STUDY OF THE *Legionella*-DEPENDENT INDUCTION OF APOPTOSIS IN THE NATURAL HOST *Acanthamoeba castellanii*

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Dedication

To my grandmother, Avó Vina, the most loving and caring woman I have ever known.
I will always miss the unconditional love I felt every time I looked into your eyes.

Fazes-me muita falta avó.

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Abstract

Legionella pneumophila, a Gram-negative bacillus first identified in 1977, was the etiologic agent responsible for an outbreak of pneumonia that occurred in the American Legion Convention in 1976, Philadelphia. Although several other species of the genus *Legionella* were subsequently identified, this species is the most frequent cause of two distinct clinical syndromes: Legionnaires' disease and Pontiac fever.

The life cycle of *Legionella pneumophila* is divided in two distinct phases: a replicative phase followed by an infectious or transmissive phase. In the replicative phase the bacterium shows a nonmotile stage with a low or nonexistent toxicity. In the infectious phase the bacterium develops a flagellum becoming motile and highly toxic.

The main hosts of *Legionella pneumophila* are amoeba and human macrophages. The interaction of the bacteria with these hosts shows several similarities, except for the death mechanism that it induces in order to evade before beginning a new infection cycle. In macrophages, this process has already been studied in great detail but in amoeba it has rarely been addressed and so far, the published data was based in only a few conditions of the infection.

For a better understanding of this process we performed a study that considered several conditions of the infection. The results obtained in our study show a significant difference in the percentage of apoptotic cells between the *Acanthamoeba castellanii* infected by *Legionella pneumophila* and the uninfected *Acanthamoeba castellanii*. This data suggests that *Legionella pneumophila* induces apoptosis in amoeba and that the process of killing and exiting the macrophages by apoptosis may have evolved from the interaction with amoeba in the environment.

Resumo

Legionella pneumophila, um bacilo Gram-negativo identificado pela primeira vez em 1977, foi o agente etiológico responsável pelo surto de pneumonia que ocorreu na Convenção da Legião Americana em 1976, em Filadélfia. Apesar de muitas outras espécies do género *Legionella* terem já sido identificadas, esta espécie é a causa mais frequente de duas síndromes clínicas distintas no Homem: a Doença dos Legionários e a Febre de Pontiac.

O ciclo de vida da *Legionella pneumophila* divide-se em duas fases: a fase replicativa seguida por uma fase infecciosa ou transmissiva. Na fase replicativa a bactéria apresenta-se numa fase não-móvel e com uma toxicidade baixa ou quase inexistente. Na fase infecciosa a bactéria desenvolve um flagelo tornando-se móvel e altamente tóxica.

Os principais hospedeiros de *Legionella pneumophila* são as amibas e os macrófagos. A interacção da bactéria com estes hospedeiros mostra várias semelhanças, à excepção do mecanismo de morte que induz para se evadir antes de iniciar um novo ciclo de infecção. Nos macrófagos este processo já foi estudado em grande detalhe mas nas amibas raramente tem sido explorado e os dados reportados até agora são baseados em poucos parâmetros da infecção.

De modo a esclarecer melhor este processo, procedemos a um estudo mais abrangente considerando várias condições de infecção. Os resultados obtidos no nosso estudo mostraram que existe uma diferença significativa na percentagem de células apoptóticas entre *Acanthamoeba castellanii* infectadas por *Legionella pneumophila e Acanthamoeba castellanii* não-infectadas. Estes dados sugerem que *Legionella pneumophila* induz apoptose nas amibas e que o processo de morte e evasão dos macrófagos por apoptose pode ter evoluído da interacção com as amibas no ambiente.

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Symbols and Abbreviations

°C - Degree Centigrade

7-AAD - 7-Aminoactinomycin D

Ac – Acanthamoeba castellanii

ACES - N-2-acetamido-2-aminoethansulfonic acid

Ca - Calcium

CFU - Colony-forming unit

DMSO - Dimethyl Sulfoxide

DNA - Deoxyribonucleic acid

ELISA - Enzyme-Linked Immunosorbent Assay

FCS - Fetal Calf Serum

HCl - Hydrochloric acid

IFA - Indirect Fluorescent-antibody Assay

LD - Legionnaires' Disease

Lp - Legionella pneumophila

ml - Milliliter

mM - Millimolar

MOI - Multiplicity Of Infection

PBS - Phosphate-Buffered Saline

PS - Phosphatidylserine

TBE - Tris-Borate–EDTA

UV - Ultraviolet

V - Volt

x *g* - Acceleration of gravity

µl - Microliter

Chapter 1. Introduction

In late July 1976, the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia, responded to an epidemic of respiratory disease that affected the attendees of the 58th annual convention of the American Legion, Department of Pennsylvania, held in Philadelphia. Although the cause and etiologic agent were unknown, the disease appeared to be characterized by flu-like symptoms [1].

By early August, the first deaths from pneumonia among men who had attended the convention began to be reported by news organizations from all over the country. Since by that time the cause of the illness was yet to be determined, the new threat was named "Legionnaires' Disease" (LD) [2].

To investigate the cause of the outbreak, the CDC sent to the field the largest team in the center's history to that date (32 people). The team was led by David Fraser and included 20 epidemiologists. The initial findings revealed that in total, 182 people met the criteria of a case, from which 29 died. Additionally, the team identified 39 people that showed a similar illness but had not attended the convention. These people had only been within a block of the hotel and the cases were called "Broad Street pneumonias" [3].

In order to identify the etiologic agent of LD, the team began to examine patients' serum and tissue specimens in a search for toxins, bacteria, fungi, chlamydiae, rickettsiae and viruses. With samples from the lungs of four patients (from a total of six) they were able to isolate a Gram-negative, nonacid-fast bacillus in guinea pigs. However, the classification of the organism was still incomplete [4].

Later on, using deoxyribonucleic acid (DNA) relatedness, Joseph McDade and colleagues showed that all strains of the LD bacterium were members of the same species and that the DNA from the LD bacterium was not significantly related to DNA from any other group of bacteria already known. Considering these data, in April 1979, they proposed that the LD bacterium should be named *Legionella pneumophila* and the type strain Philadelphia 1 [5].

1.1. *Legionella pneumophila*: Physiology and Ecology

The most well-known species, *Legionella pneumophila* (*Lp*), has a biphasic life cycle that features two phenotypically distinct phases: a replicative phase, where *Lp* shows a nonmotile shape; and a transmissive phase where *Lp* is highly virulent and flagellated [6, 7].

Legionella spp. is highly pleomorphic since it can alternate between coccoid, bacillary (~0.3 to 0.6µm by ~3µm) and long filamentous (~8 to 50µm) forms [8]. These pleomorphic changes are influenced by factors like temperature, nutrient availability, growth environment (e.g., as intracellular parasite) and the medium type [9, 10, 11, 12].

The bacterium are ubiquitous in aquatic environments and can be found in several freshwater habitats worldwide (e.g. rivers, lakes, streams, ponds, hot spring) [13, 14, 15, 16] and some species have also been found in potting mixes [17, 18, 19] and soil [20, 21, 22].

At the time of writing, the genus *Legionella* comprised approximately 56 distinct species (and many unnamed species) including at least 70 serogroups. Despite only half of them were isolated from, or identified in, clinical samples, all species are considered potential human pathogens [8].

Over 90% of the isolates associated with LD are *Lp*, and serogroup 1 (sg1) was identified in 83% of the cases [23].

In natural aquatic environments, *Lp* thrives at 25ºC to 37ºC [9], and is able to establish within biofilm communities or persist as facultative intracellular parasite that can invade and replicate inside amoebae [6]. Once ingested by the amoeba, *Lp* is able to avoid ingestion and begins to highly replicate inside the protozoan host. Once nutrients become limited, *Lp* induces the host death in order to evade and begin a new infection cycle [7].

Inside host cells *Lp* differentiates into a replicative form and when nutrients become limited into a transmissive form (Figure 1) [7]. Garduño et al. have shown that *Lp* can differentiate in vivo in *Tetrahymena tropicalis* directly from the transmissive form into a highly infectious mature intracellular form (MIF), indicating that transmissive form and MIFs constitute a differentiation continuum. In addition, MIFs are thought to be the infectious forms implicated in the transmission of LD [24].

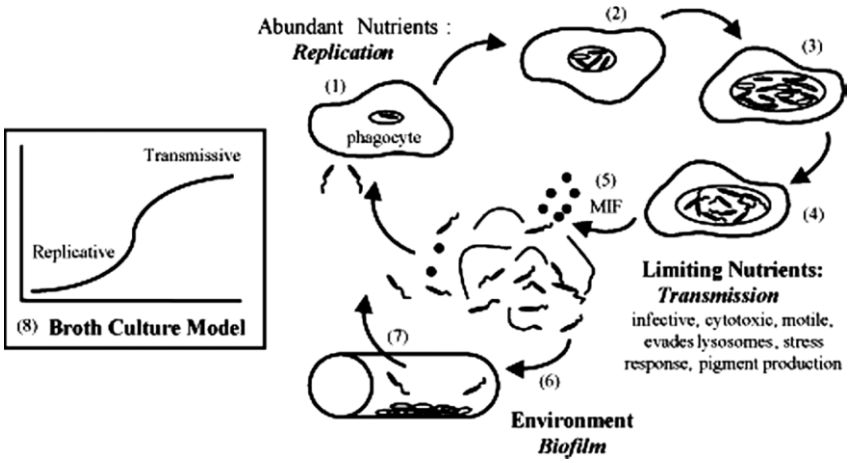


Figure 1 - The life cycle of *Lp*.

1. Free-swimming transmissive *Lp* that are engulfed by the phagocyte host (amoeba) establish vacuoles that provide protection from lysosomal digestion.

2. When nutrients and other conditions are favorable, intracellular bacteria repress transmission traits and activate pathways that promote replication.

3. As conditions in the replication compartment become unfavourable, the progeny stop dividing and coordinately express traits that promote survival in the environment and transmission to a new phagocytic host.

4. After a long period, the microbes may continue to develop into a mature intracellular form (MIF), a cell type that is highly resilient and infectious.

5. The amoeba is lysed, and the microbes are released into the aqueous environment.

6. *Lp* that do not immediately encounter a new phagocyte probably establish biofilms in both water systems and ponds, where they are resistant to biocidal agents.

7. When planktonic microbes encounter a new phagocyte, the cycle begins anew (Adapted from [7])

1.2. Epidemiology

Much has been learnt about the epidemiology of LD and *Legionella* since the organism was first identified in 1976. In the meantime, surveillance schemes for LD have been applied in several countries including the USA, Canada, New Zealand, Australia, Japan, Singapore, and Europe, where LD became a notifiable disease and coordinated European surveillance has been established since 1995 [25].

In Portugal, LD was included in the Portuguese system of mandatory notifications of infectious diseases in 1999 and in 2004 an Integrated Programme of Epidemiological Surveillance was implemented in order to improve reporting (clinical and laboratory notification) [26].

Despite being a notifiable disease, LD is likely to be underestimated in many countries mainly because of the lack of common definitions, diagnostics and surveillance

systems. This fact makes it impossible for LD to be directly comparable between countries [25].

The distribution of LD cases by age and gender is similar between countries: the disease is rare in children and more frequent in older people (74-91% of patients are ≥50 years) and males account for the majority of the notifications (1.4-4.3 male patients for every female patient) [27-31]. In Europe, the age-standardized notification rate of LD was 10.7 per million people in 2013 and the number of notifications per million inhabitants was 11.4 [23].

Several studies have shown that LD has a seasonal pattern that is associated with warmer and wetter weather conditions [32-35]. There is also evidence that suggests that the persistence of *Lp* is increased in aerosols at high relative humidity [36, 37].

There is also an association between LD cases and a history of recent travel, particularly involving overnight stays in hotel accommodations. Rooms that have not been used for a long time and the presence of large numbers of water outlets with long pipe runs can contribute to water stagnation, which will promote *Legionella* spp. growth, unless adequate control measures are applied [38, 39]. Cruise ships can also be sources of *Legionella* for similar reasons, and have already been associated with several outbreaks [40].

In view of the association with travel, specific surveillance systems for travel-related cases of LD have been implemented in order to improve source identification and public health action [40].

In Europe, the surveillance of LD is carried out by the European Legionnaires' disease Surveillance Network (ELDSNet) since 2010 and is coordinated by the European Centre for Disease Prevention and Control (ECDC). ELDSNet works in partnership with several entities like the World Health Organization (WHO), public health authorities of non-EU countries and tour operators [41].

1.3. Transmission

Transmission of LD is usually by inhalation of aerosols or aspiration of water containing *Legionella* spp. and until the present year no evidence of person-to-person transmission existed [25]. However, there has been documented recently (February,

2016) by Correia *et al* [42] a probable case of *Lp* transmission between two individuals after a cluster of cases of LD occurred in Vila Franca de Xira, Portugal, in 2014. The second patient is believed to have been infected by the first patient since the second patient was not geographically linked to the cluster epicenter and strains of both patients showed the novel ST1905 profile (identified as the causative strain in the cluster) [43].

In cases of LD where the etiologic agent is *Legionella longbeachae,* the transmission route is not well known to date, but it is believed to have a different source. In these cases, activities like exposure to potting compost or soil or poor hand-washing practices after gardening are regarded as risk factors of infection by this species [44, 45, 46].

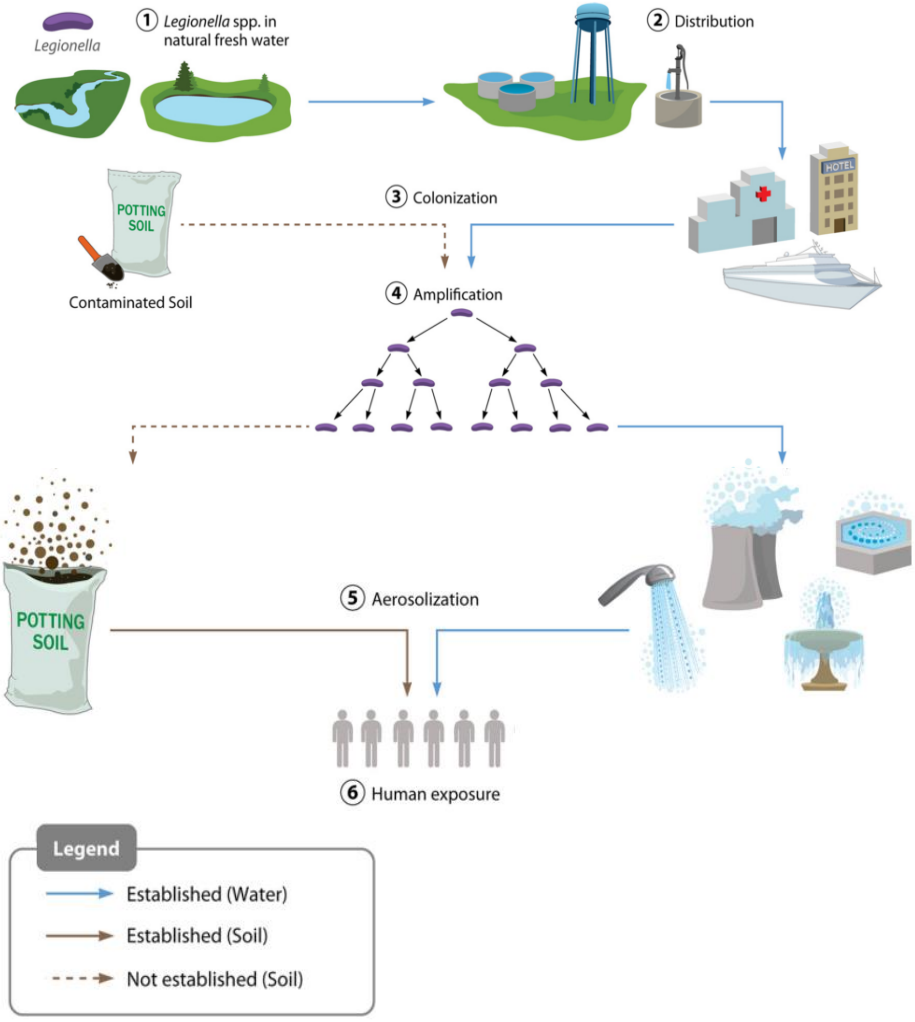


Figure 2 - Route of *Legionella* dissemination from natural waters to human exposure. *Legionella* from freshwater sources (1) is distributed at low concentrations from points of water purification (2) to colonize downstream local plumbing networks and cooling systems (among other sites) (3) and amplifies under permissive environmental conditions (4). Subsequent aerosolization (5) exposes a human population (6), leading to a potential disease spectrum. The route of LD caused by contaminated soil is less well understood but also appears to involve aerosol exposure (Adapted from [8]).

