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Differences in *Legionella pneumophila* virulence determined in *Galleria mellonella* infection model

Dissertação de Mestrado em Biologia, orientada pela Professora Doutora Joana Costa e pelo Professor Doutor António Veríssimo e apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra

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Abbreviations and Acronyms

°C - Degree centigrade

CFU - Colony-forming unit

DNA - Deoxyribonucleic acid

ELISA - Enzyme-Linked Immunosorbent Assay

ml - Mililiter

mM – Millimolar

D-PBS - Dulbecco Phosphate-Buffered Saline

µl – Microliter

BCYE - Buffered Charcoal Yeast Extract

AYE - ACES Yeast Extract

ACES - N-(2-Acetamido)-2-aminoethanesulfonic acid.

ATCC – American Type Culture Collection

PCR – Polymera Chain Reaction

^{T} - Type strain

TAE - Tris-acetate-EDTA

OD_{600} - Optical Density at 600 nm

Abstract

Legionella pneumophila is a facultative intracellular gram-negative bacteria ubiquitous in freshwater environments and in many man-made water systems, capable of inducing pneumonia in humans. Legionellae natural hosts are protozoa present in freshwater environments. The association with these organisms is the major factor regulating the presence of the bacterium in the environment. Indeed, protozoa provide the necessary conditions for the growth of legionellae, and they enhance the resistance of these organisms to adverse environmental conditions (Fields *et al.,* 2008; Richards *et al.,* 2013; Abdelhady & Garduño, 2013).

In humans the infection caused by the inhalation of legionellae is supported by the ability of these organisms to enter and to multiply within alveolar macrophages, causing the destruction of these phagocytes and damage to the pulmonary tissues. Genus *Legionella* includes several species but only some have already been associated with legionellosis. Strains belonging to *L. pneumophila* of serogroup 1 represent the majority of strains related with disease cases (Marrie *et al.,* 2008; Phin *et al.,* 2014).

It has been shown that there are clear differences between populations of clinical, man-made and natural environmental isolates, with clinical isolates showing less diversity than man-made and natural environmental isolates suggesting that the former is a subset of the latter's (Coscollá & González-Candelas, 2009; Harrison *et al.,* 2009; Harrison *et al.,* 2007; Costa *et al.*, 2010; Costa *et al.,* 2012; Costa *et al.,* 2014).

The usage of *G. mellonella* as an infection model for human pathogens has increased in the last few years, mainly due the existence of a good correlation between virulence of several bacterial species in the insect and in mammalian models (Harding *et al.,* 2013). A major component of the larvae's immunity is the presence of an innate immune system, with hemocytes, professional phagocytes like the alveolar macrophages in humans. *L. pneumophila* is able to infect, and replicate within these cells (Harding *et al.,* 2013).

The main objective of this study was to determine if unrelated *L. pneumophila* strains, isolated from different environments and with distinct genetic backgrounds, exhibited different levels of virulence, using *G.mellonella* larvae as an infection model.

In this study we concluded that *L. pneumophila* virulence in *G. mellonella* is dose-dependent and strain-specific. However all the injected larvae showed morphological alterations after injection with *L. pneumophila* which made possible to determine that all the strains tested in this study are pathogenic. Moreover, we could not establish a link between *L. pneumophila* virulence and the strains origin.

Multilocus sequence typing is a tool used to discriminate clonal groups within several bacterial species (Harb & Kwaik 1998). A scheme based on this tool has been developed for *L. pneumophila*, Sequence-based typing (SBT). This tool allowed us to conclude that the SBT profiles did not reconstruct the phylogeny of *L. pneumophila* species. Additionally, no relation could be established between the SBT profiles and the origin of strains. It was also possible to establish that the SBT profile is not related with the strains virulence in *G. mellonella*

Resumo

Legionella pneumophila é uma bactéria gram-negativa intracelular facultativa, ubíqua em ambientes de água não salina e em muitos sistemas artificias de água, capaz de provocar pneumonia em humanos. Os hospedeiros naturais da Família *Legionella*ceae são protozoários presentes em ambientes de água não salina. A associação com estes organismos é o principal fator a regular a presença desta bactéria neste tipo de ambiente. De facto, os protozoários fornecem condições necessárias para o crescimento de legionellae e aumentam a capacidade de resistência destes organismos contra condições ambientais adversas (Fields, 2008; Richards, 2013; Abdelhady & Garduño, 2013).

Em humanos a infeção causada pela inalação de legionellae é intrínseca à capacidade destes organismos para entrarem e se multiplicarem em macrófagos alveolares, causando destruição destes fagócitos e danos nos tecidos pulmonares. O género *Legionella* inclui várias espécies estando algumas envolvidas em casos de legionelose. As estirpes pertencentes ao serogrupo 1 de *Legionella pneumophila* representam a maioria das estirpes associadas a casos de doença (Marrie *et al.,* 2008; Phin *et al.,* 2014).

Comprovadamente existem diferenças evidentes entre populações de *L. pneumophila* isoladas de ambientes clínicos, artificiais e naturais. Isolados de ambientes clínicos evidenciam menor diversidade que populações isoladas de ambientes artificiais e naturais, sugerindo que os primeiros são um subgrupo dos

segundos (Coscollá & González-Candelas, 2009; Harrison *et al.,* 2009; Harrison *et al.,* 2007; Costa e*t al.,* 2010; Costa *et al*., 2012; Costa *et al*., 2014).

O uso de *G. mellonella* como modelo de infeção para agentes patogénicos humanos tem aumentado nos últimos anos, principalmente devido a uma boa correlação ente a virulência de várias bactérias no inseto e nos modelos mamíferos (Harding *et al.,* 2013). Este modelo animal é adequado ao estudo da patogenicidade de *L. pneumophila* uma vez que estas larvas possuem um sistema imunitário inato e fagócitos, células com um comportamento idêntico ao dos macrófagos alveolares em humanos, nos quais *L. pneumophila* tem a capacidade de infetar e replicar-se (Harding *et al.,* 2013).

O principal objetivo deste estudo foi determinar se estirpes de *L. pneumophila* não relacionadas, isoladas de diferentes ambientes e com diferentes *backgrounds* genéticos, apresentavam diferentes níveis de virulência, usando larvas de *G. mellonella* como modelo de infeção.

Neste estudo concluímos que a virulência de *L. pneumophila* é dependente da dose e específica para cada estirpe. No entanto todas as larvas injetadas revelaram alterações morfológicas depois da injeção com *L. pneumophila,* o que torna possível concluir que todas as estirpes testadas neste estudo são patogénicas. No entanto, não foi possível estabelecer uma relação entre a virulência e a origem das estirpes de *L. pneumophila*.

"Multilocus Sequence Typing" é uma ferramenta usada para descriminar grupos clonais em várias espécies de bactérias (Harb & Kwaik 1998). Um esquema baseado nesta ferramenta específico para *L. pneumophila* foi desenvolvido, "Sequence-Based

Typing" (SBT). Este método permitiu-nos concluir que os perfis SBT obtidos não reconstroem a filogenia da espécie *L. pneumophila*. Adicionalmente, não foi possível estabelecer uma ligação entre o perfil SBT e a origem das estirpes. Também foi possível concluir que o perfil SBT não está relacionado com a virulência de cada estirpe em *G. mellonella*.

I. Introduction

1.1 Historical Reference

In 1976 occurred the first big event related with *Legionella pneumophila.* Suddenly, 221 cases of pneumonia were registered among persons who were attending an American Legion convention in Philadelphia, United States of America. Thirty-four of those cases were fatal (Fraser *et al.,* 1977). Efforts were made to discover not only the pathogen responsible for this disease but also its source. Research proved that the pathogenic agent was transmitted through the air-conditioning system in the hotel where the convention occurred. Only months after the outbreak was possible to determine its cause trough the isolation of a pathogenic bacteria from pulmonary tissue of one deceased. The correlation between the bacteria and the infection was possible to establish due to the patients sera that contained antibodies for that specific pathogen. The high concentration of these antibodies in a convalescence phase made possible to prove that the pneumonia was caused by the isolated bacterium (Fraser & McDade, 1979). It happened to be a previously unrecognized bacterium which had been isolated, but not characterized, for the first time in 1943 by Tatlock and later in 1947 by Jackson *et al* (Fields *et al.* 2002). The newly described species was named *Legionella pneumophila* and it became a member of an also new family *Legionellaceae* (Brenner *et al.,* 1979). Nowadays there are 60 distinct *Legionella* species (Gomez-Valero *et al.,* 2014). The pneumonia caused by *Legionella* was initially named Legionnaires' Disease due to this outbreak, but currently it is known as legionellosis (Fraser & McDade, 1979).

Back in 1968 an epidemic of acute febrile illness occurred in a Health Department in Pontiac, Michigan, USA. The symptoms were mainly fever, headache and myalgia but never developed to a pneumonia condition. The incubation time was nearly 36 hours and patients self-recovered approximately in 2 to 5 days with no medication needed. Research teams concluded that the outbreak source was the air-condition system (Glick *et al.,* 1978). The pathogen behind these cases (Pontiac Fever) was never identified although sera from 32 patients were stored and analysed lately in 1977. It was then possible to detect seroconversion through the presence of *L. pneumophila* antibodies and so Pontiac Fever became one of the possible clinical presentations of *L. pneumophila* infection (Glick *et al*., 1978).

1.2 Epidemiology

1.2.1 Legionellosis and Pontiac Fever

Legionella infection in humans usually presents as two different forms. The less dangerous to human health is Pontiac Fever. This is a self-limited flu-like illness (Glick *et al.,* 1978).

Legionellosis is clinically undistinguished from other types of pneumonia *(Fields et al.,* 2002) but even there is no difference in a chest x-ray pattern between this infection and other types of pneumonia, legionellosis is usually the only one associated with alveolar infiltrates (MacFarlane *et al.,* 1984). Symptoms can include fever, cough, headache, myalgia, rigors, dyspnea, diarrhea and delirium (Tsai *et al.,* 1979) .

1.2.2 Diagnosis

The key to diagnose legionellosis is performing appropriate microbiologic testing whenever a patient is in a high-risk group: Men, >50 years old, smokers and immunosuppressive persons (Fields *et al.*, 2002). There are some different methods to diagnose this infection such as bacterial cultures, urinary antigen test, serological and Antibody-Based Assays, and Nucleic Acid-Based Molecular Diagnostics (Mercante & Winchell 2015).

1.2.2.1 Microbiological culture and isolation

Culture samples can be prevenient from the lower respiratory tract, such as sputum, pleural fluid, bronchial aspirates and bronchial alveolar lavage (Maiwald *et al., 1998)*. Semi-selective procedures enable *Legionella* resilience in presence of competing flora such as brief acid and heat exposure and/or addition to the growth media of compounds which *Legionella* is naturally resistant like glycine, polymyxin B, cycloheximide and vancomycin (Mercante & Winchell 2015). The disadvantage of this method is that it does not provide a fast result. It takes at least 9-11 days.

1.2.2.2 Urinary antigen test

This method has become the most currently used. It generally consists on based enzyme immune assay (EIA), or an ELISA, and a rapid immune-chromatographic test (ICT), in a card- or strip-based format, like a home pregnancy test. This test solves the previous method problem once it has a really fast response (15 minutes). However this

kind of tests are not really sensitive to non-serogroup 1 *Legionella pneumophila* (Mercante & Winchell 2015).

1.2.2.3 Serological and Antibody-Based Assays

Serological testing for IgG and IgM antibodies against *Legionella* was crucial in the first outbreak of legionellosis, in Philadelphia (Fraser & McDade 1979). Currently this test is not much used due to the rise of standardized culture media and techniques, and faster analyses like Urinary Antigen Test and molecular methods (Benin *et al.*, 2002).

1.2.2.4 Nucleic Acid-Based Molecular Diagnostics

Recently Real-time PCR gained some popularity among commercially marketed rapid environmental *Legionella* detection assays. This type of methods advantage mainly consist on its high sensitivity and specificity, fast results and widespread use (Mercante & Winchell 2015).

1.2.3 Treatment

All *Legionella* species tested are sensitive to commonly prescribed macrolides like rifampicin and fluoroquinolones like azithromycin and levofloxacin (Mercante & Winchell, 2015).

1.3 Transmission

Currently person-to-person transmission still hasn't been documented (Yu *et al.,* 2002), however the transmission from the environment to humans occurs through a chain of events with six different links (Fraser *et al.,*1977) (Fig.I). The first component necessary for transmission is the existence of environmental reservoirs such as rivers, lakes, hydrothermal areas and fountains (Fliiermans *et al.,* 1981) colonized with *Legionella*. The second component is the existence of man-made reservoirs, such as air-conditioning cooling towers (Negron-Alvira *et al*, 1988; Orrison *et al*., 1981), and potable water supplies (Hsu *et al.,* 1984; States *et al*., 1987; Tison & Seidler 1983; Tobin *et al.,* 1986), with propitious conditions to the bacterial amplification like the presence of biofilms, water stagnation, a range of temperatures between 30ºC e 50ºC and pH levels between 6 e 8,5.

The third component of this transmission chain is bacterial dissemination from the systems above mentioned to individuals exposed. This process occurs due the inhalation of contaminated aerosolized water (Fields *et al.* 2002). If the disseminated strain is virulent to humans, then it would be inoculated in lungs. These events correspond do the 4^{th} and 5^{th} components of the chain transmission, respectively. Finally, in order to occur infection the host has to be susceptible (Fraser *et al.,* 1977). There might be two possible clinical presentations for *Legionella* infection, Legionnaires' Disease or Pontiac Fever. It can also leads to an asymptomatic situation with no infection.

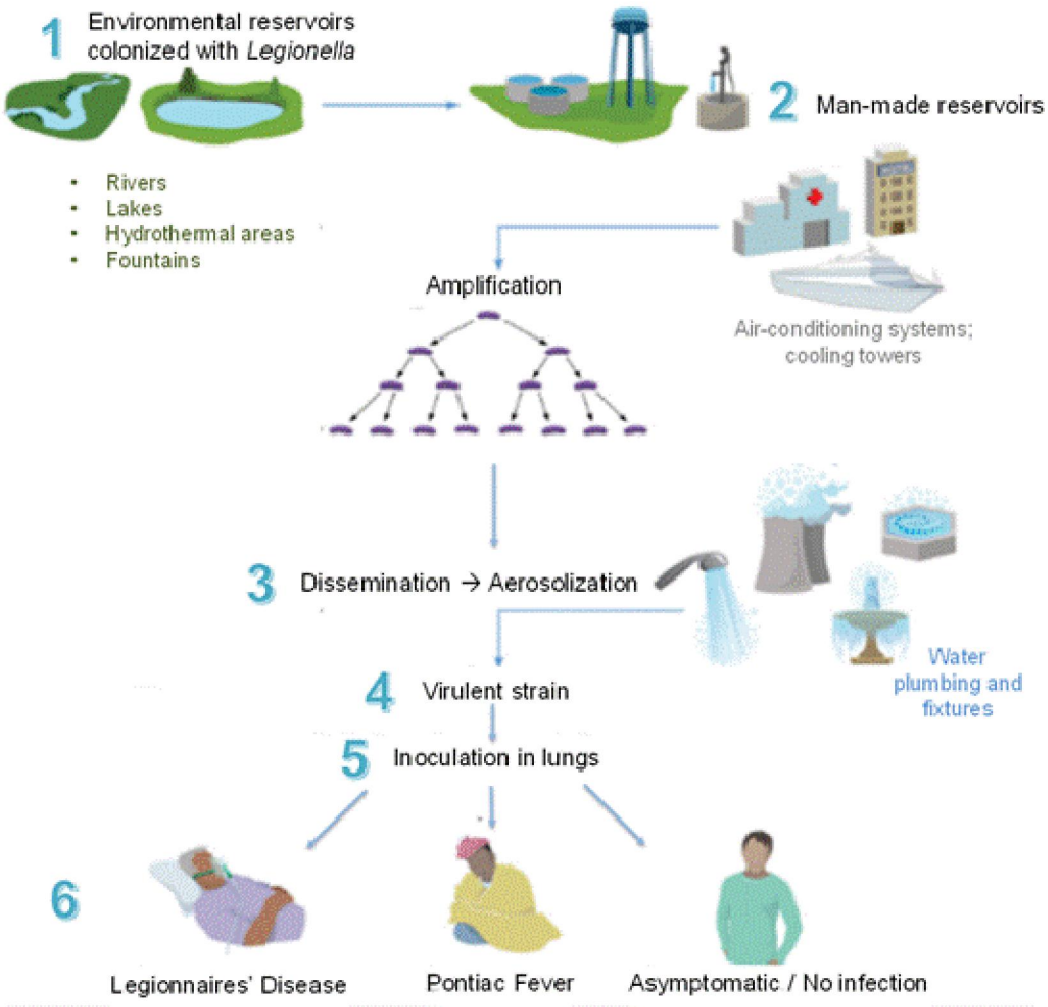


Figure 1. Schematic representation of the chain of events leading to *Legionellosis* transmission. *Legionella* present in natural sources (1) reaches at low concentrations man-made environments (2) colonizing water distribution systems and cooling towers amplifying its numbers under favourable environmental conditions. The next event on the chain is bacterial dissemination through aerosolization (3). If the strain is virulent to humans (4), the bacteria inoculates in lungs (5) and the host are susceptible to infection, then there might be two possible clinical presentations, Legionnairs'Disease or Pontiac Fever. It can also leads to an asymptomatic situation with no infection (6) (Adapted from Mercante & Winchell, 2015).

