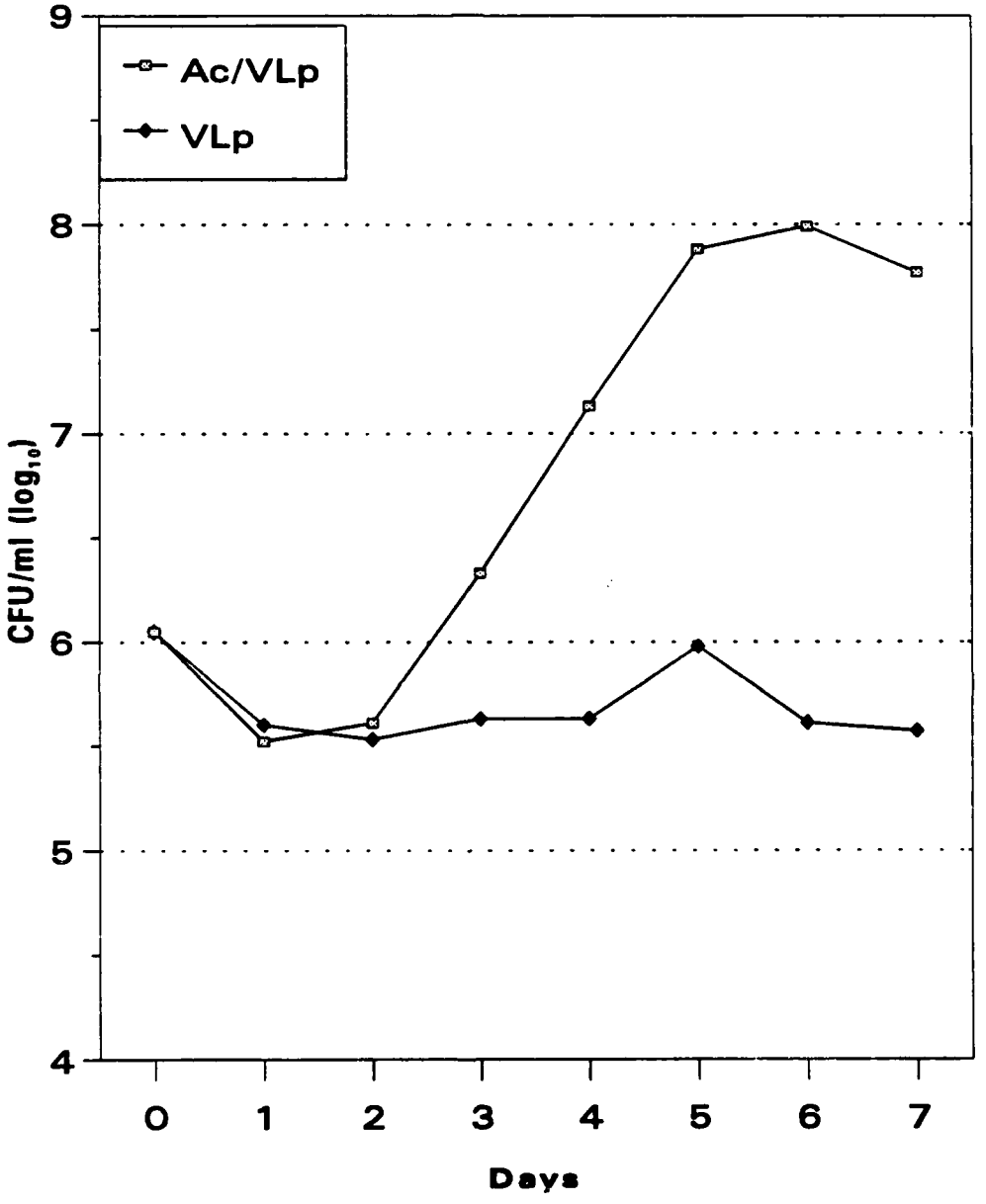


Figure 15. Initial 7 day VLp coculture (Ac/VLp) and its corresponding control (VLp).

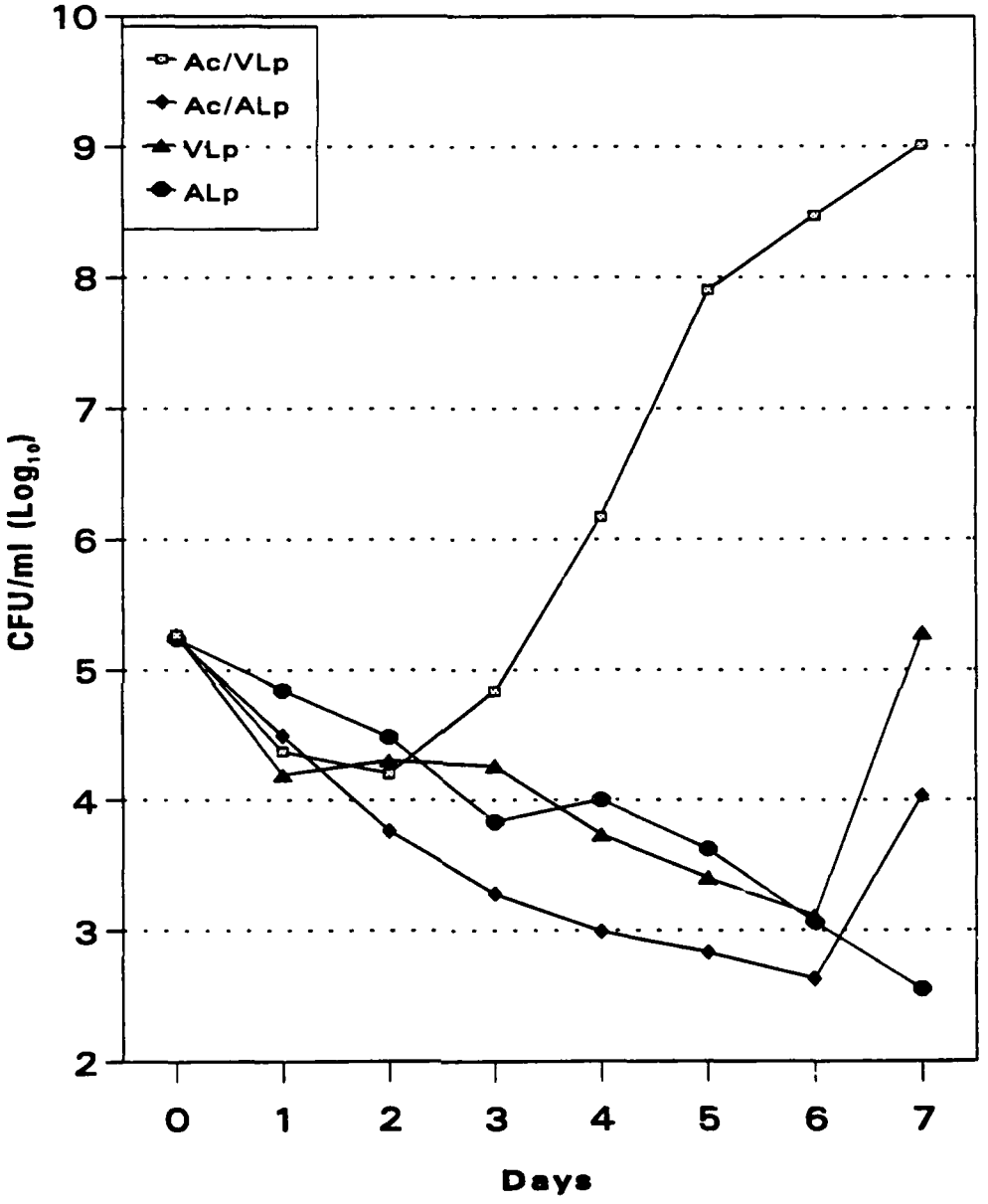


Figure 16. Seven day VLp coculture (Ac/VLp), ALp coculture (Ac/ALp) and corresponding ALp and VLp controls.

growth (Fig. 17) revealed an increase in amoeba numbers through day 5, with a plateau reached between days 5 and 7. When the monitoring period was extended to 10 days, cocultures and controls were seen to display the following results. As previously noted in the VLp in coculture, (Fig. 18) initially declined in numbers by 24 h with a subsequent increase by day 5 and a leveling off at day 7. Here, VLp control numbers remained essentially the same. Again, ALp in coculture increased by approximately 2 logs growth by day 7. A striking increase of approximately 4 logs growth was detected in the ALp control which occurred between days 9 and 10. Amoebal numbers (Fig. 19) increased through day 7 with a subsequent decline in numbers by day 9.

Next, a study was conducted involving a further examination of the *Legionella* controls which lack amoeba. Here (Fig. 20) the ALp control decreased in numbers until no viable bacteria could be recovered by day 10. Conversely, the VLp control numbers increased by approximately 4 logs growth from day 7 to day 10. At this point, the monitoring period was decreased to 5 days. In this situation, (Fig. 21) Vlp with amoeba again demonstrated the typical pattern which had been previously noted. Both VLp and ALp controls steadily decreased in bacterial numbers. Again, ALp in coculture were seen to increase approx. 1 logs growth between days 4 and 5. Further examination (Fig. 22) revealed a similar phenomenon where both VLp and ALp in coculture increased in numbers, with a longer lag phase demonstrated by ALp. Here, control ALp and VLp decreased in numbers by day 4 and remained essentially the same by day 5. Amoeba growth was seen to increase by day 5 of incubation.

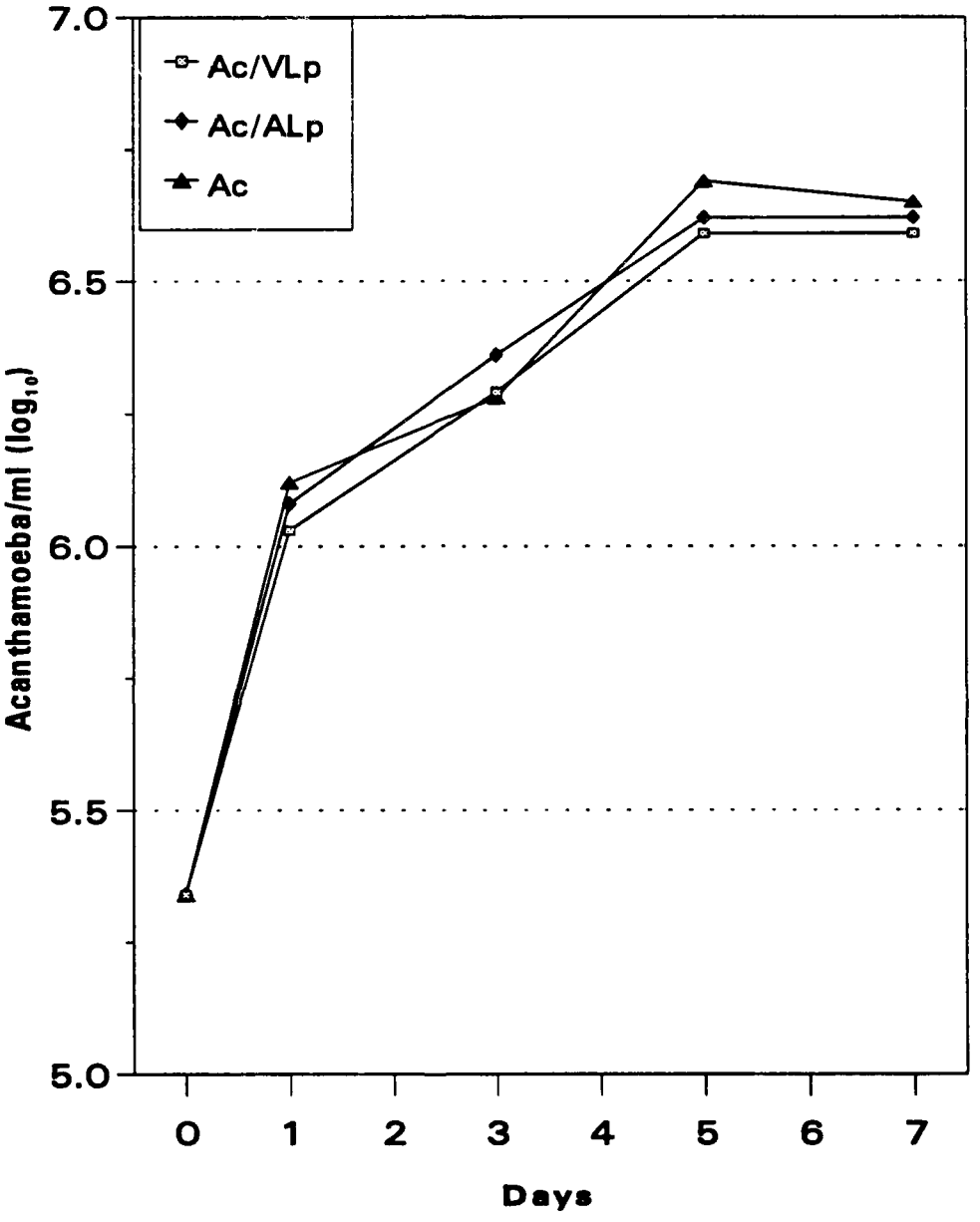


Figure 17. Fate of amoeba in 7 day coculture with VLp & ALp and amoeba control (Ac).

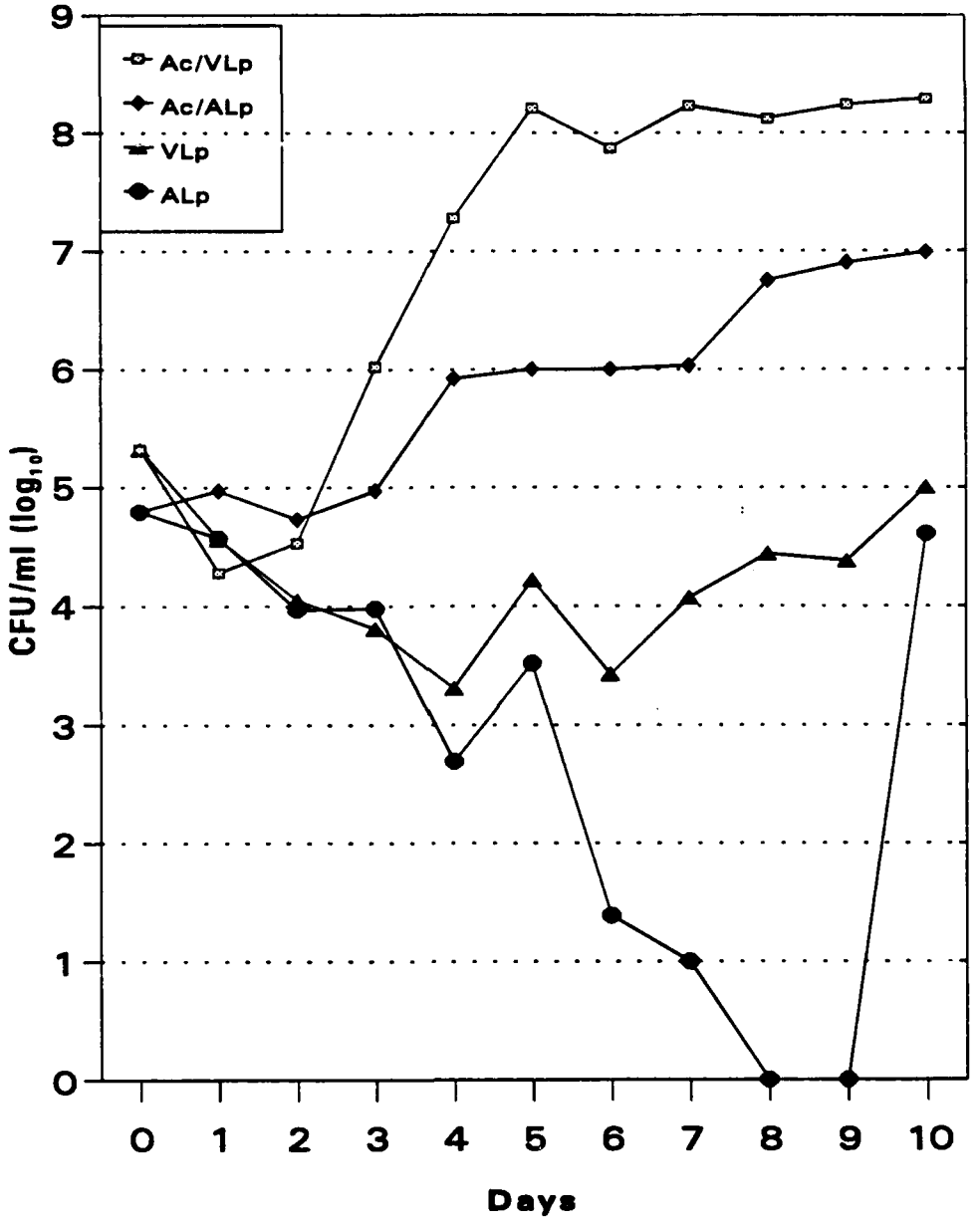


Figure 18. Extention of coculture monitoring period to 10 days.

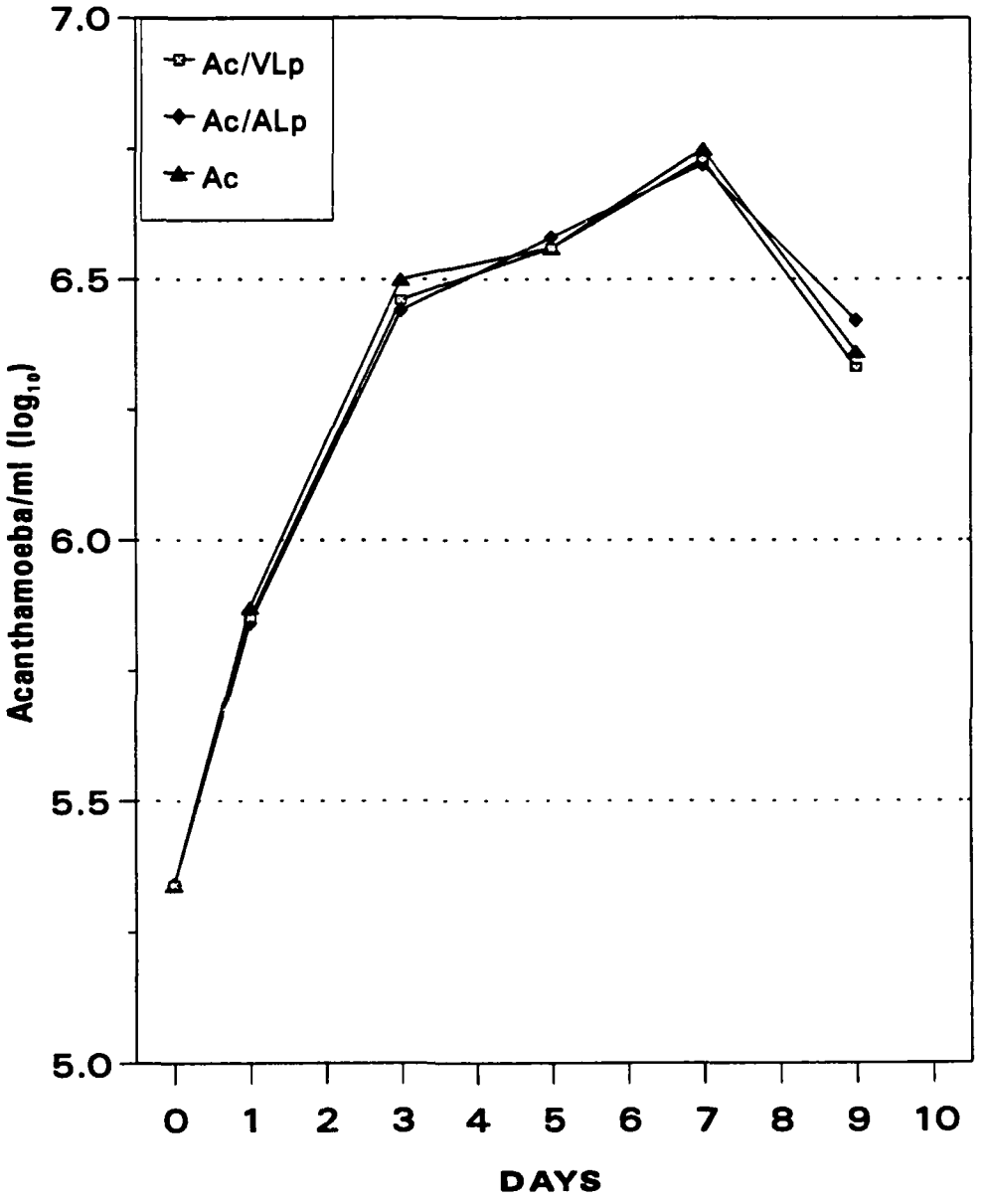


Figure 19. Fate of amoeba in 10 day coculture.