Contaminated cooling towers have been associated with the largest outbreaks of LD (Murcia, Spain, in 2001 with 449 confirmed cases and Vila Franca de Xira, Portugal, in 2014 with 334 confirmed cases) [43, 47-49]. Hot and cold water systems and whirlpool spas have also been shown to be sources of transmission [50, 51].

In fact, a great number of LD outbreaks have been shown to be linked to aerosol-producing devices as the source of *Legionella* spp. transmission and a major variety of mechanisms and settings (e.g. evaporative air conditioning units, decorative fountains and showers) have been described [52, 53].

1.4. Clinical Presentation

In humans, *Legionella* can cause two distinct clinical syndromes: Pontiac Fever (PF) and LD [54]. PF is an acute, self-limited form of legionellosis characterized by flu-like symptoms and was named after an explosive outbreak that occurred in the city of Pontiac, in Michigan, USA [55].

LD, the most severe form of legionellosis, is characterized by pneumonia that can be associated with systemic infection [25]. LD does not have specific, defining clinical features because it presents as a variety of clinical manifestations and symptoms (e.g., fever, myalgia, cough, and pneumonia) [56-58]. The incubation period of LD is thought to be 2–10 days (median 6–7 days); however, longer incubation periods have been reported [48, 51]. In the last years, the mortality from LD has decreased, probably due to earlier diagnosis associated with better treatment [59].

1.5. Diagnosis

The diagnosis of LD can be a challenging process, considering that the clinical symptoms are often indistinguishable from other causes of pneumonia. Considering this fact, laboratory confirmation becomes essential for an accurate diagnosis [60]. There are several laboratory-based methods for the LD diagnosis, which can be divided in two major groups: phenotypic and genotypic methods. The phenotypic methods currently used are urinary antigen test (UAT), serum antibody titration and culture; the genotypic methods are based on the Polymerase Chain Reaction (PCR) [61].

Figure 3 shows the anatomical locations of each specimen type used for the LD diagnosis and the possible diagnostic tests for each one.

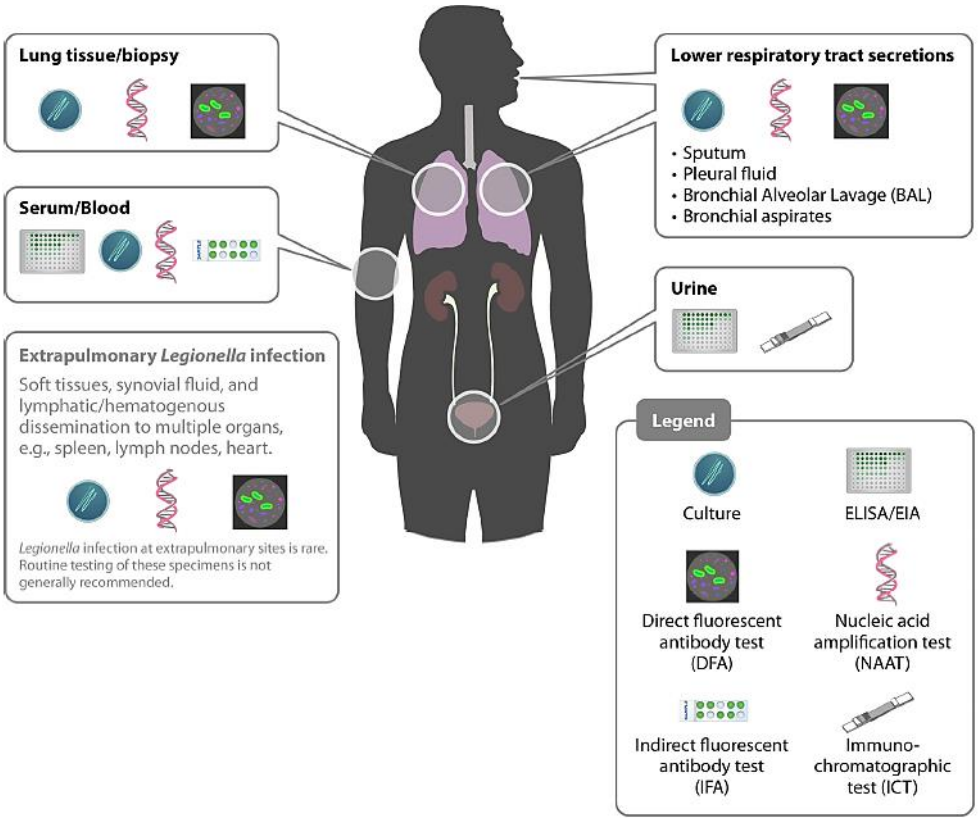


Figure 3 - Anatomical locations of each specimen type used for *Legionella* detection and the specific diagnostic tests. The clinical identification of *Legionella* can be made by several assays and different specimens. Some assays can be applied to multiple specimen types, such as culture and nucleic acid amplification. Although it is not recurrent, *Legionella* infection at extrapulmonary sites can also be observed (Adapted from [8]).

Current LD case classification is divided in probable and confirmed case. Probable case is defined when any person has pneumonia and at least one of the following four laboratory criterion: detection of *Lp* antigen in respiratory secretions or lung tissue, for example by Direct Fluorescent Antibody (DFA) staining using monoclonal-antibody derived reagents; detection of *Legionella* spp. nucleic acid in respiratory secretions, lung tissue or any normally sterile site; significant rise in specific antibody level to *Lp* other than sg 1 or other *Legionella* spp. in paired serum samples; or single high level of specific antibody to *Lp* sg 1 in serum. Confirmed case is defined when any person has pneumonia and at least one of the following three laboratory criterion: isolation of *Legionella* spp. from respiratory secretions or any normally sterile site; detection of *Lp* sg1 antigen in urine or significant rise in specific antibody level to *Lp* sg 1 in paired

serum samples [62].

In the following sections, four diagnostic tests will be described in greater detail: culture, UAT, serological assays and PCR.

1.5.1. Culture

Despite the improvements in other diagnostic tests, isolation by culture remains the gold standard for *Legionella* detection and LD diagnosis. Is the most specific test and requires special media, adequate processing of specimens, and technical expertise [63].

Legionella can be cultured from a number of specimens, including respiratory secretions (sputum, bronchial alveolar lavage (BAL), and bronchial aspirates), lung tissue/biopsy, serum, blood and stool [64, 65]. However, the preferable specimens are the lower respiratory tract secretions (e.g. sputum), which should be promptly processed since *Legionella* may survive poorly in these secretions [64].

Reports of *Legionella* infection at extrapulmonary sites are rare, but have already been verified. In those cases, the samples are collected from soft tissues, joint fluids, and blood and culture should be attempted only when other etiologies have been ruled out since the recovery of an isolate is extremely difficult [8].

The standard medium used to culture *Legionella* spp. is the Buffered Charcoal Yeast Extract (BCYE-α) agar, and contains yeast extract, activated charcoal, α-ketoglutarate, L-cysteine and soluble ferric pyrophosphate. The pH of the agar must be adjusted to 6.9 with the addition of ACES (N-2-acetamido-2-aminoethansulfonic acid) buffer in order to enhance bacterial growth [66] and the optimum growth temperature is 37ºC.

The main disadvantage of this method is the fact that it requires several days to obtain a positive result since *Legionella*, being a fastidious organism, takes at least 5 days to grow [60].

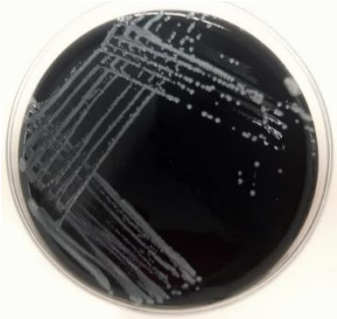


Figure 4 – Culture of *Lp* in BCYE-α agar with 4 days of incubation (photo obtained in the present study)**.**

1.5.2. Urinary Antigen Test

The UAT for the diagnosis of LD is available since 1980 [59] and it has become the first-line diagnostic test [67]. Its use has been increasing over the years, surpassing other diagnostic methods, mainly because it is fast, easy to perform and provides timely, accurate results, with high sensitivity (70%-80%) and specificity (>95%) [68].

The urinary antigen in question is a component of the cell wall LPS of *Lp* and is detected by an enzyme immunoassay (EIA), early in the course of the illness [67, 61, 69]. The test becomes positive after 48-72h of the beginning of the symptoms [67], and the antigen may continue to be excreted in detectable quantities in urine for several months, even after appropriate treatment and apparent recovery from infection, which becomes a disadvantage for diagnosis [69]. However, it has been shown that in the majority of cases antigenuria became negative within 2 months after the first diagnosis [70].

At present, the *Legionella* UAT is available in two main formats: a 96-well plate-based EIA or ELISA, and a rapid immunochromatographic test, in a card/strip-based format, similar to a home pregnancy test (Figure 5) [8].

However, despite being a valuable tool, the UAT only detects the most prevalent species and serogroup, *Lp* sg1 (the causative agent in 70%-80% of LD cases), which fails the diagnosis of LD infections caused by non-*Lp* sg1 strains [63, 59, 71].

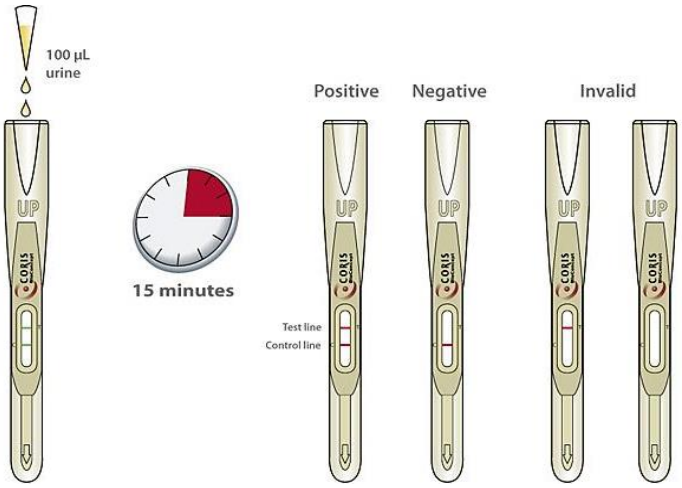


Figure 5 – Rapid diagnostic test for detection of *Lp* antigen in urine.
(Available at http://www.corisbio.com/images/Products/Procedure/full/Legionella-V-TesT.jpg).

1.5.3. Serological assays

Serological testing was one of the main methods used for LD diagnosis in the early 1980s [59]. Nowadays, this method is useful for cases where the patients have already started antibiotherapy or when it is difficult to obtain respiratory samples. It is considered a late diagnostic because it requires a paired serum samples with 2 weeks apart.

The main disadvantage of the serological assays is the possibility of cross-reactivity between different serogroups or between different species of *Legionella* spp., which may interfere with the interpretation of the results and therefore influence the diagnosis, leading to incorrect and ineffective treatment [72, 73].

Nevertheless, serology remains important for LD confirmation in the cases where the etiologic agent cannot be isolated. Furthermore, this method may be of interest for retrospective epidemiological studies, such as general seroprevalence, to identify patterns of disease or potential ongoing outbreaks [8].

1.5.4. Polymerase Chain Reaction

The first report of PCR as a method to detect *Legionella* was in 1989 [74] and since then it has become frequently used to identify *Legionella* in clinical samples [75].

PCR enables specific amplification of small amounts of *Legionella* DNA, provides rapid diagnosis (with outcome in a day) and is one of the few diagnostic tests with the potential to detect infections caused by all of the known species and serogroups of *Legionella* spp. [63, 67, 75].

It has been successfully used to detect *Legionella* DNA in a range of clinical samples, such as sputum, urine and serum. However, the reliableness of the results obtained from nonrespiratory specimens is not fully known [63].

Previous tests with samples from the lower respiratory tract have shown that PCR is highly specific and has sensitivity greater than culture, suggesting that it is suitable for use in the routine microbiological diagnostic laboratory [76].

Additionally, this method can be used when the patient have already started the antibiotherapy [77].

Despite all the advantages, the difficulty in assessing bacterial viability is the main disadvantage of all nucleic acid amplification methods, since they fail to discriminate between nucleic acids from dead or dying bacteria. Therefore, they cannot distinguish disease resolution from current disease [8].

1.6. Management and Risk Factors

When a case of LD is diagnosed, the physicians must be aware of the possible presence of other cases related in time and place, which can be crucial for identification of the potential source of infections. It is also important to have a detailed history of the recent activities of the patient, including any potential exposure to aerosolized water droplets (especially during the previous 10 days) in order to support the epidemiological follow-up, trace any other patients and identify the source of infection [25].

LD can occur in previously healthy individuals, but is more frequent in those who gather the major risk factors, such as age (≥50 years), sex (male), smoking habits, chronic cardiovascular/respiratory disease, diabetes, alcohol misuse, and immunosuppression (e.g. after solid organ transplantation or any other immunosuppressive therapy). Immunosuppressed patients might present with more severe clinical disease and frequently require intensive care, intravenous antibiotics, and a longer duration of therapy [78-81].

The empiric therapy for LD is antibiotic treatment of the infection and management of any complications [58]. The chance of recovery is higher if the appropriate antibiotics are given early [57, 82]. Since LD does not have any defining clinical features and that β-lactam antibiotics (usually used to treat bacterial community-acquired pneumonia) are unsuccessful for treatment of LD, it is wise to give effective antibiotic therapy against *Legionella* spp. in the early stage of all moderate-to-severe community-acquired and hospital-acquired pneumonias, until a specific microbiological diagnosis is made. The dose and route of administration of treatment (which can be either oral or intravenous) is guided by severity, underlying risk factors, consciousness level, and gastrointestinal disorders [25].

Because *Lp* is an intracellular pathogen residing within tissue and alveolar macrophages, successful treatment depends on use of antibiotics that achieve

therapeutic intracellular concentrations within macrophages, such as the macrolides and fluoroquinolones [83-88]. Erythromycin had been the drug of choice for treatment of LD until the 1990s but its use have been decreasing since it is bacteriostatic and has side-effects, particularly when used intravenously [89, 90]. However, the newer macrolides such as azithromycin have fewer side-effects. Regarding fluoroquinolones, they are bactericidal and *in vitro* their activity against *Legionella* spp. in animal models has been shown to be higher the one from erythromycin [91, 92].

1.7. *Legionella pneumophila* natural host: *Acanthamoeba* spp.

Amoebae are ubiquitous organisms that can be found in humid soil and water reservoirs, being the most common genus *Acanthamoeba* spp. [93, 94]. They are unicellular protozoans that can display two different phases during their life cycle, according to the environmental conditions. When the conditions are unfavorable to their growth (e.g. limited nutrients, temperature) they remain as a dormant cyst, a process called encystment. Conversely, when the conditions are suitable for their growth, they change to an active vegetative trophozoite, a process called excystment [94].

Free-living amoebae are frequently isolated from several man-made reservoirs, such as tap water, air-conditioning units, and cooling towers, where they feed on the existing microbial biofilm. However, though amoebae are bacterial predators, several bacteria have developed mechanisms to survive phagocytosis, being able to use the amoebae as hosts [95].

Transient association with amoebae has been reported for a number of different bacteria including *Lp*, many *Mycobacterium* spp., *Francisella tularensis*, and *Escherichia coli* O157, among others [96-99].

Since most of these bacteria are pathogens of humans, it has been suggested that protozoa have an important role in the development of bacterial pathogenesis and that the interaction bacteria/protozoa is significant in terms of human disease [100].

To date, only the interaction of *Lp* with free-living amoebae has been studied in greater detail [95].

1.7.1. Life cycle in *Acanthamoeba* spp.

Lp is ubiquitous in freshwater, often in close association with freshwater protozoa [95] and replicates at temperatures of 25–42°C with an optimal growth temperature of 35°C. Consistent with what *Lp* would encounter in the environment, motility and adherence to host cells are optimal at temperatures below 37°C [101].

Thermal conditions have been shown to affect the interaction between *Lp* and amoebae [102]. At temperatures over 25°C *Lp* is able to infect the trophozoite form of free-living amoebae and replicate intracellularly, increasing the number of bacteria in the water. Consequently, the chance of transmission to humans increases as well [102, 103]. In contrast, with temperatures below 20°C, the amoebae encyst and *Lp* is not able to replicate inside the host [63].

Intracellular *Lp* is capable of survival within this cysts that ensure their survival by protecting them from harsh environments. In this stage, the bacteria are not capable to proliferate and thrive to a dormant state [104].