1.4 Incidence and Prevalence

Legionellosis cases have been reported in the five continents, however it is difficult to determine its real incidence rate since most cases are not reported to the public health services. The majority of the reported cases occurs in developed countries since the ecological niches which supports the multiplication and dissemination, like air conditioning systems, are less common in countries in development (Edwards *et al.,* 2008).

The European Working Group for *Legionella* Infection (EWGLI) every year registers the detected legionellosis cases in the European Union countries. In July of 2001 a massive outbreak of Legionnaires' Disease occurred in Murcia, Spain. More than 800 suspected cases were reported and 449 of these cases were confirmed, which turned this into the world's largest outbreak of legionellosis reported to date. The case fatality-rate of this outbreak was of 1%. An environmental isolate from cooling towers of a city hospital revealed an identical molecular pattern to the clinical isolates (García-Fulgueiras *et al.,* 2003).

In September 2004 a legionellosis outbreak in a long-term care facility in Cherokee County, North Carolina caused three deaths in a total of seven disease cases. This outbreak was caused by contaminations with *Legionella* in the air-conditioning fresh-air intake (Phares *et al*., 2007).

In the summer and fall of 2005 eighteen cases of legionellosis were reported in South Dakota. Four patients needed hospitalization and one ultimately died. The strain responsible for this outbreak was Benidorm and among all the sites tested only a

decorative small fountain in a restaurant was contaminated with this strain. Clinical and environmental isolates have showed identical SBT patterns. This was the first time that a non-obvious aerosol generator was reported as the source of a legionellosis outbreak (O'Loughlin *et al.,* 2007).

In the year of 2014, 30 countries in Europe reported a total of 6 943 cases of legionellosis, 6 412 (92.4%) were confirmed. The remaining 531 (7.6%) cases were classified as probable. In this year the number of notifications per 100 000 inhabitants was of 1.4, which was the highest ever observed (Fig. 2). From 5 505 cases with known final results, 456 persons were reported to have died, giving a case fatality of 8%. *L. pneumophila* Sg 1 was the pathogen identified in the majority of cases, accounting for 81% of culture-confirmed cases. France, Germany, Italy, Portugal and Spain accounted for 74% of all the reported cases. Notification rates varied from less than 0.1 per 100 000 inhabitants in Bulgaria, Poland and Romania to 5.6 per 100 000 in Portugal. As usual, most cases (69%) were community-acquired and 20% were travel-associated. Also 8% were associated with healthcare facilities, and 3% were associated with other situations (European Center for Disease Prevention and Control. 2015. Annual Epidemiological Report. [ONLINE] Available at: http://ecdc.europa.eu/en/healthtopics/legionnaires_disease/surveillance/Pages/annual-epidemiological-report-2016.aspx. [Accessed 15 July 2016]).

The high rate reported in Portugal that year was mainly due to the large community outbreak in Vila Franca de Xira in October and November 2014. In this outbreak there were 377 confirmed cases, 14 of them were fatal. Epidemiological, environmental and microbiological analysis revealed that the source of infection was

an industrial wet cooling system. Meteorological phenomena are thought as a contribution to the scale of this outbreak (Shivaji *et al.,* 2014).

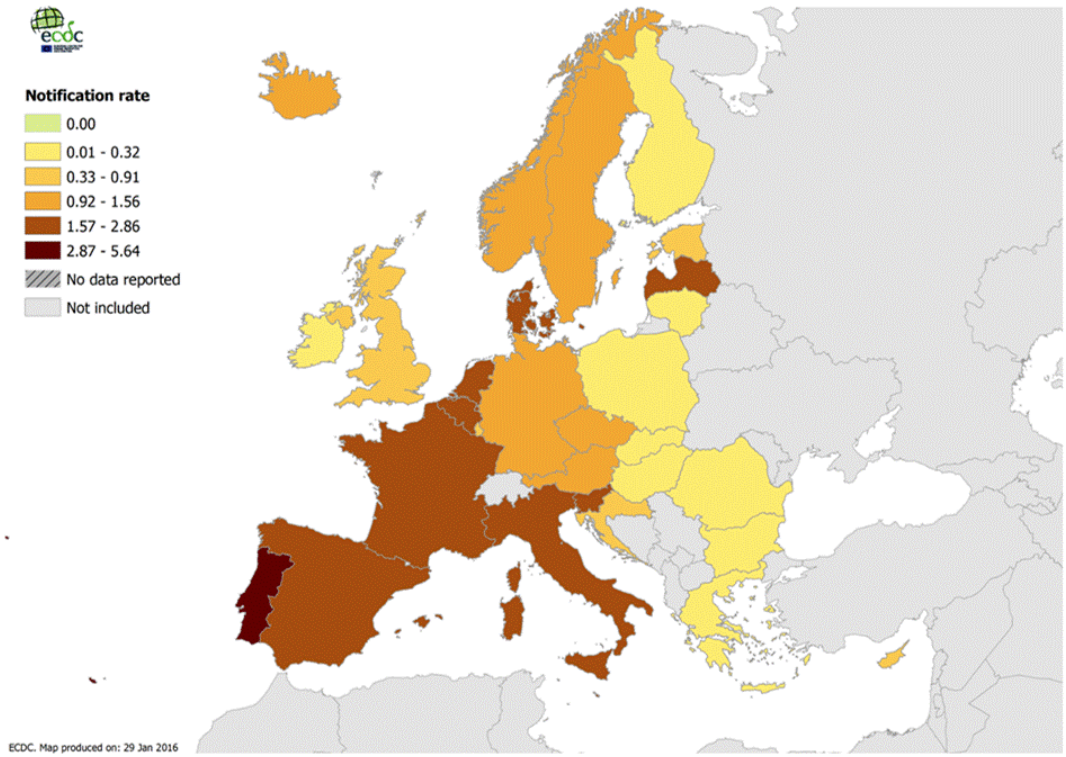


Figure 2 Rate of confirmed Legionnaires' Disease cases per 100 000 population by country, in Europe in 2014. (European Center for Disease Prevention and Control. 2015. Annual Epidemiological Report. [ONLINE] Available at: http://ecdc.europa.eu/en/healthtopics/legionnaires_disease/surveillance/Pages/annual-epidemiological-report-2016.aspx. [Accessed 15 July 2016]

1.5 *Legionella pneumophila:* classification, ecology and physiology

Family *Legionellaceae* is a monophyletic subgroup belonging to the sub-division γ of *Proteobacteria* (Benson & Fields 1998) with only one genus*, Legionella.* Based on genetic criteria, species *L. pneumophila was* divided into three subspecies: *L. pneumophila* subsp. *pneumophila*, *L. pneumophila* subsp. *fraseri* and *L. pneumophila* subsp. *pascullei* (Brenner *et al.,* 1988).

Phylogenetically the closest species to *Legionella* is *Coxiella burnetii,* the responsible agent for Q fever (Mercante & Winchell, 2015). Currently there are 60 distinct *Legionella* species divided into 70 serogroups (Allombert *et al.,* 2013).

Legionella spp. are gram-negative bacteria, aerobic, non-spore-forming, catalase-positive, and motile rods that presents polar or lateral flagella (Benson & Fields 1998; Mercante & Winchell, 2015).

These bacteria are ubiquitous in natural aqueous environments like streams, rivers and lakes (Fliiermans *et al*., 1981; Morris *et al*., 1979; Ortiz-Roque & Hazen, 1987) with the exception of *L. longbeachae* which is present mainly in compost and potting soils (Gomez-Valero & Buchrieser 2013). The presence of *Legionella* has also been reported in hydrothermal areas (Verissimo *et al,.* 1991) and groundwaters (Costa *et al.*, 2005).

Legionella species, mainly *L. pneumophila*, have also been found in man-made environments like air-conditioning cooling towers (Negron-Alvira *et al.,* 1988; Orrison *et al.,* 1981), potable water supplies (Hsu *et al*., 1984; States *et al.,* 1987; Tison &

Seidler 1983; Tobin *et al.,* 1986), plumbing and water fixtures in hospitals (Wadowsky & Yee 1983; Veríssimo *et al,.* 1990; Watkins *et al.,* 1985) and homes (Colbourne & Dennis 1985).

Legionellae are able to parasitize and multiply within protozoa including at least twenty different species of amoeba, two species of ciliated protozoa and one slime mold (Gomez-Valero & Buchrieser, 2013), but are usually associated with *Hartmannella, Acantbamoeba* and *Naegleria* species (Barker & Brown, 1994). However not all *Legionella* species grow in the same amoebal host and some of this protozoans seems to be resistant to *Legionella* infection (Gomez-Valero & Buchrieser, 2013).

The link between amoebae and legionellae may induce virulent bacterial phenotypes, assistance in distribution and also enables protection for bacteria in non-favourable environments for its survival and multiplication like excessive heat and chlorine (Mercante & Winchell, 2015). Legionellae is a very resistant organism when it comes to temperature, the most favourable values for survival and multiplication is approximately 35ºC but it can also survive in temperatures between 25°C and 42°C (Steffens & Wilson 2012). When the temperature in the aquatic environment increases, the equilibrium between bacteria and amoebae can shift, which results in fast multiplication of *Legionella*. The increase in the number of *L. pneumophila* in the water as a result of replication within protozoa increases the chance of transmission and disease manifestation. When temperature decreases or there is an exposure to environmental stress such as chlorine, the amoebae differentiate into cyst, and intracellular *L. pneumophila* has the ability of surviving within the cyst (Richards *et al.,* 2013). Regarding other environmental conditions propitious for *Legionella spp*. survival and proliferation it varies between pH levels of 5.5-8.1 and dissolved oxygen in the

water of 0.2-15 ppm. Also water stagnation, the presence of sediments and the existence of microbial communities capable of providing essential nutrients, are factors that promote *Legionella* development (Fliiermans *et al.,* 1981).

Primary sources of energy and carbon for *Legionella* are amino acids. There is a large number of toxic metals for legionellae, however iron and zinc in low concentrations can stimulate growth in these bacteria. All the organisms of the Family *Legionellaceae* require an exogenous source of cysteine to grow, with the exception of *L. oakridgensis.* This characteristic was for a long time a problem concerning isolation and growth *in vitro* (Verissimo *et al.*, 1991).

Despite the fastidious character of *L. pneumophila*, it can be commonly found in oligotrophic aquatic environments, which has a low rate of nutrients. This implies that this organism is capable of obtaining the necessary amino acids and organic carbon from this type of environment, specifically, from the microbial community existing in biofilms (Declerck 2010).

Biofilms are defined as complex microbial communities characterized by cells bound to a substrate via a matrix of polymeric extracellular self-produced substances (EPS). Due to its dynamic profile, a biofilm community can change over time and space providing a better survival and growth of the microorganisms. For this reason it is easy to understand that in the majority of natural environments, associations in biofilms are the predominant lifestyle for bacteria. *L. pneumophila* is associated with pre-established biofilms as a secondary colonizer. In other words, these bacteria create an association with other microorganisms already in the biofilm. This transitory association facilitate *L. pneumophila* to find a place to establish in the community. Bacterial release from the biofilms into the environment is an intrinsic part of the

dynamic profile of life in microbial communities associated with biofilms (Declerck 2010).

L. pneumophila was first described as an intracellular parasite of amoebae by Rowbotham (1980) (Garduño 2007). However evidences of *L. pneumophila* growth outside host cells, either in nutrient-rich media *in vitro* or within microbial communities theoretically defines it as a facultative intracellular pathogen. Nevertheless, in nature, *L. pneumophila* acts more like an obligate intracellular pathogen than like a facultative one. Therefore extracellular replication does not represent an important role regarding the maintenance of *L. pneumophila* populations in freshwater when compared to growth inside natural hosts (Robertson *et al.*, 2014). *L. pneumophila* is thought to co-evolved with protozoa in order to replicate and to optimize the acquisition of intracellular nutrients (Robertson *et al.*, 2014).

Legionella spp. possesses a rather large number and wide variety of secretion systems to provide an efficient and fast deliverance of effector molecules into the phagocytic host cells (De Buck *et al.,* 2007). This bacteria's unique physiology has been proved to be primarily adapted for survival and replication within protozoa organisms, and secondly as a free-living bacterium or associated with biofilms (Mercante & Winchell 2015; Taylor *et al. ,* 2009).

1.6 *L. pneumophila* life cycle

L. pneumophila life cycle was first described by Rowbotham (1986). Briefly, it starts with the invasion of an amoebal host usually made through coiling phagocytosis (Atlas 1999). A series of intracellular events occur including inhibition of phagosome-lysosome fusion and the alteration of organelle traffic. *L. pneumophila* replicates until nutrients become insufficient inside of a ribosome–decorated vacuole (*Legionella* containing vacuole – LCV). This structure then associates with the endoplasmic reticulum (ER) and obtains ER markers. Bacteria are now in a transmissive phase (mature intracellular phase – MIF) that is characterized by the repression of cell multiplication and augmentation of the expression of several traits such as motility and virulence factors that promotes host cell lysis and enables *L. pneumophila* to kill and escape from its host cell. At that moment bacterial progeny is released from the wasted host cell and *L. pneumophila* is now ready to infect a new host, completing the cycle (Garduño 2007; Robertson *et al.,* 2014; Allombert *et al*., 2013).

L. pneumophila has a life cycle associated with bacterial differentiation (Garduño, 2007). In the differentiation process one developmental form can lead to another (and then back to the original form) in a linear way (biphasic cycle), or more than two developmental forms can sequentially differentiate into each other (multiphasic cycle). Although if the differentiation links are not sequentially circular a developmental network is established (Robertson *et al.*, 2014).

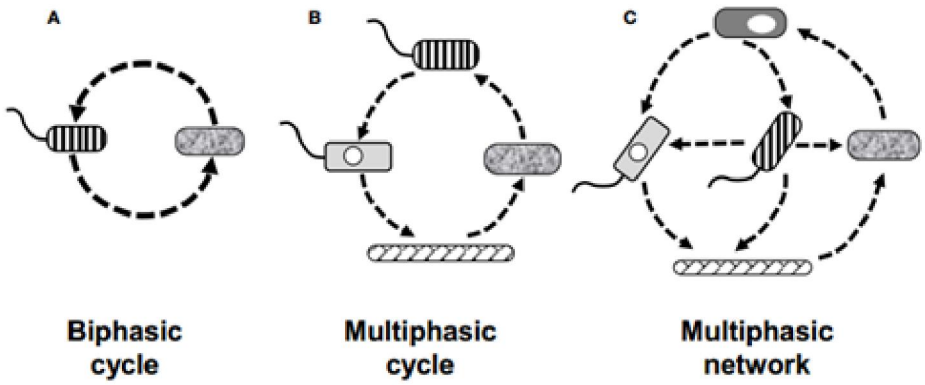


Figure 3. Schematic representation of different types of developmental cycles and developmental network. A – One form simply alternate into another (Biphasic cycle); B – Four forms originating each other in a linear way (multiphasic cycle); C – Five forms with non-linear differentiation links (multiphasic network). (From Robertson *et al.,* 2014)

The type of cycle varies depending on the replication site. Intracellularly *L. pneumophila* presents a multiphasic cycle. However morphological observations has proven that *L. pneumophila* reaches different development endpoints in different host cells, therefore is thought that *L. pneumophila* possesses many intracellular multiphasic developmental cycles, one for each host cell type. Currently is known that *L. pneumophila* has a single developmental program integrated into its life cycle that includes 14 developmental forms reported to date. Since there are so many developmental forms the existence of a developmental network was established (Robertson *et al.,* 2014).

L. pneumophila is also able to grow on agar plates as well as in nutrient-rich liquid media. In this conditions the growth is extracellular and alternates between an exponential phase, that is able to actively replicate and a post-exponential phase, that

eventually stop replication until placed in fresh medium where growth is reinitiated. In this conditions *L. pneumophila* performs a biphasic cycle (Garduño, 2007).