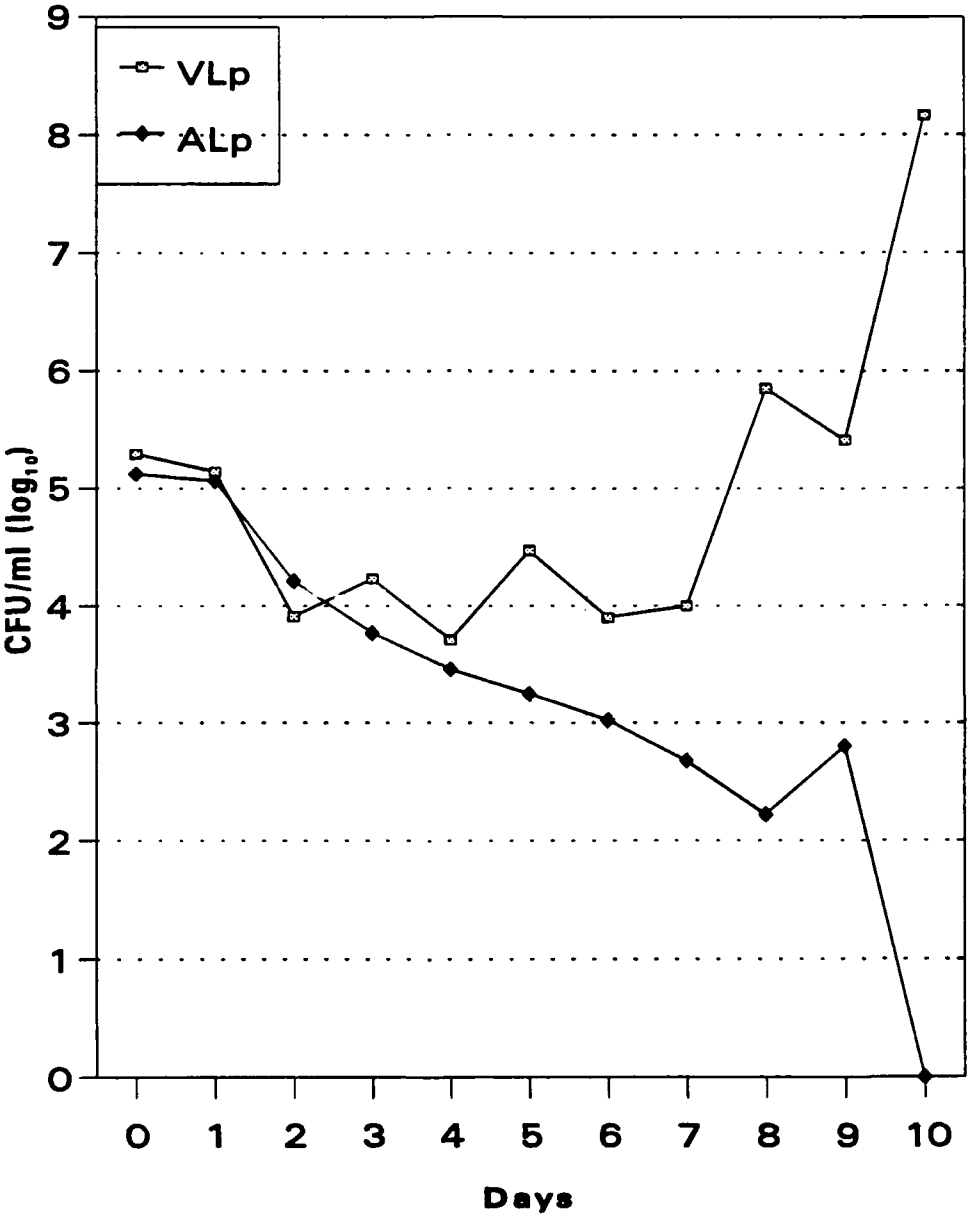


Figure 20. Ten day amoeba-free ALp and VLp culture.

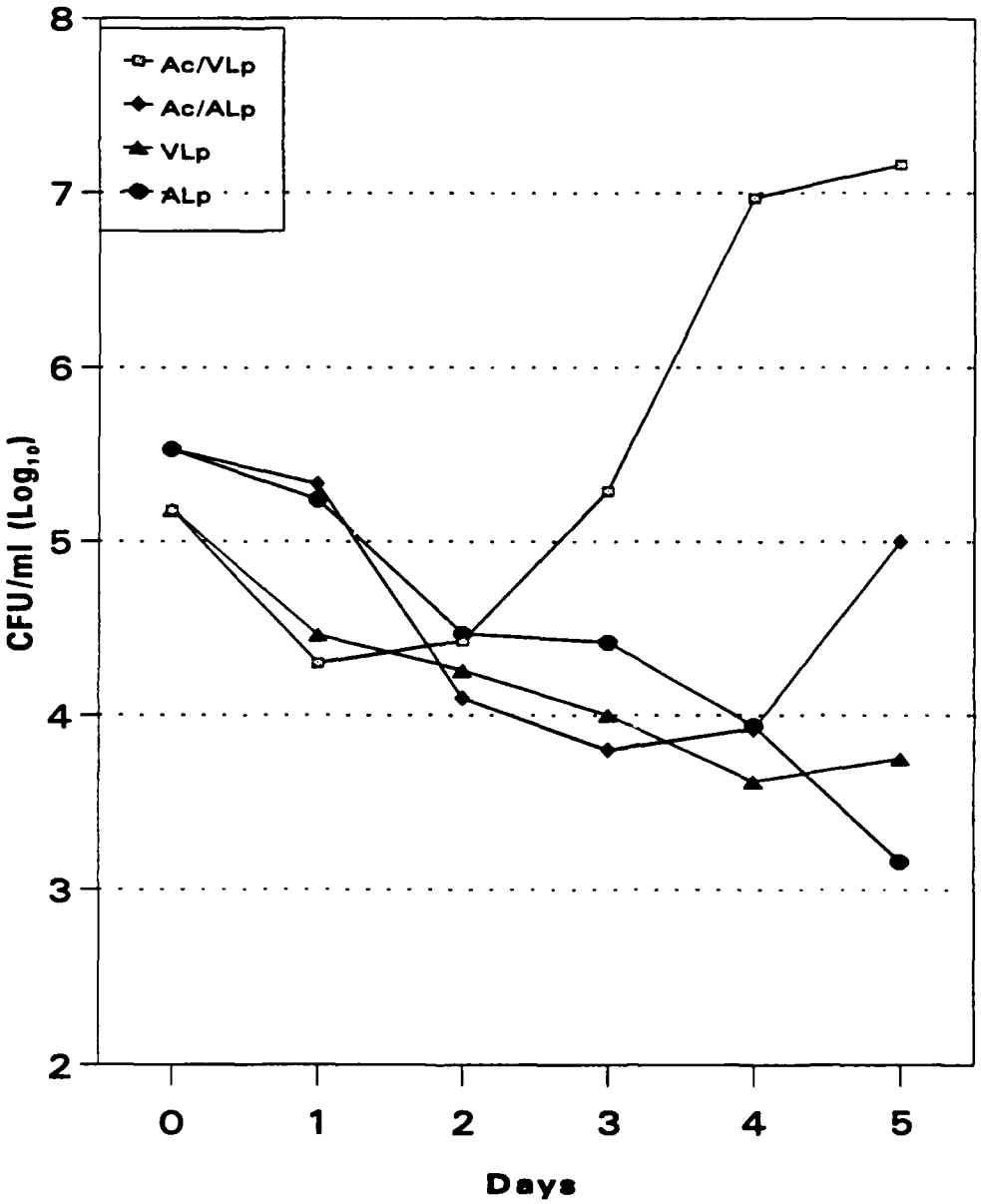


Figure 21. Five day ALp and VLp coculture with their corresponding controls.

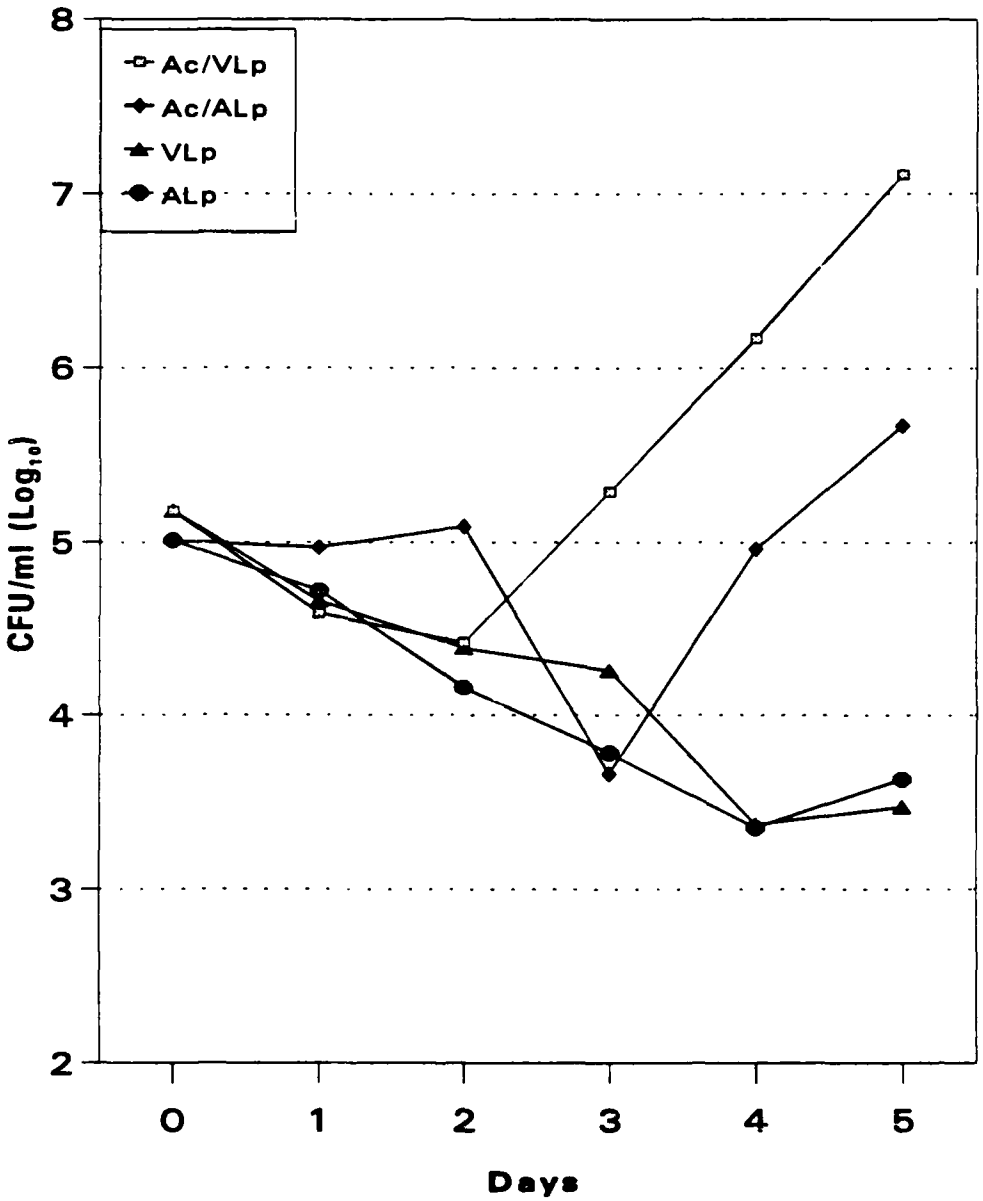


Figure 22. Repeat 5 day ALp and VLp coculture with their corresponding controls.

Multiplicity of Infection

An investigation was conducted which examined the effects of increasing the *Legionella* inoculum size while keeping amoeba numbers at the same level. Here, VLp was inoculated at the usual MOI of 1 bacterium to 2 amoebas (0.5:1). Additionally, *Legionella* inoculums were increased by approximately 100 time resulting in an MOI of 50:1. At the lower MOI of 0.5:1 (Fig. 23), VLp in coculture increased by day 5 in a typical fashion. ALp numbers steadily decreased by day 5. At the higher MOI, VLp numbers increased less than 1 log by day 5. Alternately, ALp numbers rose initially from days 1 to 3 and decreased thereafter through day 5. Again, less than a log increase was noted. The corresponding ALp and VLp controls exhibited a steady decline in numbers by day 5. In examining amoeba growth (Fig. 24) the results are fairly unremarkable for all conditions except for those in colculture with VLp at a MOI of 50:1. Here, a rapid reduction in numbers of amoeba is noted by day 3. At day 5 amoebas are seen to be at a level 3 logs lower than the other conditions.

Filtration Treatment of ALp

Three separate experiments were carried out which examined possible effects on ALp virulence by adjusting bacterial size to that which approximated typical VLp. In all three experiments (Figs. 25, 26 & 27)) the corresponding controls reacted in basically the same fashion, except for a slight increase in VLp numbers in one study. In each case the VLp in coculture increased in number by approx. 2 logs growth by day 5. The filtered ALp (ALp5) and

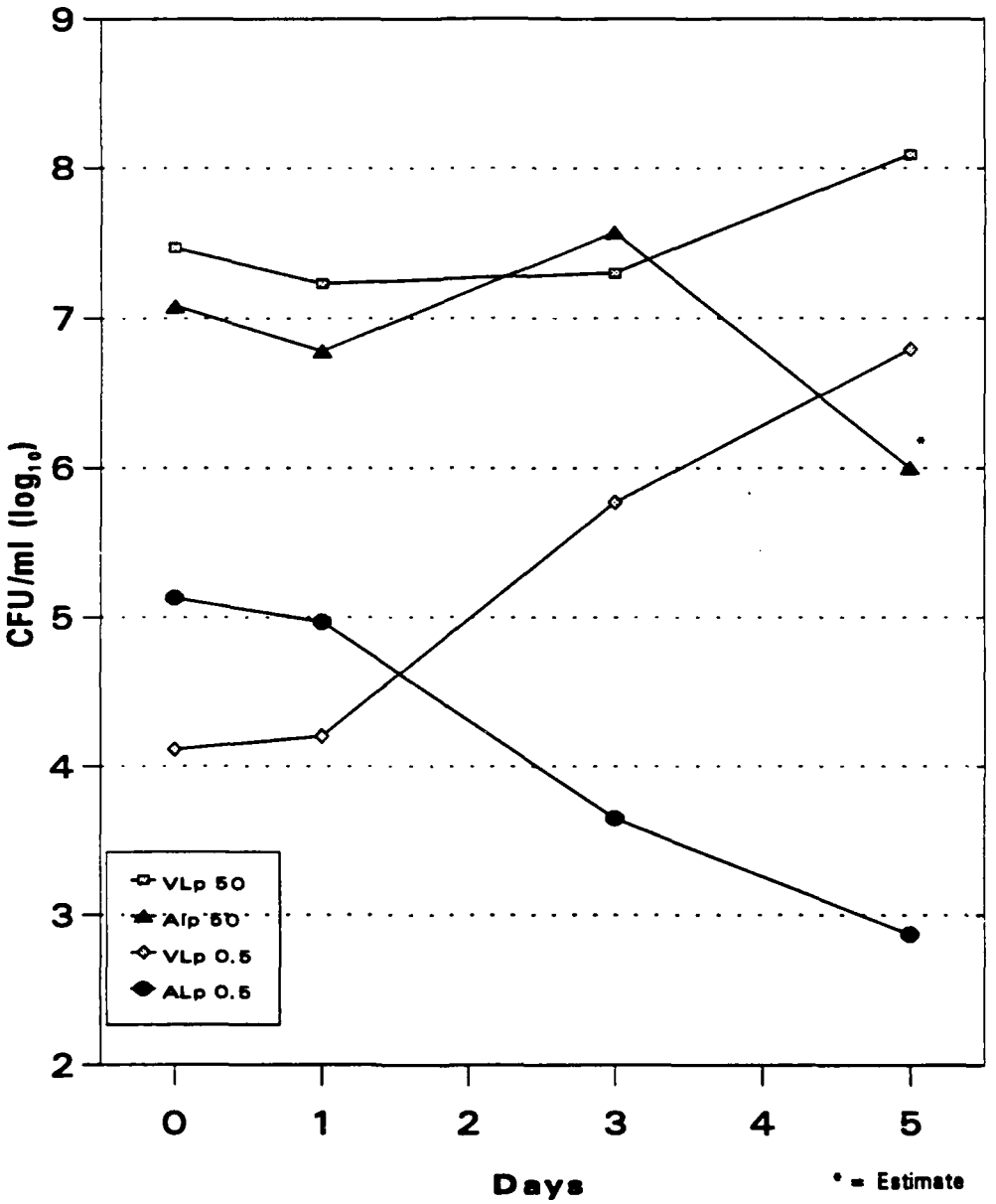


Figure 23. Multiplicity of infection (MOI) study. ALp or VLp were inoculated into amoeba at an MOI of 50:1 (VLp50 & ALp50) or at an MOI of 0.5:1 (VLp0.5 & ALp0.5).