Fourteen species of amoebae, with *Hartmannellae* and *Acanthamoeba* being the most prominent, and two species of ciliated protozoa have been shown to support intracellular replication of *Lp* [95]. When compared with bacteria grown *in vitro*, bacteria grown in amoebae have an increased resistance to harsh conditions [105, 106] and show changes in biochemistry, physiology, and virulence potential [103]. From those changes it has been verified: an enhanced resistance to chemical disinfectants, treatment with biocides and antibiotics [95]; shorter size and larger diameter; different protein expression [107]; enhanced intracellular survival and replication and increased ability to infect not only amoebae but also mammalian cells [108]. It has also been shown that amoeba are capable of resuscitate viable non-culturable *Lp*, for instance after disinfection [95, 109, 110]. Additionally, it is believed that the enhanced infectivity of *Lp* after growth within amoebae may compensate the low concentration of bacteria usually detected in the aquatic reservoirs from which the bacteria are transmitted to humans during LD outbreaks [111-114].

Besides enhancing the pathogenicity of *Lp*, amoebae are also responsible for their persistence in the environment. Since *Legionella* species cannot multiply extracellularly, the presence of amoebae as hosts in their life cycle is fundamental

(Figure 6) [103].

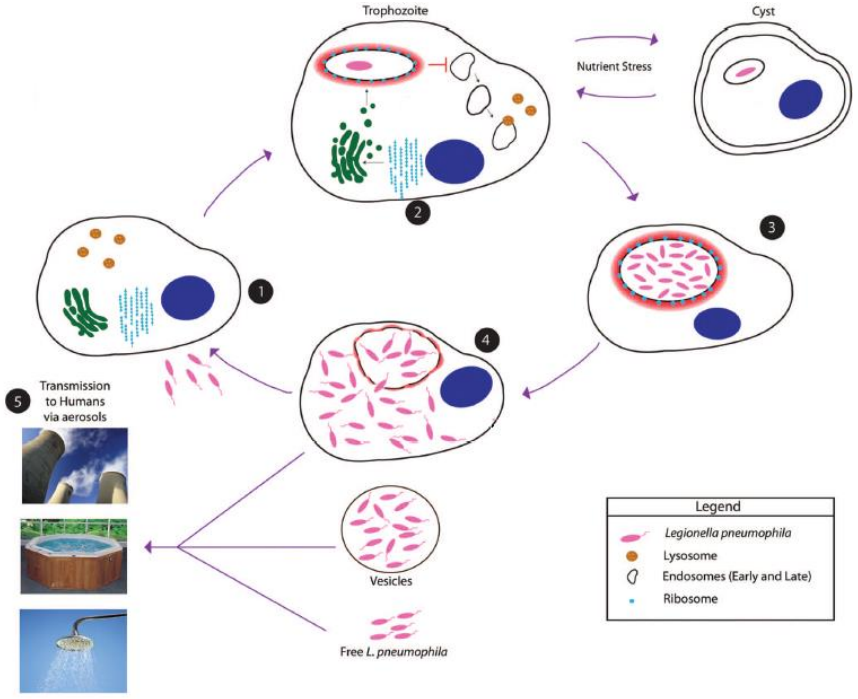


Figure 6 - The environmental life cycle of *Lp* within protozoa.

1. Flagellated *Lp* infects protozoa in the aquatic environment.
2. The *Legionella*-containing vacuole (LCV) evades the default endosomal–lysosomal degradation pathway and becomes rapidly remodeled by the ER through intercepting ER-to-Golgi vesicle traffic.
3. Under unfavorable stress conditions, such as nutrient deprivation, amoeba encysts, and bacterial proliferation will not occur due to nutrient limitation.
4. During late stages of infection, the LCV becomes disrupted leading to bacterial egress into the cytosol where the last 1–2 rounds of proliferations are completed. Nutrient depletion triggers a phenotypic transition into a flagellated virulent phenotype followed by lysis of the amoeba and bacterial escape from the host cell. Excreted vesicles filled with bacteria are also released. The infectious particle is not known but may include excreted *Legionella*-filled vesicles, intact *Legionella*-filled amoeba, or free *Legionella* that have been released from host cell.
5. Transmission to humans occurs via aerosols generated from man-made devices and installations, such as cooling towers, whirlpools, and showerheads (Adapted from [115]).

1.7.2. From Amoebae to Macrophage

Lp are able to infect, multiply within, and kill human macrophages, as well as free-living amoebae [116]. This ability is thought to be a consequence of previous interaction with several protozoan hosts that allowed *Lp* to adapt to intracellular growth within macrophages [117]. This adaptation process is thought to include the acquisition of eukaryotic genes during its co-evolution with amoebae [118], which is supported by

findings that show that all the genes that are required for intracellular growth in human macrophages are the same required for intracellular growth in *Acanthamoeba castellanii* (*Ac*) [116].

Additionally, the life cycle of *Lp* in macrophages strongly resembles the one observed in amoebae, mainly in the process by which *Lp* is able to avoid digestion. In both phagocytes vacuoles containing *Lp* neither acidify nor fuse with the lysosomal compartment, allowing the association between the phagosome and the endoplasmic reticulum which leads to the high intracellular replication of *Lp* [119].

Moreover, the similar cell biology between amoebae and macrophages can also be an indicator that the virulence of *Lp* for macrophages is in fact a consequence of its evolution as an intracellular parasite of protozoa [119].

Lp and amoebae have been isolated from the same source of infection during outbreaks of LD [95] which shows that amoeba are a natural reservoir for the opportunistic pathogens of macrophages [119].

1.7.3. Evasion of the host for new infection cycle

The ability to lyse and exit host cells after intracellular replication is an essential step in the life cycle of all intracellular pathogens [120]. After this step, the released pathogens are able to infect other cells within the same host or be transmitted to a new susceptible host [120].

It is known that *Legionella* has the ability to kill a wide variety of host cells (e.g. human phagocytic cells, amoeba and ciliated protozoa) and it has been suggested that the bacteria induce apoptosis, or programmed cell death, in the host [121].

Furthermore, it has been shown that *Lp* is able to induce major changes in the nuclear morphology of the host as well as increase the proportion of fragmented DNA, which correlates with the cell death process that occurs in macrophage-like HL-60 cells [122].

Even though this issue has already been addressed, apoptotic death of amoeba infected with *Lp* has not been observed yet [121].

1.8. Apoptosis

The first studies dedicated to cell death date back to the nineteenth century and were generally related to the metamorphosis of tadpoles and insects, and later with transient embryonic structures [123, 124]. The subject of "programmed cell death" – in which "apoptosis" is included as one type of cell death - appeared later in the 1970s when scientists first began to observe a sequence of events in the cell that once established could lead to its death. These observations showed that cell death during development is not of accidental nature but instead follows a sequence of controlled steps that lead to self-destruction of the cell [124].

The term "apoptosis" was then proposed in 1972 to name one type of programmed cell death that plays an important role in the regulation of animal cell populations. Moreover, it was shown that the apoptotic process could be initiated or inhibited by a variety of environmental stimuli, both physiological and pathological [125].

Since then, the field of apoptosis research began to grow exponentially with a new set of mind in which cell death is not an incidental part of life, but instead a highly controlled and medically important element of existence [124, 126].

Although apoptosis is the most frequent form of programmed cell death, it is important to note that there are other non-apoptotic types of programmed cell death that have already been described and have biological significance [127, 126].

1.8.1. Morphological features of apoptosis

When a cell is triggered to suffer apoptosis it means that a cascade of molecular events has been activated, resulting in the total disintegration of the cell [128].

One of the first events of this type of "programmed cell death" is the loss of intracellular water, which leads to a smaller and denser cell. Later, one of the most characteristic features of apoptosis occurs: the condensation of the nuclear chromatin to heterochromatin (in one or more masses in the nucleus). The nuclear membrane of the cell begins to disintegrate and lamin proteins undergo proteolytic degradation, followed by nuclear fragmentation. Activation of endonuclease(s) results in a selective degradation of DNA, in fragments up to 50–300kb, and is followed (in many but not all

cell systems) by internucleosomal DNA cleavage. As a result, many nuclear fragments (that resemble with DNA droplets of different sizes) are then scattered throughout the cytoplasm. Afterwards, those nuclear fragments, together with constituents of the cytoplasm (e.g. undamaged organelles), are packaged and enclosed by fragments of the plasma membrane. These structures, named ‘‘apoptotic bodies’’, are then shed from the dying cell (Figure 7) [124, 128].

During this process, there are two important characteristics that are exclusive of apoptosis: the activation of endonuclease(s) that preferentially cleave DNA at the internucleosomal sections and the preservation of the structural integrity of the plasma membrane as well as some cellular organelles, such as mitochondria and lysosomes [128].

Opposing to apoptosis, there is a different type of cell death that can be interpreted as an “accidental death”, named necrosis [126]. In this case, the cell death pathway begins with the mitochondrial swelling that eventually leads to the rupture of the plasma membrane, which causes the releasing of the cytoplasmic constituents. At the end of this process, nuclear chromatin shows irregular areas of condensation and the nucleus is slowly dissolute [128].

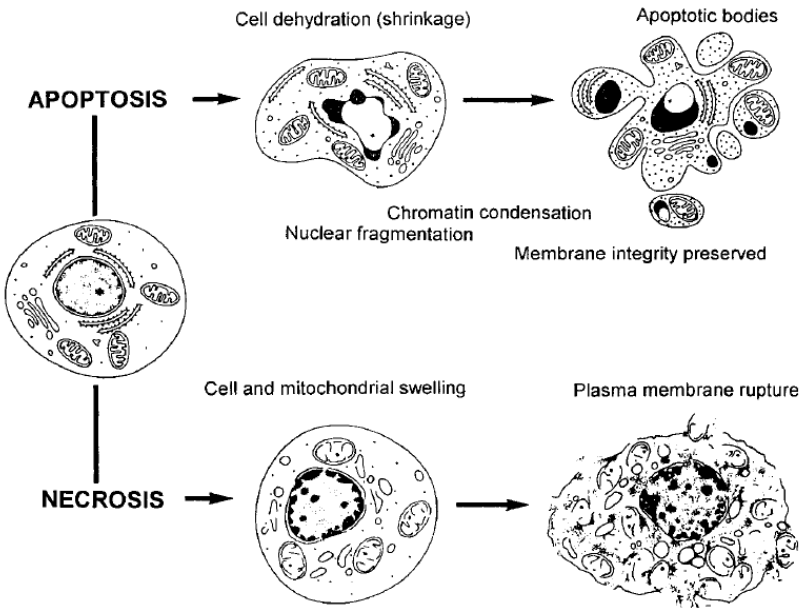


Figure 7 – Morphological and biochemical changes that occur during apoptosis and necrosis. The apoptotic pathway begins with the loss of intracellular water and increase in the concentration of ionized calcium in the cytoplasm, resulting in the cell shrinkage. Subsequently, the chromatin condensation occurs followed by nuclear disintegration and formation of apoptotic bodies. The integrity of the plasma membrane is preserved to the late stages of apoptosis. In contrast, the necrotic pathway begins with the swelling of mitochondria as well as swelling of the whole cell, combined with marginal chromatin condensation. Finally, the rupture of the plasma membrane results in the releasing of the cytoplasmic content of the cell (Adapted from [128]).

1.8.2. Mechanisms of apoptosis

The apoptotic machinery is tightly controlled and can be initiated by two main alternative pathways: the death-receptor pathway (usually named "extrinsic pathway"); or the mitochondrial pathway (usually named "intrinsic pathway") [129, 126].

Both pathways have in common the presence of initiator cysteine aspartyl-specific proteases, called caspases (caspase-8 for the extrinsic pathway and caspase-9 for the intrinsic pathway) that once activated cleave and activate the 'executioner' caspases, such as caspase-3, which starts the execution pathway and is common to both extrinsic and intrinsic pathways. The active executioner caspases then cleave each other and an amplifying proteolytic cascade of caspase activation is started. Ultimately, the active executioner caspases cleave cellular substrates leading to the characteristic biochemical and morphological changes of apoptosis (Figure 8) [129, 126].

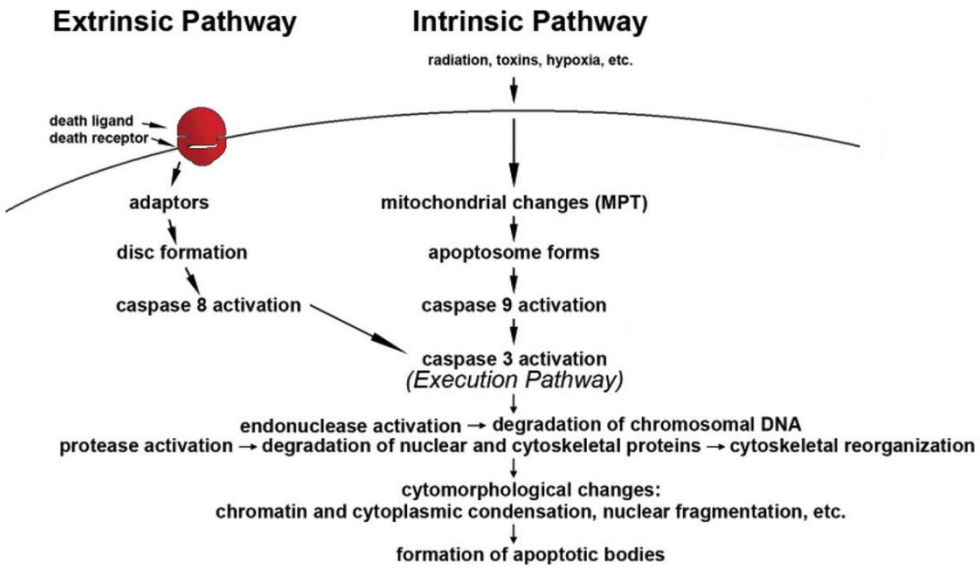


Figure 8 - The two main apoptotic signalling pathways. Apoptosis can be initiated by two alternative pathways: either through death receptors on the cell surface (extrinsic pathway) or through mitochondria (intrinsic pathway). Both of them require specific triggering signals to begin an energy-dependent cascade of molecular events. Each pathway activates its own initiator caspase (8 and 9) which in turn will activate the executioner caspase-3. The execution pathway results in characteristic cytomorphological features including cell shrinkage, chromatin condensation, formation of cytoplasmic blebs and apoptotic bodies and finally phagocytosis of the apoptotic bodies by adjacent parenchymal cells, neoplastic cells or macrophages (Adapted from [126]).

1.8.3. Biochemical features

There are several biochemical hallmarks of apoptosis (e.g. DNA degradation, protein cleavage, protein cross-linking, phagocytic recognition) that can be used to identify this mode of cell death [126].

One of those biochemical features is the expression of cell surface markers in the outer leaflet of the cell membrane. This membrane alteration allows the apoptotic cells to be recognized by adjacent cells, allowing a quick phagocytosis [126, 130].

Phosphatidylserine (PS) is an anionic phospholipid of the cellular membrane that in normal cells is only present in the inner membrane. During apoptosis, the amount of phosphatidylserine (PS) on the outer surface of the membrane increases, becoming exposed outside the cell [130].

For the study of apoptotic cells, a recombinant phosphatidylserine-binding protein named Annexin V can be used to detect this type of cells. Annexin V has been shown to interact strongly and specifically with phosphatidylserine residues, which makes it a useful tool for apoptotic cell detection [131-133].

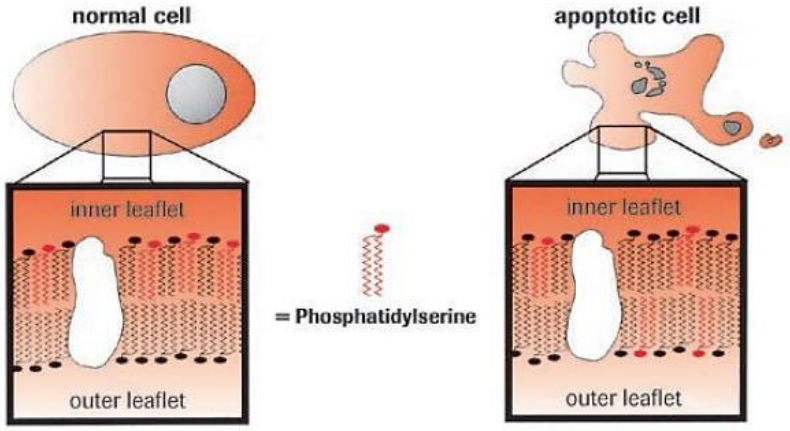


Figure 9 – Phosphatidylserine exposure during apoptosis. During apoptosis, the distribution of neutral phospholipids (black symbols) and anionic phospholipids such as PS (red symbols) in the cell membrane changes. PS is present in the outer membrane of apoptotic cells, but not of normal cells. An exogenously added molecule specific for PS, such as Annexin V-FITC, will bind to PS on the outer membrane of apoptotic cells, but cannot react with the PS of normal cells (Adapted from [130]).