Developmental cycles are essential to the pathogenesis and ecology of obligate intracellular bacterial pathogens with an extracellular phase, like *L. pneumophila*. Differentiation into a highly infectious and environmentally resistant form capable of surviving for extended periods of starvation in freshwater, in order to have the opportunity to encounter a new host, is a characteristic evolved by *L. pneumophila* and has become an intrinsic part of its natural history (Garduño, 2007).

In humans *L. pneumophila* use alveolar macrophages as a host. These cells phagocytise the bacteria and it becomes the major site for bacterial replication causing respiratory disease. Bacteria reaches the lungs trough inhalation of contaminated aerosolized water (Fields *et al.,* 2002), once in the alveolar macrophages the multiplication process of *L. pneumophila* occurs the same way as it does in its natural hosts and it can lead to a pneumonia (legionellosis) (Allombert *et al.*, 2013).

One hypothesis regarding person-to-person transmission relies on the fact that in vivo MIF's are the transmissive form of *L. pneumophila* and has been shown that in macrophage infection there is a low number of MIF's. This happens due to early apoptosis of *Legionella* – infected macrophages or the lack of appropriate signals leading to an incomplete differentiation of *L. pneumophila.* Furthermore, differentiation-deficient *rpoS* and *letA* mutants, which are not capable of growing in amoebae, grow well in HeLa cells and macrophages. Therefore this results suggest that *L. pneumophila* is under strong selective pressure to differentiate within protozoa but not inside mammalian cells, and also supports the idea that *L. pneumophila* can't

complete its developmental cycle in cultured macrophages. So the hypothesis above mentioned suggests that there are not enough MIF's produced in alveolar macrophages during human infection, thus preventing person-to-person transmission (Abdelhady & Garduño, 2013).

1.7 Pathogenicity

Legionella genus includes several species but only few had already been associated with legionellosis. Strains belonging to *L. pneumophila* of serogroup 1 represent the majority of strains related with disease cases. This strains are responsible for 70% of legionellosis in United States, 92% in Europe and 50% in Australia and New Zealand (Yu *et al.,* 2002). *L. longbeachae* is the second species most associated with legionellosis, causing approximately 2 to 7% of cases worldwide, except for Australia and New Zealand where this species is associated to 30% of the disease cases (Yu *et al.*, 2002). *Legionella micdadei, Legionella bozemanii, Legionella dumoffii, Legionella anisa, Legionella wadsworthii* and *Legionella feelei* have also been associated with some cases of legionellosis, but very rarely. However, species presente different distribution patterns in clinical cases and in the environment. A study in England showed that in 97.6% of disease cases isolates were identified as *L. pneumophila* Sg1, however this serogroup represents only 55.8% of isolates from the environment (Harrison *et al.,* 2009). Interestingly other species like *L. anisa*, *L. dumoffi* or *L. feeleii* are commonly found in the environment and rarely associated with clinical cases, as mentioned before (Costa *et al.,* 2010).

The remaining *Legionella* species have never or only once been isolated from humans (Yu *et al.*, 2002). These facts justify that most studies regarding pathogenicity has been made with *L. pneumophila* strains.

Studies have shown the presence of many eukaryotic-like genes encoding proteins in *L. pneumophila* (Cazalet *et al.*, 2004). These genes are thought to be acquired during the co-evolution of *L. pneumophila* within its natural hosts and were proposed to provide effective molecular tools for bacteria to mimic eukaryotic molecular activities such as small GTPase-controlling activities (Allombert *et al.,* 2013) as following described.

Processing of *L. pneumophila* by protozoa is similar with its processing by mammalian macrophages and monocytes in humans, showing the biogenesis of a *Legionella* containing vacuole (LCV) as described in section 1.6 (Mercante & Winchell, 2015; Atlas 1999). After intracellular replication, *L. pneumophila* re-programs its genetic expression in order to be able to support the production of virulence characteristics, such as motility and virulence factors, to promote host cell lysis and to infect other phagocytic cells. LCV biogenesis allows the bacteria to escape endocytic pathways and to generate a niche tolerant for intracellular replication. It requires complex molecular mechanisms that typically mimic host-cell mechanisms and are strictly dependent on a Type IV Secretion System (T4SS) and its exceptionally high number of effectors (Allombert *et al.,* 2013).

Type IV Secretion System, *dot/icm* ("Defective in organelle trafficking" / "Intracellular multiplication") is crucial for entering and controlling the host-cell. This is a multi-protein complex which main function is to translocate virulence effectors

(Costa *et al.,* 2010; Allombert *et al.,* 2013). Currently more than 300 substrates have been identified but only a few have been functionally characterized (Allombert *et al.*, 2013). *dotA* gene is one of the included genes of this complex. Bacteria lacking the *dotA* gene are defective in all virulence activities that require the *dot/icm* transporter and lose the ability to maintain the integrity of their phagosomes after entry into the mammalian and protozoan cells (Costa *et al.*, 2010). Although several effectors are injected into the host cell, with only few exceptions, deletion of individual effectors does not result in reduced intracellular proliferation, suggesting potential functional redundancy (Richards *et al.,* 2013), so even that the majority of individual *dot/icm*-secreted substrates are genetically unnecessary for the intracellular replication of *L. pneumophila*, fundamental components for intracellular replication within animals cells have been identified. Only *SdhA*, *SidJ* and *AnkB* have been shown as indispensable for greatest intracellular replication, indicating that certain proteins in *L. pneumophila* selectively offer an advantage to the pathogen in certain hosts. Additionally both *sdhA* and *sidJ* are conserved among known genome sequences of strains of *Legionella pneumophila* and *Legionella longbeachae* (Costa *et al.,* 2014).

Type II protein secretion system (T2SS) has also been proved to play an important role in *L. pneumophila* pathogenesis (Costa *et al.*, 2012). T2SS is present in several but not all Gram-negative bacteria, suggesting its role as an important secretion system. It was shown that the system is effective in both pathogens and non-pathogens, playing significant roles in pathogenesis and/or contributing to bacterial fitness in different ecological niches. In *L. pneumophila* T2SS is crucial for intracellular infection of protozoa cells and stimulates the intracellular infection of lung epithelial

cells, reduces the cytokine output from infected macrophages and epithelia, and restricts the levels of cytokine transcripts in infected macrophages. Nowadays at least 25 proteins have been shown to be T2SS substrates (Costa *et al.* 2012).

Besides these secretion systems above mentioned other secretion systems types are present in *L. pneumophila*, although they are not all characteristic of every *L. pneumophila* strains. For example, different type IVB secretion systems are present in *many L. pneumophila* strains but a new type IVA system has only been found in *L. pneumophila* strain Corby. Also a type V secretion system could only be identified in *L. pneumophila* strain Paris. These differences could be explained by the noteworthy genome plasticity that has been revealed from comparing the genomes between sequenced *L. pneumophila* strains (De Buck *et al.*, 2007).

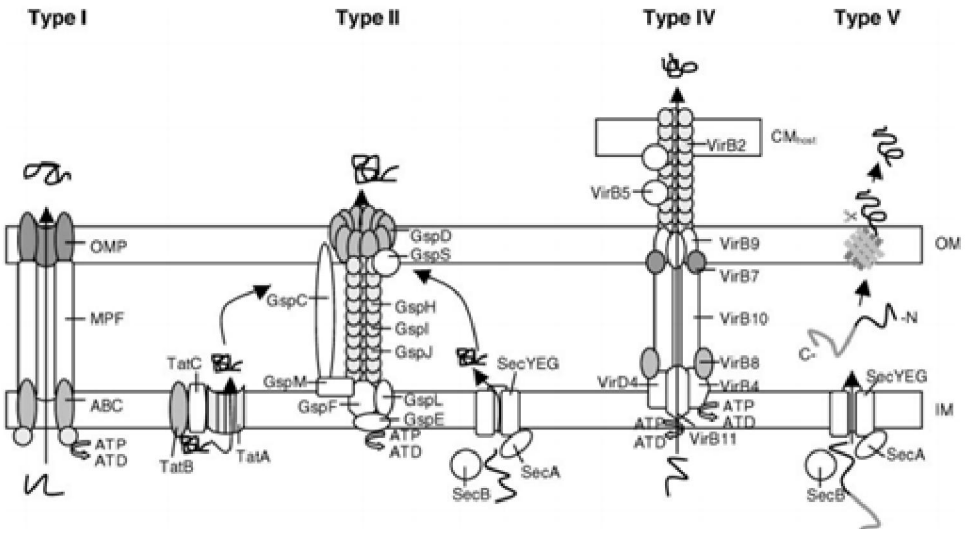


Figure 4. Schematic representation of the main secretion systems in *L. pneumophila.* OM - outer membrane; IM - inner membrane; CM_{host} - cytoplasmic membrane of host cell; OMP - outer-membrane protein; MPF - membrane fusion protein; ABC – ABC transporter. (From De Buck *et al*., 2007)

Despite the efforts made in the last decades it is still not completely clear how *L. pneumophila* pathogenic mechanisms work and why some specific species are the major cause of disease. It is possible that some of these organisms could have greater intrinsic virulence or they could just be more abundant in the environments that promote bacteria dispersal to humans (Costa *et al.*, 2005).

1.8 *L. pneumophila* Sequence-Based Typing and genetic diversity

Genomes from *L. pneumophila* strains Philadelphia, Paris, and Lens were the first *Legionella* genomes being sequenced. Comparative genomics revealed that ~300 genes (~10%) are specific for each strain. Considering that these strains belong to the same species and serogroup, the genomic diversity between them is relatively high. Additional analysis of the gene content of 217 *L. pneumophila* strains established that the gene content of the *L. pneumophila* genome is extremely diverse. These studies suggested that the principal source of diversity between *Legionella* genomes is mobile genetic elements and horizontal gene transfer, amongst *L. pneumophila* strains, as well as among strains belonging to different *Legionella* species, other bacterial species, and probably also among *Legionella* and their eukaryotic hosts (Cazalet *et al.*, 2004; Gomez-Valero *et al.,* 2014).

Recently five new *L. pneumophila* genomes were sequenced providing the possibility to study deeply their diversity and evolution. Comparative analyses of the yet available genomes showed that the core genome contains 2405 orthologous groups of genes, and each genome once compared with the seven others contains

between 154 and 271 strain-specific genes (Gomez-Valero & Buchrieser, 2013). Besides, more than 1000 genes are not present in all eight genomes and therefore belong to the flexible gene content or accessory genome (Gomez-Valero & Buchrieser, 2013).

Comparison of the genomes of human disease-related strains with non-disease-related strains has given some understanding into the genomic specificities related to adaptation and host-pathogen interactions of *L. pneumophil*a bacterium. *L. pneumophila* and *L. longbeachae*, contain a set of genes that seems to increase their successful infection of mammalian cells (Gomez-Valero *et al.,* 2014), as previously mentioned in section 1.7.

Multilocus sequence typing is a tool used to discriminate clonal groups within several bacterial species based only on the analysis of house-keeping genes (Harb & Kwaik 1998). A scheme based on this tool has been developed for *L. pneumophila* - Sequence-based typing (SBT), and nowadays it uses the sequence of genes: *flaA, pilE, asd, mip, mompS, and proA* (Gaia *et al.,* 2005) and *neuA* (Ratzow *et al.,* 2007). This tool is not only based on house-keeping genes, but also in genes under selective pressure like virulence-related genes (*Legionella pneumophila* Sequence-Based typing. [ONLINE] Available at: http://www.hpa-bioinformatics.org.uk/legionella/legionella_sbt/php/faq.php. [Accessed 10 July 2016]).

European Working Group for *Legionella* Infections (EWGLI) maintains an allele online data base that allows researchers to inquire sequence data and obtain an allelic profile (SBT profile) and a final combined sequence type for each isolate. An SBT profile

includes a set of numbers referred to the number of individual alleles for each gene separated by commas (Ratzow *et al.*, 2007; ***Gaia*** *et al.,* 2005).

This method is very important and largely used in epidemiological studies. One advantage is the direct sequence comparison which prevents interpretational subjectivity of other methods and differences over time or between laboratories (Scaturro *et al.*, 2005). This kind of method is also important because it allows to identify shared phylogenies between clinical and environmental strains (Mercante & Winchell, 2015). One hypothesis regarding this topic is that the high percentage of *L. pneumophila* Sg1 strains related with human disease is not due to its predominance in the environment but maybe because these strains presents a higher virulence for humans (Costa *et al.*, 2012). It was already shown that there are clear differences between populations of clinical and man-made environmental isolates, and clinical isolates showed less diversity than man-made environmental isolates (Harrison *et al.,* 2007; Harrison *et al.,* 2009; Coscollá & González-Candelas 2009). These results reinforced the hypothesis above mentioned where the major premise is that *L. pneumophila* strains present in clinical and man-made environments represent a subgroup of clones existing in nature that specially adapted to niches related with this environmental types (Coscollá & González-Candelas 2009).

1.9 *Galleria mellonella* as an infection model for *L. pneumophila*

Since *L. pneumophila* natural hosts are amoebae, like *Acanthamoeba castellanii* or *Hartmannella vermiformis*, some of these organisms have been used as models to study molecular aspects of *Legionella* pathogenesis (Kwaik 1996; Hilbi *et al.,* 2007). *Dictyostelium discoideum* has proved to be a useful protozoan model organism due to its capability to be easily genetically modified (Solomon *et al.*, 2000).Since amoebae have a much less complex antimicrobial mechanisms than mammalian cells, these models may not be the most appropriate to study *L. pneumophila* infection *(*Harding *et al.*, 2012).

Another alternative of model organisms used to study *L. pneumophila* is the nematode *Caenorhabditis elegans* which presents an innate immune system and is also used as a model for other species studies (Brassinga *et al.,* 2010). However this is also not an ideal model once bacteria replicates in the intestinal lumen and the epithelial cells stay intact. This fact restrain research focused on the virulence determinants needed for the intracellular lifestyle of *Legionella* (Harding *et al.,* 2012).

Legionella infection in humans is usually studied with mammalian hosts such as guinea pigs. Also some mouse strains have been used but this organisms seems to be resistant to *Legionella* infection, with some exceptions (Harding *et al.* 2012). Mammalian hosts has some strings attached due to ethical reasons and also because it involves high cost and maintenance difficulties. Therefore some alternatives have been searched.

Insects have been introduced as a model organism. Particularly *Drosophila melanogaster* has been used to study bacterial pathogenesis (Scully & Bidochka, 2006). The insect innate immune system reveals many similarities to human's. Most insect species use some specialized cells to phagocyte pathogens and posteriorly form aggregates which encapsulate and neutralize those microorganisms. This cells are called hemocytes. When activated hemocytes can lead to a series of mechanisms that end on the production of antimicrobial compounds. Besides this kind of responses there is another where antimicrobial peptides are produced and secrete by the insect body (organ similar to mammalian liver)(Harding *et al.,* 2012).

D. melanogaster is currently not the only insect model adopted, also the larvae of the greater wax moth *Galleria mellonella* has been widely used as a model to study a large number of human pathogens such as *Campylobacter jejuni* (Champion *et al.,* 2010), *Listeria spp* (Joyce & Gahan 2010), *Yersinia pseudotuberculosis* (Champion *et al.,* 2009), *Streptococcus pyogenes* (Olsen *et al.,* 2011), *Burkholderia cepacia* (Silva *et al.,* 2011) and several pathogenic fungi such as *Candida albicans* (Mowlds *et al.,* 2008).

G. mellonella can be easily maintained, resists incubation at 37ºC and can reproduce the strain-to-strain variations in virulence observed in mammalian cell culture and animal models. This aspects along with the presence of an innate immune system makes *G. mellonella* larvae an appropriate model for study *L. pneumophila* infection.

1.10 Objective

The main objective of this study was to determine if unrelated *L*. *pneumophila* strains, isolated from different environments and with distinct genetic backgrounds, exhibited different levels of virulence, using *G.mellonella* larvae as an infection model.

II. Materials and Method

2.1 *Legionella pneumophila* strains

L. pneumophila strains were selected from several others in order to capture the maximum genetic variability, since they represented the allelic diversity determined in early studies from *dot/icm* and T2S related genes (Costa *et al.,* 2010; Costa *et al.,* 2012; Costa *et al.*, 2014). These included 16 isolates comprising 10 natural and manmade environments, and 6 clinical-related *L. pneumophila* type and reference strains, 12 belonging to *L. pneumophila* subsp. *pneumophila*, 2 from *L. pneumophila* subsp. *fraseri* strains and 2 L. *pneumophila* subsp. *pascullei* (Table I).