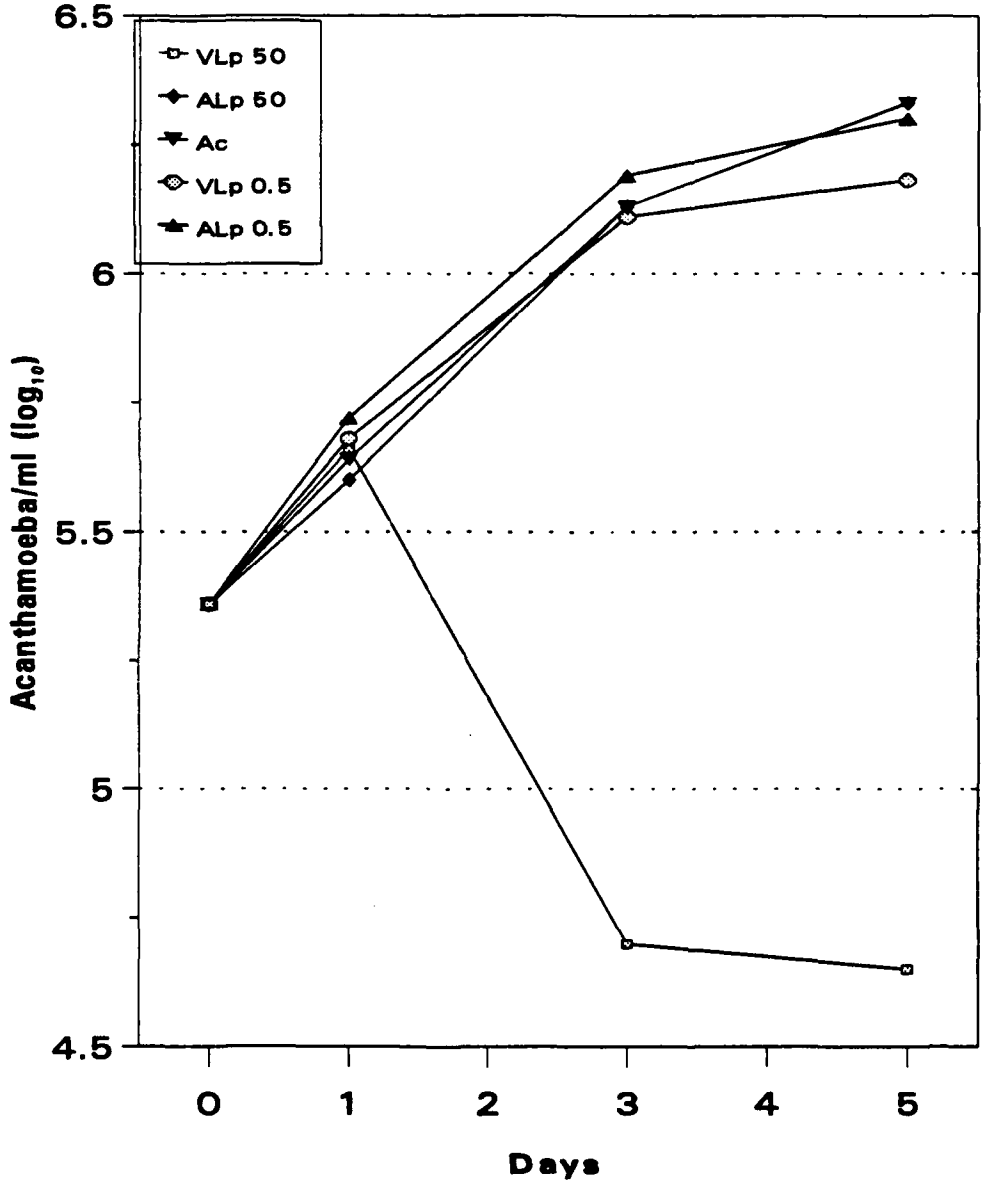


Figure 24. Effect of increasing ALp and VLp MOI on amoebal growth.

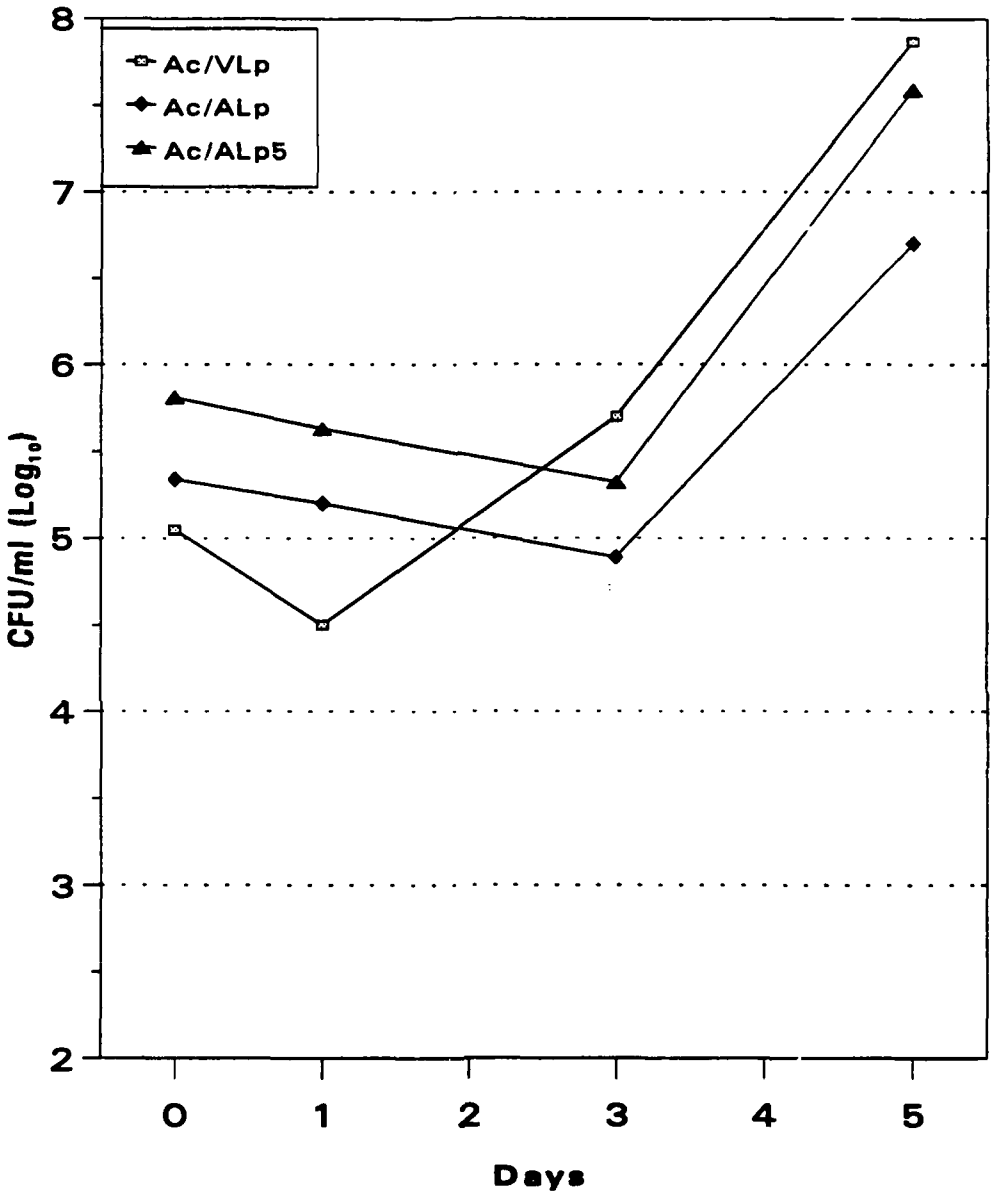


Figure 25. First coculture utilizing ALp filtrate (ALp5).

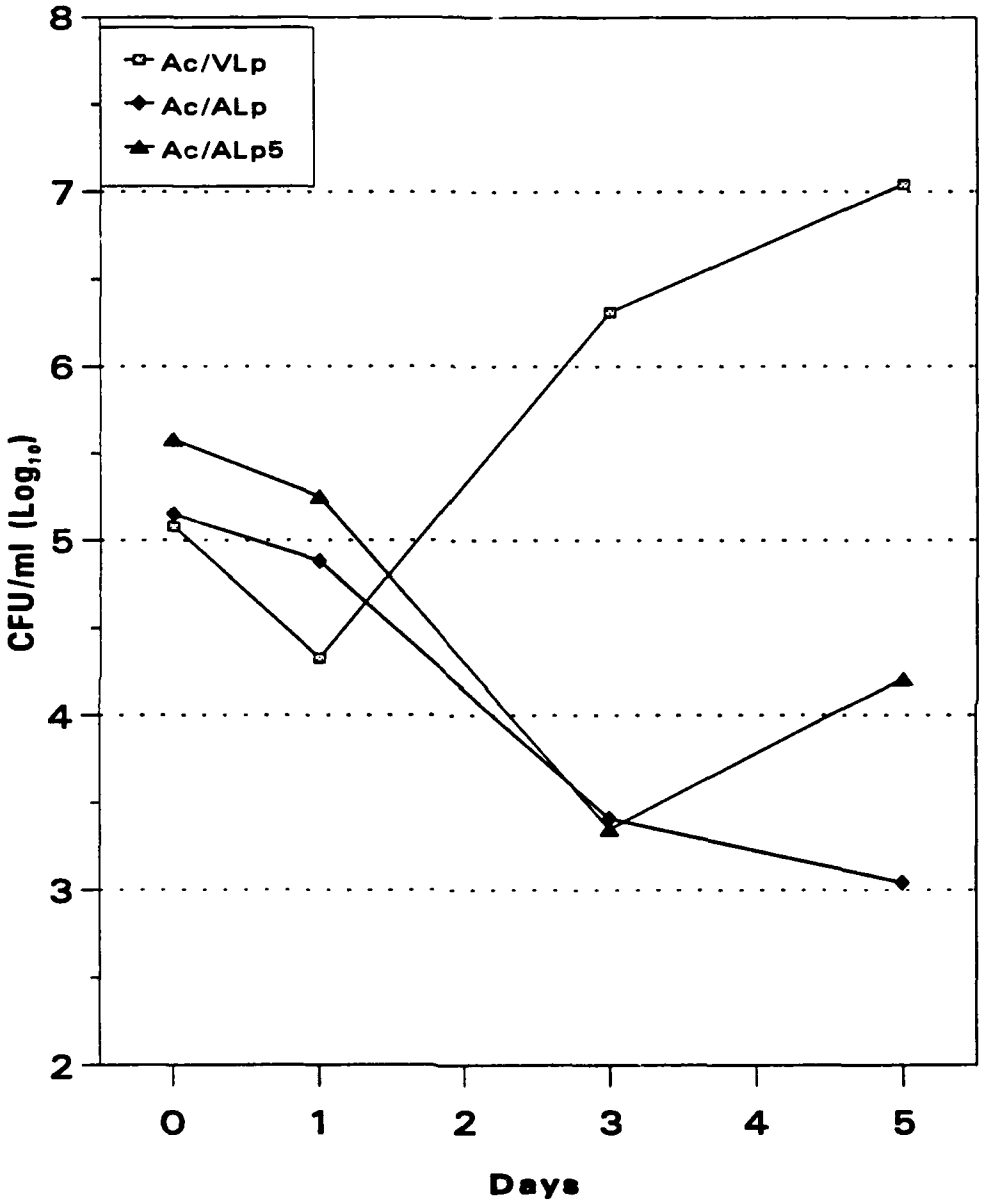


Figure 26. Second coculture utilizing ALp filtrate.

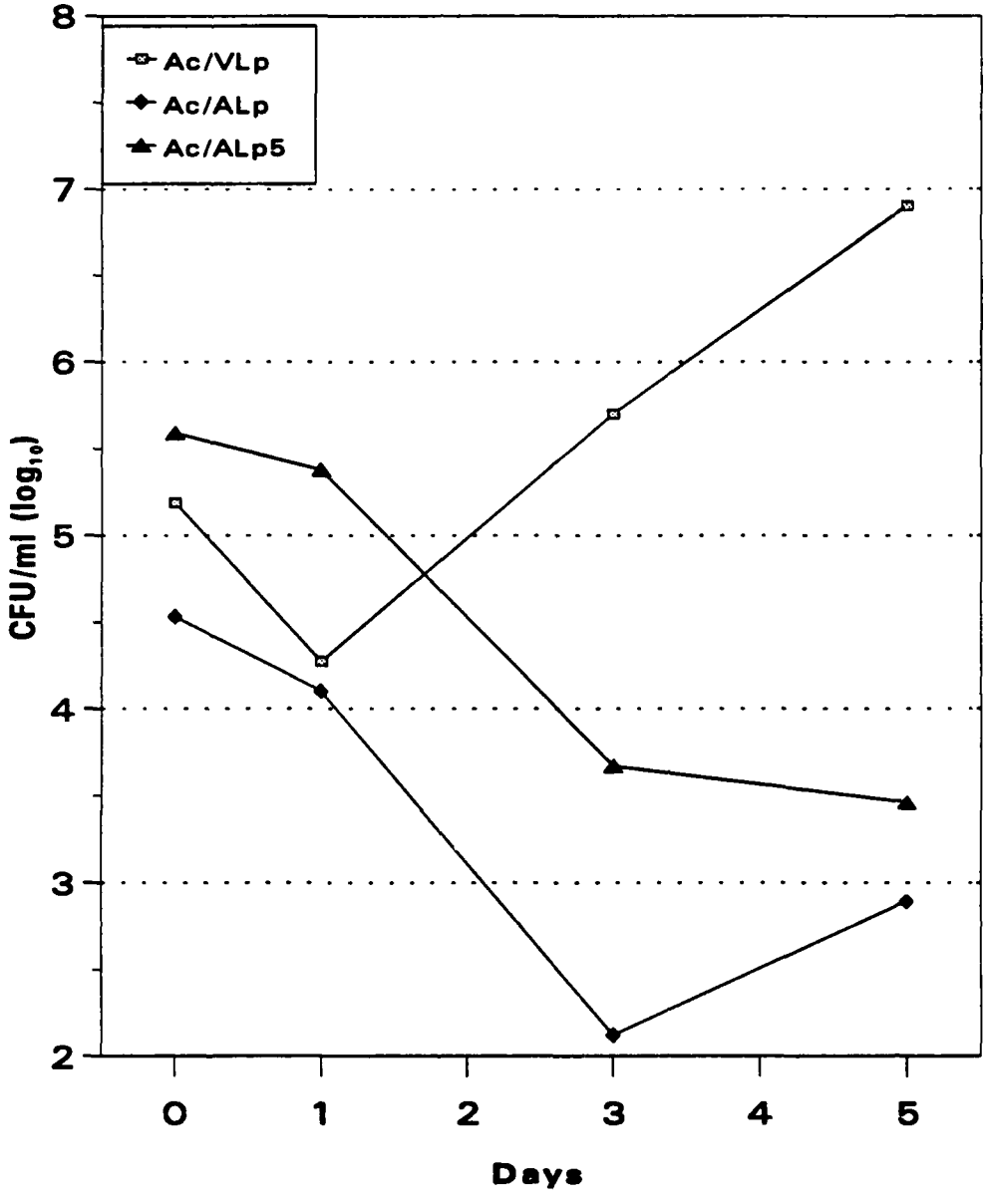


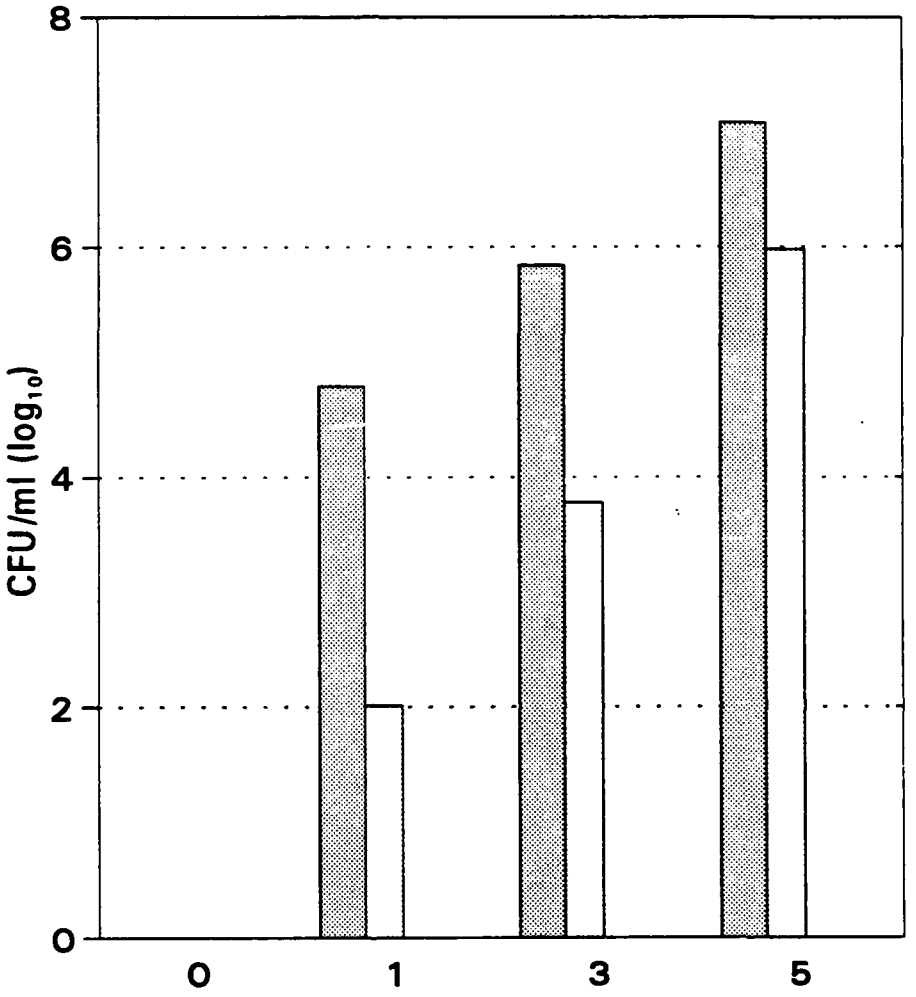
Figure 27. Third coculture utilizing ALp filtrate.

unfiltered ALp demonstrated parallel rises or declines in numbers through day 5. Corresponding amoeba counts indicated an increase of approximately 1 log in all monitored conditions by day 5 of incubation.

2 h Gentamicin Treatment

A preliminary time study was conducted in which cocultures were subjected to a gentamicin pre-treatment and sampled at various days of incubation. Both the extracellular fraction and total bacterial numbers were determined. Here, total VLp numbers (Fig. 28) increased by day 5, after the initial dip at 24 h as seen in previous studies . The extracellular VLp numbers fell approximately 3 logs by 24 h, but rebounded by almost 4 logs growth by day 5. Viable bacteria could not be recovered from either the total or extracellular ALp cocultures. The amount of extracellular VLp (Fig. 29) steadily increased from days 1 to 5.