1.8.4. Apoptosis measurement

Since apoptosis has so many characteristic changes that appeared this type of cell death, those changes have become markers that are used to identify this mode of cell death by several laboratory methods [128].

There are several commercial kits available to detect and count apoptotic cells and since many features of apoptosis and necrosis can overlap, it is crucial to employ two or more distinct assays to confirm that cell death is occurring via apoptosis. The assays should be based on a different principle in order to have a more complete approach. Other methodology for apoptosis detection that is becoming very popular is the multiplex, in which the same sample allows to gather more than one set of data [126].

Therefore, there are apoptosis assays based on several methodologies, such as cytomorphological alterations; DNA fragmentation; detection of caspases, cleaved substrates, regulators and inhibitors; membrane alterations; detection of apoptosis in whole mounts and mitochondrial assays [126].

All assays have advantages and disadvantages regarding the object of study. Consequently, it is crucial to understand the pros and cons of each assay and to define the best combination of assays to allow a more complete and accurate study [126].

1.9. Research objectives

The molecular mechanisms by which *Lp* kills protozoan host cells are largely unknown. In 1998, Hagele *et* al have demonstrated that *Lp* is able to induce apoptosis in human monocytes and that this process depends on the multiplicity of infection (MOI), i.e. the proportion between *Lp* and the host, and the time post-infection. However, by studying infection in *Ac* at 24h post-infection and a MOI of 50, they concluded that *Lp* was not able to induce apoptosis in *Ac*. This finding suggested that *Lp* induces different mechanisms of cell killing to evade the main hosts: protozoan and human cells [121].

More recently, Gao and Kwaik demonstrated that intracellular *Lp* kills and exits *Acanthamoeba polyphaga* preferentially by induction of necrosis. They have also confirmed that, as in the mammalian cells, PS is distributed in the inner leaflet of the plasma membrane in *Acanthamoeba* and that specifically *Acanthamoeba polyphaga*

possesses a functional apoptotic pathway [133].

The main objective of this study was to clarify if *Lp* is able to induce apoptosis in the natural host *Ac.*

Since the molecular mechanisms by which *Lp* kills protozoan host cells remains uncertain and the published studies were based only in a few conditions of the infection, we proposed to clarify whether *Lp* induces apoptosis in *Ac*, using different MOIs and time post-infection.

The apoptosis detection in *Ac* was assessed by membrane alterations (PS externalization) using flow cytometry analysis and DNA fragmentation using electrophoresis.

Chapter 2. Material and Methods

All the experimental procedures were performed in a tissue culture room, in a Class II Type A2 Biological Safety Cabinet that maintained the sterility of cell lines and protected both the user and the experiment. In the tissue culture room, an exclusive disposable lab coat was always used, proper aseptic techniques were assembled in order to eliminate possible contaminants and all the materials were disposable plastic.

2.1. Microorganisms, culture media and growth conditions

In this study, we used *Lp* strain Paris (*Lp* Paris), isolated from patients and the environment in the area of Paris, France, from 1987 to 1997 (courtesy of the Pasteur Institute) and a line of *Acanthamoeba castellanii* (*Ac*) strain Neff (ATCC® 30010™).
A stock culture of both strains was preserved at -80ºC. *Lp* was stored in skim milk and *Ac* was stored in 10% DMSO + 90% FCS.

2.1.1. Growth of *Legionella pneumophila* strain Paris

After thawing, *Lp* Paris was cultured on Buffered Charcoal Yeast Extract (BCYE, Appendix 2) agar supplemented with ACES Buffer/potassium hydroxide, ferric pyrophosphate, L-cysteine HCl and α-ketoglutarate (*Legionella* BCYE Growth Supplement SR0110A, Oxoid), and incubated at 37°C, for 48h.

2.1.2. Growth of *Acanthamoeba castellanii*

Ac was grown in axenic static culture in culture T-flasks with a surface area of 25cm^2, in 6ml of Peptone-Yeast Extract-Glucose medium (PYG, Appendix 3) at room temperature.

Additionally, to ensure that the cultures remained viable for an extended period of time, we had to follow some procedures that will be described below:

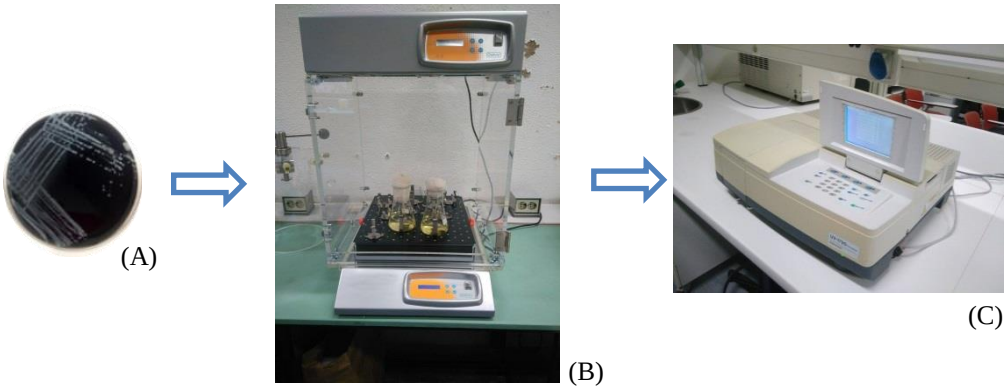
- Monitor the culture regularly, to confirm its viability and healthy morphology, using an inverted microscope (Meiji Techno, TC-5300).
- When the culture reaches the peak density, i.e., confluent layer of *Ac* on the bottom surface of the flask, the culture was split to a new culture flask. In order to do this, first, the confluent layer of *Ac* was washed with 3ml of PYG, for three times, to assure that the wastes resulting from metabolism and unviable *Ac* were rejected. After this, the *Ac* were harvested in 3ml of PYG with a cell scrapper and approximately 0.25ml of the *Ac* suspension was transferred to a new flask. Repeat the procedure in 2-3 days intervals.
- Prepare fresh PYG on a monthly basis in order to maintain this procedure.

2.2. Infection Protocol of *Acanthamoeba castellanii* with *Legionella pneumophila* strain Paris

For the infection protocol of *Ac* with *Lp* Paris, both microorganisms required previous growth conditions and specific procedures before the infection. The preparation of each microorganism for infection will be described below.

2.2.1. Preparation of *Legionella pneumophila* strain Paris liquid culture for infection

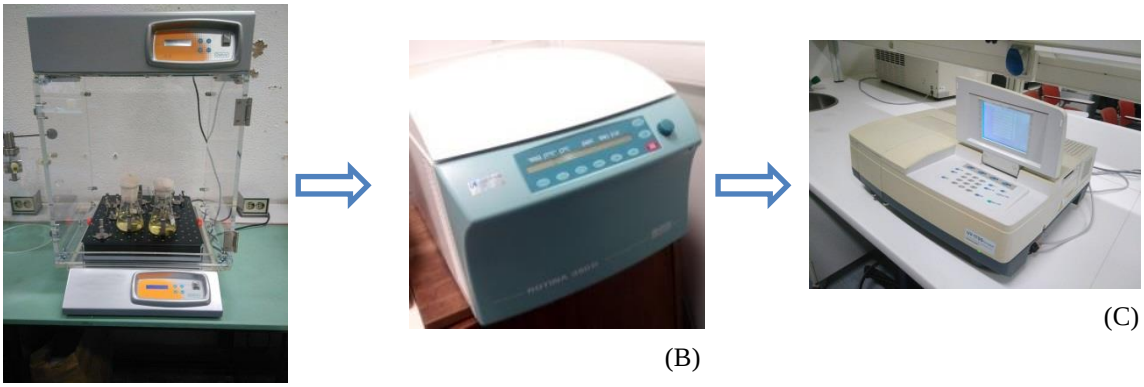
For each infection, three to four colonies from a new 48h passage culture in BCYE agar, were cultured to 100ml of ACES-buffered yeast extract broth (AYE, Appendix 4) and incubated at 37ºC with shaking at 170 rpm (incubator I10-C+ACOP. E, OVAN). To monitor the growth of the liquid culture we used a spectrophotometer (Shimadzu, UV-1700 PharmaSpec) to measure the optical density at 600nm (OD_{600}), at hourly intervals. These measures were used to build the *Lp* Paris growth curve and determine when the culture reached the beginning of the stationary phase, i.e. when the difference between two consecutive measures was 0.05 (previously determined by the research group).



(A) (B) (C)

Figure 10 – *Lp* Paris was inoculated, in duplicate, on AYE **(A)** and incubated at 37ºC, 170 rpm **(B)**. The *Lp* Paris growth was monitored by measure of OD_{600} until the culture reached the stationary phase **(C)**.

Once the culture was in the stationary phase, 5ml of the suspension were collected to a 15ml sterile Falcon tube and centrifuged (centrifuge ROTINA 380R, Hettich) for 15 minutes at 3095 x *g*. The supernatant was discarded and the pellet was resuspended in 10ml of Minimum Medium (MM, Appendix 5). The OD_{600} of the suspension was measured and adjusted to 1.2, which represents a concentration of 10^9 CFU/ml (previously determined by the research group).



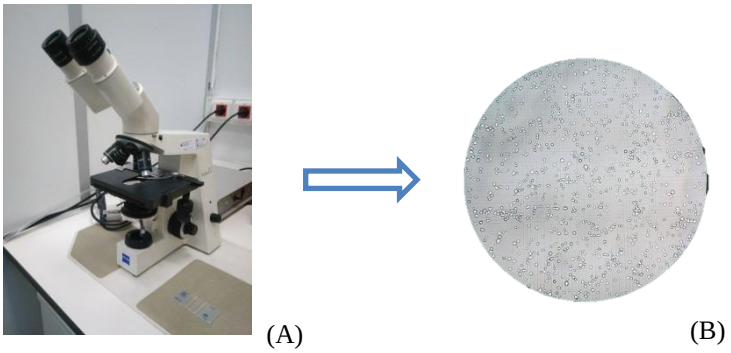
(A) (B) (C)

Figure 11 – The *Lp* Paris liquid culture in the stationary phase was collected **(A)** and centrifuged for 15 minutes at 3095 x *g* **(B)**. For the infection, the concentration of *Lp* Paris was measured by spectrophotometry **(C)**.

2.2.2. Preparation of the *Acanthamoeba castellanii* confluent layer for infection

For each infection, we used a confluent layer of *Ac* with 48h of growth. The *Ac* monolayer was washed with 3ml of minimum medium (MM, Appendix 5), for two

times. To detach the *Ac* from the bottom layer of the flask, we used a cell scrapper and 1,5ml of MM. A counting chamber (Thoma, 0.1mm) was used in order to count the *Ac* (Transmitted Light Microscope Standard 25 ICS, Carl Zeiss) and determine the concentration of the suspension.



(A) (B)

Figure 12 – The *Ac* concentration for infection was determined by counting on a Thoma Chamber with a transmitted light microscope (A) at 10x (B).

2.2.3. Infection of *Acanthamoeba castellanii* with *Legionella pneumophila* strain Paris

For the infection protocol, we tested three different MOIs: 10, 50 and 100 (this values were previously established by the research group). In each infection, we prepared the required culture T-flasks with an *Ac* monolayer of $3x10^6$ *Ac*/ml. After this, we calculated the required volume of the *Lp* Paris suspension to infect the *Ac* (considering the intended MOI). Once the *Lp* Paris was pipetted into each flask, the flasks were incubated at 37ºC for one hour. After the incubation time, we washed each flask with 3ml of MM, for three times, to remove the extracellular *Lp*. After the washing process, we added a final volume of 6ml of MM and the flasks were incubated at 37ºC. The infection is considered to be started at this point.

Each infection assay had a negative control (flask with uninfected *Ac* in MM, incubated at 37ºC).

2.3. Apoptosis analysis

The apoptosis analysis was performed using two techniques: flow cytometry and

agarose gel electrophoresis. In this study, we analyzed the *Ac* at different time post-infection: 8, 12, 14, 16, 18, 20 and 24 hours. Each assay had a negative control (the same referred in 2.2.3) and a positive control. The positive control chosen for *Ac* apoptosis was heat shock and had to be optimized. To optimize the positive control, we tested different incubation time (1, 2.5, 3.5, 4, 4.5, 5 and 5.5 minutes) at 56ºC followed by one hour at 37 ºC and analyzed using three techniques: agarose gel electrophoresis, fluorescent microscopy and flow cytometry.

2.3.1. Flow cytometry

Flow cytometry is a biophysical technology that allows a rapid analysis of both qualitative and quantitative characteristics of single cells. Those characteristics include cell size, cytoplasmic complexity, DNA or RNA content, and a wide range of membrane-bound and intracellular proteins [134].

In this method, cells are fluorescently labelled and then flow in front of a laser which will excite them to emit light at varying wavelengths. By measuring the emitted fluorescence, it is possible to determine the amount and type of cells present in a sample [135].

2.3.1.1. Staining of *Acanthamoeba castellanii* with Annexin V-FITC and 7-AAD

As referred in the Introduction chapter (1.8.3), one of the biochemical features of the apoptotic process is the expression of cell surface markers in the outer leaflet of the cell membrane, such as PS. By flow cytometry, we can detect the apoptotic cells by the binding of the externalized PS with the highly fluorescent Annexin V. In this study we used Annexin V-FITC, a member of the annexin family of intracellular proteins that binds to PS in a calcium-dependent manner.

Although it will not bind to normal living cells, Annexin V-FITC will bind to the PS exposed on the surface of apoptotic cells. Thus, Annexin V-FITC has proved suitable for detecting apoptotic cells, for instance by multicolor flow cytometry or fluorescence microscopy. Additionally, since necrotic cells are labeled upon rupture of their plasma membrane, it is important to control the membrane integrity of the PS-positive cells by double-staining with membrane-impermeable DNA dyes such as 7-AAD. In these

assays, healthy cells are doubly negative to Annexin V-FITC and 7-AAD, whereas cells in the early phases of apoptosis are Annexin V-FITC-positive but 7-AAD-negative, and secondary necrotic cells are doubly positive to Annexin V-FITC and 7-AAD [130].

For the staining of *Ac* with Annexin V-FITC and 7-AAD we used the following protocol (previously established by the research group):

1. Harvest the *Ac* of each condition (the infected *Ac* of each hour post-infection, the corresponding negative control and the positive control).
2. Centrifuge the tubes for 5 minutes at 2000 x *g*.
3. Discard the supernatants and wash the pellets in 1ml of PBS (1x).
4. Divide the total volume into two Eppendorf tubes (stained and unstained).
5. Centrifuge the tubes for 5 minutes at 2000 x *g*.
6. Discard the supernatants and resuspend the pellets in 190µl of PBS + Ca^{2+}, 5µl of Annexin V-FITC (ImmunoTools) and 5µl of 7-AAD (SIGMA) - for the stained tubes - and 200µl de PBS + Ca^{2+} - for the unstained tubes.
7. Incubate the tubes for 15 minutes in the dark, at room temperature.
8. After the incubation period add 800µl of PBS (1x) to each tube.

After the staining protocol, the *Ac* were analyzed in a 3-laser 9-color flow cytometry analyzer (CyAn ADP, Beckman Coulter).

2.3.1.2. Strategy to analyze flow cytometry results

For the analysis, the *Ac* were distributed in four groups considering the detected fluorescence: live, early apoptotic, late apoptotic and necrotic.

Table 1 - Distinguishing apoptosis from necrosis using Annexin V-FITC and 7-AAD staining.

	Live	Early Apoptotic	Late Apoptotic	Necrotic
Annexin V-FITC staining	-	+	+	-
7-AAD staining	-	-	+	+

These four groups were delimited by four gates, created based on acquisition of positive control cells that were single staining (with Annexin V-FITC only or 7-AAD only) and considering characteristics such as the fluorescence intensity, the cell size and

granularity (Figure 13).

The *Ac* that were Annexin V-FITC positive were considered the early apoptotic cells and the *Ac* that were 7-AAD positive were considered the necrotic cells. For this analysis, we used the Summit® Software v4.3.01 from Beckman Coulter.