Table I. *L*. *pneumophila* unrelated strains, isolated from distinct environments, type and reference strains included in this study

Strain designation	Environmental Type	Subspecies	Reference
Agn2	Natural, Italy	*L. pneumophila* subsp. *pneumophila*	Costa *et al.* (2010)
Aço5	Natural, Azores	*L. pneumophila* subsp. *pneumophila*	Veríssimo *et al*. (1991)
Aço12	Natural, Azores	*L. pneumophila* subsp. *pneumophila*	Veríssimo *et al*. (1991)
Aço22	Natural, Azores	*L. pneumophila* subsp. *pneumophila*	Veríssimo *et al*. (1991)
NMex1	Natural, New Mexico, USA	*L. pneumophila* subsp. *pneumophila*	Marrão *et al.* (1993)
Ger10	Natural, Northern Portugal	*L. pneumophila* subsp. *pneumophila*	Costa *et al.* (2010)
HRD2	Lung aspirate	*L. pneumophila* subsp. *pneumophila*	Costa *et al.* (2010)
Ice27	Natural, Iceland	*L. pneumophila* subsp. *pneumophila*	Costa *et al.* (2010)
HUC1	Man-made	*L. pneumophila* subsp. *pneumophila*	Costa *et al.* (2010)
Por3	Man-made	*L. pneumophila* subsp. *pneumophila*	Costa *et al.* (2010)
IMC23	Man-made/ Disease-related	*L. pneumophila* subsp. *pneumophila*	Veríssimo *et al.* (1990)
Philadelphia1 (ATCC 33152^{T})	Disease-related	*L. pneumophila* subsp. *pneumophila*	Chien *et al.* (2004)
Los Angeles1(ATCC 33156^{T})	Disease-related	*L. pneumophila* subsp. *fraseri*	McKinney *et al* (1979)
Lansing3 (ATCC 35251)	Disease-related	*L. pneumophila* subsp. *fraseri*	Brenner *et al.* (1988)
U8W (ATCC 33737^{T})	Disease-related	*L. pneumophila* subsp. *pascullei*	Brenner *et al.* (1988)
MICU B (ATCC 33735)	Disease-related	*L. pneumophila* subsp. *pascullei*	Brenner *et al.* (1988)

2.2 Culture media

2.2.1 Buffered Charcoal Yeast Extract (BCYE)

Table II. Buffered Charcoal Yeast Extract (BCYE) reagents and quantities.

Reagents	Quantity
Activated charcoal	2 g/L
Yeast extract	10 g/L
Agar	13 g/L
N-(2-acetamido)-2- aminothanesulfonic acid (ACES)	10 g/L
α-ketoglutarate	1 g/L
L-cysteine	0.4 g/L
Ferric pyrophosphate	0.25 g/L

ACES and α-ketoglutarate were diluted in 980 ml of distilled water and pH was adjusted to 6.9 using KOH or H_2SO_4 at 5% if required. This solution was used to hydrate the yeast extract, activated charcoal and the agar.

The final solution was autoclaved (121ºC for 15 minutes). After sterilization the temperature was stabilized using a water bath at 55ºC. Previously prepared and sterilized (syringe filtration using a 0.22 µm filter) solutions of L-cysteine (4%) and ferric pyrophosphate (2.5%) were added to the media (10 ml each).

2.2.1.1 L-cysteine solution

A solution of L-cysteine 4% (Sigma) was prepared, in demineralized water, sterilized with a 0.22 µm filter. The final solution was separate into aliquots and stored at 20º C.

2.2.1.2 Ferric pyrophosphate solution

A solution of ferric pyrophosphate 2,5% (Sigma) was prepared in demineralized water, sterilized with a 0.22 µm filter. The final solution was separate into aliquots and stored at -20º C.

2.2.2 ACES Yeast Extract (AYE)

Table III. ACES Yeast Extract (AYE) reagents and quantities.

Reagent	Quantity
Bovine serum albumin (BSA)	5 g/ L
Yeast extract	10 g/ L
Ferric pyrophosphate	0.25 g/L
L-cysteine hydrochloride	0.4 g/L
ACES	10 g/L

BSA, yeast extract and ACES were dissolved in 980 ml of distilled and autoclaved water. The pH was adjusted to 6.9 using KOH or H_2SO_4 at 5% if required. This solution was

sterilized by a vacuum filtration system with a 0.22µm filter. Ferric pyrophosphate and L-cysteine solutions were added later like described above (steps 2.2.1.1 and 2.2.1.2).

2.3 Preparation of *L. pneumophila* for injection in *G.mellonella*

The selected strains (Table I) were cultured out of cryopreserved suspensions preserved at -80ºC. After thawing *L. pneumophila* strains were cultured in BCYE (Edelstein, 1981) plates and incubate at 37ºC for 3-4 days.

2.3.1 Determine the growth rate of *L. pneumophila* strains

For the infection experiments in *G. mellonella,* the selected *L. pneumophila* strains had to be in the post-exponential phase of growth (Harding *et al*., 2013). OD_{600} was measured using a spectrophotometer (Jenway 6405 W/Vis) during 36 hours in order to monitor the growth of all *L. pneumophila* strains and assesses the corresponding growth rates.

2.3.2 *L. pneumophila* CFU/ml determination

Determining *L. pneumophila* CFU/ml values corresponding to an OD_{600} of 1 allowed to prepare solutions with pre-determined concentrations to be tested (injected) in *G.mellonella*.

2.3.2.1 Drop plate method

For each *L. pneumophila* strain one full loop of bacteria from BCYE plates was suspended in 3 ml of AYE (Horwitz & Silverstein 1983) and incubated for 21 hours at 37ºC with 200 rpm in a shaking incubator. A tube with AYE was used as control to ensure sterility of the media. *L. pneumophila* cultures were serial diluted in D-PBS. Dilutions of 10^{-5}, 10^{-6} and 10^{-7} were pipetted (25 µl) on BCYE plates. Three drops per dilution were plated. After the drops dried, the petri plates were inverted and incubated at 37ºC for 1-2 days.

To calculate *L. pneumophila* strains CFU/ml values only drops corresponding to the concentrations of 10^{-6} were reckoned. Since triplicates were made, average and standard deviation were calculated and those results were used to finally establish the relation between OD_{600}=1 and the CFU/ml for each strain.

Table IV. D-PBS reagents and quantities.

Reagents	Quantity
NaCl	8 g/L
KCl	0.2 g/L
Na2HPO4•2H2O	1.44 g/L
KH2PO4	0.2 g/L

All the reagents were dissolved in 1 liter (L) of distilled water. pH was adjusted to 7.2 - 7.4 with NaOH or HCl. The final solution was sterilized by autoclaving (15 min at 121ºC) or microfiltering (syringe with a filter of 22 µm).

2.3.3 *L. pneumophila* growth for injection

For each *L. pneumophila* strain one full loop of bacteria from BCYE plates was suspended in 3 ml of AYE (Horwitz & Silverstein 1983) and incubated for 18-19 hours at 37ºC with 200 rpm in a shaking incubator. A tube with AYE was used as control to ensure sterility of the media.

After incubation, the OD_{600} was measured using a spectrophotometer (Jenway 6405 W/Vis) and new inoculums were prepared in 3 ml of AYE to a final OD_{600} of 0.1. Tubes were left in a shaking incubator as described above, this time for 21 hours. After this incubation time, OD_{600} was measured again. Since bacteria should be grown to post-exponential phase for infection, growing for 21 hours allows experiments to be standardized. According to the predetermined relation between OD_{600} and CFU/ml values calculated for each strain as described on section 2.3.2, the used injection concentration was constant for the tested strains. The inoculum was plated as described in section 2.3.2.1 as a control to ensure that the expected CFU/ml is present in the inoculum.

2.4 *Galleria mellonella* infection

G. mellonella larvae were kindly provided by Professor Leonilde Moreira and her research group at Instituto Superior Técnico, Lisbon.

2.4.1 Larvae preparation

To be suitable for injection larvae were at the 5th or 6th instars stage which corresponded approximately to 2-3 cm in length and shouldn't show any signs of darkening (Fig. 5 and 6) (Harding *et al.*, 2013).

Figure 5. *Galleria mellonella* larvae suitable for injection. (Photo obtained in this study).

Figure 6. *Galleria mellonella* larvae with signs of darkening (Photo obtained in this study).

2.4.2 *G. mellonella* injection

All the following experiments regarding *G. mellonella* infection with *L. pneumophila* were performed as previously described by Harding *et al.,* 2013 with some adjustments.

After choosing healthy larvae according to the number of strains to be tested the injection site (the hindmost left proleg) (Fig. 7) was disinfected with ethanol. The injection was performed using a micrometer adapted to control the injection volume onto a micro-syringe(Mil-Homens *et al.*, 2010). With this device *G. mellonella* larvae were injected with 10 µl of bacterial suspension prepared as previously described on section 2.3.3 and were incubated at 37°C in the dark. As a control, 10 larvae were injected with D-PBS alone, and 10 untreated insects were included with every experiment. Assays were allowed to proceed for only 72 hours as pupa formation could occasionally be seen by day 4. At least three independent replicates of each experiment were performed. The corresponding average values and standard deviations were calculated. Ten larvae were injected for each strain of *L. pneumophila* tested.

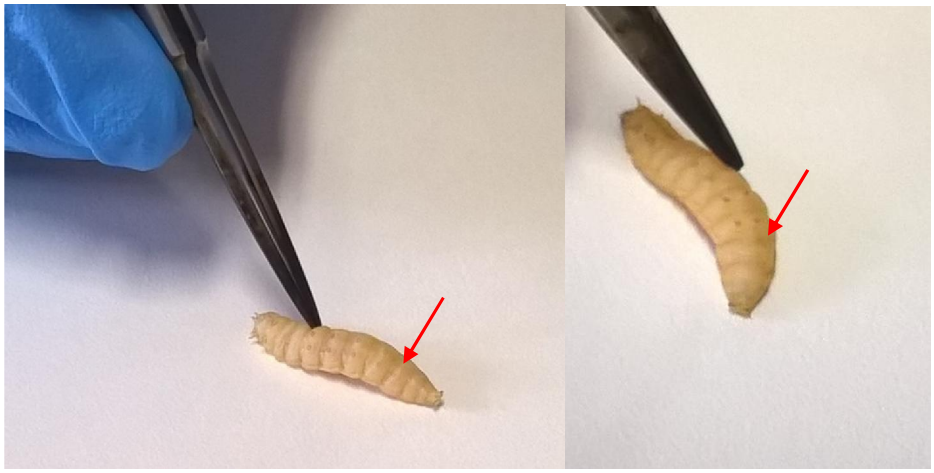


Figure 7. The injection site on *G. mellonella* is marked by the red arrow. (Photo obtained in this study).

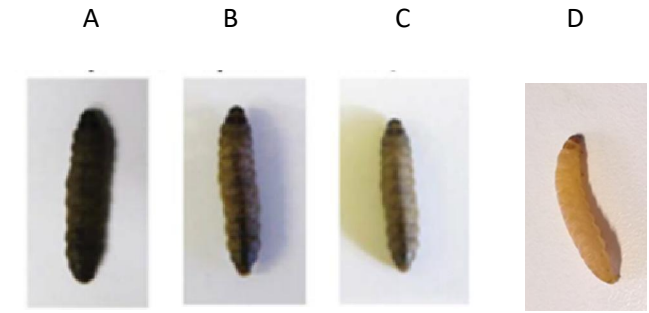
2.4.3 Larvae monitorization after injection

For each *L. pneumophila* strain the injected larvae were individually examined for some phenotypic characteristics and the death time was recorded after 18, 24, 48 and 72 hours after injection, respectively.

The phenotypic parameters evaluated are presented on table V and depicted on Fig.8. A distinct table was filled for each replica.

Table V. Matrix table used to evaluate the larvae phenotypic parameters observed *after L. pneumophila* infection.

Hours post injection	Survivers	Colour				Cocoon formation		Movement		
		Normal	Darkening	Dark	Very dark	Yes	No	Normal	Slow	Very slow



F

igure 8. G. mellonella larvae phenotypic parameters. (A) - very dark; (B) – dark; (C) – darkening; (D) - normal colour; (E) - cocoon formation; (F) - no cocoon formation. (A),(B) and (C) adapted from Tsai, Loh, and Proft 2016. (D), (E) and (F) are photos obtained in this study.

2.4.4 Confirmation of the injected L. pneumophila CFU/ml

To confirm the inject CFU/ml of *L. pneumophila*, the injected solutions were plated in BCYE plates as described in step 2.3.2.1.

2.5 Statistical analysis

All quantitative data was obtained from at least three independent assays. Standard deviation was used to calculate error bars. The Mantel-Cox test to determine *P*-values was performed using GraphPad Prism 7.01 software. Differences were considered to be statistically significant if the *P*-value was lower than 0.05.

2.6 *Legionella pneumophila* Sequenced-Based Typing

Sequenced-Based Typing (SBT) protocol was performed as previously described based on the partial sequence of seven genes (*flaA, pilE, asd, mip, mompS* and *proA* (Gaia et al. 2005), *neuA*(Ratzow et al. 2007) and *NeuAh* (Mentasti *et al.,* 2012) in order to type the *L. pneumophila* strains used in this project.

2.6.1 DNA extraction

DNA extraction was performed according to an adaptation of the Wiedmann-al-Ahmad *et al.*, 1994 method. Briefly the selected strains (Table I) were cultured from cryopreserved suspensions, in BCYE at 37ºC for 72 hours. The cultures were observed with the help of a magnifying glass to confirm its purity. One colony from each strain was suspended in 50 µl of lysis buffer and boiled at 100ºC for 5 minutes in order to get a complete lyse of cells. The remaining cell components were sediment through centrifugation for 10 minutes at 13000 rpm. The supernatant containing DNA was removed to a new tube and conserved at -20ºC.

2.6.1.1 Lysis Buffer

Table VI. Lysis buffer reagents and quantities.

Reagents	Quantity
Tween 20 2% (Sigma)[a]	500 µl
10x concentrated solution of NH_4 (Bioline)	100 µl
Ultrapure water[b]	400 µl

[a] Previously sterilized by a 0.22 µm filter; [b] sterilized by autoclaving – 121ºC 15 minutes.

2.6.2 Amplification of the SBT genes by Polymerase Chain Reaction (PCR)

L. pneumophila SBT genes amplifications were performed according to Mentasti *et al.,* 2012 with some adjustments. Briefly, reaction mixtures were prepared by sequential addition of the reagents in the final concentrations described on Table VIII to an "Eppendorf" tube of 200µl.

Table VII. PCR amplification reagents, final concentrations and used volumes.

Reagent	Final concentration	Volume (µl)
Ultrapure water		19
MasterMix NZYTech	1x	25
Primer F	4 pmoles	2
Primer R	4 pmoles	2
DNA		2
Final Volume		50

Each oligonucleotide was resuspended in ultrapure water (sterilized by filtration and autoclaving – 121ºC 15 minutes) in order to achieve a final concentration of 1 µl/µg. Work aliquots were prepared with a final concentration of 0,1 µl/µg through the addition of 10 µl the stock solution and 90 µl of ultrapure water (sterilized by filtration and autoclaving – 121ºC 15 minutes)

The characteristics of the primers used for the SBT gene amplification on this study are described on the Table VIII.

We were not able to amplify *neuA* with the *neuA* standard primers in strains Ger10, Los Angeles1 (ATCC 33156^T), U8W (ATCC 33737^T) and Agn2. This could be explained by the presence of an homolog (N-Acylneuraminate Cytidyltransferase homolog - *neuAh*) gene found in some non-serogroup 1 strains(Mentasti *et al.,* 2012). To amplify the *neuAh* gene from the strains above mentioned a distinct set of primers was used

Table VIII. Amplification primers used in this study and its characteristics (Mentasti *et al.,* 2012.; Farhat *et al.*, 2011)

Gene	Primer name	Position	Primer sequence	Annealing temperature	Fragment size (bp)
flaA	flaA-587F flaA-960R	568-587 981-960	GCG TAT TGC TCA AAA TAC TG CCA TTA ATC GTT AAG TTG TAG G	55 °C	414
pilE	pilE-35F pile-453R	12-35 471-453	CAC AAT CGG ATG GAA CAC AAA CTA GCT GGC GCA CTC GGT ATC T	55 °C	460
asd	asd-511F asd-1039R	487-511 1062-1039	CCC TAA TTG CTC TAC CAT TCA GAT G CGA ATG TTA TCT GCG ACT ATC CAC	55 °C	576
mip	mip-74F mip-595R	58-74 616-595	GCT GCA ACC GAT GCC AC CAT ATG CAA GAC CTG AGG GAA C	55 °C	559
mompS	mompS-450F mompS-1116R	430-450 1140-1116	TTG ACC ATG AGT GGG ATT GG TGG ATA AAT TAT CCA GCC GGA CTT C	55 °C	711
proA	proA-1107F proA-1553R	1090-1107 1570-1553	GAT CGC CAA TGC AAT TAG ACC ATA ACA TCA AAA GCC	55 °C	481
neuA	neuA-196F neuA-634R	176-196 634-611	CCG TTC AAT ATG GGG CTT CAG CGA TGT CGA TGG ATT CAC TAA TAC	55 °C	459
neuAh	neuAh – L neuAh - R		ATCCAGCAGTTTTTAMAAATTTAGG TGGCTGCATAAAYTAATTCTTTAGCCA		791-794

The amplification reactions were performed on a thermal cycler with the parameters described on Table IX.