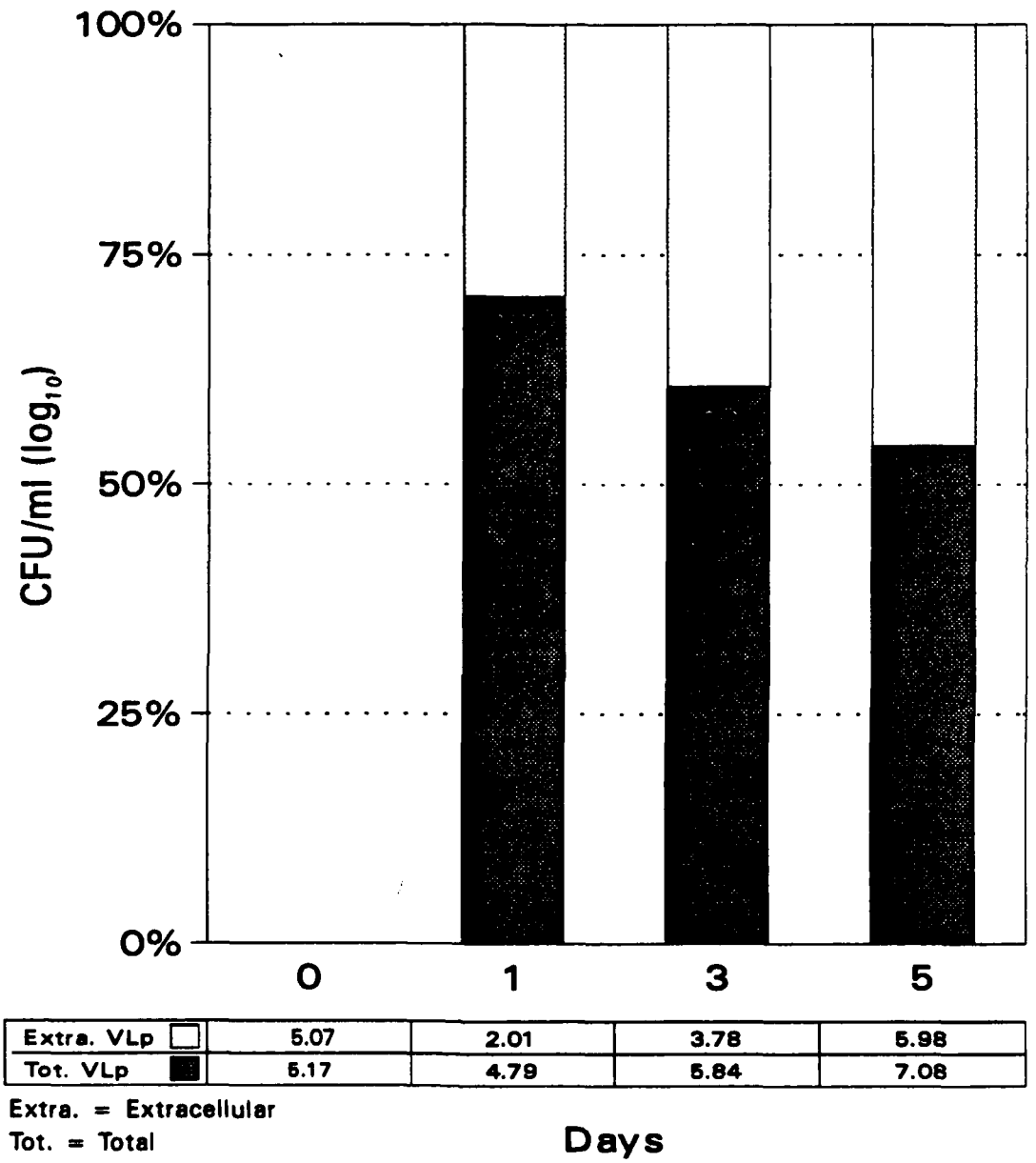
As a result a further time study was initiated to determine the short term localization of VLp and ALp in coculture. In this study, sampling was conducted at 1, 2, 4 and 24 h. Total VLp recovered from cocultures (Fig. 30) remainedconsistent at 1, 2 and 4 h and increased slightly by 24 h. Alternately, total ALp numbers decreased by less than 1 log throughout the same time periods. External VLp numbers (Fig. 31) were seen to decrease approximately 1 log by 24 h. When examining internalized *Legionella*, the VLp coculture (Fig. 32) displayed approximately a 5 log increase in recovered bacterial numbers by 24 h incubation. As seen previously, viable ALp could not be recovered during the sampling periods. The following observations were made in examining



	0	1	3	5
Tot. VLp	5.17	4.79	5.84	7.08
Extra. VLp	5.07	2.01	3.78	5.98
Tot. ALp	5.10	0	0	0
Extra. ALp	5.18	0	0	0

Tot. = Total
Extra. = Extracellular

Figure 28. Two hour gentamicin pre-treatment on ALp and VLp growth in coculture.



Extra. VLp	5.07	2.01	3.78	5.98
Tot. VLp	5.17	4.79	5.84	7.08

Extra. = Extracellular
Tot. = Total

Figure 29. VLp location in 5 day coculture.

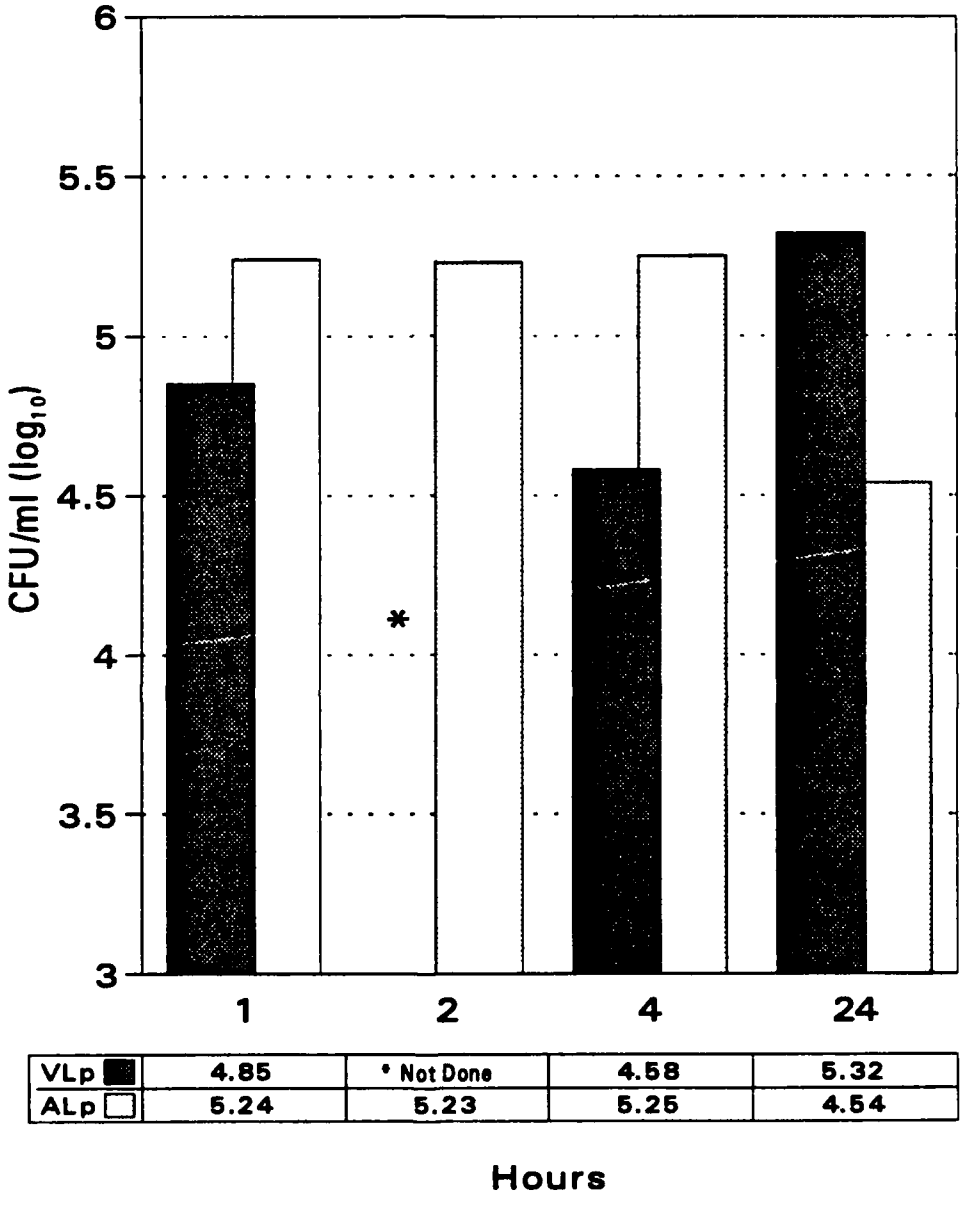


Figure 30. Total VLp and ALp recovered in 24 h coculture.

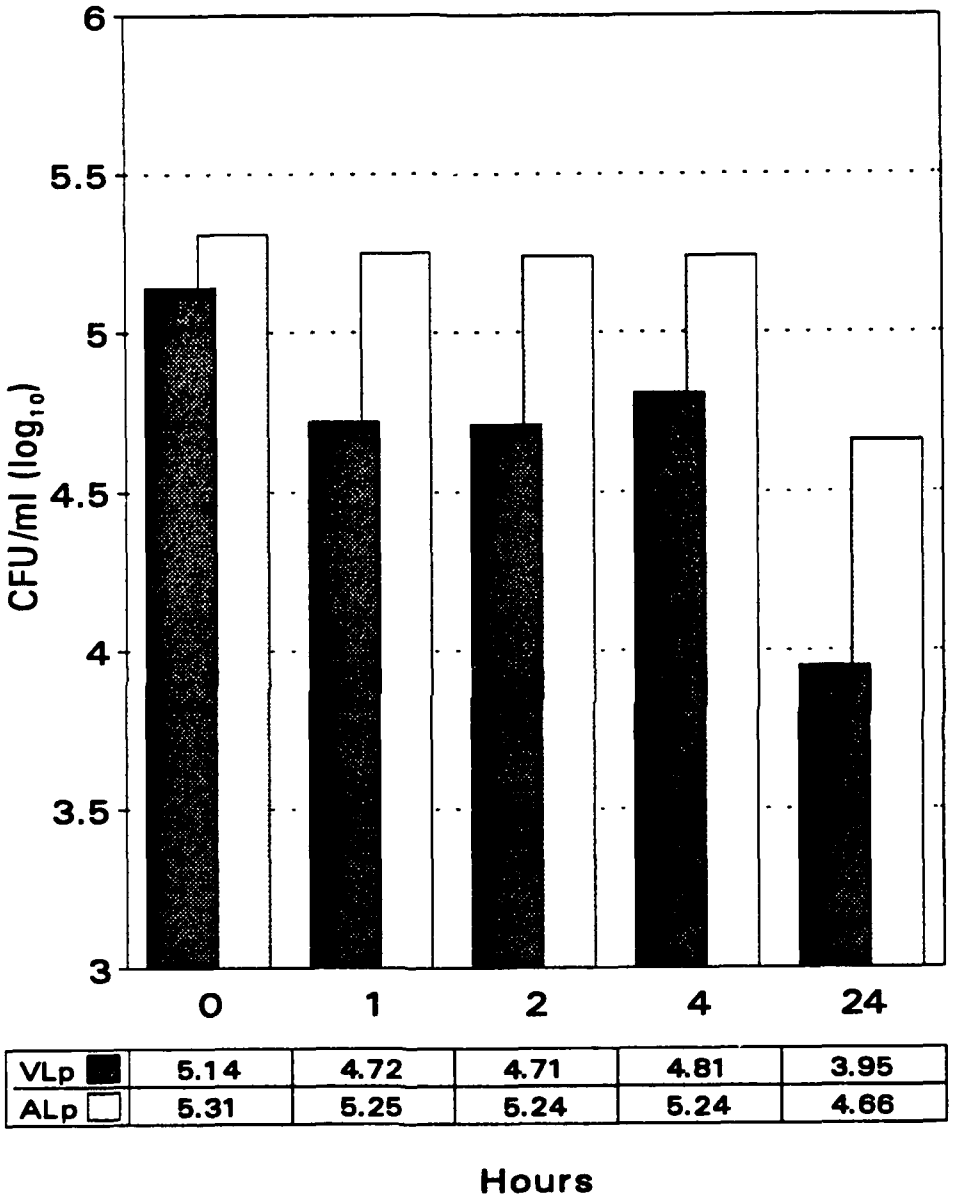
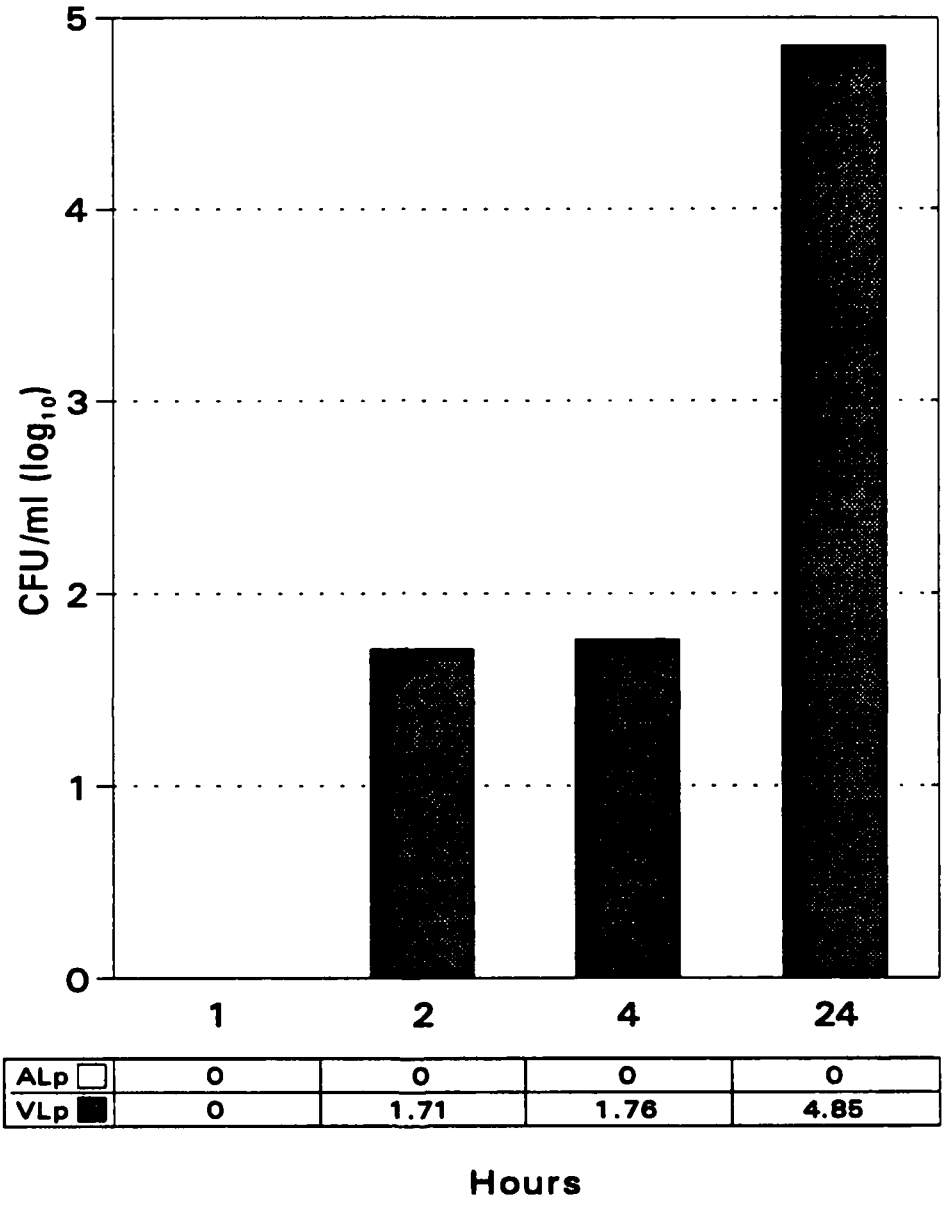


Figure 31. External VLp and ALp recovered in 24 h coculture.



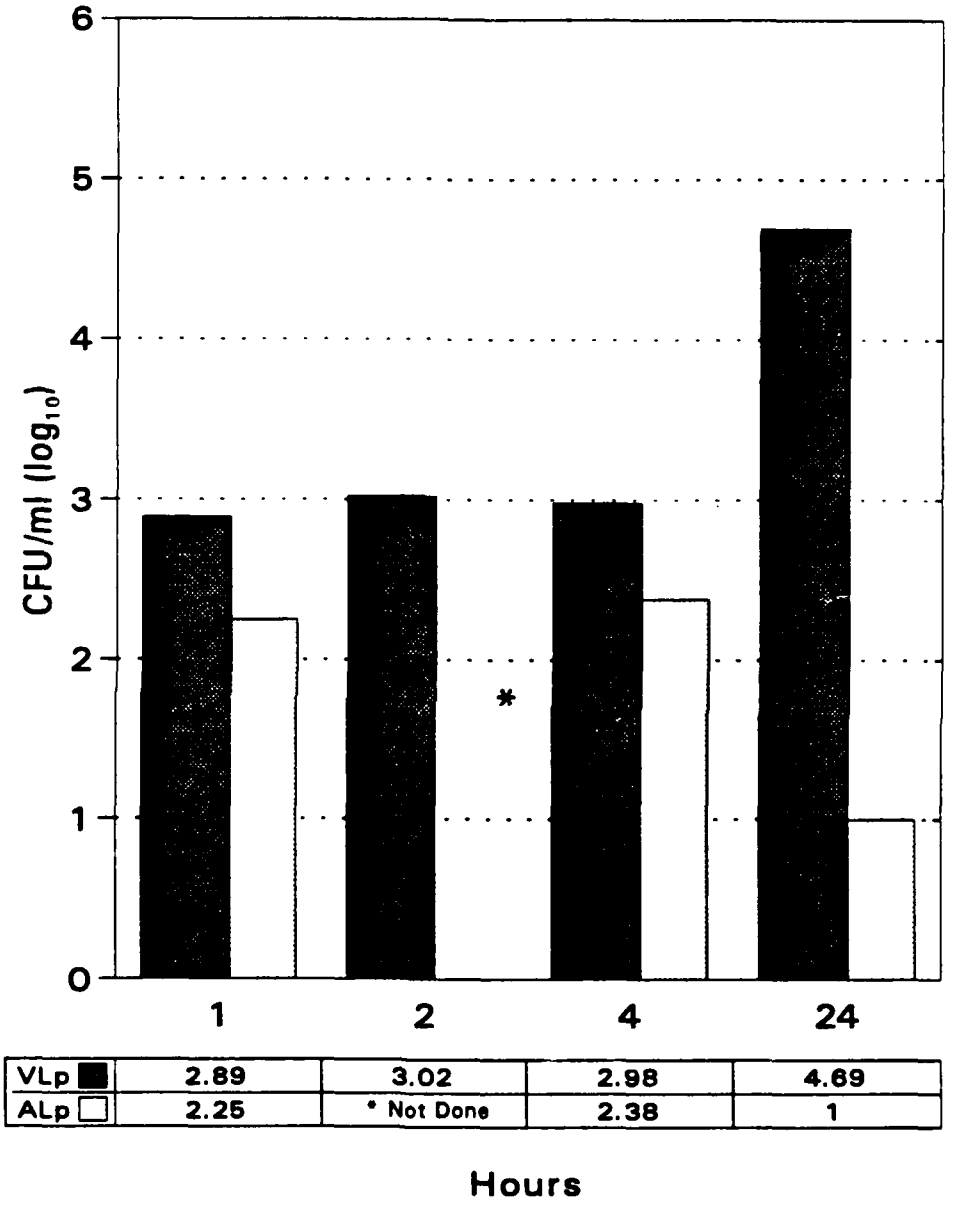
ALp □	0	0	0	0
VLp ■	0	1.71	1.76	4.85

Figure 32. Internalized VLp recovered in 24 h coculture.

Legionella association with amoeba. VLp association (Fig. 33) remained constant at approximately 3 logs at 1, 2, and 4 h, but increased to 4.7 logs by 24 h incubation. The ALp coculture demonstrated a similar association number at 1 and 4 h but decreased approximately 1 log by 24 h. The corresponding control flasks both revealed a decrease of less than 1 log by 24 h incubation. Since the previous time study had indicated the largest increase in internalized VLp numbers occurred between 4 and 24 h, further refinements of sampling time periods were necessary. Two additional time periods were added between 4 and 24 h. This study was also conducted using duplicate flasks in order to check the reproducibilty of sampling from different cocultures under the same conditions. As previously noted, the ALp containing cocultures (Fig. 34) did not allow recovery of viable bacteria during any sampling period. Conversely, cocultures containing VLp demonstrated a consistent increase in internalized bacteria by 24 h. Recovered VLp numbers from duplicate coculture flasks indicates that the sampling process is reproducible at each time period. Fluorescent anti-*Legionella* antibody staining of both ALp and VLp cocultures reveals that both bacteria associate with the amoeba but possibly in differing numbers. Vlp amoeba association (Table 2) increases from 35% at 30 min to 87% at 4 h. Alternately during the same time periods Alp association remains between 34% to 37.5%.