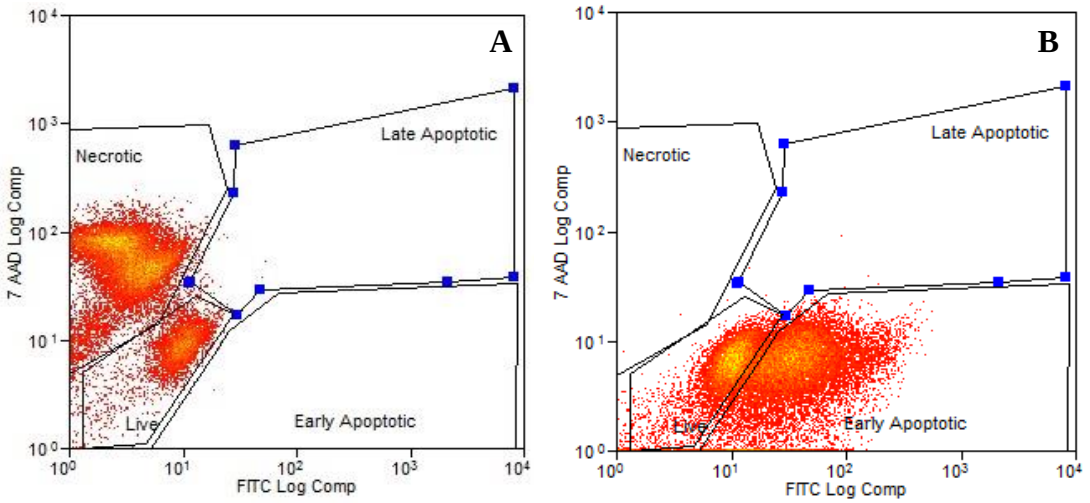


Figure 13 – Definition of the gates according to the emitted fluorescence. **(A)** Positive control - *Ac* treated with heatshock, single stained with 7-AAD; **(B)** Positive control - *Ac* treated with heatshock, single stained with Annexin V-FITC.

2.3.1.3 Statistical analysis

The flow cytometry results were statistically analyzed using the software GraphPad Prism® 6 (version 6.01). A Student t test analysis was done in order to compare each sample with the negative control (paired samples). We considered P values < 0,05 statistically significant (* P < 0,05; ** P < 0,01 and *** P < 0,001).

2.3.2. Agarose gel electrophoresis to assess DNA fragmentation

As referred in the Introduction chapter (1.8.1), one characteristic feature of apoptosis is the activation of endonuclease(s) that preferentially cleave DNA at the internucleosomal sections. As a result, the products of DNA degradation are nucleosomal and oligonucleosomal DNA sections (with approximately 180 base pairs) that generate a characteristic ‘‘ladder’’ pattern that is possible to identify during an agarose gel electrophoresis [128].

Therefore, after the extraction of DNA from a lysed cell homogenate we performed an agarose gel electrophoresis to visualize whether endonuclease cleavage products,

characteristic from apoptosis, were formed.

For this technique, we compared three different DNAs: *Lp*, *Ac* from the negative and positive control and infected *Ac*.

Lp Paris DNA was extracted using the InstaGene™ Matrix (Bio-Rad) and *Ac* DNA was extracted using the QIAamp® DNA Mini Kit (QIAGEN).

2.3.2.1. Agarose gel electrophoresis

DNA fragments were separated via agarose gel electrophoresis (1.5%). The gels were prepared by dissolving the agarose in TBE buffer (0.5x). To visualize the DNA, 2 µl of (10 mg/ml) ethidium bromide (BioRad) was added to the agarose solution. To load the samples, 5µl of DNA was mixed with 2µl of the agarose gel loading buffer. Electrophoresis was performed for 40 minutes at 140 V (Horizon 58, Life Technologies). The DNA was detected using UV light linked to photographic system (Kodak EDAS 290) and the size of the DNA was determined using 1Kb DNA ladder (Invitrogen™).

2.4. Analysis of the intracellular *Legionella pneumophila* strain Paris replication

To measure the entry and survival of *Lp* Paris in *Ac*, we performed an infection assay with a MOI of 100 for 24 hours at 37ºC. After, we plated the initial *Lp* suspension (0h) and the intracellular *Lp* (obtained from the *Ac* monolayers lysis, Appendix 6) from 1 and 24 hours post-infection into BCYE agar plates in order to determine the CFU/ml of *Lp* from each time point.

The distribution of the bacteria in the plates was made by spread and drop plate method.

2.4.1. Spread and drop plate method

For bacterial enumeration by spread and drop plate method, successive dilutions of *Lp* (from each hour of infection) were pipetted into BCYE agar plates. For the spread method, we pipetted 100µl of each dilution to a specific plate. For the drop method, all plates were divided in quadrants (four dilutions each) and two different volumes of drop were tested: 10 and 30µl. For the spread method duplicates of each dilution were made

while for the drop method were made triplicates of each dilution.

After the drops on the agar dried, the petri plates were inverted and incubated at 37ºC until colonies were formed for counting.

2.4.2. Counting and CFU calculation

After appropriate incubation (approximately four days) the plates were inspected and the colonies of each dilution were counted. To calculate the CFUs/ml we considered the dilutions with 30 to 300 CFUs/plate - for the spread method - and the dilutions with 3 to 30 CFUs/drop - for the drop method.

Chapter 3. Results

3.1. Study of the death mechanisms induced by *Legionella pneumophila* in *Acanthamoeba castellanii*

There are several factors that can affect the timeline of biochemical events associated with cellular death. Therefore, in order to clarify which death mechanisms are inducted by *Lp* Paris in *Ac* we evaluated the following conditions:

3.1.1. *Legionella pneumophila* strain Paris culture for infection

For the infection protocol, we were interested in having *Lp* Paris from the stationary phase.

In order to determine when the cultures reached this phase, we monitored the growth of the all cultures by spectrophotometry and built a growth curve.

Table 2 – OD_{600nm} measures of *Lp* Paris in AYE.

Incubation hours	OD_{600nm}		
	1	2	3
0.5	0.156	0.043	0.112
1.5	0.279	0.193	0.200
3.5	0.372	0.323	0.308
5.5	0.556	0.608	0.523
6.5	0.702	0.835	0.699
7.5	0.903	1.137	1.040
8.5	1.235	1.418	1.287
9.5	1.507	1.533	1.509
10.5	1.579	1.565	1.573

We observed that *Lp* Paris in AYE would usually take up to 10 hours to reach the stationary phase (Figure 14).

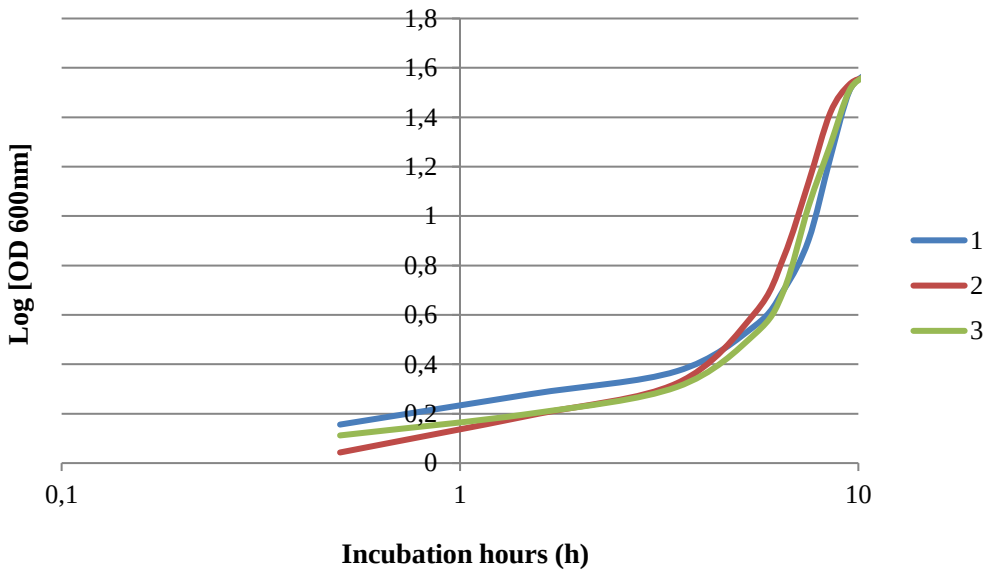


Figure 14 – *Lp* Paris growth curves in AYE.

To infect the *Ac* with stationary phase-*Lp* Paris culture and then evaluate the experiments regarding the earlier hours post-infection, we opted to grow *Lp* Paris for 24h in BCYE agar plate, due to practical reasons. Nevertheless, we first verified that growing *Lp* Paris either in liquid medium or agar plates gives rise to the same results, concerning the infection process.

3.1.2. Optimization of the positive control for apoptosis

After submitting the *Ac* to heat shock (treated *Ac*), DNA from each time point was purified and analyzed through agarose gel electrophoresis (1.5%). By this technique, we were able to detect the presence of endonuclease cleavage products characteristic from apoptosis (has referred in 2.3.2) at all incubation times tested (Figure 15).

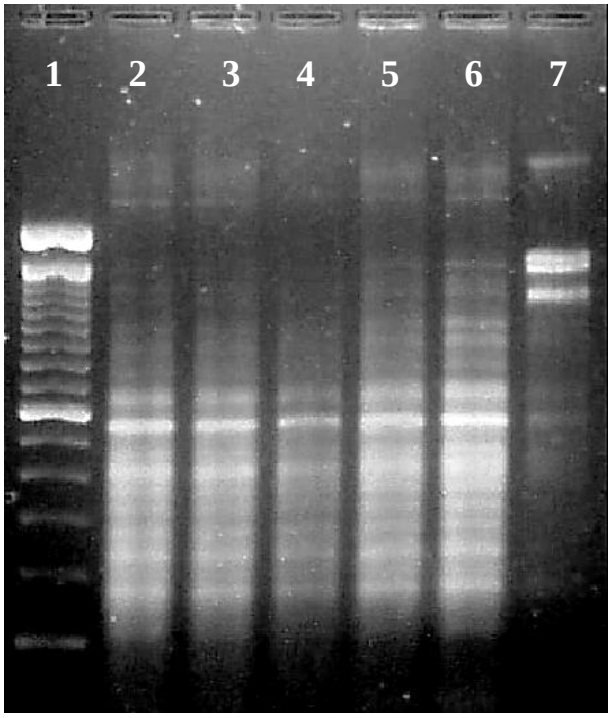


Figure 15 – Agarose gel electrophoresis (1.5%). **(1)** 1 kb DNA Ladder; **(2)** *Ac* treated for 3.5 minutes; **(3)** *Ac* treated for 4 minutes; **(4)** *Ac* treated for 4.5 minutes; **(5)** *Ac* treated for 5 minutes; **(6)** *Ac* treated for 5.5 minutes; **(7)** Negative control (untreated *Ac*).

Then, we performed the double staining of the treated *Ac* with Annexin V-FITC and 7-AAD in order to analyze by fluorescent microscopy and flow cytometry.

By fluorescent microscopy, we verified a difference between the negative control (untreated *Ac*) and treated *Ac* (1, 2.5, 3.5, 4, 4.5, 5 and 5.5 minutes at 56ºC). In the negative control, we only observed unspecific fluorescence around the *Ac* membrane (yellow fluorescence, Figure 16 **A**), related to the *Ac* autofluorescence. In the treated *Ac*, we observed green fluorescence (Figure 16 **B**) around the *Ac* membrane as a result of the binding of the Annexin V-FITC with the externalized PS in the surface of the treated *Ac*. This result showed that treated *Ac* are able to externalize PS to the membrane surface, which is one of the membrane alterations that occurs during the apoptotic process.

Moreover, we observed red fluorescence in the center of the cell, meaning that the 7-AAD was able to bind with the DNA of the *Ac.* This assay validated the use of Annexin V-FITC and 7-AAD for the purpose of our study.

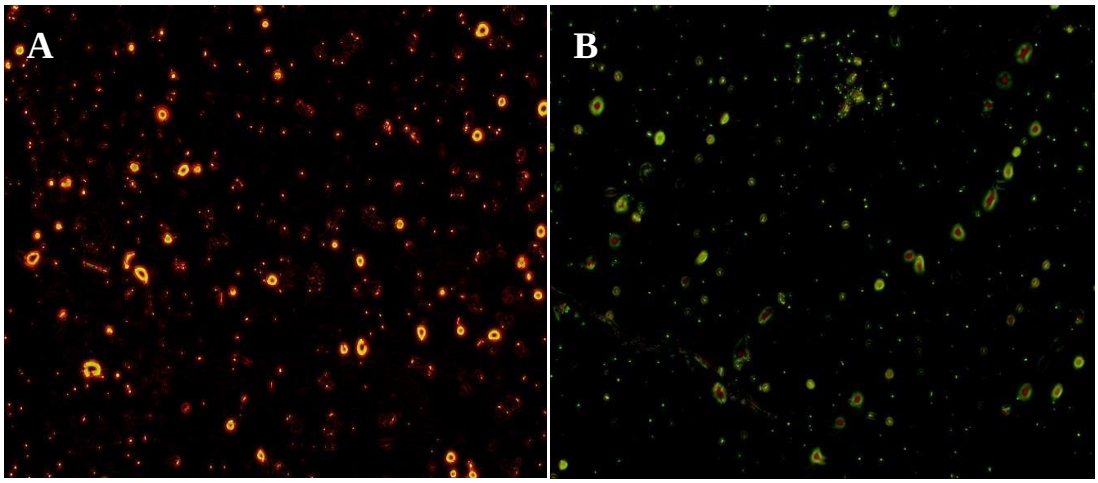


Figure 16 – Fluorescent microscopy analysis of *Ac* double stained with Annexin V-FITC and 7-AAD. **(A)** Normal *Ac*; **(B)** *Ac* submitted to heat shock at 56ºC for 5 minutes.

By flow cytometry, we obtained the percentages of untreated and treated (1, 2.5, 4 and 5 min of incubation at 56ºC) *Ac* in each stage (live/encysted, early apoptotic, late apoptotic and necrotic). The obtained results are presented in Table 3.

Table 3 – Apoptosis detection by flow cytometry after heat shock with different times at 56ºC. The results are presented in percentages.

	Untreated *Ac*	Treated *Ac*		
		1 minute	2.5 minutes	5 minutes
Live/Encysted	86.45	68.43	1.21	0.87
Early Apoptotic	0.01	3.5	27.5	58.2
Late Apoptotic	0.32	14.8	70.3	40.7
Necrotic	13.22	13.27	0.99	0.23

Considering these results, we concluded that heat shock was a good positive control for *Ac* apoptosis and that the best protocol was incubation for 5 minutes at 56ºC followed by 1 hour incubation at 37ºC.

3.1.3. Different conditions of infection

As referred in 1.9, in order to clarify the cell death process that occurs in *Ac* after infection with *Lp* Paris, we performed the infection protocol considering different times post-infection and MOI values. Following the infection protocol, *Ac* were double

stained with Annexin V-FITC and 7-AAD and analyzed by flow cytometry to divide the infected *Ac* population in four main groups considering their viability: live/encysted, early apoptotic, late apoptotic and necrotic. This way, we were able to understand the different stages that infected *Ac* went through during infection and how the time post-infection and MOI values would influence those stages.

In each assay, the infected *Ac* were compared with a negative control (uninfected *Ac*, as described in 2.2.3) in order to validate that the obtained results were a consequence of the infection by *Lp* Paris. In order to ease reading, since the obtained values for the negative control were very similar in all experiments, the means of the percentages from each stage of the *Ac* from the negative control are shown in Figure 17.

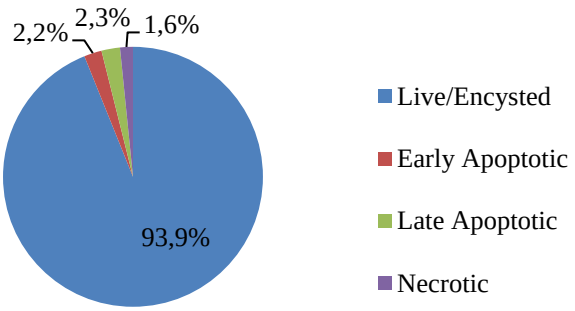


Figure 17 - Means of the obtained percentages from each stage (live/encysted, early apoptotic, late apoptotic and necrotic) of the uninfected *Ac* (negative control), from all the infection assays.

The first infection assay was performed with two different MOI values (10 and 25) and the infected *Ac* were analyzed at 20 hours post-infection (Figure 18).