Table IX. Thermal cycler parameters for PCR amplification (Mentasti *et al.,* 2012)

Step	Temperature	Time	Stage	No. cycles
1	95	5 min	Initial denaturation	1
2	95	30 sec	Denaturation	35
	55	30 sec	Annealing	
	72	45 sec	Extension	
3	72	10 min	Final Extension	1
4	4	∞	Hold	

To amplify the *neuAh* gene from strains Agn2, Ger10, Los Angeles1(ATCC 33156^{T}), U8W (ATCC 33737^{T}) and MICU B (ATCC 33735) the thermal cycle parameters were the same as described on Table IX with exception of step 2 at 72 ^{0} C where the extension time was 60 seconds instead of 45. (2016, May 29) Retrieved from http://www.hpa-bioinformatics.org.uk/*Legionella*/*Legionella*_sbt/php/sbt_homepage.php

2.6.3 Analysis of PCR amplification products by agarose gel electrophoresis

After each PCR reaction, and in order to determine the presence and quality of the amplified genes, an electrophoresis was performed on 2% agarose gel in Tris-acetate-EDTA (TAE) buffer for 30 minutes at 90 V (Gibco BRL ST 504). In each well was deposited 5 µl of the amplification product to each was previously added 1 µl of

loading buffer. In each gel one well was reserved for the molecular weight marker (DNA ladder NZYTech III) (4 µl). After electrophoresis, gels were observed under UV light using a Gel Doc XR System (BioRad, Hercules, A, EUA) system linked to a computer.

2.6.3.1 Agarose gel

Table XI. 2% Agarose gel reagents and quantities.

Reagents	Quantity
TAE buffer 1X	30ml
Agarose	0.6g
Green Safe NZYTech 2x	0.5 µl

Agarose was hydrated with the TAE (50x) solution. To ease the dilution process the suspension was heated in a microwave for a few minutes at 500W. The solution was cooled and finally Green Safe was added.

2.6.3.2 TAE (50x) stock solution

Tris- HCL was diluted in an aqueous solution of EDTA and the acetic acid was add to the solution. pH was adjusted to 8.0 using a solution of NaOH 5 M. Finally, the solution volume was adjusted to 500 ml with ultrapure water and stored at room temperature in a dark flask.

2.6.4 Purification of the amplification products

PCR products were purified using NZYTech Purification Spin Kit according to the manufacturing instructions. Briefly the volume reaction mixture was transferred into a 1.5ml microcentrifuge tube and 5 volumes of Binding Buffer were added. The above mixture was added to the NZYTech spin column and centrifuged for 30 seconds. The flow-through was discard. Following, 600 µl of Wash Buffer were added and centrifugation was performed for 1 minute. The flow-through was discard. In order to dry NZYTech spin membrane of residual ethanol one more minute of centrifugation was needed. The NZYTech spin column was placed into a new 1.5ml microcentrifuge tube. 50 µl of ultrapure water was added to the spin column and centrifuged for 1 minute to elute the DNA. The purified DNA was stored at -20ºC.

The constitution of the solutions used in the purification procedure was omitted by the manufacture.

2.6.5 Sequence determination and analysis

The sequence of the purified products was determined by sanger sequencing by a commercial sequencing provider (GATC Biotech)

2.6.5.1 Sequence analysis

The quality of the sequences was manually checked using the Sequence Scanner software: (https://products.appliedbiosystems.com). Alignment against the corresponding reference genes available on the SBT database (http://www.hpa-bioinformatics.org.uk/*Legionella*/*Legionella*_sbt/php/sbt_homepage.php) was performed using the multiple alignment CLUSTAL software, included on MEGA5 package.

2.6.5.2 Allelic profiling

Designation of alleles was performed according to the EWGLI SBT database (http://www.hpa.org.uk/cfi/bioinformatics/dbases.htm#EWGLI), and the combination of the identified alleles is represented as an ordered numerical vector corresponding to a Sequence Type (ST) (Edwards *et al.*, 2008).

2.6.5.3 Sequence Type Clustering

The similarity between the ST profiles was determined by calculating the Pearson's coefficient with correction: d = (1 - r) x 100. Clustering was performed using the Unweighted Pair Group Method with Arithmetic Averages (UPGMA), employing the DendroUPGMA computer program (http://genomes.urv.cat/UPGMA/) (Garcia-Vallvé *et al.,* 1999).

III. Results and Discussion

3.1 Determine the growth rate of *L. pneumophila* strains

For the infection experiments in *G. mellonella,* the selected *L. pneumophila* strains had to be in the post-exponential phase of growth (Harding *et al*., 2013). This was confirmed by monitoring the growth of all *L. pneumophila* strains for 36 hours and assesses the corresponding growth rates. From the obtained results it was possible to confirm that after 21 hours of incubation all *L. pneumophila* strains used in this study were in post-exponential phase (data not shown).

3.2 *L. pneumophila* CFU/ml determination

Determining *L. pneumophila* CFU/ml values corresponding to an OD_{600} of 1 was mandatory to prepare the solutions for injection with a known pre-determined concentration in order to standardize the experiment. To perform this step the values obtained in the previous section were associated with the *L. pneumophila* CFU counting through the drop plate method (Figure 9), as described on section 2.3.2.

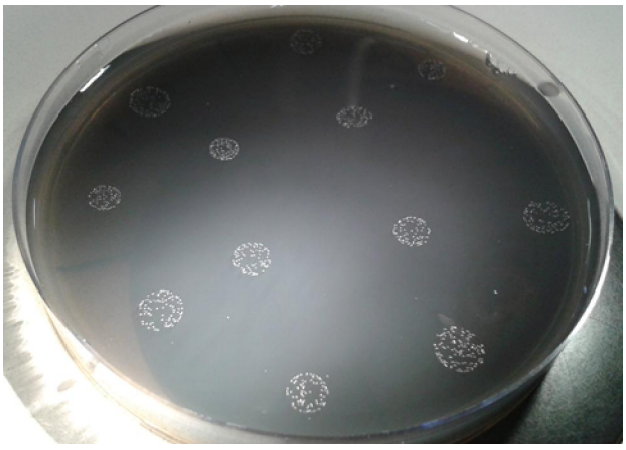


Figure 9. Drop plate method used to determine de CFU/ml of *L. pneumophila* strains. Photo obtained in this study.

Differences between the CFU/ml values corresponding to an OD_{600} of 1 were observed between strains. The most notorious one was from strain Por3 with a CFU/ml value of $2.59x10^8$, one order of magnitude lower than all other strains (Table XII). These results were taken in account in order to guarantee that the injection concentration was the same for all *L. pneumophila* strains.

Table XII. *L. pneumophila* CFU/ml corresponding to an OD_{600} of 1 assessed by the drop plate method.

***L. pneumophila* strain**	**CFU/ml**
Philadelphia1	$1,89x10^9$
Los Angeles1	$4,26x10^9$
Lansing3	$5,48x10^9$
U8W	$2x10^9$
MICU B	$2x10^9$
HRD2	$3,82x10^9$
HUC1	$5,82x10^9$
Por3	$2,59x10^8$
IMC23	$7,79x10^9$
Agn2	$6,12x10^9$
Aço5	$6,46x10^9$
Aço12	$3,13x10^9$
Aço22	$3,47x10^9$
NMex1	$3,9x10^9$
Ger10	$6,31x10^9$
Ice27	$3,2x10^9$

3.3 Survival of *L. pneumophila* – infected *G. mellonella* is dose dependent

As previously mentioned one of the goals of this study was to determine if there were differences between *L. pneumophila* strains regarding its capability to kill larvae. In order to do that three different injection concentrations were tested for three distinct strains. This allowed us to choose the injection concentration that was responsible for greater differences on larvae survival between strains to be used in the following experiments.

From the tested conditions, no mortality was observed for the 10^5 CFU per larvae injection concentration, similar to what was observed for the D-PBS injected larvae serving as control (Fig.10).

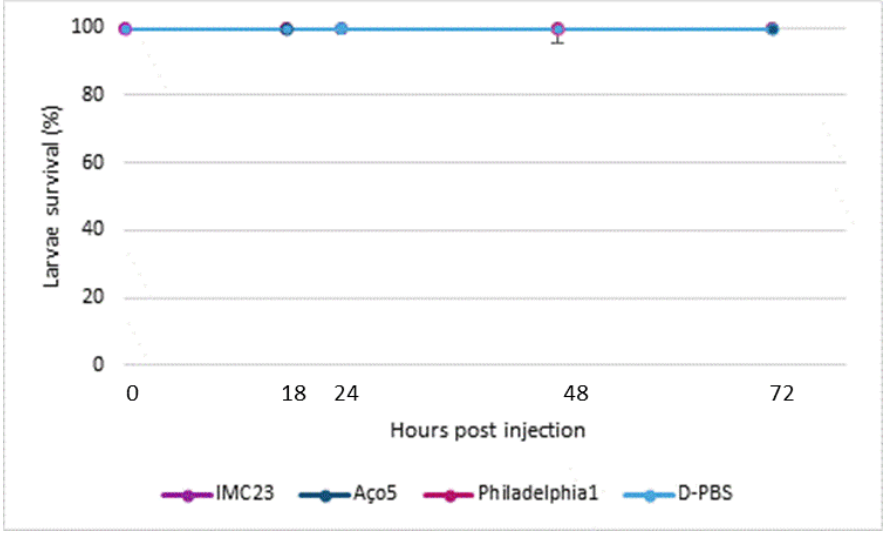


Figure 10. Larvae survival (%) after injection with 10^5 CFU per larvae. *G. mellonella* survival was observed at 18, 24, 48 and 72 hours post-infection. Injection with D-PBS was used as control. Results represent the mean of at least three separate experiments, ± standard deviation with ten larvae per condition.

Differences on larvae survival between strains were observed after injection with 10^6 CFU per larvae. In the first 24 hours after injection, 100% of larvae survival were

registered for all strains. The differences between strains were more pronounced with the time. Namely, for IMC23 and Philadelphia1 a larvae mortality of 20% occurred at 48 hours, while for strain Aço5 this value was of 10%. In addition, Philadelphia1 – infected G*. mellonella* had a larvae mortality of 60% after 72 hours post-injection while for strains IMC23 and Aço5 the mortality values were of 80%. Nevertheless we did not observe 100% of mortality on larvae injected with this concentration. Also no mortality was observed for the D-PBS injected larvae serving as control (Fig.11).

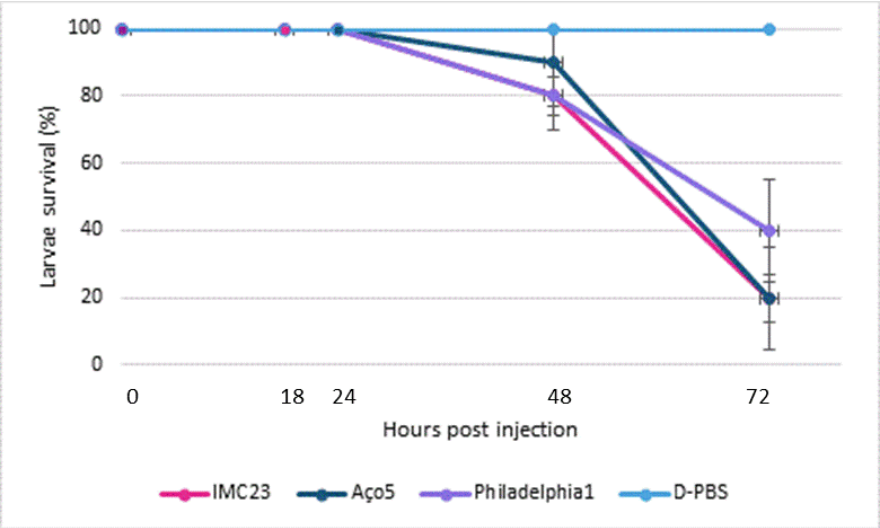


Figure 11. Larvae survival (%) after injection with 10^6 CFU per larvae. *G. mellonella* survival was observed at 18, 24, 48 and 72 hours post-infection. Injection with D-PBS was used as control. Results represent the mean of at least three separate experiments, ± standard deviation with ten larvae per condition.

No major differences on larvae survival were observed between strains after injection with 10^7 CFU per larvae. The only difference was observed after 18 hours of the injection, where strain Philadelphia1 presents a larvae mortality of 20% and strain Aço5 and IMC23 both represent a larvae mortality of 40%. At all the other observation times, all strains showed the same mortality values. Larvae mortality of 70/ was induced after 24 hours. After 48 hours larvae mortality was 90%. At the end of the

experiment all the strains induced 100% of larvae mortality. No mortality was observed for the D-PBS injected larvae serving as control (Fig.12).

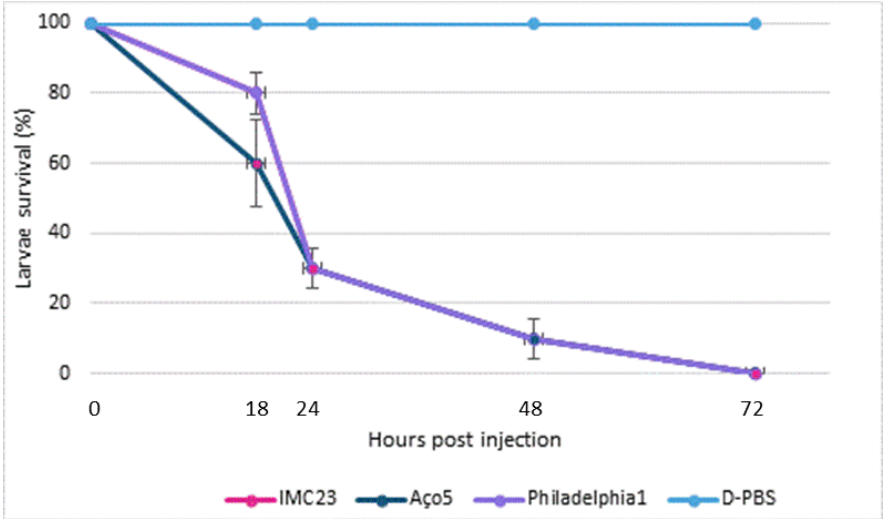


Figure 12. Larvae survival (%) after injection with 10^7 CFU per larvae. *G. mellonella* survival was observed 18, 24, 48 and 72 hours post-infection. Injection with D-PBS was used as control. Results represent the mean of at least three separate experiments, ± standard deviation with ten larvae per condition.

Relevant differences in larvae survival were observed between the three injection concentrations tested. These evidences suggest that the survival of *G. mellonella* is dependent on *L. pneumophila* dose. From the obtained results (Fig. 10 to 12) the concentration that produced the most noticeable effect on larvae was 10^6 CFU per larvae and that was the one used on the following experiments.

3.4 Survival of *L. pneumophila* – infected *G. mellonella* is strain dependent

In order to determine the intrinsic capability of *L. pneumophila* strains to cause death and induce morphological alterations in *G. mellonella*, 10 larvae were injected with 10^6 CFU per larvae of each tested strain. A control group was also tested by injecting 10 µl of D-PBS. The larvae morphologic characteristics observed and registered were: colour (normal, darkening, dark, very dark), cocoon formation and movement (normal, slow, very slow), as described in section 2.4.3. The results for each individual strain are described below.

The *L. pneumophila* type strain Philadelphia1 only induced larvae mortality 48 hours post-infection (30%). At the end of the experiment, this strain was responsible for 60% of larvae mortality. No mortality was observed for the D-PBS injected larvae serving as control (Fig. 13).