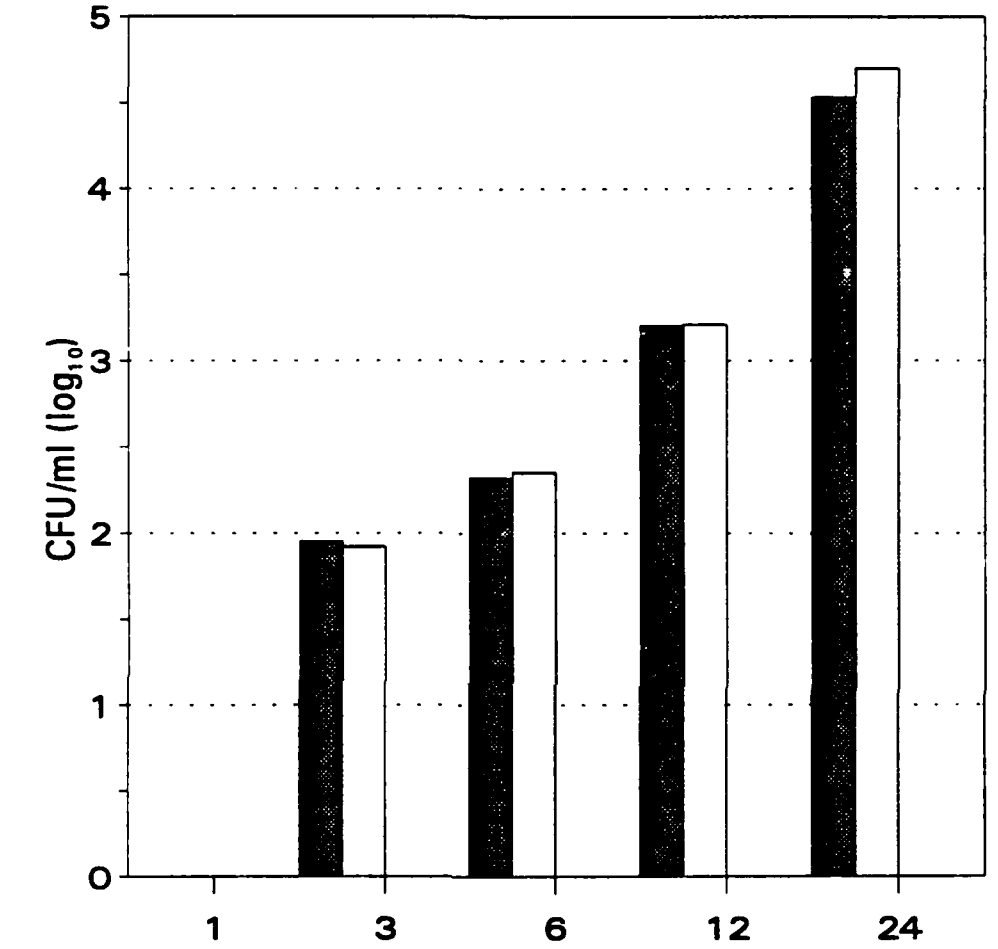
Passage Results

The ALp coculture initiated on day 0 could not be passed at day 5 due to an inability to recover viable bacteria. Alternately, the VLp coculture (Fig. 35)



VLp ■	2.89	3.02	2.98	4.69
ALp □	2.25	* Not Done	2.38	1

Figure 33. Association of VLp and ALp with amoebas in 24 h coculture.



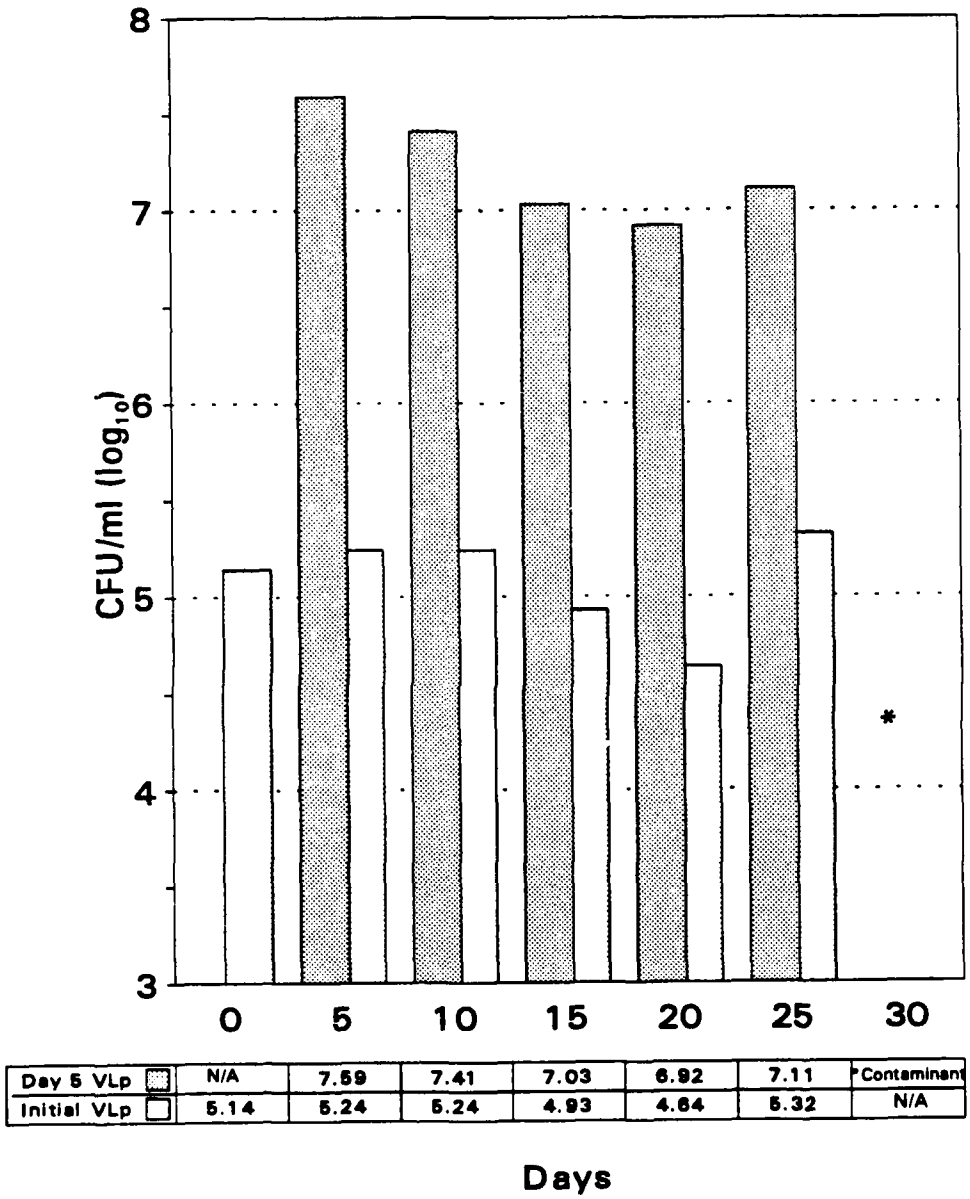
VLP1 ■	0	1.95	2.32	3.2	4.53
VLp2 □	0	1.92	2.35	3.21	4.7
ALp1 □	0	0	0	0	0
ALp2 ■	0	0	0	0	0

Figure 34. Twenty-four hour coculture of internalized VLp and ALp utilizing duplicate flasks.

Table 2. Examination of ALp and VLp association with amoeba using a fluorescent monoclonal anti-*Legionella* antibody.

Hour	Ac/VLp	Ac/Alp
0.5	35	37
1.0	64	34
4.0	87.5	34.5

Numbers represent % of amoeba demonstrating ALp or VLp association.



Day 5 VLp	N/A	7.59	7.41	7.03	6.92	7.11	*Contaminant
Initial VLp	5.14	5.24	5.24	4.93	4.64	5.32	N/A

Figure 35. Thirty day passage experiment in which begining and ending VLp numbers were determined at 5 day intervals.

demonstrated approximately a 2 log increase in recovered bacteria by day 5 of incubation. This same growth pattern reoccurred throughout the fifth passage on day 25. Unfortunately, the day 30 total VLp coculture was contaminated, although similar results could be anticipated based upon the previous five passages. Amoebal growth was seen to occur in each 5 day cycle, resulting in approximately a 1 to 2 log increase as previously noted.

EJ1 Clinical *Legionella* Isolate

A virulence potential assessment of the EJ1 clinical isolate with amoeba resulted in the following observations. Both ALp and VLp cocultures were also initiated and monitored in addition to EJ1 cocultures. VLp in coculture (Fig. 36) exhibited its typical growth pattern yet increased at a more rapid rate than previously noted. The ALp coculture demonstrated rapid growth increases resulting in a similar bacterial number by day 7. No apparent lag phase was noted for ALp cocultures. The EJ1 coculture showed a slight decrease at 24 h with a subsequent rate of increase paralleling that of the VLp coculture. All three coculture conditions revealed peak bacterial numbers between days 5 and 7. A slight decrease in bacterial numbers was seen at day 7 in the ALp and EJ1 cocultures. The corresponding control flasks demonstrated different reactions. Both the VLp and EJ1 controls (Fig. 37) increased in bacterial numbers from days 1 to 7. The EJ1 control did so a slower rate which was not as dramatic as the VLp control. Conversely, the ALp control dropped off rapidly by day 5, but rebounded slightly between days 5 and 7. Corresponding amoeba counts (Fig. 38)

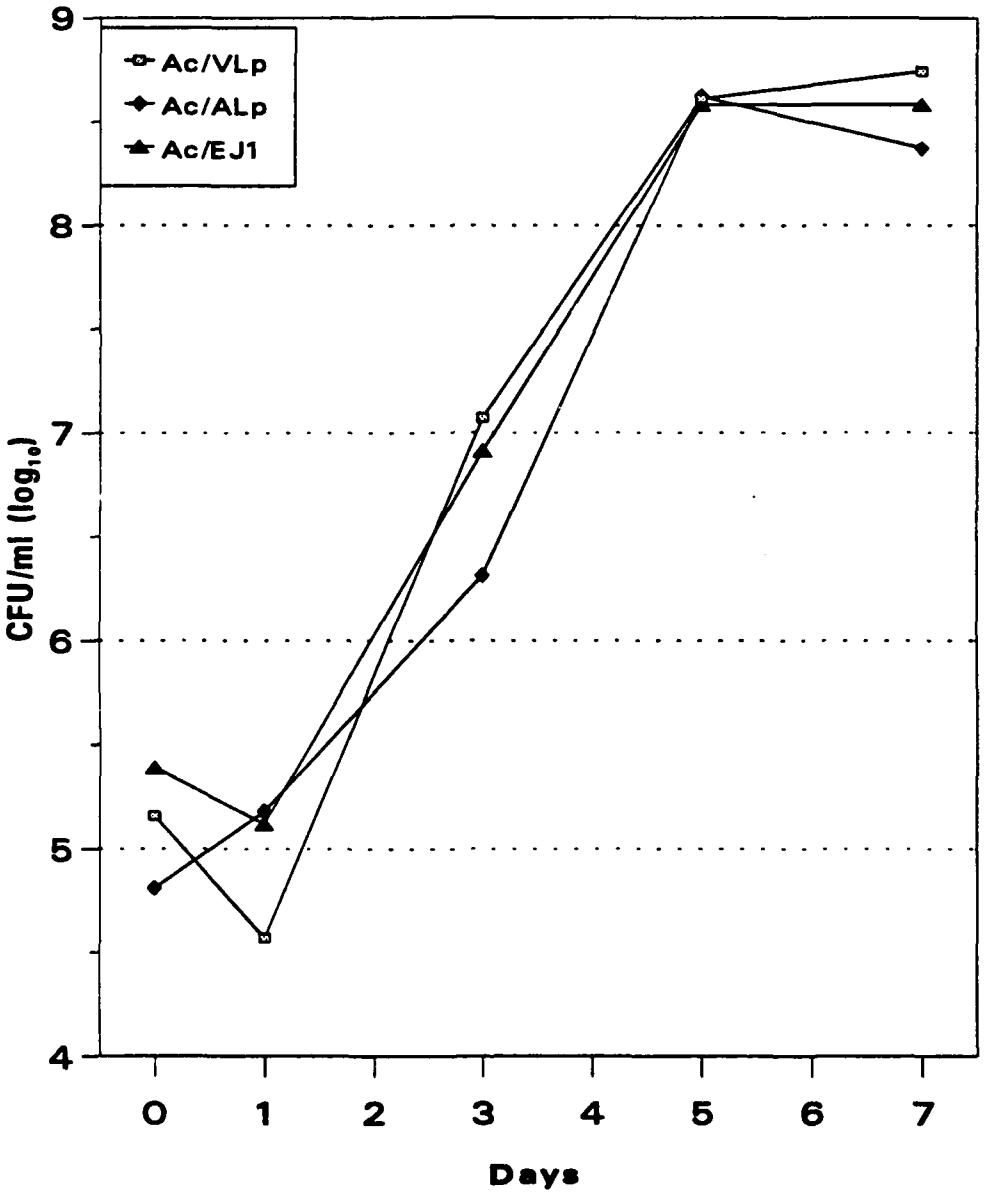


Figure 36. Seven day coculture utilizing a clinical *Legionella* isolate (EJ1).

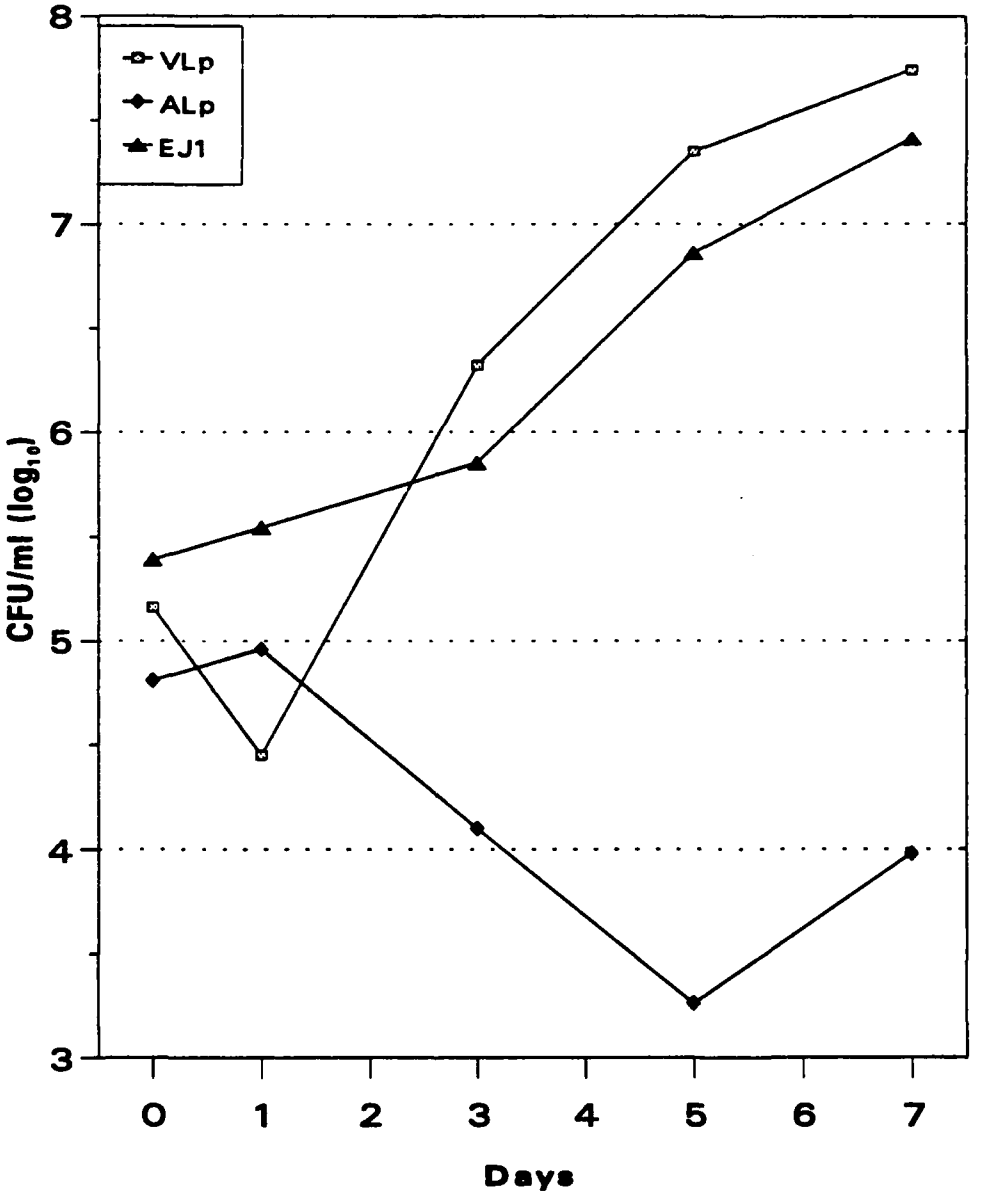


Figure 37. Corresponding ALp, VLp and patient (EJ1) controls of 7 day coculture.