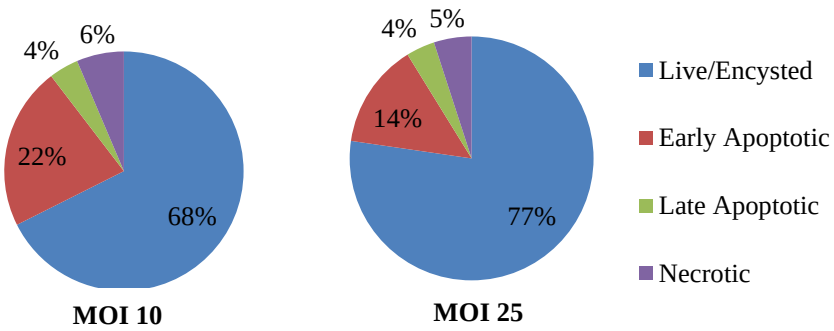


Figure 18 – Flow cytometry analysis of the *Ac* death mechanism inducted by *Lp* Paris at 20 hours post-infection.

These results showed a higher percentage of apoptotic *Ac* (26% *vs* 18%) when the infection was performed with a MOI of 10. Compared with the negative control (Figure 17), here we observed an increase of 10 fold in the percentage of apoptotic *Ac* (Figure 18).

After this infection assay, we maintained the MOI of 10 and analyzed the infected *Ac* at 18, 20 and 23 hours post-infection in order to understand the progression of the death process of the infected *Ac* at this time points (Figure 19).

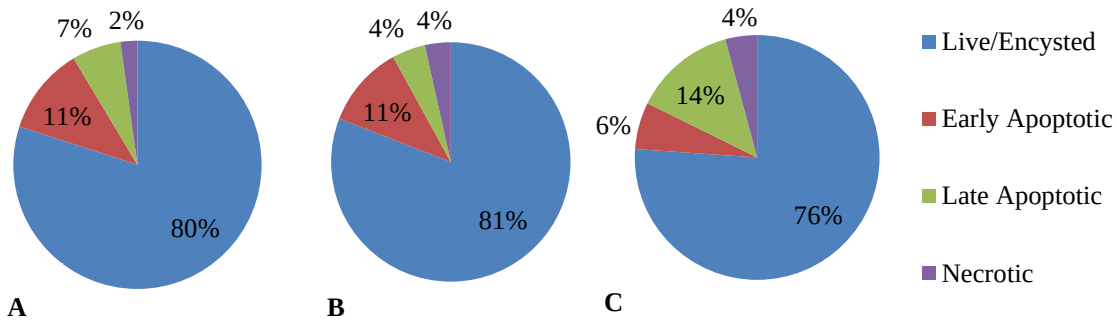


Figure 19 - Flow cytometry analysis of the *Ac* death mechanism inducted by *Lp* Paris: (A) at 18h post-infection; (B) 20h and (C) 23h.

The obtained results revealed a higher percentage of apoptotic *Ac* (20%) at 23 hours post-infection. However, this percentage was higher for the late apoptotic stage, meaning that at this time point the infected *Ac* were already at the end of the apoptotic process.

We were able to conclude that with lower MOI values, the infected *Ac* reach the final stages of apoptosis at the latest hours post-infection.

In the following infection, we were interested in analyzing the effect of higher MOI values on the infected *Ac*. Therefore, we analyzed the 18.5, 20 and 21.5 hours post-infection with a MOI of 100 (Figure 20).

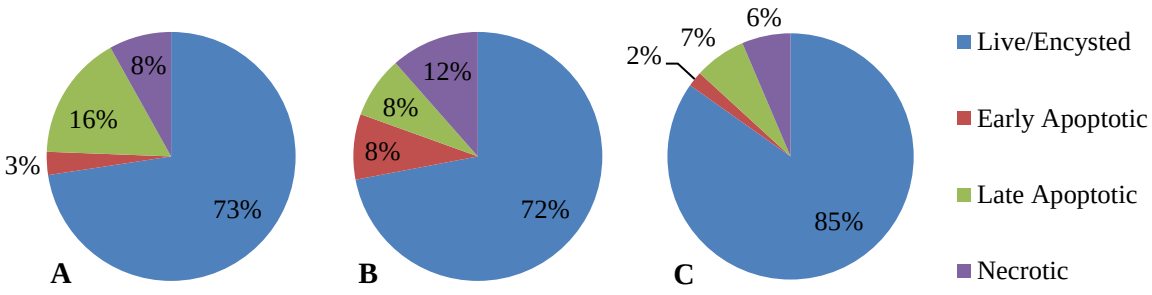


Figure 20 - Flow cytometry analysis of the *Ac* death mechanism inducted by *Lp* Paris: (A) at 18.5 h post-infection, (B) 20h and (C) 21.5 h.

The obtained results showed a decrease in the apoptotic *Ac* between the 18.5 and 21.5 hours post infection. Therefore, we concluded that between those hours the infected *Ac* are already reaching the final stages of their death process.

Since we wanted to clarify if *Lp* Paris induces apoptosis in *Ac*, we decided to analyze the infected *Ac* at the earlier hours post-infection in order to understand when the induction process begins and determine the percentage of infected *Ac* that undergoes apoptosis at that time point. Therefore, we choose to focus our analysis in the infected *Ac* at 12 hours post-infection, with a MOI of 100 (Figure 21).

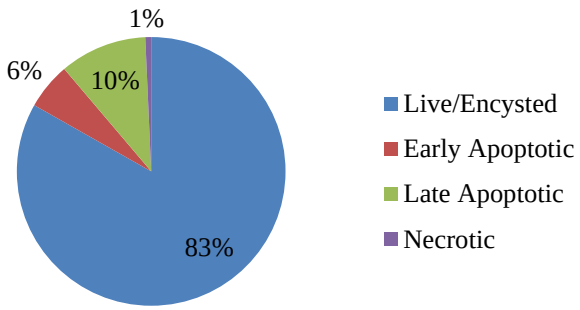


Figure 21 - Flow cytometry analysis of the *Ac* death mechanism inducted by *Lp* Paris at 12h post-infection, with a MOI of 100.

Compared with the negative control (Figure 17), here we observed an increase of seven-fold in the percentage of apoptotic *Ac* (Figure 21). Moreover, this assay showed similar percentages between early and late apoptotic *Ac* and had the lowest percentage of necrotic *Ac*, indicating that apoptotic process must be induced before this time point.

While we performed the infection assays, we noticed that the infected *Ac* suffered several morphological changes during the infection course. One of those morphological changes was the loss of their adherence, and in the same flask we would have the total population of *Ac* separated in two subpopulations: one at the bottom of the flask and another one in suspension. This separation could indicate that the death pathway of *Ac* had been activated. So, we performed several infection protocols in order to determine the time post-infection when this change would begin. Figure 22 shows the infected *Ac* at 3 hours post-infection with three different MOI values (100, 50 and 25).

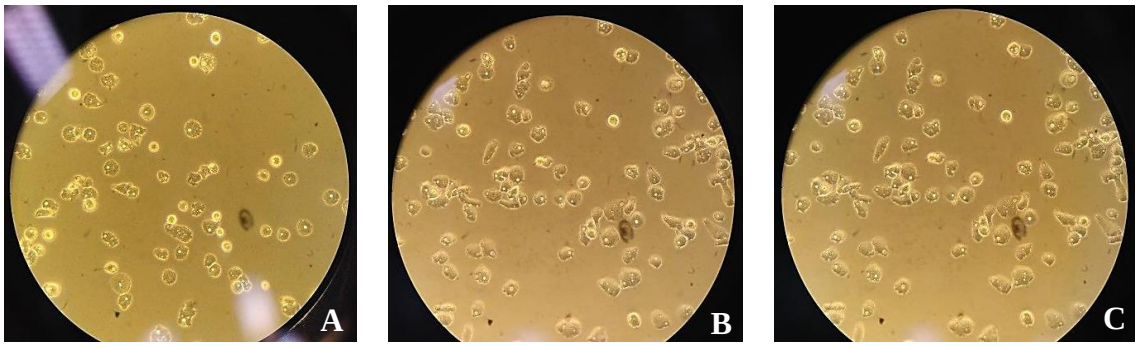


Figure 22 – Light microscopic images of the infected *Ac*, 3 hours post-infection, with three different MOI values (40x). **(A)** MOI 100; **(B)** MOI 50; **(C)** MOI 25.

With the microscopic observations, we verified that at a MOI of 100, the *Ac* began to lose their adherence around 8 hours post-infection.

Acknowledging the previous microscopic observations, we performed an assay where we analyzed the *Ac* after 8 hours post-infection with a MOI 100. In this assay, we analyzed, separately, the total *Ac* population (amoebic shape *Ac*) (Figure 23) and the suspended subpopulation (round shape *Ac*) (Figure 25).

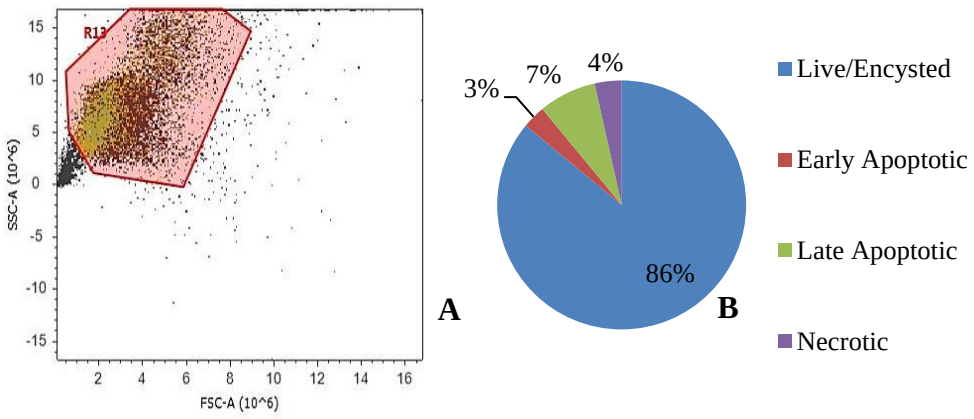


Figure 23 - Flow cytometry analysis of the *Ac* death mechanism inducted by *Lp* Paris at 8h (total population). (A) We can observe by the graphic (FSC vs SSC) that the total population has two subpopulations. (B) Percentages of the total *Ac* population.

However, according to the graphic that gives the morphological parameters - size (FSC) *vs* internal complexity (SSC) - of the infected *Ac*, we realized that we could divide the total population in two subpopulations (Figure 24).

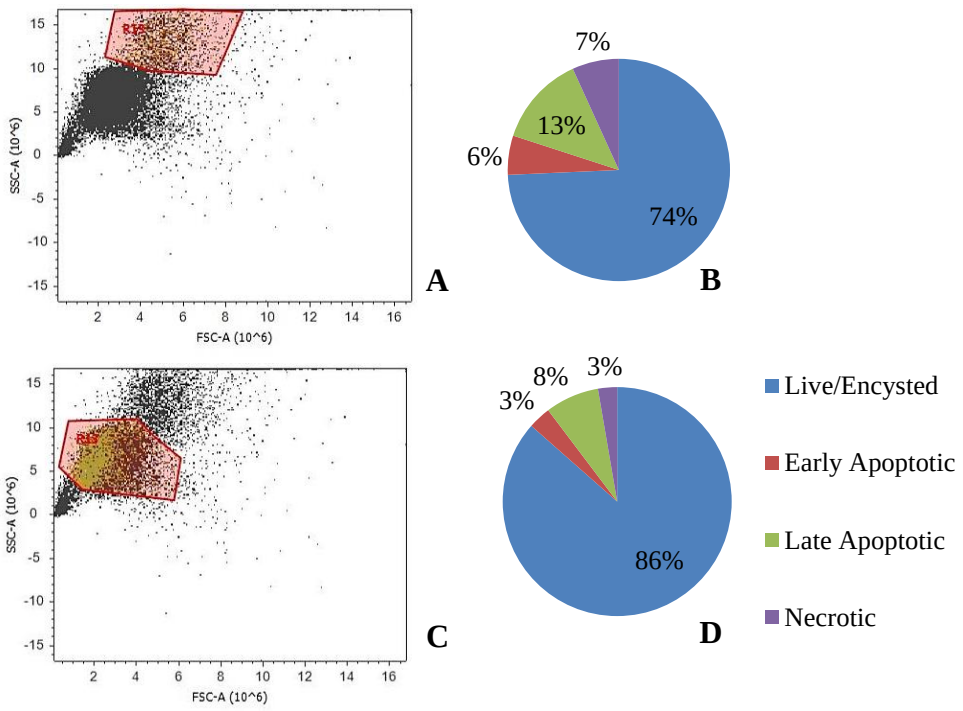


Figure 24 - Flow cytometry analysis of the two sub-populations, separately. A and B represent the first subpopulation; C and D represent the second subpopulation.

With these results, we verified that the first subpopulation (A) gathered the major part of apoptotic *Ac*, while the second subpopulation (B) was made of *Ac* that were still in the beginning of their apoptotic process.

Although the *Ac* were all infected at the same time, it is possible that some differences occurred considering the timing when *Lp* Paris induces the death of the *Ac*. In the total population, we can see *Ac* with different sizes and internal complexity.

When we analyzed the suspended *Ac* population alone, we verified a higher percentage of late apoptotic *Ac* (Figure 25).

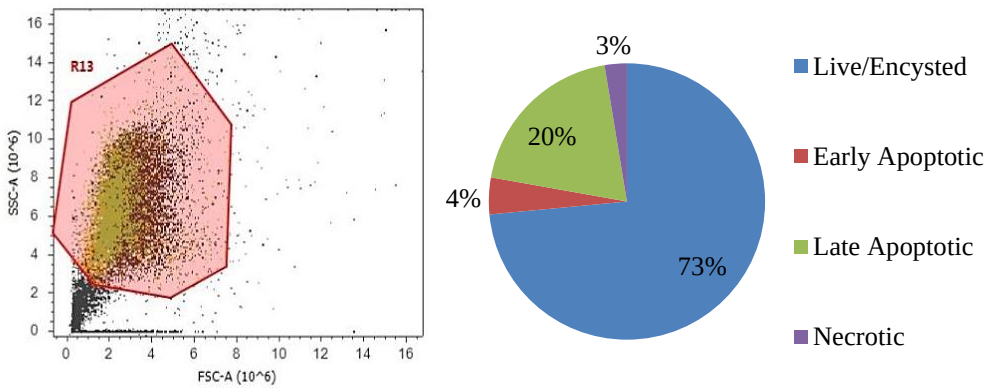


Figure 25 - Flow cytometry analysis of the suspended *Ac* population at 8h post-infection.

All the previous analyses were performed using Attune® Acoustic Focusing Cytometer (Applied Biosystems). However, due to technical reasons (unavailable flow cytometer), we had to continue our study in a different flow cytometer, the CyAn™ ADP Analyzer (Beckman Coulter) at IGC (Instituto Gulbenkian de Ciência).

For the first assay in this new cytometer, we kept the previous conditions for the infection protocol (infected *Ac* at 8 hours post-infection with a MOI 100). The obtained results are shown in Table 4.

Table 4 - First flow cytometry analysis of the infected *Ac* with MOI 100 at 8h post-infection, in the CyAn™ ADP Analyzer.

Assay		Live/Encysted (%)	Early Apoptotic (%)	Late Apoptotic (%)	Necrotic (%)
1º	Negative Control	73.08	5.03	10.7	7.17
	Total	43.22	14.25	31.77	5.98
	Suspension	37.94	9.59	45.34	4.4

According to these results, 46.02% of the total population of *Ac* are apoptotic (14.25% Early Apoptotic + 31.77% Late Apoptotic), opposing with the negative control where only 15.73% of the total population of *Ac* is in that stage (5.03% + 10.7%).

In order to verify if the activation of the apoptotic process in the *Ac* is in fact induced by *Lp* Paris, we carried out triplicates of this assay. The results are summarized in Table 5. The negative control included the total population of uninfected *Ac* in order to validate the results of the total *Ac* population and the suspended subpopulation.

Table 5 – Flow cytometry analysis of the triplicates with MOI 100 at 8h post-infection, in the CyAn™ ADP Analyzer.

Assay		Live/Encysted (%)	Early Apoptotic (%)	Late Apoptotic (%)	Necrotic (%)
2º	Negative Control	84,41	1,22	3,63	8,39
	Total	54,85	12,08	21,66	6,28
	Suspension	58,49	8,67	23,75	5,05
3º	Negative Control	79,87	5,34	5,85	4,92
	Total	39,29	17,79	34,07	3,71
	Suspension	56,31	15,71	18,96	5,53
4º	Negative Control	87,48	2,16	3,18	4,16
	Total	58,23	15,05	18,33	4,86
	Suspension	70,16	6,38	16,77	3,34

The triplicates were statistically analyzed, considering a Student's t-test distribution, in order to determine if the differences between the negative control (uninfected *Ac*) and the infected *Ac* were statistically significant.

The statistical analysis showed significant differences in the percentage of live/encysted and apoptotic *Ac* between the negative control and the infected *Ac*, whether considering the total population (Figure 26) or the suspended subpopulation (Figure 27).