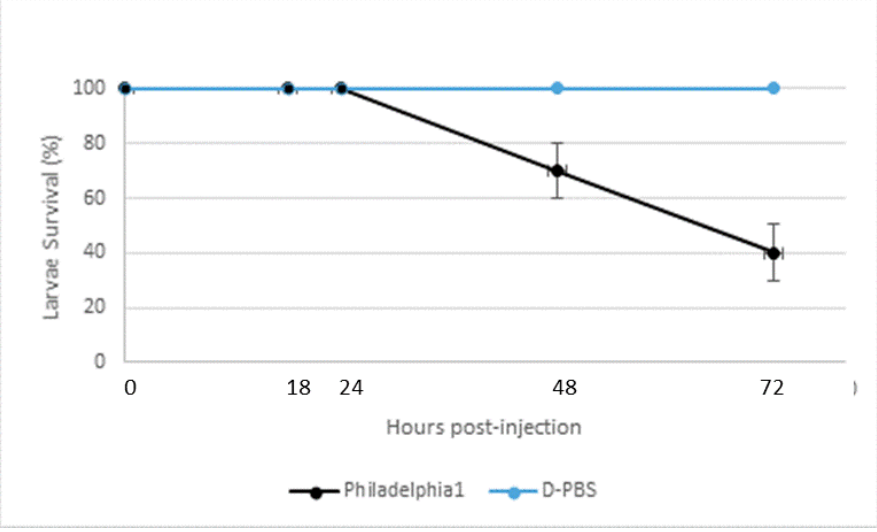


Figure 13. Survival of *L. pneumophila* - infected *G.mellonella* with 10^6 CFU of Philadelphia1 per larvae. *G. mellonella* were observed at 18, 24, 48 and 72 hours post-infection. A negative control with only D-PBS was used. Results are the mean of at least three separate experiments, ± standard deviation with ten larvae per condition.

Since the ability of this strain to infect *G. mellonella* (Harding *et al.,* 2012; Harding *et al.*, 2013) has already been tested by other authors, we decide to use it as a reference strain for comparison purposes. Therefore six replicas were performed instead of three. A *p*-value of 0.8517 (Mantel-Cox test) reveals no significant differences among Philadelphia1 replicas made during this study. This is an important result since not all the injection experiments were performed at the same time.

Observing the morphological characteristics of Philadelphia1 – injected *G. mellonella* it was noticeable that alterations such as the increase of darkening, slow and not forming cocoon larvae increased over time, suggesting that the infection mechanism induced by *L. pneumophila* is time-dependent (Table XIII).

Table XIII. Larvae morphological characteristics after injection with 10^6 CFU per larvae of *L. pneumophila* strain Philadelphia1.

Hours post injection	Survivers	Color				Cocoon formation		Movement		
		Normal	Darkening	Dark	Very dark	Yes	No	Normal	Slow	Very slow
18	10	9	1			10		10		
18	10	10				10		10		
18	10		10			8	2	9	1	
18	10	8	2			9	1	8	2	
18	10	7	3			8	2	8	2	
18	10	9	1			7	3	5	5	
24	10	6	4			5	5	4	6	
24	10	5	5			4	6	1	9	
24	10	5	4	1		3	7	2	8	
24	10	6	4			4	6	2	8	
24	10	6	3	1		5	5	3	7	
24	10	5	5			4	6	2	8	
48	8	2	6			3	8	2	6	
48	6	1	4	1		1	5	1	4	1
48	7		6	1		1	6		7	
48	6		6				6		5	1
48	6		5	1		1	5		4	2
48	8	1	5	2		1	7		7	1
72	6			6			6			6
72	3			3			3			3
72	4		1	2			4			4
72	4		1	3			4			4
72	5		1	4			5		1	4
72	4			4			4		1	3

The *L. pneumophila* type strain Los Angeles1 induced 20% of larvae mortality 18 hours after injection. At the end of the experiment, this strain was responsible for 40% of larvae mortality. No mortality was observed for the D-PBS injected larvae (Fig.14).

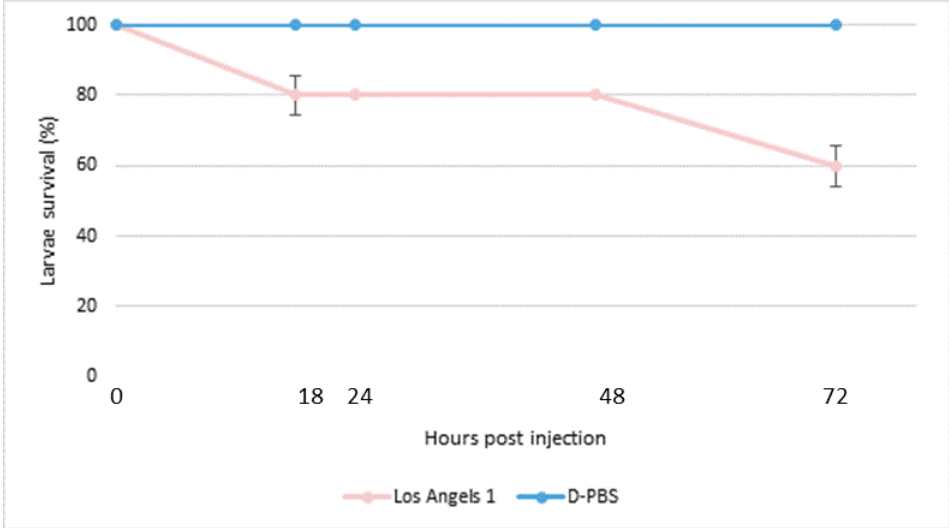


Figure 14 Survival of *L. pneumophila*- infected *G.mellonella* with 10^6 CFU of Los Angeles1 per larvae. *G. mellonella* were observed at 18, 24, 48 and 72 hours post-infection. A negative control with only D-PBS was used. Results are the mean of at least three separate experiments, ± standard deviation with ten larvae per condition.

Examining the morphological characteristics of Los Angeles1 – injected *G. mellonella* it was evident that the majority of the alterations occurred during the first 48 hours post-infection. Between 48 and 72 hours after injection the number of darkening larvae stabilized. The only parameter that increased through the whole time of observation was movement with the number of very slow larvae increasing. Cocoon formation seemed to be equally affected at all the observed times These results allow us to conclude that infection with *L. pneumophila* Los Angeles1 induced clear morphological alterations in the first 48 hours after injection (Table XIV).

Table XIV. Larvae morphological characteristics after injection with 10^6 CFU per larvae of *L. pneumophila* strain Los Angeles1.

Hours post injection	Survivers	Color				Cocoon formation		Movement		
		Normal	Darkening	Dark	Very dark	Yes	No	Normal	Slow	Very slow
18	8	4	4			3	5		5	3
18	8	7	1			3	5	8		
18	9	1	7		1	7	2	8		1
24	8	4	2	2		4	4			8
24	8	6	2			3	5			8
24	8	7	1			4	4			8
48	8		7	1		4	4		6	2
48	8		7		1	5	3	5	2	1
48	8		7		1	4	4	4	3	1
72	6		4		2	4	2			6
72	7		7			5	2			7
72	6		6			3	3			6

Regarding strain Lansing3, the larvae mortality occurred between 18 and 48 hours after injection, with a mortality of 40% and 60%, respectively. No mortality was observed control larvae (Fig. 15).

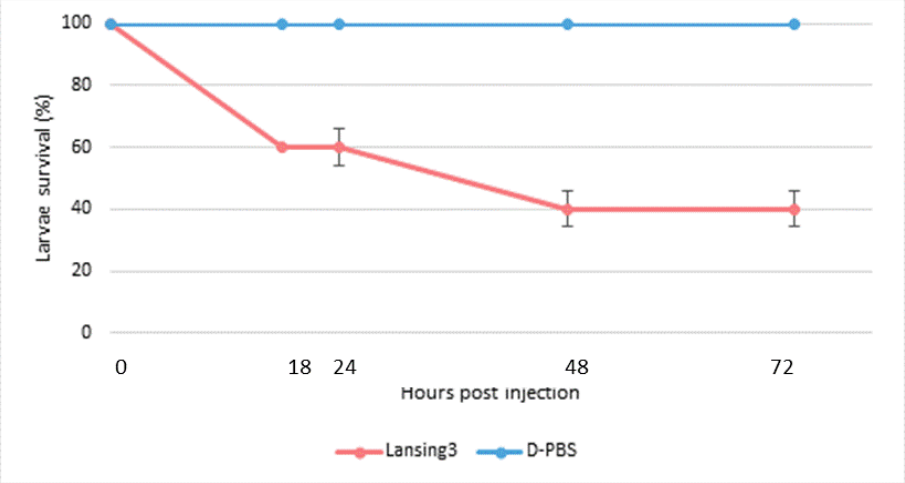


Figure 15. Survival of *L. pneumophila*- infected *G.mellonella* with 10^6 CFU of Lansing3 per larvae. *G. mellonella* were observed at 18, 24, 48 and 72 hours post-infection. A negative control with only D-PBS was used. Results are the mean of at least three separate experiments, ± standard deviation with ten larvae per condition.

Analysing the morphological characteristics of Lansing3 – injected *G. mellonella* it was clear that alterations in all the observed parameters increased over time (Table XV).

Table XV. Larvae morphological characteristics after injection with 10^6 CFU per larvae of *L. pneumophila* strain Lansing3.

Hours post injection	Survivers	Color				Cocoon formation		Movement		
		Normal	Darkening	Dark	Very dark	Yes	No	Normal	Slow	Very slow
18	6	1	5			5	1		3	3
18	6	6				6		6		
18	6	1	4	1		4	2	4		2
24	5	2	2	1		1	4			5
24	6	3	3			2	4	6		
24	6	2	3	1		2	4		4	2
48	4		3	1		2	2		1	3
48	5	3	2			3	2		5	
48	4	1	3			4				4
72	4		2	2		1	3			4
72	5		2	3		2	3			5
72	4		1	3		4				4

The larvae mortality induced by *L. pneumophila* strain U8W increased over time, namely inducing 10% mortality at 18 hours, 40% mortality at 48 hours and 90% mortality at 72 hours after injection. No mortality was observed for the D-PBS injected larvae serving as control (Fig. 16).

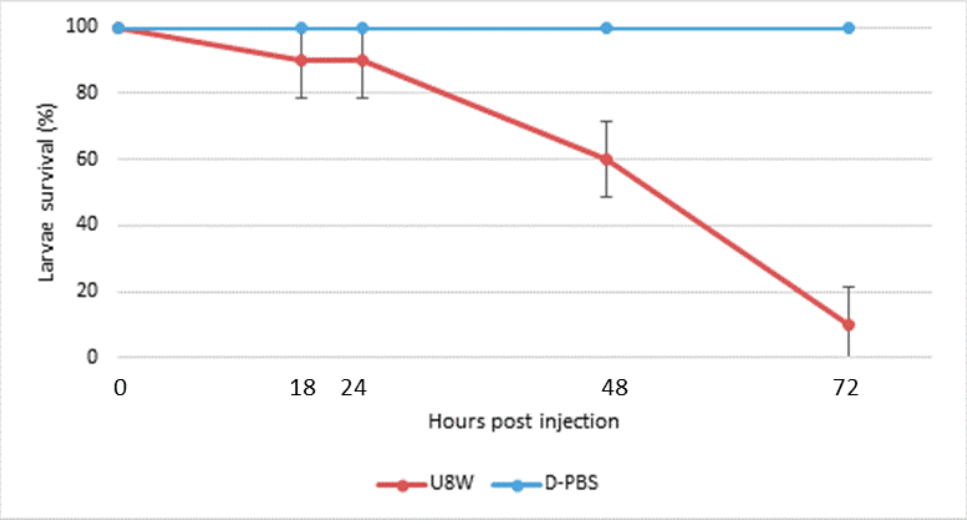


Figure 16. Survival of *L. pneumophila*- infected *G.mellonella* with 10^6 CFU of U8W per larvae. *G. mellonella* were observed at 18, 24, 48 and 72 hours post-infection. A negative control with only D-PBS was used. Results are the mean of at least three separate experiments, ± standard deviation with ten larvae per condition.

Observing the defined morphological characteristics results regarding this strain it was perceptible that alterations increased over time, as mentioned for other studied strains. (Table XVI).

Table XVI. Larvae morphological characteristics after injection with 10^6 CFU per larvae of *L. pneumophila* strain U8W.

Hours post injection	Survivers	Color				Cocoon formation		Movement		
		Normal	Darkening	Dark	Very dark	Yes	No	Normal	Slow	Very slow
18	8	7	1			6	2			8
18	10	7	3			7			10	
18	10	6	4			10			10	
24	8	6	2			4	4			8
24	10	8	2				10		10	
24	10	7	3				10		5	5
48	4		4			4			2	2
48	6	3	3				6			6
48	7		5	2			7			7
72	0									
72	0									
72	2				2		2			2

In contrast with all the above mentioned strains, MICU B induced 100% larvae mortality 18 hours post-infection. No mortality was observed for the D-PBS injected larvae serving as control (Fig. 17).

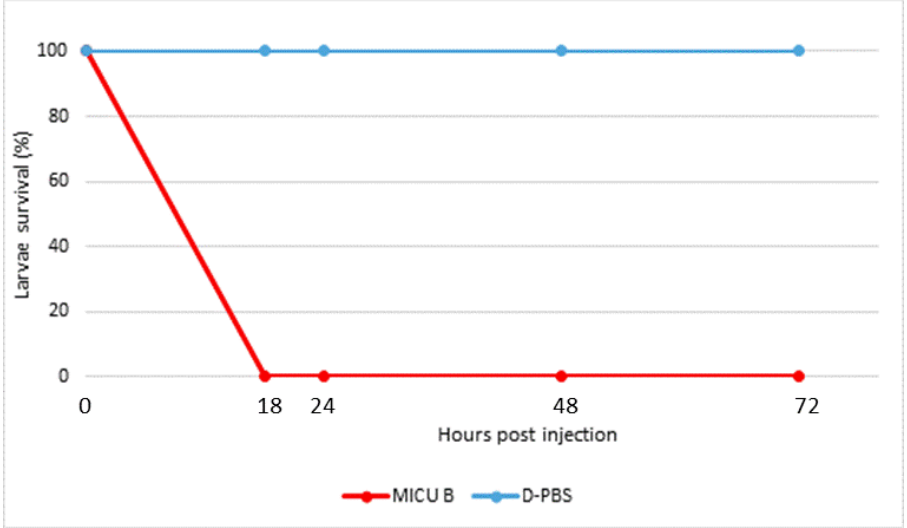


Figure 17. Survival of *L. pneumophila*- infected *G.mellonella* with 10^6 CFU of MICU B per larvae. *G. mellonella* were observed at 18, 24, 48 and 72 hours post-infection. A negative control with only D-PBS was used. Results are the mean of at least three separate experiments, ± standard deviation with ten larvae per condition.

Since no larvae survival was observed at the first time of observation there are no results regarding morphological characteristics to show.

Similarly to strain MICUB, strain HRD2 induced 100% mortality after 24 hours post-infection. Until this time, larvae mortality was 0% despite the observation of clear alterations on the morphological characteristics (Table XVII). No mortality was observed for the control larvae (Fig. 18).

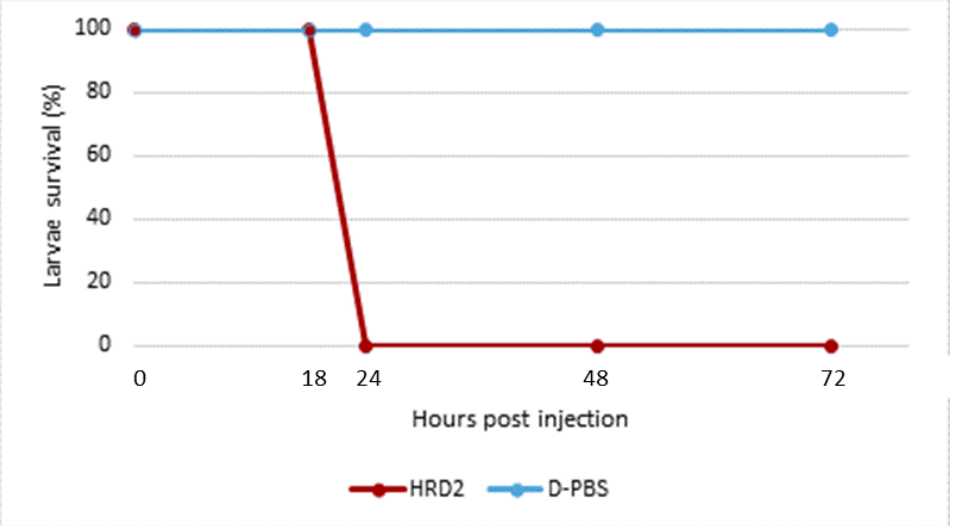


Figure 18. Survival of *L. pneumophila*- infected *G.mellonella* with 10^6 CFU of HRD2 per larvae. *G. mellonella* were observed at 18, 24, 48 and 72 hours post-infection. A negative control with only D-PBS was used. Results are the mean of at least three separate experiments, ± standard deviation with ten larvae per condition.