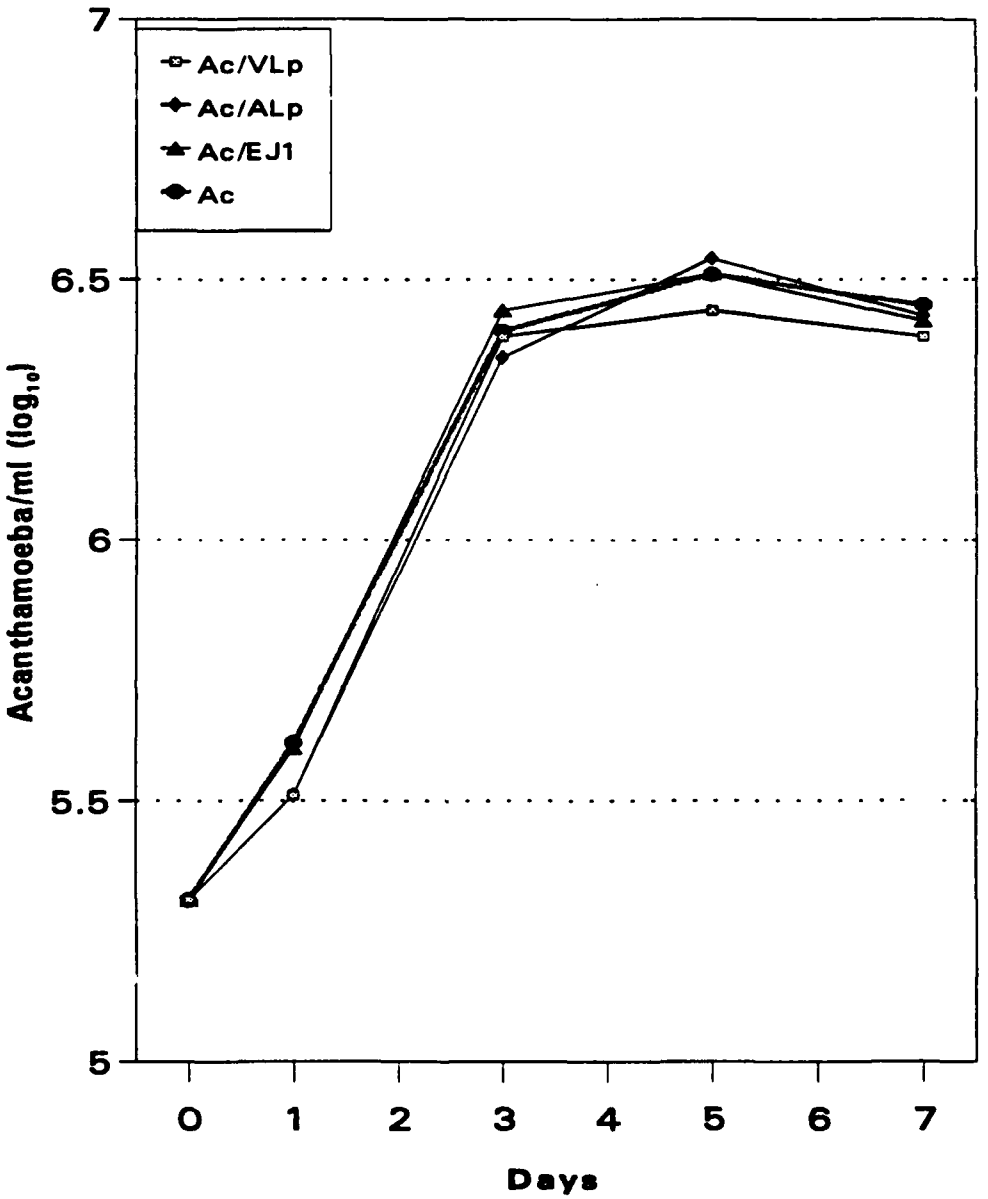


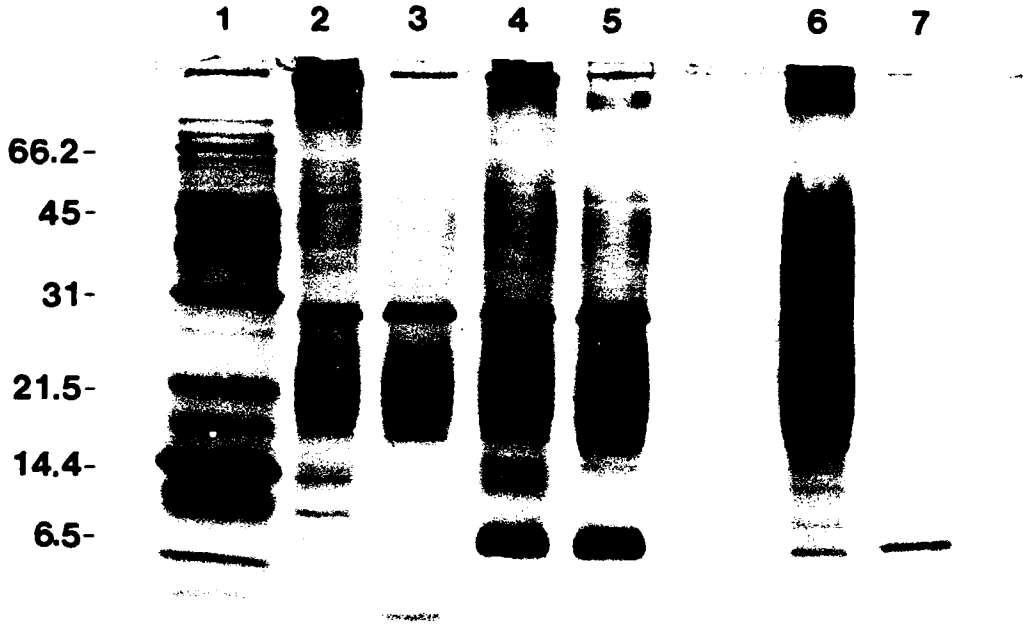
Figure 38. Corresponding amoeba counts of ALp, VLp and EJ1 of 7 day coculture.

demonstrated sustained growth until day 5 and a subsequent plateau at day 7.

SDS-PAGE Results

General Characteristics

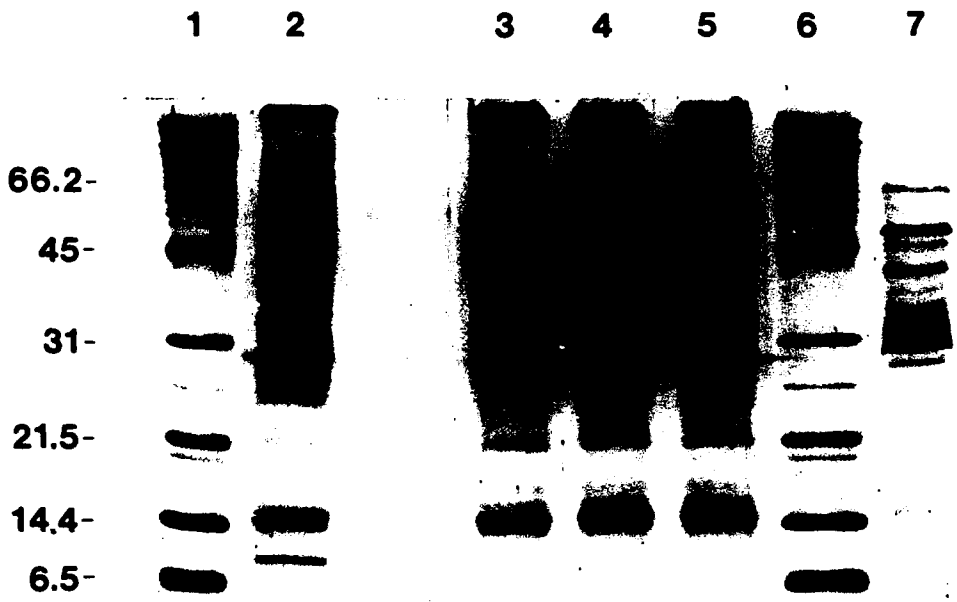
Examination of membrane preparations of each *Legionella* organism using SDS-PAGE reveals several differences in each strain. When a sliver stain is used, both total and outer membrane preparations of ALp demonstrate a broad, intensely staining band (Fig. 39) slightly below the 6.5 kDa molecular weight marker. A corresponding band is not readily discernable in the VLp lanes or EJ1, although a slight band can be seen. Molecular weights were determined by plotting log molecular weight v. relative migration. The total membrane preparations of ALp and VLp appear quite similar, except for the presence of a small band at 10 kDa. Both the LPS region, 16.5 to 27.5 kDa, and the MOMP, 29 kDa of ALP and VLp are identical in banding patterns. Similarly, the outer membrane preparations of VLp and ALp are basically the same, except for the aforementioned ~6.5 kDa broad band exhibited by ALp. The EJ1 clinical isolate appears very similar to VLp, except that the MOMP is located at 30.2 kDa. An intensely staining band appears at 27.4 kDa which is not readily apparent in either ALp or VLp. The mid-to-high molecular weight regions, >30 kDa, do not exhibit differences in banding patterns. Coomasie-blue stained gels (Fig 40), run in tandem with silver stained gels, did not demonstrate the broad band seen at ~6.5 kDa in the ALp organism. However, the 27.4 kDa band seen in EJ1 was barely



Lane Assignments

1& 7. Molecular weight markers
2. VLp Total membrane
3. VLp Outer membrane
4. ALp Total membrane
5. ALp Outer membrane
6. EJ1 Outer membrane

Figure 39. Silver-stained SDS-PAGE gel (13%) demonstrating total and outer membrane preperation of ALP and VLp with outer membrane preperation of EJ1. Note the appearance of an ALp band slightly below 6.5 kDa.



Lane Assignments

1&6. Molecular weight markers
2. VLp Total membrane
3. ALp Total membrane
4. ALp Total membrane
5. ALp Total membrane
7. EJ1 Outer membrane

Figure 40. Coomassie-blue stain SDS-PAGE gel (13%) to demonstrate total and outer membrane preperation of ALP and VLp with outer membrane preperation of EJ1.

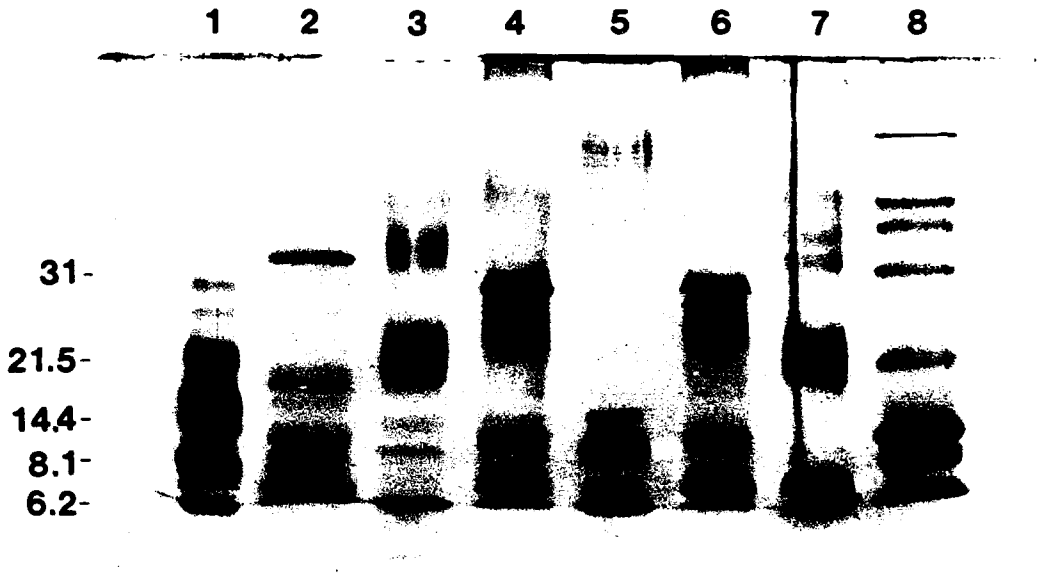
detectable in ALp and VLp when gels are loaded at similar protein concentrations.

Proteinase K Digests

Silver stained outer membrane preparations (Fig. 41) showed proteinase-K digestion to completely remove the MOMP in both ALp and VLp. However, during this process the ALp low molecular weight band of ~6.5 kDa remained intact. The ability to detect completeness of the MOMP digestion appears to differ when periodate oxidation is substituted for the normal Bio-Rad potassium dichromate/nitric acid oxidizer. Here, carbohydrates (Fig. 42) are better demonstrated in the *Salmonella* control at the same sample load concentration. A major drawback to this substitution is the inability to pick-up the MOMP, except as a negatively-stained band. Furthermore, molecular weight markers do not appear to stain well at comparable sample loads, as they do with the Bio-Rad oxidizer.

Passage Gels

Both silver and Coomassie-blue stained gels (Figs. 43 & 44) demonstrated no apparent changes in the outer membrane preparations, during any time period. However, there did appear to be a noticeable difference in the amount of MOMP seen in the 20 day sample when compared to other sampling times.



Lane Assignments

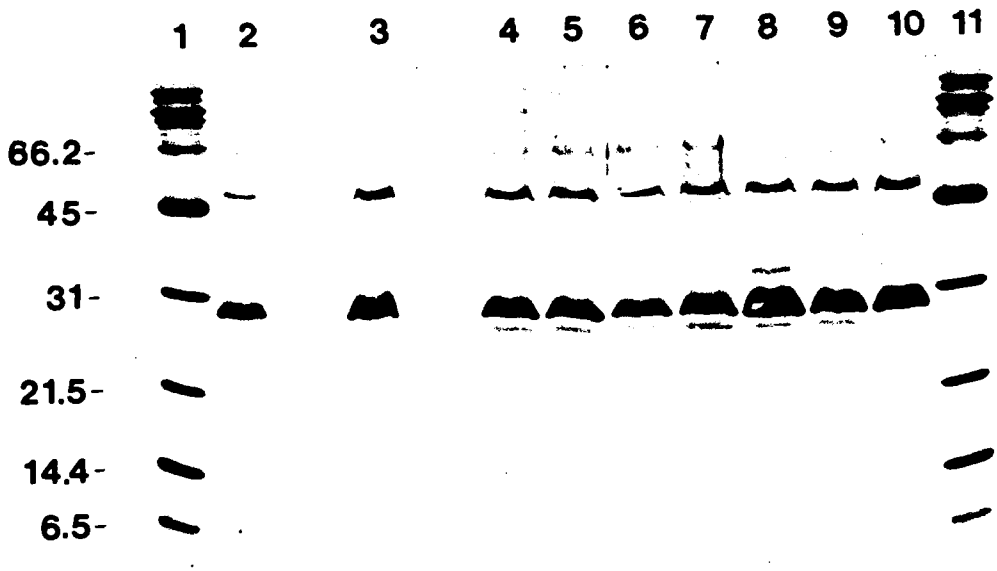
1&8. Molecular weight markers
2. Proteinase-K control
3. Proteinase-K treated VLp Outer membrane
4. VLp Outer membrane
5. *Samonella minnesota* LPS
6. ALp Outer membrane
7. Proteinase-K treated ALp Outer membrane

Figure 41. SDS-PAGE gel (12%) using dichromate oxidation to demonstrate proteinase K digestion of VLp and ALp outer membrane preperations. Note the presence of the ~6.5 kDa band still present in ALp after digestion procedure.

Lane Assignments

1&8. Molecular weight markers
2. Proteinase-K control
3. Proteinase-K treated VLp Outer membrane
4. VLp Outer membrane
5. *Samonella minnesota* LPS
6. ALp Outer membrane
7. Proteinase-K treated ALp Outer membrane

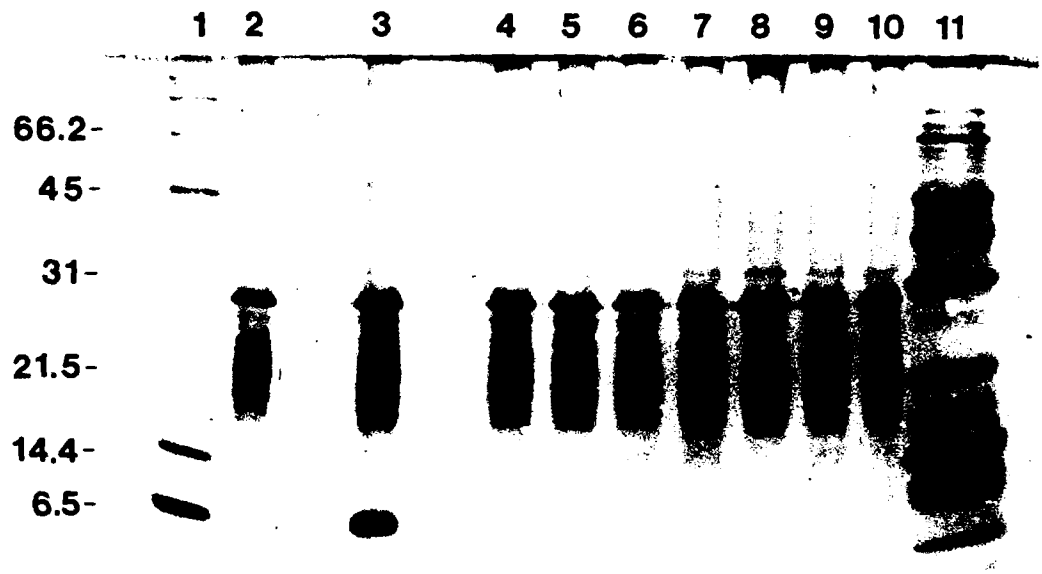
Figure 42. SDS-PAGE gel (12%) using periodate oxidation to demonstrate proteinase K digestion of VLp and ALp outer membrane preperations. Note the presence of the ~6.5 kDa band still present in ALp after digestion procedure.



Lane Assignments

1&11. Molecular weight markers
2. VLp Outer membrane
3. ALp Outer membrane
4. 24h VLp Outer membrane
5. Day 5 VLp Outer membrane
6. Day 10 VLp Outer membrane
7. Day 15 VLp Outer membrane
8. Day 20 VLp Outer membrane
9. Day 25 VLp Outer membrane
10. Day 30 VLp Outer membrane

Figure 43. Coomassie-blue stain of SDS-PAGE gel (13%) demonstrating VLp outer membrane preperations recovered during passage experiment.



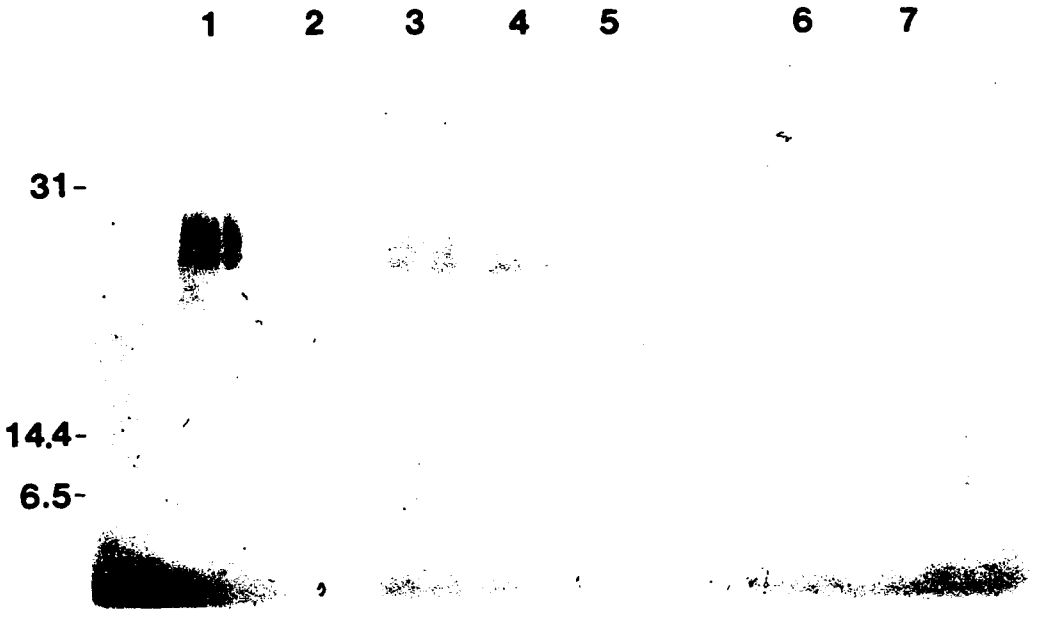
Lane Assignments

1&11. Molecular weight markers
2. VLp Outer membrane
3. ALp Outer membrane
4. 24h VLp Outer membrane
5. Day 5 VLp Outer membrane
6. Day 10 VLp Outer membrane
7. Day 15 VLp Outer membrane
8. Day 20 VLp Outer membrane
9. Day 25 VLp Outer membrane
10. Day 30 VLp Outer membrane

Figure 44. Silver stain of SDS-PAGE gel (13%) demonstrating VLp outer membrane preperations recovered during passage experiment.

PAS Gel

Examination of total membrane preparations by PAS staining (Fig. 45) reveals a positively-stained band at ~6.5 kDa in all three lanes of varying concentrations of ALp. Neither the VLp total membrane preparation nor the EJ1 outer membrane preparation demonstrated a similar band.