According to these results we can conclude that the apoptotic process detected in the *Ac* was due to the infection with *Lp* Paris.

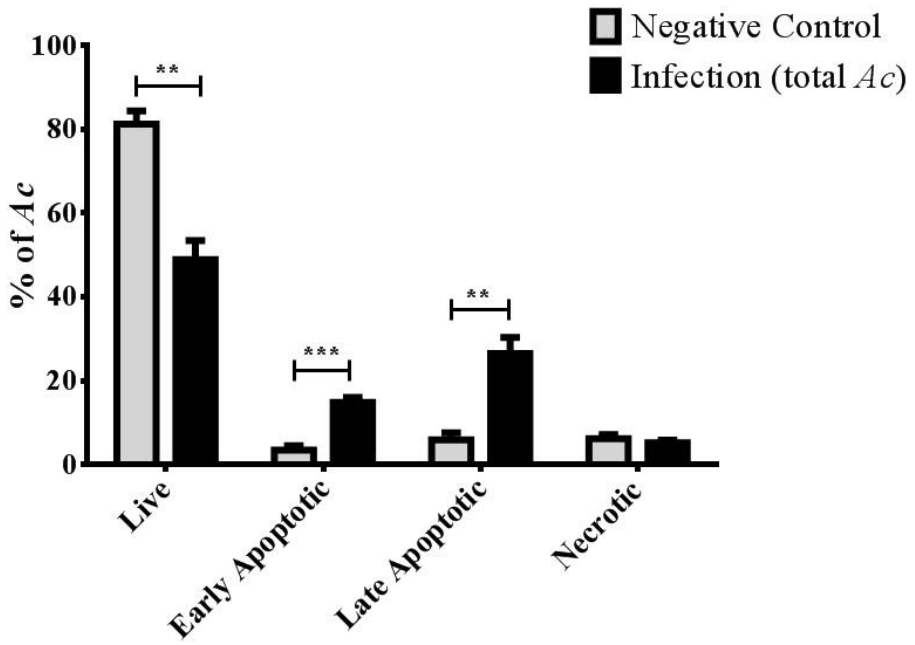


Figure 26 - Graphic where the *Ac* from the negative control are compared with the total population of infected *Ac*. Results from the triplicates are represented in percentages, and statistical differences (*, P < 0,05; ** P < 0,01 and *** P < 0,001) are shown, referring to the difference between live, early apoptotic, late apoptotic and necrotic *Ac* after infection with *Lp* Paris and the negative control.

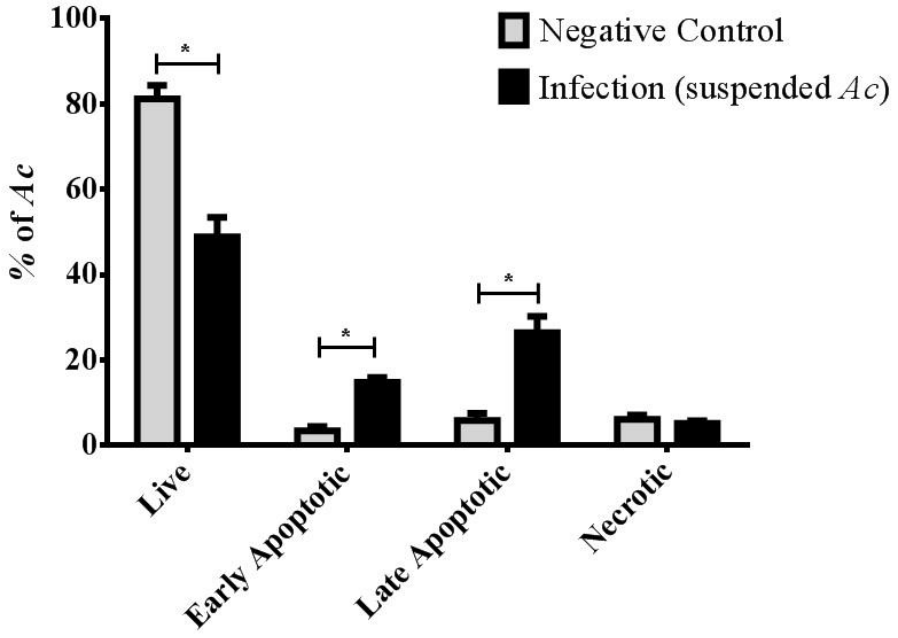


Figure 27 - Graphic where *Ac* from the negative control are compared with the suspended subpopulation of infected *Ac*. Results from the triplicates are represented in percentages, and statistical differences (*, P < 0,05; ** P < 0,01 and *** P < 0,001) are shown, referring to the difference between live, early apoptotic, late apoptotic and necrotic *Ac* after infection with *Lp* Paris and the negative control.

3.1.4. Detection of apoptosis using agarose gel electrophoresis

After the analyses by flow cytometer, DNA of the infected *Ac* after 8 hours post-infection with a MOI of 100 was purified and analyzed through agarose gel electrophoresis, as a complement assay for apoptosis detection. For this analysis were also considered the DNA from *Lp*, negative control and *Ac* alone. The obtained results are shown in Figure 28.

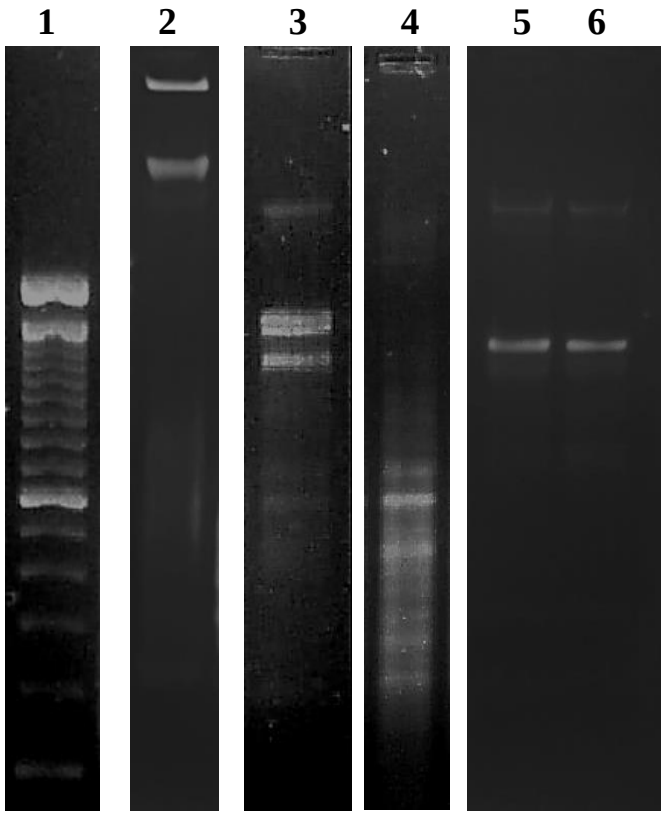


Figure 28 - Agarose gel electrophoresis (1.5%). **(1)** 1 kb DNA Ladder; **(2)** *Lp* Paris; **(3)** Untreated *Ac*; **(4)** Positive control (heat shock); **(5)** Uninfected *Ac*; **(6)** Infected *Ac* (8h, MOI 100).

By this technique, we were not able to detect the presence of endonuclease cleavage products characteristic from apoptosis in the infected *Ac*. With this result, we concluded that DNA cleavage does not seem to be involved in the apoptotic process after infection with *Lp*.

3.2. Intracellular replication of *Legionella pneumophila* strain Paris

After infection, *Lp* Paris replicates within the *Ac* and then evades the host (approximately 24h post-infection). In order to determine the intracellular replication rate of *Lp* Paris in *Ac*, we plated different dilutions of *Lp* Paris suspensions obtained from lysed *Ac* at two different hours post-infection (1 and 24h).

3.2.1. Validation of the drop plate method for bacteria enumeration

For the determination of the intracellular replication of *Lp* Paris, we had to compare the accuracy and fidelity of drop plate method *vs* spread plate method, the "gold standard" method for enumerating bacteria. For this comparison, we plated the *Lp* Paris from infected *Ac* at different hours post-infection with both methods.

Each dilution was plated in duplicate and for the drop method two different volumes of drop were tested: 10 and 30µl. After plating, the petri plates were incubated at 37ºC until colonies were formed for counting (approximately 4 days). To calculate the CFUs/ml we considered the dilutions with 30 to 300 CFUs/plate - for the spread method - and the dilutions with 3 to 30 CFUs/drop - for the drop method. After calculation of the CFUs/ml for each method, we obtained the results are presented in Table 6.

Table 6 – Calculation of CFU/ml of spread and drop plate methods.

Post-infection (h)	Dilution	Method	CFUs/ml
0	10^{-9}	Spread	$2.1*10^{12}$
		Drop 10µl	$2.0*10^{12}$
		Drop 30µl	$1.2*10^{12}$
1	10^{-3}	Spread	$1.4*10^{6}$
		Drop 10µl	$1.7*10^{6}$
		Drop 30µl	$1.1*10^{6}$
24	10^{-6}	Spread	$2.05*10^{9}$
		Drop 10µl	$1.6*10^{9}$
		Drop 30µl	$0.44*10^{9}$

The results of the drop plate method were similar to the results of the spread method. Considering these results, we chose to use the drop plate method for bacteria

enumeration instead of the spread method. We also standardized our plating protocol to drops with a volume of 30µl. Examples of both plating methods are shown in Figures 29 and 30.

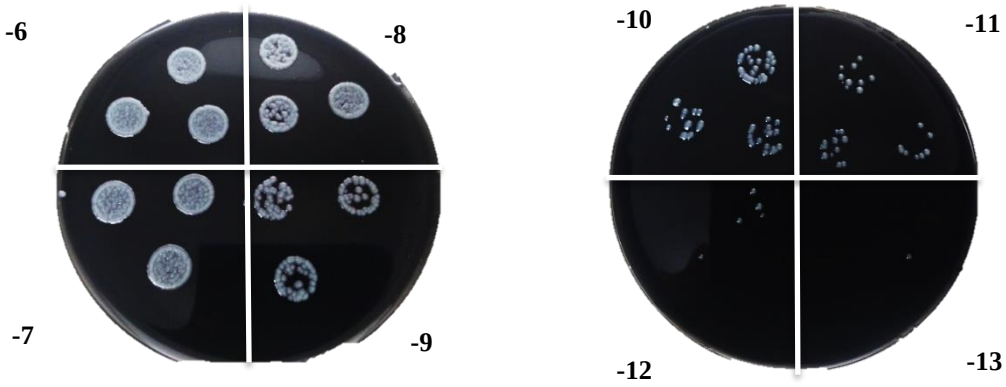


Figure 29 – Example of plates with the drop plate method. Two BCYE agar plates with different dilutions (specified in each quadrant) of the *Lp* Paris liquid culture by the drop plate method with drops of 30µl (photo obtained in the present study)**.**

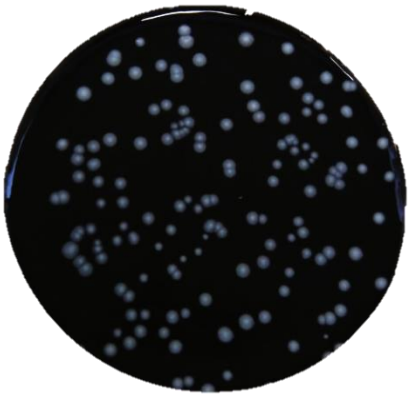


Figure 30 - Example of a plate with the spread method. A BCYE agar plate with a dilution of -10 of the *Lp* Paris liquid culture by the spread method (photo obtained in the present study).

3.2.2. Determination of the intracellular replication rate of *Legionella pneumophila* strain Paris

To determine the intracellular replication rate of *Lp* Paris in *Ac*, we plated the initial *Lp* suspension (0h) and intracellular *Lp* from two different hours post-infection (1 and 24 hours). The results are presented in Table 7.

Table 7 – Drop plate of initial suspension (0h) and 1 and 24 hours post-infection

Post-infection (h)	Dilution	CFUs/ml
0	10^{-8}	$1.6*10^{10}$
1	10^{-3}	$3.5*10^{5}$
24	10^{-8}	$7.5*10^{10}$

The percentage of infectious bacteria was calculated by the following equation:

$$\text{Ratio of infectivity} = \frac{\text{(intracellular } \textit{Lp} \text{ Paris at 1h)}}{\text{(}\textit{Lp}\text{ Paris added at 0h)}} \times 100$$

Ratio of infectivity = 0.002%

The relative number of CFU/ml of *Lp* at 1 and 24 hours post-infection was calculated by dividing the CFU at each time point by the CFU at the first time point.
The yield of CFU for *Lp* Paris increased approximately 10^5-fold during a 24h infection.

Chapter 4. Discussion and Conclusions

It has been suggested that the infectious particle for Legionnaires' disease is an amoeba infected with *Lp* [6]. Initially it was believed that the *Lp* transmission occurred by inhalation of the free-living bacteria but several authors have been verifying that the transmission is made by inhalation of MIFs (mature intracellular form) [96, 11].

Has been said trough this dissertation, the interaction between *Lp* and free-living amoebae shows several similarities with the one that occurs during infection of human alveolar macrophages by *Lp.* Although the interaction between *Lp* and mammalian cells have already been studied in great detail, the processes that are involved in interaction between *Lp* and prokaryotes hosts such as amoebae has rarely been addressed. One of those processes is the death mechanism induced by *Lp* in order to evade the amoeba and begin a new infection cycle.

Kwaik *et al* [133] have shown that at a MOI of 0.5, 5, or 50, *Lp* induces apoptosis in macrophages and alveolar epithelial cells within a few hours of infection in a dose-dependent manner. Later on, the group examined the molecular mechanisms by which *Lp* kills the protozoan host *Acanthamoeba polyphaga* (*Ap*) and demonstrated that *Ap* undergoes apoptosis upon induction by actinomycin D (an apoptosis inducer used has a positive control) but, unlike in mammalian cells, *Lp* does not induce apoptosis in this protozoan host. Despite the ability of *Ap* to undergo apoptosis, intracellular surface exposure of PS has a result of *Lp* infection did not occur. In addition, the group showed that intracellular *Lp* kills *Ap* preferentially by the induction of necrosis. Additionally, we noticed that among these studies, few infection conditions parameters such as MOI and time post-infection of the analyzed protozoan, were considered. In some cases the analyses were made considering only one MOI value to infect the protozoan or only one time point post-infection was analyzed.

Considering that the published literature on the evasion process of *Lp* from *Ac* is very limited and vague, in this study we wanted to clarify this question by analyzing the infection process of *Lp* in *Ac,* considering different conditions from the ones that have already been studied.

Since the ability to undergo apoptosis of the protozoan *Ap* has been demonstrated and that in mammalian cells *Lp* has been shown to induce apoptosis, we had to start our study by analyzing the *Ac* ability to undergo apoptosis. This study was performed based on two biochemical processes that may occur during apoptosis: DNA fragmentation and intracellular surface exposure of PS. To induce the apoptotic process we used heat shock as a positive control.

The DNA fragmentation process was verified by agarose gel electrophoresis which is a good methodology since is easy to perform, has a sensitivity of 1×10^6 cells and is useful for tissues and cell cultures with high numbers of apoptotic cells per tissue mass or volume, respectively. By this technique, we were able to visualize the systematic cleavage of DNA into oligonucleosomal multimers of 180-200 bp, which is considered the "hallmark" of apoptosis [126]. With these results we were able to conclude that DNA fragmentation is one of features that occur when *Ac* undergoes apoptosis when submitted to heat shock.

Moreover we wanted to verify if the *Ac* were able to externalize the PS located in the inner membrane when undergoing apoptosis. Under normal physiologic conditions, PS is predominantly located in the inner leaflet or cytosol-facing part of the plasma membrane. Upon initiation of apoptosis, PS loses its asymmetric distribution in the phospholipid bilayer and is translocated to the extracellular membrane leaflet where it identifies cells as targets for phagocytosis [136]. We visualized this feature by fluorescent microscopy and flow cytometry. By fluorescent microscopy we were able to visualize the difference between the fluorescence emitted by the negative control (*Ac* in normal conditions) and the *Ac* submitted to heat shock. For the negative control, we only visualized unspecific fluorescence, resulting from the *Ac* autofluorescence. The *Ac* submitted to heat shock, revealed green fluorescence, which is known to be the result of the binding between the recombinant Annexin V conjugated to green-fluorescent FITC dye (Annexin V-FITC) and the PS present in the surface of the *Ac*. After, this result was confirmed by flow cytometry where the higher percentage of cell death corresponded to apoptotic *Ac*, opposing to the percentage of necrotic ones. These assays combined showed that *Ac* are able to externalize the PS located in the inner membrane when undergoing apoptosis.