Table XVII. Larvae morphological characteristics after injection with 10^6 CFU per larvae of *L. pneumophila* strain HRD2.

Hours post injection	Survivers	Color				Cocoon formation		Movement		
		Normal	Darkening	Dark	Very dark	Yes	No	Normal	Slow	Very slow
18	10		8	2		7	3		10	
18	10		7	3		9	1		10	
18	10		6	4		9	1		10	
24	0									
24	0									
24	0									

Regarding strain HUC1, larvae mortality occurred at 18 hours (30%), 24 hours (60%) and 48 hours (100%) after injection. No mortality was observed for the D-PBS injected larvae serving as control (Fig. 19).

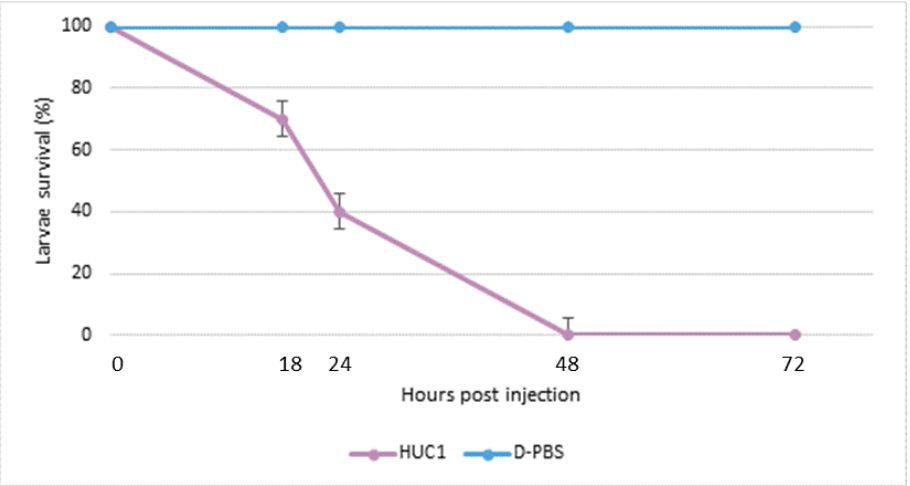


Figure 19 Survival of *L. pneumophila*- infected *G.mellonella* with 10^6 CFU of HUC1 per larvae. *G. mellonella* were observed at 18, 24, 48 and 72 hours post-infection. A negative control with only D-PBS was used. Results are the mean of at least three separate experiments, ± standard deviation with ten larvae per condition.

Observing the morphological characteristics results regarding this strain it was visible that morphologic alterations increased until 48 hours after injection when larvae mortality reached 100% (Table XVIII). These results confirmed a correlation between the increase of morphological disease-related parameters and larvae death.

Table XVIII Larvae morphological characteristics after injection with 10^6 CFU per larvae of *L. pneumophila* strain HUC1.

Hours post injection	Survivers	Color				Cocoon formation		Movement		
		Normal	Darkening	Dark	Very dark	Yes	No	Normal	Slow	Very slow
18	8	1	4	3		4	4		4	4
18	7		5	2		3	4		2	5
18	7	1	4	2		4	3		7	
24	5		5			0	5			5
24	4		3	1		0	4			4
24	4	1	2	1		0	4			4
48	1			1		0	1			1
48	0									
48	0									
72	0									

L. pneumophila strain Por3, induced larvae mortality at all the observation times. Larvae mortality was 10%, 20%, 40% and 50% at 18, 24, 48 and 72 hours after injection, respectively. No mortality was observed for control larvae (Fig. 20).

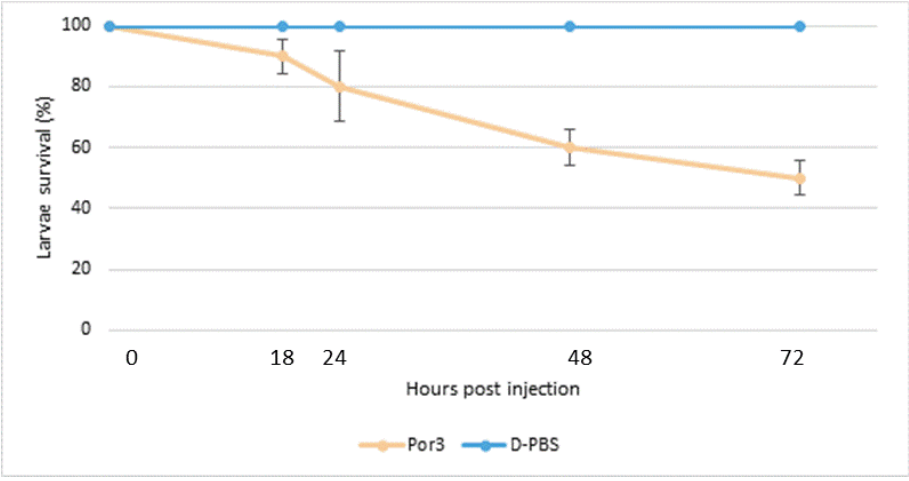


Figure 20. Survival of *L. pneumophila*- infected *G.mellonella* with 10^6 CFU of Por3 per larvae. *G. mellonella* were observed at 18, 24, 48 and 72 hours post-infection. A negative control with only D-PBS was used. Results are the mean of at least three separate experiments, ± standard deviation with ten larvae per condition.

Observing the morphological characteristics results regarding Por3 it was noticeable that until 48 hours of infection larvae presented more alterations such as the increase of darkening, slow and non-forming cocoon larvae. Between 48 and 72 hours after injection the number of darkening larvae stabilized and larvae show some

signs of recovery. Indeed, the number of “very slow” larvae decreases through time with a concomitant increase in the number of larvae with normal movement. The same happened with cocoon formation and colour. All the morphological parameters seemed to be restored with time with the exception of colour that just got stable. These observations suggest that most larvae start recovering from *L. pneumophila* Por3 infection after 48 hours of injection (Table XIX).

Table XIX. Larvae morphological characteristics after injection with 10^6 CFU per larvae of *L. pneumophila* strain Por3.

Hours post injection	Survivers	Color				Cocoon formation		Movement		
		Normal	Darkening	Dark	Very dark	Yes	No	Normal	Slow	Very slow
18	9	6	3			7	2	4	2	3
18	9	7	2				9	9		
18	8		8			4	4	4	4	
24	7	2	5			0	7		4	3
24	9		9			0	9		9	
24	7		7			1	6		5	2
48	6		6			1	5		2	4
48	7	1	6			1	6		4	3
48	6		6			1	5			6
72	5	3	1	1		3	2		1	4
72	6		5	1		3	3		6	
72	5		5			4	1		4	1

Regarding *L. pneumophila* strain IMC23, larvae mortality occurred 48 hours after injection (20%) and at the end of this experiment (72 hours after injection) larvae mortality was 80%. No mortality was observed for the D-PBS injected larvae serving as control (Fig. 21).

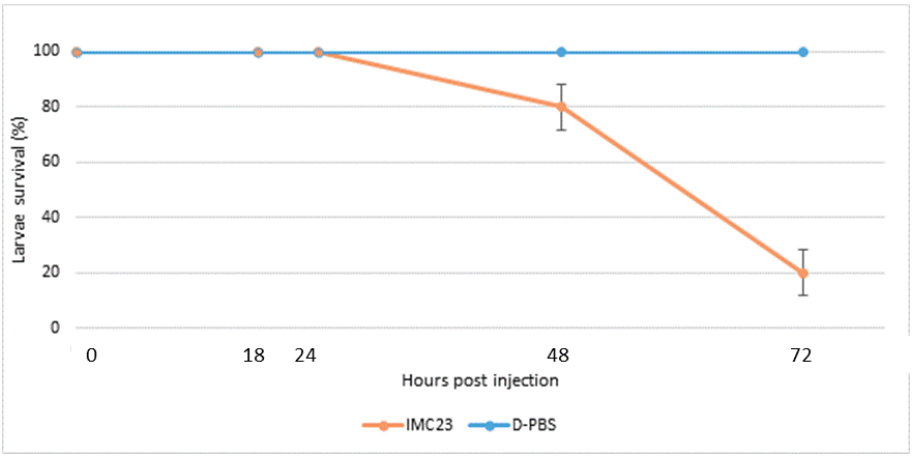


Figure 21. Survival of *L. pneumophila*- infected *G.mellonella* with 10^6 CFU of IMC23 per larvae. *G. mellonella* were observed at 18, 24, 48 and 72 hours post-infection. A negative control with only D-PBS was used. Results are the mean of at least three separate experiments, ± standard deviation with ten larvae per condition

Observing the morphological characteristics results regarding this strain it was evident that alterations such as the increase of darkening larvae, slow larvae and not forming cocoon larvae increased over time (Table XX).

Table XX. Larvae morphological characteristics after injection with 10^6 CFU per larvae of *L. pneumophila* strain IMC23.

Hours post injection	Survivers	Color				Cocoon formation		Movement		
		Normal	Darkening	Dark	Very dark	Yes	No	Normal	Slow	Very slow
18	10	1	9			7	3	2	8	
18	10	10				10		7	3	
18	10	5	5			8	2	8	2	
24	10	8	2			6	4	4	6	
24	10	6	4	1		4	6	3	7	
24	10	3	6	1		4	6	4	5	1
48	8		4	2	2		8		7	1
48	9		6	2	1	2	7		5	4
48	8		3	4	1		8		5	3
72	2			1	1		2			2
72	4			2	2		4			4
72	2			1	1		2			2

L. pneumophila strain Agn2, induced larvae mortality of 50%, 80% and 100% at 18, 24 and 48 hours after injection, respectively. No mortality was observed for the D-PBS injected larvae serving as control (Fig. 22).

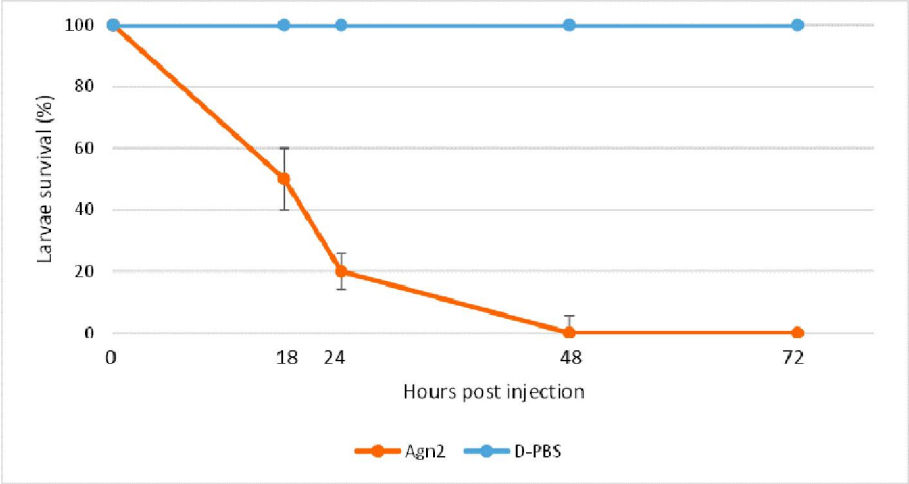


Figure 22. Survival of *L. pneumophila*- infected *G.mellonella* with 10^6 CFU of Agn2 per larvae. *G. mellonella* were observed at 18, 24, 48 and 72 hours post-infection. A negative control with only D-PBS was used. Results are the mean of at least three separate experiments, ± standard deviation with ten larvae per condition.

Observing the morphological characteristics results regarding this strain it was noticeable that morphologic alterations increased until 48 hours after injection when larvae mortality reached 100% (Table XXI).

Table XXI. Larvae morphological characteristics after injection with 10^6 CFU per larvae of *L. pneumophila* strain Agn2.

Hours post injection	Survivers	Color				Cocoon formation		Movement		
		Normal	Darkening	Dark	Very dark	Yes	No	Normal	Slow	Very slow
18	4	1		3		2	2		1	3
18	6		4	2		3	3			6
18	5		4	1		1	4			5
24	2	1			1	0	2			2
24	3		3			0	3			3
24	2		2			0	2			2
48	0									
48	1		1			0	1			1
48	0									

L. pneumophila strain Aço5 only induced larvae mortality after 48 hours post-injection (10%) and in the end of this experiment (72 hours after injection) larvae mortality was 80%. No mortality was observed for the D-PBS injected larvae serving as control (Fig. 23).

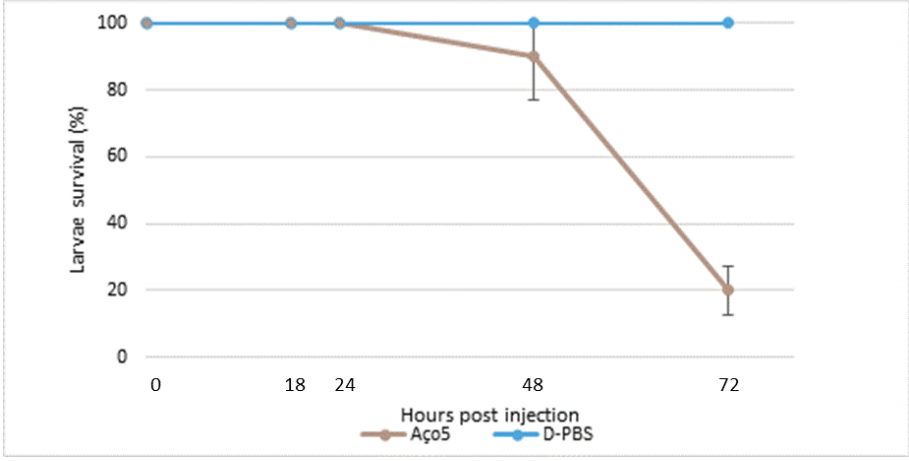


Figure 23. Survival of *L. pneumophila*- infected *G.mellonella* with 10^6 CFU of Aço5 per larvae. *G. mellonella* were observed at 18, 24, 48 and 72 hours post-infection. A negative control with only D-PBS was used. Results are the mean of at least three separate experiments, ± standard deviation with ten larvae per condition.

Observing the morphological characteristics results regarding this strain it was noticed that alterations increased over time (Table XXII).

Table XXII Larvae morphological characteristics after injection with 10^6 CFU per larvae of *L. pneumophila* strain Aço5.

Hours post injection	Survivers	Color				Cocoon formation		Movement		
		Normal	Darkening	Dark	Very dark	Yes	No	Normal	Slow	Very slow
18	10	9	1			10		6	4	
18	10	10				10		7	3	
18	10	8	2			9	1	6	4	
24	10	8	2			5	5	5	5	
24	10	9	1			6	4	4	6	
24	10	7	2	1		5	5	5	5	
48	9		5	5		1	8	2	4	3
48	10		7	3		2	8	1	9	
48	8		6	2		1	7		5	3
72	3			3			3		1	2
72	2			2			2			2
72	2			2			2			2

L. pneumophila strain Aço12 like, strain Aço5, only induced larvae mortality at 48 hours post-injection (20%). At the end of the experiment this strain was responsible for 70% of larvae mortality. No mortality was observed for the control larvae (Fig. 24).

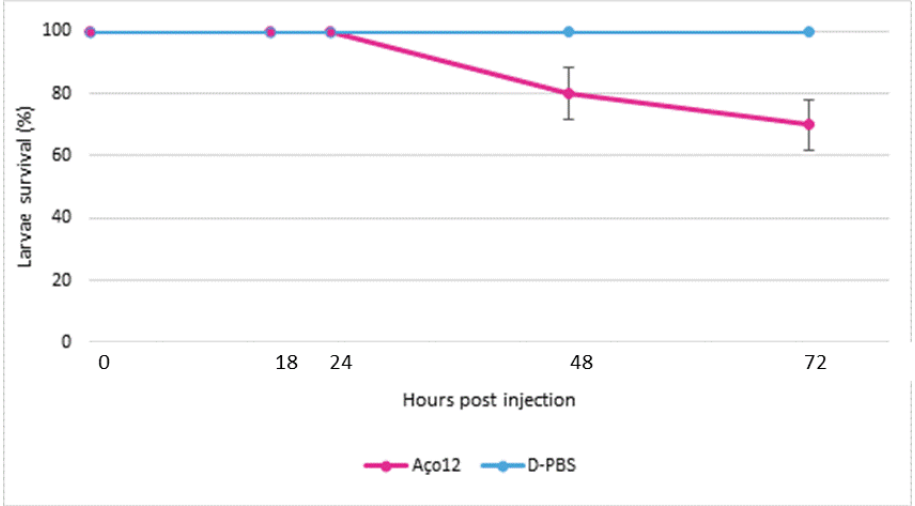


Figure 24. Survival of *L. pneumophila*- infected *G.mellonella* with 10^6 CFU of Aço12 per larvae. *G. mellonella* were observed at 18, 24, 48 and 72 hours post-infection. A negative control with only D-PBS was used. Results are the mean of at least three separate experiments, ± standard deviation with ten larvae per condition.