Lane Assignments

1&6. Molecular weight markers
2. VLp Total membrane
3. ALp Total membrane
4. ALp Total membrane
5. ALp Total membrane
7. EJ1 Outer membrane

Figure 45. Periodic acid schiff stain of SDS-PAGE gel (13%) demonstrating VLp and ALp total membrane preperations.

DISCUSSION

Establishment of *Legionella* infection is dependent upon the virulence of the organism. VLp is highly virulent and is characterized as a lethal human pathogen. It was repeatedly isolated in Burlington, VT during outbreaks of Legionnaires' disease in 1977 and again in 1980 (83).

The ability of *L. pneumophila* to infect both animal and cell culture models is well established (50). Specifically, the ability of *L. pneumophila* to infect a variety of amoeba hosts with its consequential environmental implications has gained renewed interest. Guinea pigs are the most widely accepted animal model in evaluating *Legionella* virulence (34). The resulting pneumonia closely resembles what is seen in human disease (25). Consequently this model was utilized to confirm previously noted differences in virulence potential between ALp and VLp (Sommers dissert., 1987; Lawrence dissert.,1990). Male Hartley guinea pigs, which received $2.5x10^8$ cfu/ml of VLp by intraperitoneal injection, experienced an overwhelming respiratory infection similar to Legionnaires' disease. This result is quite remarkable considering that organisms used to prepare working stocks were recovered from guinea pig spleens frozen for eight years (-70°C). Alternately, guinea pigs in the ALp group, who received $2.0x10^9$ cfu/ml, survived throughout the duration of the monitoring period. This result suggests that ALp infection is

readily resolved by immunocompetent animals even at an inoculum size approximately 10X greater than those animals receiving VLp. Variation seen in animal reactions to either organism suggests differences in phagocytic clearance mechanisms. Dissimilarities in ALp and VLp attachment, uptake or intracellular survival mechanisms are most likely to account for variations seen in the outcome of guinea pig infection. First, ALp attachment to guinea pig phagocytes may be inhibited due to a missing or altered surface-receptor responsible for active binding to potential host cells. To date, a receptor responsible for initiating *Legionella* uptake by amoeba or human phagocytic cells has not been characterized (50). Secondly, viable ALp may not be actively ingested by guinea pig phagocytes. More likely, the process may occur at a much slower rate. Thirdly, unlike its virulent counterpart, ALp phagosomes appear to be destined for lysozyme fusion (49). Taken alone, these events would be sufficient to preclude intracellular multiplication of ALp. However, in guinea pigs it appears that a combination of these factors are ultimately responsible for the noted variation.

As previously mentioned, VLp were recovered from all guinea pig organs during the current virulence study. However, similar forms were not recovered in the ALp group. In retrospect, the two colonies recovered from differing organs in two guinea pigs were probably ALp. If true, this result would indicate that certain ALp forms did establish a low grade infection in the tested guinea pigs. Given sufficient time, this infection seed may result in febrile illness, or possibly develop into an overwhelming disease process.

As previously indicated, noticeable differences are seen in VLp and ALp at several levels. While VLp is typically seen as a short gram-negative rod, ALp demonstrates numerous filamentous forms. Of particular interest is the presence of small bacilli interspersed between lengthy ALp filaments. The ALp filaments appear to be the result of defective cross-wall formation which occurs during bacterial division. This phenomenon would explain an apparent elongation of these cells to sizes greater than 50 μm in length. Using chemically defined liquid medium, Pine, *et al.* noticed a progressive change in *L. pneumophila* cellular morphology in broth cultures (120). Large masses of filaments or bacilli chains were seen, which would break into shorter filaments and ultimately result in single and double cells. This observation may explain the presence of shorter ALp forms. It appears that ALp filaments break in a similar fashion, considering that cultures are usually 72 h old at time of usage. The difficulties encountered in spectrophotometrically adjusting ALp inoculum size to approximate VLp amounts, generated interest about each organism's physical characteristics. Optical interference caused by the presence of ALp filaments may account for such difficulties, assuming that organism viability is similar. Additionally, a standard deviation three times greater than VLp occurs when adjusting ALp numbers from cultures which are similarly incubated. An explanation for this discrepancy may involve bacterial breakage into shorter forms during vigorous mixing when preparing ALp suspensions.

During early adjustment attempts, ALp organisms were not as readily suspended as was VLp in either saline or PYG medium . This phenomenon could

be related to the "stickiness" observed in attempting to lift ALp colonies off agar medium. As a result, each organism was examined for surface characteristic variations. Overall, no apparent differences were noted in either VLp or ALp surfaces. However, ALp filament formation was readily visible in all fields examined by SEM. Several ALp exhibited a "pinched" area which appears to be an attempt at cell wall division.

A report by Ormsbee, *et al.* suggests that *L. pneumophila* virulence can be reestablished after prolonged passage on SMH agar (114). Passage of the avirulent organism through embryonated egg yolk sacs restored its ability to cause infection in guinea pigs. Unfortunately this investigation did not include information relating to the size of the inoculated and recovered bacteria. However, Ormsbee, *et al.* did indicate that bacilli of similar lengths had been recovered from both guinea pig organs and embryonated yolk sacs (114). Bornstein, *et al.* were the first investigators to demonstrate a loss in virulence potential with increasing *L. pneumophila* length (16). Organisms recovered from guinea pig spleens ranged from 0.3 to 2.0 μm in length. When placed onto agar medium, the overall bacilli length increased from 2.0 to 10 μm. Therefore, it can be proposed that had Bronstein, *et al.* examined the cell lengths of bacteria recovered from embryonated yolk sacs a shorter rod form would have been noted.

Examination of transmission electron micrographs (TEM) reveal discrepancies both in internal density and the presence of cytoplasmic vacuoles. The occurrence of vacuoles in VLp suggests the presence of a lipid substance

which inhibits infiltration of plastic embedding medium. These vacuoles may be the result of large cytoplasmic β-hydroxybutyrate granules. β-hydroxybutyrate granules are known to be a common storage polymer in bacteria (152). Similar vacuoles were not seen in ALp organisms. However, in rare instances a small vacuole could be detected. When seen, vacuoles are not considered to be artifacts. They have been commonly documented in numerous TEM studies utilizing both VLp and other *Legionella* isolates (Chan dissert. 1982; Sommer dissert. 1987; 46; 76; 136; 1; 150). VLp demonstrate well defined double membrane structures. Similar membranes are seen in ALp organisms. The internal spaces are densely packed with small granular material. Alternately, ALp displays similar granular material which is less densely packed than VLp. Occasionally, an ALp form that is as densely granulated as VLp is found. Whether or not there is a direct correlation between virulence, granular density and the presence of vacuoles remains to be seen. This relationship would prove difficult to examine when using agar-derived avirulent forms. Organism suspensions must be prepared which contain many *Legionella* colonies in order to recover enough organisms for subsequent coculture testing. Consequently, genetically-derived avirulent mutants appear to be better suited for studying phenotypic variations.

When TEMs are examined, a difference is seen in the cell membranes of VLp internalized by amoeba. Phagosome-bound VLp at 24 h coculture incubation display a wavy cell membrane. This result suggests that internalized VLp undergo changes in their membrane structures. This change may be the result of bacterial

adaptation to an inhospitable intracellular environment presented by the amoeba. Similarly, Kwaik, *et al.* demonstrated that *Legionella* internalized in human macrophages are subjected to a stressful microenvironment (91). Phenotypic changes are regarded as a global stress response. The expression of new bacterial proteins are selectively induced upon macrophage infection.

Transmission electron micrographs of amoeba grown in PYG medium demonstrate evenly distributed mitochondria and little RER. When ALp are placed in the presence of amoeba relatively few ALp are ingested even after 24 h coculture incubation. Various states of bacterial degradation suggest that ingested ALp are readily degraded by amoeba. Alternately, internalized VLp cause an influx of mitochondria to the phagosome area and are seen to be surrounded by RER. Similarly, the encirclement of *L. pneumophila* with RER was recently noted by Fields, *et al.* in cocultures containing *Hartmanella veriformis* (49). This group suggests that the *Legionella*-containing endosome fuses with host cell endoplasmic reticulum. In the current study, a similar union would provide VLp with both valuable bacterial nutrients and prevent lysosomal exposure.

Coculture of VLp with amoeba results in a consistent growth pattern. Alternately, ALp demonstrated three different growth patterns which did not occur in a reproducible fashion. Generally, ALp numbers did increase but at a much slower rate than VLp. Wong, *et al.* also noted that intracellular replication of virulent *L. pneumophila* takes place at a faster rate than seen with avirulent forms (157). Multiplication of virulent forms in human fibroblast cocultures was evident as early as 2 days, whereas avirulent growth could not be noted until 10

days incubation. Jacobs, *et al.* noted that an agar-derived avirulent mutant could multiply when coincubated with human alveolar macrophages (80). Replication was seen to occur at a much slower rate than the virulent parent strain. A similar result was seen in the present study when ALp were coincubated with *Acanthamoeba castellanii*. Here, the replication process appears to take longer than 5 days incubation.

The ability of avirulent *Legionella* mutants to survive in human cells has been previously documented (Horwitz 1987). Intracellular survival of ALp in *A. castellanii* does not occur. Evidence provided by Wadowsky, *et al.* suggests that avirulent *L. pneumophila* do not infect and multiply within *H. veriformis* (150). A similar result was seen in the current study with the rare occurrence of an ALp located inside an amoeba by TEM. When noted, these forms appeared to be completely degraded by 24 h coincubation. The resulting ALp replication is more likely to occur in the extracellular spaces. Again, Wadowsky, *et al.* proposed that the ability of an avirulent *L. pneumophila* strain to multiply is solely due to amoebal action (150). The coculture medium was suspected to contain an inhibitory agent to *L. pneumophila* growth which may be neutralized by *H. veriformis*. Here again, the amoeba-free controls did not multiply in culture. Although plausible, this explanation may only apply when cultures are maintained at 37°C. Had these cultures been incubated at 30°C rather than 37°C growth may have been seen by 7 days incubation. This relies upon the assumption that PYNFH (ATTC 1034) medium is similar in composition to PYG medium, since both media contain yeast extract. Close examination of ALp growth in the current

coculture system does suggest that ALp growth may be enhanced by the amoeba in comparison to the amoeba-free control. *Acanthamoeba* metabolism of coculture medium may provide abundant precursor substrates for extracellular ALp growth. Although the presence of amoeba may enhance ALp growth, it does not appear to be a growth requirement since ALp replicates in amoeba-free PYG medium.

Wadowsky, *et al.* described the multiplication of 4 agar derived avirulent *L. pneumophila* mutants in coculture with *H. veriformis* (150). None of the tested strains multiplied in amoeba-free cultures. The ability of VLp and ALp to multiply in amoeba-free cultures has not been previously reported. When noted, ALp and VLp amoeba-free controls increased in numbers between 6 to 10 days incubation. The majority of amoeba-*Legionella* studies incubated cultures at 35 or 37°C, whereas the current system utilizes a 30°C incubation temperature. Consequently, ALp and VLp growth in amoeba-free PYG medium may be a temperature dependent phenomenon. Unfortunately this result is not a reproducible event and varies between coculture experiments. Alternately, this phenomenon may indicate media adaptation by either organism over an extended time period. Thus, PYG medium may provide essential nutrients for *Legionella* growth due to its basic composition. The presence of peptones and yeast extract elements readily provide ample precursor substances for bacterial growth. Additionally, the glucose component would provide a readily available carbon source which has been shown to be metabolized by *L. pneumophila* organisms (101). Possibly, a reduction in the glucose concentration when added to PYG

coculture medium may inhibit ALp and VLp growth in amoeba-free culture. This result suggests that an increase in the number of extracellular *Legionella* is not the result of intracellular bacterial multiplication. Employment of a suitable wash procedure would remove unattached *Legionella* from cocultures. However, this procedure would not remove surface-associated bacteria. Alternately, an antibiotic pre-treatment could be used to kill all extracellular bacterial forms including surface-associated bacteria. Either procedure would be sufficient in removing extracellular forms which resulted from replication in culture medium. Gentamicin pre-treatment appears to inhibit the recovery of viable ALp in coincubation. This result is similar to that seen by Ott, et al. and also by Moffit and Thompkins (115; 103). Both studies utilized *A. castellanii* as the potential amoeba host. Here, antibiotic pre-treatment completely inhibited the recovery of avirulent *L. pneumophila* organisms. A similar pre-treatment was used in the current study which prevented the recovery of ALp during sampling periods. Gentamicin treatment as early as 1 h after coculture initiation resulted in a failure to recover viable ALp forms. Even after 5 days coincubation, ALp could not be recovered. This result indicates that rarely phagocytosed ALp can not survive within the amoeba for even a short period of time. These internalized organisms appear to be readily degraded by the amoeba when viewed by TEM. This occurrence readily explains the inability to passage ALp cocultures at 5 day intervals into fresh amoeba cultures.

With time, amoeba-associated ALp and VLp exhibit an increase in recovered VLp with a corresponding decrease in ALp. Recovered bacterial

counts include both attached and internalized forms. Both ALp and VLp attach to the amoeba host at similar rates of 2 to 3 logs up to 4 h incubation. At 24 h, VLp association has increased to approximately 4.7 logs, while ALp association has declined to 1 log. This indicates ALp still remains attached to amoeba, even after 24 h coincubation when taken in conjunction with an inability to recover ALp at this time period. A study by Fields, *et al.* examined *L. pneumophila* binding to *H. veriformis* supports this position (50). Here, a virulent *L. pneumophila* and its avirulent counterpart were inoculated into monolayers of *H. veriformis* cells at 4°C to prevent bacterial phagocytosis by the amoeba. In both forms, *L. pneumophila* attachment appears to occur at similar rates up to 8 h coincubation. At 12 h, the virulent form exhibited a 1 log greater attachment potential than the avirulent form. In the present study, VLp attachment to the amoeba was approximately 4.7 logs greater than ALp at 24 h coincubation. Using fluorescent antibody staining, VLp association with the amoeba is 2.5 times greater than ALp by 4 h coincubation. Similarly, Fields, *et al.* examined *L. pneumophila* association with *H. veriformis* using a similar fluorescent antibody technique (50). Here, at 4 h coincubation the virulent form demonstrated twice the association potential of its agar-derived avirulent mutant. This result closely approximated the 2.5 value seen with VLp. By 12 h the number of associated virulent bacteria were 75 times that exhibited by the avirulent form.

Ott, *et al.* investigated the effect of temperature variation on *L. pneumophila* replication in *A. castellanii* (115). These cocultures received a gentamicin pre-treatment similar to that used with the current study. As with

ALp, here too the avirulent mutant did not replicate. However, an incubation temperature of 37°C was used. When the incubation temperature was lowered to 30°C the avirulent organism demonstrated appreciable growth. The authors suggest that multiplication of the avirulent Philadelphia-1 mutant occurs by intracellular replication. This result appears to conflict with a failure to recover ALp organisms after a similar gentamicin pre-treatment. However, the authors' pre-treatment consisted of 80 μg/ml gentamicin suspended in PYG medium and coculture incubation for 1 h at 37°C. The current study used 100 μg/ml gentamicin suspended in Ac buffer with a coculture incubation for 2 h at 35°C. The difference in gentamicin concentrations and incubation times may be sufficient to account for the noted differences. However, the Philadelphia-1 strain may actually possess the capability to undergo intracellular replication in amoeba. The method by which an agar-derived avirulent *Legionella* form is generated may determine its virulence potential in both amoeba and guinea pig models. Both the agar type and number of passages used in achieving avirulent form propagation vary widely between coculture and animal model studies. An additional study by States, *et al.* examined temperature dependent survival of *L. pneumophila* when coincubated with *H. veriformis*. Coculture temperatures varied from 10 to 44°C. A much lower MOI of 1:50 (*Legionella*:amoeba) was used rather than the currently used 1:2 ratio. Peak bacterial growth was seen at 37°C of 4.92 logs with growth at 30°C at 4.45 logs. These results indicate that recovery of *L. pneumophila* in amoeba coculture does not significantly differ at either temperature. Consequently, the current 30°C coculture temperature may not

drastically alter potential recovery of either organism.

In all coculture experiments, amoeba numbers increased by approximately 1 to 1.5 logs by days 5 through 7, ex ept when the MOI was increased. When the MOI was changed to 50:1 (*Legionella*:amoeba) approximately a 2 log drop was observed in amoeba numbers in VLp coculture. This drop appears to correlate well with the relatively small increase in VLp numbers seen between days 3 and 5 at the higher MOI. A substantial decrease in potential amoeba hosts would apparently affect the ability of VLp to amplify its numbers via intracellular replication. Similarly, a study conducted by Holden, *et al.* demonstrated a difference in *L. pneumophila* growth rates when the MOI is slightly manipulated (69). Extracellular *L. pneumophila* numbers increased by approximately 3.5 logs at 48 h in coculture when an MOI of 1:1 (*Legionella*:amoeba) was used. Changing the MOI to approximately 2:1 afforded only a 2 log increase in the same time period. Unfortunately, results were not given to indicate the affect of a slight MOI change on the amoeba.

Using dialyzed yeast extract to prepare charcoal yeast extract (CYE) agar, Norwiki, *et al.* reduced filament formation of an agar-derived avirulent mutant (112). Resulting LD_{50} of these shorter forms demonstrated increased virulence when compared to organisms grown on non-dialyzed agar. Agar-adapted *Legionella* are known to lose their virulence potential in guinea pigs. They can be recovered after repeated passage of the virulent parent on supplemented Mueller-Hinton agar (99). As previously indicated, ALp is an agar-derived organism. It was produced by multiple passage on Feeley-Gorman agar (Personal

communication W. C. Winn, Jr, 1992). The ability to increase agar-derived *L. pneumophila* virulence by shortening bacilli length through *in vivo* culturing warranted further examination. Consequently, coculture studies were initiated in which a dual-step filtration technique enabled a physical reduction in ALp size to an average of 2.3 μm in length. Separately, in three experiments VLp exhibited its typical 2.0 log increase by 5 days incubation. Here, unfiltered ALp in coculture demonstrated differing results as previously stated, but ultimately increased in numbers in 2 of 3 experiments. The ALp filtrate exhibited growth characteristics which generally paralleled its unfiltered counterpart. These results suggest that a shortening of ALp forms by filtration does not affect ALp virulence potential, at least in amoeba. However, this does not predict the effect of rod-shortening using physical techniques on ALp virulence potential in guinea pigs.