In conclusion, our results indicate that DNA fragmentation and externalization of PS to

the outer leaflet of the cell membrane, which are characteristic processes observed during apoptotic death in eukaryotes, can be identified in the process of heat-induced cell death in *Ac.*

After verifying the *Ac* ability to undergo apoptosis, we were able to begin to study the death mechanism that was induced by *Lp* during the evasion from *Ac*. Our analyses were made by flow cytometry and agarose gel electrophoresis.

Compared to the alternative methods (e.g. analysis of cell morphology, DNA gel electrophoresis) flow cytometry is rapid, objective, and very sensitive. However, improper use of flow cytometry in analysis of cell death and in data interpretation is possible and it is due to some errors like misclassification of nuclear fragments and individual apoptotic bodies as single apoptotic cells or assumption that the apoptotic index represents the rate of cell death [128]. Because of the lack of previous studies of *Ac* death mechanism using flow cytometry, in our initial analyses we had to adapt a protocol that allowed us to analyze this kind of cells. Everything had to be tested several times before we reached an ideal protocol for flow cytometry analysis and data interpretation. Several alterations to the infection protocol conditions were also tested, in order to determine the ideal value of MOI and the time post-infection that would allow us to study the death process activated on *Ac* infected by *Lp.*

In our first assays, we assumed that the activation of the death mechanism of *Ac* by *Lp* would occur near the timing when *Lp* evaded the *Ac* (approximately 24 hours post-infection). However, by that time, a great part of the infected *Ac* had already reached the final stage of their death process, losing all their integrity and ability to bind with the Annexin V-FITC or the 7-AAD. After this conclusion, we had to start analyzing the previous hours post-infection.

By microscopic observation, we verified that at 8 hours post-infection, with a MOI of 100, the infected *Ac* start to change their amoebic morphology and begin to appear the first *Ac* suspended in the medium. These observations lead us to believe that these alterations are somehow linked to the moment when *Lp* induces the death of *Ac* in order to evade. This hypothesis would also imply that induction of the *Ac* death is done hours before the *Lp* evades *Ac*, contrary to our first hypothesis that the *Lp* evasion from Ac would only occur when *Lp* was already in the virulent stage and ready to begin a new infection cycle.

By flow cytometry analysis of the infected *Ac* in the conditions (8 hours post-infection with a MOI of 100), our results showed that in fact, in these conditions, almost 50% of the infected *Ac* were apoptotic, opposing to the negative control where only about 15% were apoptotic. After performing triplicates of this assay we were able verify by statistical analysis that there were in fact significant differences between the negative control and the infected *Ac* regarding the apoptotic *Ac* and the ones that were live or encysted.

In 1998, Hägele *et al* [121] verified that *Lp* did not induce apoptosis in the protozoan host *Ac.* However, in that study only the 3, 5 and 24 hours post-infection were considered, the infection was performed only with a MOI of 50 and only 10000 cells were analyzed. Additionally, some questions remained at the end of this study since the group were not able to say if the failure of *Lp* to induce apoptosis in *Ac* was due to: (1) the inability of *Lp* itself to induce apoptosis; (2) the use of a different killing mechanism; or (3) the fact that *Ac* do not possess the adequate genetic program.

With this study, we tried to clarify these answers and in order to do that we had to analyze the infected *Ac* in different conditions. Our results show that *Ac* has in fact the cellular machinery to undergo apoptosis and that after infection with *Lp* there is a high percentage of apoptotic *Ac* compared with the ones from the negative control.

Although there are several remarkable similarities in the models of intracellular infection of macrophages and amoeba by *Lp,* the published literature affirms that the induction of apoptosis for evasion of the host only happens in macrophages. Considering our results, we can say that *Lp* may use similar molecular mechanisms to manipulate host cell processes of macrophages and protozoa and therefore hypothesize that the *Lp* process of killing and exiting the macrophages by apoptosis has evolved from the interaction with protozoa in the environment.

By agarose gel electrophoresis, we were not able to detect DNA fragmentation of the infected *Ac.* However, according to Darzynkiewicz *et al* [128], the lack of evidence of apoptosis, detected by a particular method, is not evidence of the lack of apoptosis. There are numerous examples in the literature where cells die by a process resembling apoptosis which lacks one or more typical apoptotic features. Most frequently, DNA degradation stops after creation of 50–300-kb fragments, meaning there is no internucleosomal fragmentation seen and, therefore, fewer in situ DNA strand breaks

compared with classical apoptosis. The DNA laddering on gels for identification of apoptosis, fails to identify atypical apoptosis in such a situation. This is why application of more than one method, each based on a different principle stands a better chance of detecting atypical apoptosis than any single method.

Considering this, our results do not exclude the possibility that the infected *Ac* are in fact undergoing apoptosis.

For bacterial enumeration, the most commonly used direct plating method is the spread method, which consists in the spreading of one dilution of a bacterial suspension into a plate. Another plating method is the drop plate method, were the bacterial suspension is plated in drops and therefore one plate can have several dilutions. This method has some advantages over the spread plate method: it uses fewer materials; less time and effort are required to dispense the drops onto an agar plate than to spread an equivalent total sample volume into the agar plate and by distributing the sample in drops, colony counting can be done faster and perhaps more accurately. Even though it has been present in the laboratory for many years, the drop plate method has not been standardized. The objective of this research was to validate the use of the drop plate method for bacteria enumeration and to standardize a plating protocol. After counting and calculation of the CFU/ml for each method, the obtained results were similar which allowed us to conclude that the drop plate method could be used instead of the spread method for colony counting. Beside validation of the drop plate method, we tested the volume of the drops for 10 and 30µl. We chose the 30µl volume since it allows easier counting and to have four different dilutions per plate.

For the determination of the intracellular replication rate of *Lp* Paris in *Ac*, we started by calculating the percentage of infectious bacteria which was 0.002%. This is a low infectivity value when compared with published data where an infectivity of 8% has been verified for *Lp* [137]. This value can be explained with the fact that after infection there was a high number of extracellular bacteria remaining in the medium. However, this value can also be due to the fact that the *Ac* lysis protocol may not have been effective on lysing the *Ac* completely, influencing the number of *Lp* considered for the calculus. Regarding the intracellular replication rate of *Lp* Paris in *Ac*, we verified that the yield of CFU for *Lp* Paris increased approximately 10^5-fold during a 24h infection in *Ac*. This value reflects a high rate of *Lp* replication in the *Ac* that is consistent with

our microscopic observations, where we were able to visualize a great number of bacteria after 24 hours post-infection. Additionally, we also know that this result was not influenced by the high number of extracellular bacteria because we have verified that *Lp* is not able to grow in the minimum medium used for the infection assays. However, if the *Ac* lysis protocol was not in fact effective it could have also influenced this result.

Numerous methods have been employed to attempt to eradicate *Lp* from aquatic environments, with little success. These attempts, which include chemical biocides, overheating water, and UV irradiation, have been successful for short periods after which the bacteria can be again detected. It has been suggested that in order to eradicate *Lp* from aquatic environments continuous treatments effective against both the bacteria and the protozoan host should be employed [115].

Since free-living amoebae seem to play a crucial role for persistence and dispersal of *Legionellaceae* in the environment, and there is convincing evidence that intracellular multiplication of *Lp* in free-living amoebae is a prerequisite for the infection of humans [95], we believe that future studies should examine in greater detail the apoptotic process induced in *Ac* by *Lp.*

Being this study a contribution in this field, we believe that a better understanding of such processes would help to develop novel strategies and targets to eradicate *Lp* from aquatic environments. The modulation of the *Ac*'s regulatory machinery, specifically their propensity to die in response to *Lp* infection through blocking the *Ac* apoptosis process, may help preventing bacteria evasion and the beginning of a new infection cycle.

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Appendix 1

Estudo do processo de morte celular induzido por *Legionella pneumophila* em *Acanthamoeba castellanii*

14 ENCONTRO NACIONAL DE ATUALIZAÇÃO EM INFECIOLOGIA 14-16 OUT 2015

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Introdução:

A *Legionella pneumophila* é uma bactéria ubíqua em ecossistemas de água doce, onde vive em associação com protozoários, nomeadamente a amiba *Acanthamoeba castellanii*. Após a multiplicação da *Legionella pneumophila* na amiba, a bactéria induz a morte celular do hospedeiro, evade-se e inicia um novo ciclo. Este processo de transmissão da *Legionella pneumophila* é o responsável pelo aumento do inóculo nos reservatórios artificiais e consequentemente a origem dos vários surtos de Doença dos Legionários que têm ocorrido nos últimos anos.

Objectivo:

O presente trabalho pretende estudar o processo de morte celular da *Acanthamoeba castellanii* induzido por *Legionella pneumophila*.

Materiais e Métodos:

Fig. 1 – Cultura de *Ac* com 48h de crescimento em frasco de cultura com meio PYG (ampliação 20x).

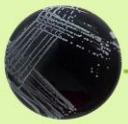


Fig. 2 – Cultura de *Lp* em meio BCYE α com 48h de incubação.

Controlo positivo
Ac sujeitas a choque térmico:
5 min – 56ºC
60 min – 37ºC

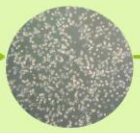


Fig. 3 – *Lp* e *Ac* após 8h de infecção *(Lp:Ac* = 1:100).

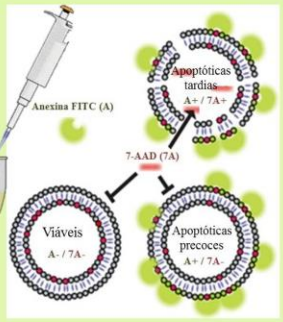


Fig. 4 – Ligação do 7-AAD ao DNA das células necróticas e da anexina à fosfatidilserina externalizada pelas células apoptóticas.

Fig. 5 – Citómetro Attune

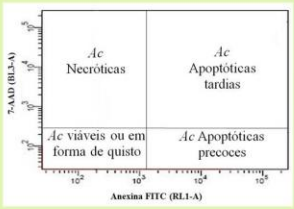


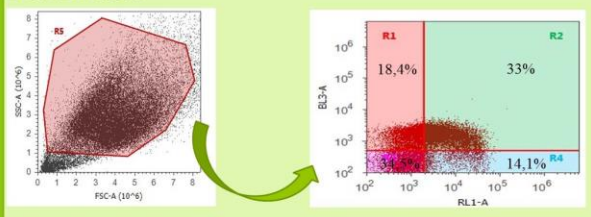
Fig. 6 – Distribuição das *Ac* pela viabilidade celular em função da ligação à anexina FITC e ao 7-AAD.

Infecção das *Acanthamoeba castellanii* (*Ac*) *Por Legionella pneumophila* (*Lp*)

Marcação das *Ac* com anexina FITC e 7-AAD

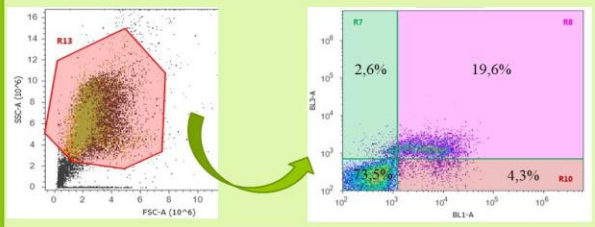
Análise das *Ac* por Citometria de Fluxo

Resultados:



Controlo Positivo

- Verificou-se que *Acanthamoeba castellanii* externaliza a fosfatidilserina presente no interior da membrana (ligação da anexina FITC) durante o processo apoptótico.
- Por choque térmico obteve-se 47,1% de *Ac* apoptóticas.



Infecção (marcação das *Ac* após 8h)

- Verificou-se que o processo de morte celular induzido por *Legionella pneumophila* em *Acanthamoeba castellanii* é sobretudo a apoptose.
- Por infecção com *Legionella pneumophila* obteve-se 23,9% de *Acanthamoeba castellanii* apoptóticas e apenas 2,6% necróticas.

Conclusão:

A dupla marcação com anexina FITC e 7-AAD sugere que o principal mecanismo de morte celular induzido por *Legionella pneumophila* em *Acanthamoeba castellanii* é a apoptose. Conhecendo esta etapa do ciclo de vida de *Legionella pneumophila* em *Acanthamoeba castellanii* será possível optimizar os métodos já existentes para redução do inóculo infectante nos reservatórios artificiais e assim contribuir para a diminuição da probabilidade da ocorrência de surtos.

Referências:
1. Richards, A. M., Von Dwingelo, J. E., Price, C. T. & Abu Kwaik, Y. Cellular microbiology and molecular ecology of *Legionella*-amoeba interaction. *Virulence* **4**, 307–14 (2013).

Appendix 2

Preparation of the Buffered Charcoal Yeast Extract (BCYE) agar

1. Suspend 2.77g of Legionella CYE Agar Base (OXOID) in 100ml of distilled water.
2. In a heat plate, bring to the boil to dissolve completely. Keep agitating.
3. Once the solution is boiling, sterilize it in an autoclave at 120ºC, for 20min.
4. After the sterilization, wait for the temperature to decrease to 60ºC.
5. Add the Legionella growth supplement (OXOID).
6. Distribute the medium into Petri dishes (20ml of medium/plate). The following step has to be done in a laminar flow cabinet or near a Bunsen burner to assure the sterility of the medium.
7. Store the plates at 2 – 8ºC.
8. Sterility test: incubate one of the plates at 37ºC, for 2 days for monitoring.

Appendix 3

Preparation of the Peptone-Yeast Extract-Glucose (PYG) medium

1. Suspend in 950ml of distilled water:
    - 1g Trisodium citrate (AppliChem)
    - 20g Bactotrypone (Biokar Diagnostics)
    - 1g Yeast extract (Biokar Diagnostics)
    - 18g Glucose (AppliChem)
2. Add to the previous solution:
    - 8ml $CaCl_2$ 0.05M
    - 10ml KH_2PO_4 0.25M
    - 10ml $MgSO_4$ 0.4M
    - 10ml Na_2HPO_4 0.25M
    - 10ml Iron Pyrophosphate 0.005M
3. Agitate.
4. Sterilize the solution using a vacuum filtration system, with a 0.22µm filter (Millipore).
5. Sterility test: after filtration, incubate 3ml of the medium at 37ºC, for one week for monitoring. During this period, the medium should be kept in quarantine.

Appendix 4

Preparation of the ACES-buffered yeast extract (AYE) broth

1. Suspend in 450ml of distilled water:
    - 5g ACES (AppliChem)
    - 5g Yeast extract (Biokar Diagnostics)
2. Set the pH to 6.9.
3. Add 0.2g of L-cystein (AppliChem).
4. Separately, in a heat plate, dissolve 0.125g of Iron Pyrophosphate (Sigma) in 50ml of distilled water.
5. Add the Iron Pyrophosphate solution to the solution of step 2.
6. Agitate.
7. Sterilize the solution using a vacuum filtration system, with a 0.22µm filter (Millipore).
8. Sterility test: after filtration, incubate 3ml of the medium at 37ºC, for one week for monitoring. During this period, the medium should be kept in quarantine.

Appendix 5

Preparation of the Minimum Medium (MM)

1. Suspend 1g of Trisodium citrate (AppliChem) in 950ml of distilled water.
2. Add to the previous solution:
    - 8ml $CaCl_2$ 0.05M
    - 10ml KH_2PO_4 0.25M
    - 10ml $MgSO_4$ 0.4M
    - 10ml Na_2HPO_4 0.25M
    - 10ml Iron Pyrophosphate 0.005M
3. Agitate.
4. Sterilize the solution using a vacuum filtration system, with a 0.22µm filter (Millipore).
5. Sterility test: after filtration, incubate 3ml of the medium at 37ºC, for one week for monitoring. During this period, the medium should be kept in quarantine.

Appendix 6

Protocol for *Ac* monolayers lysis

For 1 hour post-infection:

1. Harvest the *Ac* and divide the volume into 3 Eppendorf tubes of 1.5ml.
2. Centrifuge for 5 minutes at 19280 x *g*.
3. Vortex at maximum speed for 1 minute.
4. Pass the suspension through a syringe of 27G gauge.
5. Collect the total volume to a sterile Falcon tube of 15ml.

For 24 hours post-infection:

1. Harvest the *Ac* and divide the volume into 3 Eppendorf tubes of 1.5ml.
2. Vortex at maximum speed for 1 minute.
3. Centrifuge for 5 minutes at 19280 x *g*.
4. Vortex at maximum speed for 1 minute.
5. Collect the total volume to a sterile Falcon tube of 15ml.