Several reports of avirulent to virulent conversion have been documented. Wong, *et al.* reported that virulence could be restored by cultivation of an agar-derived avirulent *L. pneumophila* in human embryonic lung fibroblast cultures (157). Virulence potential in guinea pigs was seen to increase as bacteria were sequentially passaged through host cells. ID_{50} decreased from 7.5 logs at 3 passages to approximately 5.7 logs by passage 14. In a similar study, Elliot & Johnson were able to recover a virulent form after 6 passages through guinea pigs of an agar-derived *L. pneumophila* (39). Additionally, virulence potential for guinea pigs and embryonated eggs seen at 12 passages approximated that of the virulent organism. Ormsbee, *et al.* reported a restoration of virulence potential in guinea pigs of three separate *L. pneumophila* species (114). Full restoration of

virulence potential was achieved with one test strain, while partial restoration was noted in the remaining strains. Taken together, these results suggest that a suitable host can select for virulent *L. pneumophila* forms originating from avirulent cultures. Consequently, it could be proposed that not all the bacterial forms present in an avirulent culture are avirulent. Subpopulations of virulent organisms may remain even after extensive bacterial passage. This observation may help to explain the VLp-appearing forms seen in ALp using TEM. Both Elliot and Johnson as well as Wong, *et al.* reported maintenance of *L. pneumophila* virulence in guinea pigs after 12 passages on CYE agar (39; 157). Catrenich and Johnson were able to estimate that only 4.5% of the virulent *L. pneumophila* convert to avirulent forms even after 30 successive passages on CYE agar (21). It was proposed that virulent cells convert to avirulent forms in low numbers due to an inherent instability of the virulent cells on agar medium. In contrast to previous reports, these individuals suggest that virulence conversion is a one-way phenomenon only occurring in the direction of virulence to avirulence. Here, a virulent *L. pneumophila* form was sequentially passaged 5 times on CYE agar, 5 times on SMH agar, and through 6 guinea pigs and was subsequently inoculated into a seventh guinea pig resulting in its death. However, when using the same passage regime but with only one incubation on SMH agar, the seventh guinea pig survived. This result suggests that avirulent form conversion appears to vary, even when selected from the same parent cell population. McDade and Shepard demonstrated that a loss of *L. pneumophila* virulence in guinea pigs would occur within the first 10 passages on SMH agar (99). Consequently, the

present study examined the effect of long term association of VLp and ALp with amoebas on organism virulence potential. Here, only VLp could be passaged into a new amoeba culture. No apparent increase or decrease in VLp virulence potential was noted. However, the appearance of an increased amount of MOMP was seen in outer membranes recovered at the fourth passage. The reason for this increase is unknown. Alternately, ALp could not be recovered from 5 day cultures. It appears that extracellular ALp were completely removed during wash procedures prior to amoeba disruption.

The outer membrane of ALp typically demonstrates a broad band at ~6.5 kDa. This low molecular weight band is the only significant difference seen in the ALp outer membrane when compared to a similar preparation of VLp. A question may arise as to why this band has not been widely noted. The only known report of its existence has not been published (Lawrence, dissertation). In this study, it was preliminarily characterized to be approximately 7 kDa. Current study results indicate that it is slightly less than 6.5 kDa. The LPS region of both ALp and VLp, 16.5 kDa to 27.5 kDa, was previously characterized to contain 14 identical oligosaccharide side chains. Current results demonstrate only 7 to 8 well-defined bands. The additional bands Lawrence noted were in viewing whole cell-lysates of both organisms after proteinase K digestion. The current study subjected only ALp and VLp outer membrane preparations to proteinase K digestion. Consequently, the additional bands may be attributed to protein comigration in total membrane preparations as seen in both *Salmonella* LPS by Hitchcock & Brown and in *L. pneumophila* LPS by Hindal & Iglewski (66; 65).

Additionally, the inability to partition *L. pneumophila* LPS by hot phenol-water extraction indicates extensive cross-linkage of the MOMP by covalent bonding supports protein comigration (111). It was further proposed by Lawrence that this band was the LPS core region of ALp and that the VLP core region migrated further. Although in the present study, the ~6.5 kDa band is characterized as a carbohydrate containing substance, since insufficient evidence was offered to support the core region concept and evidence was not found in relevant literature. Additionally, the area proposed to be the core region of VLp consistently was seen to migrate beyond the dye front. This band, although noticeable, is also seen in silver-stained ALp outer membrane preparations. A simple explanation can be offered as to why this band has never been reported by other researchers. This band may be an organism specific trait only displayed by ALp. Additionally, it may only appear in agar-derived avirulent mutants. These mutant forms have not been extensively studied by electrophoresis. Thus, adaptation to solid-agar medium may result in band production. Furthermore, the areas of apparent interest have traditionally been at the higher molecular weights, especially in the LPS and MOMP regions. The use of various electrophoretic conditions and SDS-PAGE gel strengths may preclude detection of this band. In the current study a 12% resolving mini-gel was inadequate in demonstrating this band. The band would appear at the dye front unless gel running time was appreciably decreased. These conditions will result in decreased band resolution. By increasing the resolving gel strength to 13% the ~6.5 kDa band was detected as separate from the dye-front. The choice of molecular weight markers may affect the ability to

detect such a low molecular weight band. For instance, widely used commercial low molecular weight markers such as Bio-Rad incorporate a 14.4 kDa protein as their lowest molecular weight marker. In achieving maximal band resolution normally be the dye-front is allowed to migrate to near the end of the resolving gel. Under such conditions a low molecular weight moiety of ~6.5 kDa usually will be lost. Recently, a broad molecular-weight marker has been introduced by Bio-Rad which incorporates a 6.5 kDa protein marker. Consequently, researchers using this molecular weight marker may find a similar band when examining agar-derived avirulent *L. pneumophila* membrane preparations.

The relationship of the ~6.5 kDa band seen in ALp to virulence currently is not known. It may directly contribute to the failure of ALp to cause guinea pig infection. This substance may be produced due to the availability of a medium-derived substrate which the organism is not usually exposed to. Excessive production of this substance may interfere in amoeba uptake mechanisms, although initial attachment was not affected in the current study. The location of this band in the outer membrane may be of some significance. If outwardly exposed as a membrane bound component, this substance may be responsible for a decreased specific receptor-mediated ALp uptake, but it does not appear to inhibit amoeba attachment.

Examination of ALp and VLp total membrane preparations reveals additional differences in protein band patterns. A small 10 kDa protein band can be detected in both Coomassie blue and silver stained gels of VLp membrane preparations which is barely discernable in ALp total membrane preparations. Of

greater interest is a small band at approximately 6.5 kDa in VLp total membrane samples, but not in VLp outer membrane samples. Silver staining of the ALp total membrane preparation masks the appearance of a similar band in this region. The corresponding Coomassie blue stained gel revealed a barely visible band in this area at recommended protein sample loads. This band corresponds to one seen in both VLp and ALp membrane preparations. A similar band is not noted in VLp outer membrane samples even after silver staining. This result suggests that the protein is situated in the VLp inner membrane region and is not normally expressed in VLp outer membranes. The location of this VLp protein may be more than coincidental. It is very similar to the ~6.5 kDa band seen in ALp total membrane and outer membrane preparations. It is likely that the ~6.5 kDa band seen in ALp membrane preparations is an over expression of a glycosylated protein normally expressed in the inner membrane of VLp and to a lesser degree in ALp. Propagation on agar-based medium may modify protein expression resulting in the highly glycosylated form expressed in ALp outer membranes. This proposal is further supported by the retention of this band after proteinase K digestion indicating a non-protein component. Additionally, PAS positive staining of this band indicates a carbohydrate component.

This thesis has advanced the knowledge of the relationship of *L. pneumophila* with the free-living amoeba *Acanthamoeba castellanii* as follows:

1. ALp filaments appear to result from defective cross-wall formation during organism division as supported by TEM studies.

2. ALp filaments interfere with accurate spectrophotometric

adjustments of desired inoculum size.

3. VLp organisms are densely packed with granulated material whereas ALp are less dense.

4. The presence of cytoplamic VLp vacuoles are associated with intracellular replication potential of *L. pneumophila*.

5. Amoeba ingestion of ALp is a rare event with subsequent degradation of internalized bacteria.

6. Amoeba internalized VLp establish an overwhelming infection which ultimately results in amoeba death.

7. Transmission electron microscopy supports the concept of bacterial endosome fusion with host cell endoplasmic reticulum.

8. Replication of ALp in coculture is not due to intracellular multiplication but may be enhanced by the presence of amoeba.

9. ALp and VLp will replicate in amoeba-free PYG medium when cultures are incubated at 30°C for greater than 5 days.

10. Repeated passage of recovered VLp organisms into axenic amoeba cultures does not result in a change in outer membrane expression.

11. The ~6.5 kDa band noted in ALp membrane preperations is a highly glycosylated protein which is normally expressed only at the inner membrane of VLp.

Overall, intracellular multiplication of virulent *L. pneumophila* in free-living amoeba is indicative of a corresponding virulence in guinea pigs. The ability of this bacterium to establish an infection of amoeba widely dispersed in aquatic and

soil environments presents ample opportunity to amplify *Legionella* numbers. Consequently, prevention and control of human *Legionella* infections presents a formidable task to health care personnel.

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APPENDIX A
PEPTONE YEAST GLUCOSE (PYG) BROTH
(ATCC 712)

INGREDIENTS:

STOCK SOLUTIONS/OTHER REAGENTS:

0.4 M $MgSO_4 \circ 7H_2O$	9.84 g/100 ml dist. water
0.05 M $CaCl_2$	0.595 g/100 ml dist. water
0.005 M $Fe(NH_4)_2SO_4$	0.196 g/100 ml dist. water
0.25 M Na_2HPO_4 (dibasic)	6.70 g/100 ml dist. water
0.25 M KH_2PO_4 (monobasic)	3.40 g/100 ml dist. water
Yeast Extract	1.00 g
Proteose Peptone	20.0 g
2M Glucose	36.4 g/100 ml dist. water
Sodium Citrate	1.00 g

PROTOCOL:

1. Prepare above stock solutions aseptically by filtering through a 0.45u, 150 ml capacity flask. (Store a 4-6 °C for 6 months)

2. Place the Yeast extract and Proteose Peptone in a 1 liter flask and disolve in 900 mls dist. water.

3. Add the stock solutions in the following order:

0.4 M $MgSO_4 \circ 7H_2O$	10 ml
0.05 M $CaCl_2$	8-10 ml
0.005 M $Fe(NH_4)_2SO_4$	10 ml
0.25 M Na_2HPO_4	10 ml
0.25 M KH_2PO_4	10 ml

4. Autoclave for 25 min at 15 psi and let cool to RT.

5. Meanwhile, prepare the 2 M Glucose solution (fresh), add 1g Sodium Citrate and filter through a 0.45u filter assembly.

6. Add 50 ml of 2M Glucose solution to cooled medium.

7. Dispense in 50-100 ml aliquots and store at 4-6^{0}C.

APPENDIX B
BUFFERED CHARCOAL YEAST EXTRACT AGAR (BCYE)

INGREDIENTS:	500 ml	1,000 ml
BCYE dehydrated media (BBL)	19.5 g	38.3 g
L- cysteine $HClOH_2O$ (Sigma)	0.2 g	0.4 g

PROTOCOL:

1. Suspend 19.15 gms of BCYE agar in 450 ml (900 ml) of deionized water (DI).
2. Adjust to pH 6.9 using 1N KOH (approx 20 mls for 500 ml).
3. Bring total volume to 495 ml (990 ml).
4. Add 2 drops (4) POURRITE•
5. Autoclave BCYE agar at 121°C for 15-20 min.
6. Prepare fresh solution of L-cysteine 0.2 g in 5 mls DI H_2O (0.4 g in 10 ml).
7. Allow BCYE agar to cool to 50°C.
8. Add L-cysteine solution to BCYE agar using a 10 ml syringe through a 0.2 um filter (Milipore)
9. Mix agar well to resuspend charcoal particles and pour plates.
10. Occasionally, flame bottle and gently mix agar between pouring plates.

NOTE: 500 ml will be sufficient for pouring approx. 30-35 plates.

SOURCE: BBL/Becton Dickinson, Cockeysville, MD.

APPENDIX C
BCYE SELECTIVE AGAR WITH PAC*

INGREDIENTS: Approximate formula per L of H_2O (Commercially prepared plates)

Yeast Extract	10.0 g
L- cysteine HCl	0.40 g
Ferric Pryophosphate, Soluble	0.25 g
ACES Buffer	10.0 g
Activated Charcoal	2.0 g
Alpha-Ketoglutaric Acid	1.0 g
Agar	15.0 g
Polymixin B*	80,000 units
Anisomycin*	80.0 mg
Cefamandole*	40.0 g

SOURCE: BBL/Becton Dickinson, Cockeysville, MD.

APPENDIX D
ACANTHAMOEBA BUFFER

INGREDIENTS:

STOCK SOLUTIONS:

0.4 M $MgSO_4$*$7H_2$09.84 9g/100 ml dist. water

0.05 M $CaCl_2$	0.595 g/100 ml dist. water
0.005 M $Fe(NH_4)_2SO_4$	0.1›6 9g100 ml dist. water
0.25 M Na_2HPO_4 (dibasic)	6.70 g/100 ml dist. water
0.25 M KH_2PO_4 (monobasic)	3.40 g/100 ml dist. water

OTHER INGREDIENT:

Sodium Citrate	1.00 g

1. Prepare above stock solutions aseptically by filtering through a 0.45u, 150 ml capacity flask. (Store a 4-6 °C for 6 months)

2. Disolve in 950 mls dist. water.

3. Add the stock solutions in the following order:

	Volume	Final molarity
$MgS0_4$-$7H_20$ (0.4 M)	10 ml	4 mM
$CaCl_2$ (0.05 M)	8-10 ml	0.4 mM
$Fe(NH_4)_2SO_4$ (0.005 M)	10 ml	3.4 mM
Na_2HPO_4 (0.25 M)	10 ml	2.5 mM
KH_2PO_4 (0.25 M)	10 ml	2.5 mM

4. Add 1 g Sodium Citrate .

5. Autoclave for 25 min at 15 psi and let cool to RT.

6. Dispense in 50-100 ml aliquots and store at 4-6°C.

REFERENCE:

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APPENDIX E
OUTER MEMBRANE PROTEINS

REAGENTS:

10 mM HEPES BUFFER

(HEPES M.W. = 238.3)

Hepes	0.238 g

1. Add to 100 ml ddH_20 and adjust pH to 7.4

10 mM HEPES BUFFER with 1% SARKOSYL

Sarkosyl	0.5 g

Add to 50 ml of 10 mM HEPES buffer or 1.0 g Sarkosyl to 100 ml of 10 mM HEPES buffer

10 mM HEPES BUFFER with PROTEASE INHIBITORS

Iodoacetamide	92.5 mg
200 U/ml Aprotinin soln.	0.1 ml
PMSF stock soln.	1.0 ml

1. To make a 200 U/ml Aprotinin stock

Aprotinin	0.816 mg *(at 245 iU/mg)

Add to 1 ml H_20 * (Check activity and adjust for between lot differences)

2. To make a 0.1M PMSF stock (M.W. = 174.2)

PMSF	0.174 g

Add to 10 ml ethanol

1. Add reagents to 90 ml of 10 mM HEPES and adjust to pH 7.4. QS to 100 ml with 10 mM HEPES.

APPENDIX F
POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

REAGENTS:

1. 30% Acrylamide/0.8% bis-acrylamide

Acrylamide	30.0 g
N,N'-methylene-bis-acrylamide	0.8g

   a. Dissolve in 70 ml ddH_20 then QS to 100 ml. Filter solution through 0.45 um filter.

   b. Store at 4°C in dark colored bottle. Discard after 30 days.

2. 10% $(NH_4)_2S_20_8$ (Ammonium Persulfate = APS)

   0.1 g into 1 ml ddH_2O (fresh solution or store 4°C for 2 days only)

3. 4X - 0.5M TRIS*HCL with 0.4% SDS (Stacking gel buffer)

Tris base	6.05 g
SDS	0.4 g

   a. Dissolve into 40 ml of ddH_20 and adjust to pH 6.8 with 5N HCl first then 1N HCl when nearing desired pH.

   b. Add ddH_20 to final volume of 100 ml. Filter solution through a 0.45 um filter and store at 4°C.

4. 4X -1.5M TRIS*HCL w;th 0.4% SDS (Separating gel buffer)

Tris-base	36.4 g
SDS	0.8 g

a. Dissolve into 120 ml of dd$H_2$0 and adjust to pH 8.8 with 1 N HCl.

b. QS to final volume of 200 ml.

c. Filter solution through a 0.45 um filter and store at 4°C.

5. 10X - SDS RUNNING BUFFER (ELECTROPHORESIS)

Tris base	30.2 g
Glycine	144.0 g
SDS	10.0 g

a. Dissolve into 800 ml of dd$H_2$0 and QS to 1 L.

b. Do not adjust pH as diluted buffer will be pH 8.3 (1:10)

NOTE: Dilute 1:10 with ddH_2O before running gel.

6. 2X SAMPLE BUFFER

To 15 ml dd H20 add:

Tris-base	0.757 g (0.25M)
SDS	2.0 g (8%)
Glycerol	5.0 ml (20%)
2-Mercaptoethanol	2.5 ml (10%)
Bromophenol blue	0.0025 g (0.01 %)

Adjust to pH 6.8 with HCl and add dd $H_2$0 to 25 ml.

GEL PREPERATION:

SEPARATING GEL (Lower gel)	LARGE	MINI

	12%	12%	12%	13%	15%
30 Acrylamide/0.8% bisacryl	40.0	30.0	8.0	8.7	10.0
4X-TRIS/SDS buffer (pH 8.8)	25.0	18.75	5.0	5.0	5.0
dd H_20	35.0	26.25	6.9	6.2	4.9
Total ml	100	75.3	20.0	20.0	20.0

Degas in small side arm flask for 10-15 mins then add:

10 % APS(ul)	250	332	100	100	100
TMED	67.0	50.0	10	10	10

1. Swirl gently to mix then pour between clean glass plates and overlay with isobutyl alcohol.
2. Allow to polymerize for 30 min at RT.

STACKING GEL (Uppe. gel)

3-9% gel (ml)	Full size	Mini
30% Acrylamide/0.8% BIS soln	3.25	1.3
4X-TRIS*HCl/SDS buffer (pH 6.8)	6.25	2.6
ddH_2O	15.25	6.1

Degas in small side arm flask for 10-15 mins then add:

10% APS (ul)	125	50
TEMED	25	10

1. Swirl gently to mix then pour on top of seperating gel.
2. Allow to polymerize for 30-45 min at RT.

APPENDIX G
COOMASSIE BLUE STAIN SOLUTIONS

PROCEDURE:

1. Fix gel in a suitable containerfor 30 min to overnight with slow rotation before staining.

2. Remove fixative and replace with staining solution adding enough stain to cover the gel and rotate slowly for at least 30 min.

3. Remove stain and wash gel with small amount of destaining solution.

4. Remove destain wash and replace with sufficient amount of destain to cover gel.

5. After 1 h, remove disclored destain solution and replace with fresh destain. and rotate slowly overnight.

6. Replace destain until the stain background is completely cleared.

REAGENTS:

(ml)	100	200	300	400
FIX SOLUTION:				
50% MetOH	50	100	150	200
10% HAc	10	20	30	40
ddH_20	40	80	120	160
STAINING SOLUTION:				
50% MetOH	50	100	150	200
10% HAc	10	20	30	40
0.05% Coom. Blue	0.05	0.1	0.15	0.2 g
ddH_20	40	80	120	160

DESTAIN SOLUTION:

7% HAc	7	14	21	28
ddH_20	88	176	264	352

REFERENCE:

Ausubel et al., (1989) Current Protocols in Molecular Biology, John Wiley &Sons, New York, N.Y. pg 10.6.1 - 10.6.3

VITA

Terrence James Ravine was born on July 30, 1955 in Canton, Ohio. He attended the University of Akron in Akron, Ohio where he graduated cum laude in 1983 with a Bachelor of Science degree in Medical Technology. In 1985 he received a Master of Science degree in Biology also from the University of Akron. In the same year, he joined the medical service branch of the U.S. Air Force as a Biomedical Laboratory Officer. While in this position, he distinguished himself as an outstanding clinical laboratory officer. He was subsequently selected to pursue a Ph.D. in Microbiology/Pathology by the Air Force Institute of Technology (AFIT) starting in August of 1990.