Observing the morphological characteristics results regarding this strain it was noticed that until 48 hours of infection larvae presented more alterations such as the increase of darkening, no cocoon formation and slow larvae. Although 72 hours after injection larvae show some signs of recovery. Indeed, the number of darkening larvae decreases through time with a concomitant increase in the number of normal colour larvae. The same happened with cocoon formation and movement. All the normal morphological parameters seemed to be restored with time. These observations suggest that most larvae start recovering from Aço12 infection after 48 hours of injection (Table XXIII).

Table XXIII. Larvae morphological characteristics after injection with 10^6 CFU per larvae of *L. pneumophila* strain Aço12.

Hours post injection	Survivers	Color				Cocoon formation		Movement		
		Normal	Darkening	Dark	Very dark	Yes	No	Normal	Slow	Very slow
18	10	8	2			10		9	1	
18	10	9	1			9	1	8	2	
18	10	10				10		10		
24	10	7	3			8	2	1	9	
24	10	8	2			6	4	7	3	
24	10	7	3			7	3	5	5	
48	7	2	5			3	4	1	6	
48	8	1	7			3	5	3	5	
48	9		9			2	7		9	
72	6	4	2			5	1		6	
72	7	5	2			5	2	1	6	
72	8	4	4			4	4	2	6	

Regarding strain Aço22, larvae mortality occurred only 48 hours after injection (30%).At the end of this experiment (72 hours after injection) this strain was responsible for 70% of larvae mortality. No mortality was observed for the D-PBS injected larvae serving as control (Fig. 25).

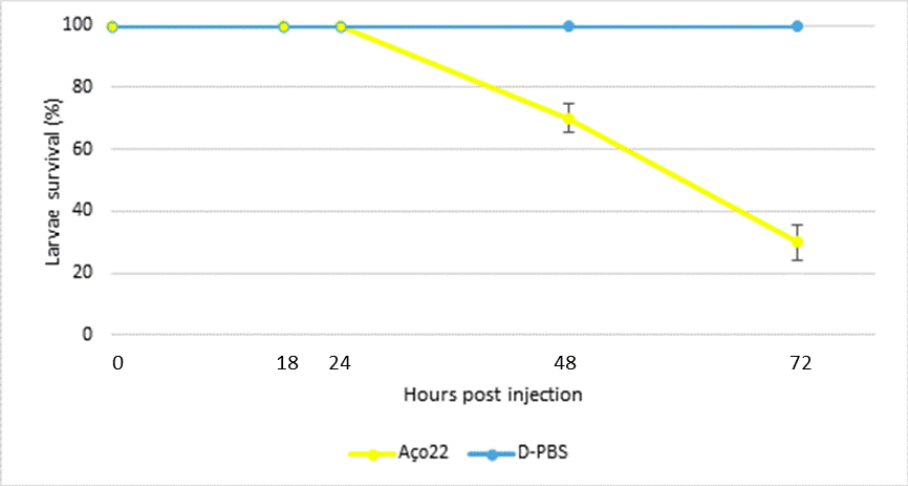


Figure 25. Survival of *L. pneumophila*- infected *G.mellonella* with 10^6 CFU of Aço22 per larvae. *G. mellonella* were observed at 18, 24, 48 and 72 hours post-infection. A negative control with only D-PBS was used. Results are the mean of at least three separate experiments, ± standard deviation with ten larvae per condition.

Observing the morphological characteristics results regarding this strain it was observed that negative alterations such as the increase of darkening, slow and not forming cocoon larvae increased over time. (Table XXIV).

Table XXIV. Larvae morphological characteristics after injection with 10^6 CFU per larvae of *L. pneumophila* strain Aço22.

Hours post injection	Survivers	Color				Cocoon formation		Movement		
		Normal	Darkening	Dark	Very dark	Yes	No	Normal	Slow	Very slow
18	10	7	3			10		3	7	
18	10	10				10		9	1	
18	10	9	1			9	1	8	2	
24	10	7	3			9	1	9	1	
24	10	9	1			10			10	
24	10	8	2			9	1	2	8	
48	8	4	4			3	5	1	6	1
48	7	3	3	1		2	5	2	5	
48	7	2	5			3	4	1	4	2
72	3		1	2			3		1	2
72	4		1	3			4			4
72	3			3			3		1	2

L. pneumophila strain NMex1 induced 100% of larvae mortality 24 hours after injection, similarly to strains HRD2 and MICU B. No mortality was observed for the D-PBS injected larvae serving as control (Fig. 26).

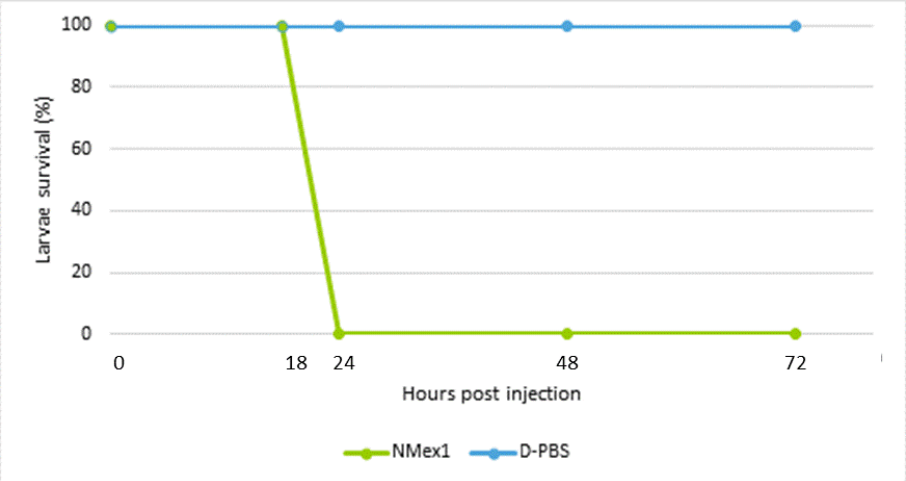


Figure 26. Survival of *L. pneumophila*- infected *G.mellonella* with 10^6 CFU of NMex1 per larvae. *G. mellonella* were observed at 18, 24, 48 and 72 hours post-infection. A negative control with only D-PBS was used. Results are the mean of at least three separate experiments, ± standard deviation with ten larvae per condition.

Observing the morphological characteristics results regarding this strain it was observed that 18 hours after injection larvae presented negative alterations such as the increase of darkening, slow and not forming cocoon larvae. Since all larvae died between 18 and 24 hours after the injection, this results could suggest that infection with *L. pneumophila* NMex1 somehow develops faster in this period of time (Table XXV).

Table XXV. Larvae morphological characteristics after injection with 10^6 CFU per larvae of *L. pneumophila* strain NMex1.

Hours post injection	Survivers	Color				Cocoon formation		Movement		
		Normal	Darkening	Dark	Very dark	Yes	No	Normal	Slow	Very slow
18	10		7	3		7	3		10	
18	10		7	3		8	2		10	
18	10		8	2		9	1		10	
24	0									
24	0									
24	0									

L. pneumophila strain Ger10, induced larvae mortality at all the observation times, similar to strain Por3.A 20% larvae mortality was observed 18 hours after injection and 50 % after 24 hours. After 48 hours post-injection larvae mortality was 60% and at the end of the experiment this strain induced 100% of larvae mortality. No mortality was observed for the control larvae (Fig. 27).

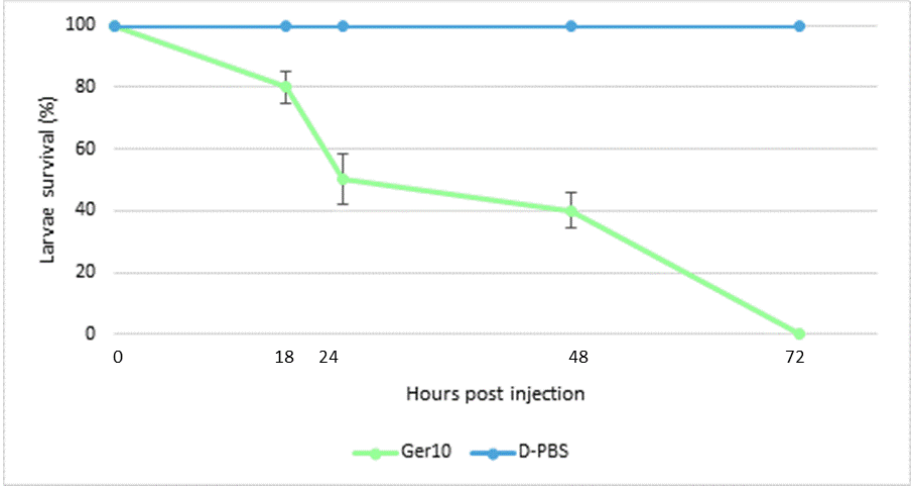


Figure 27 Survival of *L. pneumophila*- infected *G.mellonella* with 10^6 CFU of Ger10 per larvae. *G. mellonella* were observed at 18, 24, 48 and 72 hours post-infection. A negative control with only D-PBS was used. Results are the mean of at least three separate experiments, ± standard deviation with ten larvae per condition.

Observing the morphological characteristics results regarding this strain it was noticeable that alterations increased with time as expected (Table XXVI)

Table XXVI. Larvae morphological characteristics after injection with 10^6 CFU per larvae of *L. pneumophila* strain Ger10.

Hours post injection	Survivers	Color				Cocoon formation		Movement		
		Normal	Darkening	Dark	Very dark	Yes	No	Normal	Slow	Very slow
18	8	6	2							8
18	8		7	1		3			7	1
18	8	2	5	1		2	6	4	4	
24	4			4		4				4
24	6	4	2			6				6
24	5		5			2	3		5	
48	3					2				3
48	4		4			1			2	2
48	4		3	1		0	4		3	1
72	0									
72	0									
72	0									

Regarding strain Ice27, larvae mortality occurred between 18 hours and 48 hours after injection with 20% and 40%, respectively. At the end of this experiment (72 hours after injection) this strain was responsible for 50% of larvae mortality. No mortality was observed for the D-PBS injected larvae serving as control (Fig. 28).

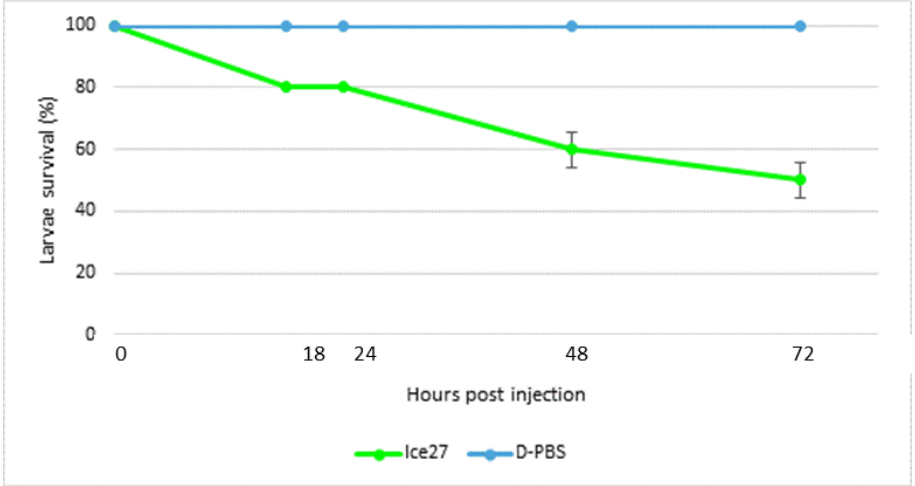


Figure 28. Survival of *L. pneumophila*- infected *G.mellonella* with 10^6 CFU of Ice27 per larvae. *G. mellonella* were observed at 18, 24, 48 and 72 hours post-infection. A negative control with only D-PBS was used. Results are the mean of at least three separate experiments, ± standard deviation with ten larvae per condition.

Observing the morphological characteristics results regarding this strain it was noticed that morphologic alterations increased over time. (Table XXVII).

Table XXVII. Larvae morphological characteristics after injection with 10^6 CFU per larvae of *L. pneumophila* strain Ice27.

Hours post injection	Survivers	Color				Cocoon formation		Movement		
		Normal	Darkening	Dark	Very dark	Yes	No	Normal	Slow	Very slow
18	8	6	1	1		6		6	2	
18	8	8				8		8		
18	8	8				8		8		
24	8	6	1	1			8		7	1
24	8	6	2	1	1		8		7	1
24	8	8				1		8		
48	6		5	1		3		2	3	1
48	5	3	2			3			5	
48	6		2			2			6	
72	5	1	3	1			5			5
72	5	1	2	2		2	3		4	1
72	6		3	3			6		6	

The comparison between the survivals of *L. pneumophila* – infected *G. mellonella* with different strains is shown below. A *p*-value <0,0001 (Mantel-Cox test) supported significant differences on the capability of causing death between strains. Indeed, some *L. pneumophila* strains were not able to cause 100% mortality within the

experience time frame, while other managed to kill all the larvae although at distinct rates (Fig. 29).

Namely, MICU B-infected larvae had 100% of mortality after only 18 hours post-injection while NMex1-infected larvae and HRD2-infected larvae showed the same results but after 24 hours. Agn2 and HUC1 also induced 100% mortality but in these cases after 48 hours. Finally, Ger10 hit 100% mortality at 72 hours.

All other strains did not cause 100% larvae mortality with relevant differences between them. Nevertheless, all *L. pneumophila* strains induced morphologic alterations in the injected larvae at a given point.

Strains Aço12 and Por3 induced 30% and 50% of larvae mortality at the end of the experiment, respectively. However between 48 and 72 hours after injection larvae showed some signs of recovery suggesting that most larvae start recovering from *L. pneumophila* Por3 and Aço12 infection after 48 hours of injection.

The other strains showed some differences between them regarding larvae mortality, namely, U8W (90%), IMC23 and Aço5 (80%), Aço22 (70%), Philadelphia1 and Lansing3 (60%), Ice27 (50%) Los Angeles1 (40%) but all this strains have a common characteristic. They were capable of inducing an increase of larvae morphological alterations over time. These results suggest that the infection mechanism induced by *L. pneumophila* is time-dependent and if the observations would have been extended for more hours we might have observed a continuous decrease on larvae survival however it was not possible to verify this hypothesis since the infection model used in this study should not be used for periods longer than 72 hours due to larvae transformation into pupae.

Results showed in this section allow us to conclude that larvae mortality rate and the infection profile is strain-dependent.

L. pneumophila strains	Hours after injection			
	18	24	48	72
Aço12	100	100	80	70
Los angels 1	80	80	80	60
Por 3	90	80	60	50
Ice 27	80	80	60	50
Philadelphia 1	100	100	70	40
Lansing 3	60	60	40	40
Aço 22	100	100	70	30
Aço 5	100	100	90	20
IMC 23	100	100	80	20
U8W	90	90	60	10
Ger 10	80	50	40	0
HUC 1	70	40	0	0
Agn 2	50	20	0	0
Nmex 1	100	0	0	0
HRD 2	100	0	0	0
MICU B	0	0	0	0

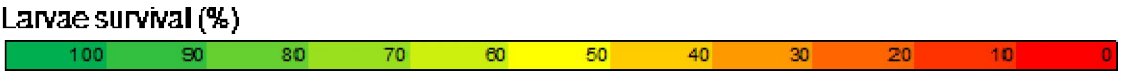


Figure 29. Survival (%) of *L. pneumophila* – infected *G. mellonella* with different strains over time. Ten larvae were injected with 10^6 CFU for each strain. *G. mellonella* were observed at 18, 24, 48 and 72 hours post-infection. A negative control with only D-PBS was used. Results are the mean of at least three separate experiments.

3.5 Relation between survival in *L. pneumophila* – infected *G.mellonella* and strains origin

In order to determine if *L. pneumophila* ability to infect and kill *G. mellonella* is related with the origin of the strains, this is if they were isolated from natural, man-made environments or disease-related, the results above showed were grouped

according to their environmental origin and a statistical analysis was performed. Moreover this is the first time that natural environmental *L. pneumophila* strains were tested for ability to infect *G. mellonella*.

Regarding strains from natural environments (Table I), a *p*-value <0,0001 (Mantel-Cox test) supports the existence of significant differences on the ability to kill larvae among strains. For instance, strain NMex1 induced 100% of larvae mortality 24 hours after injection while strain Aço12 only induced 70% of larvae mortality and in a longer period of time (72 hours) (Fig.30).

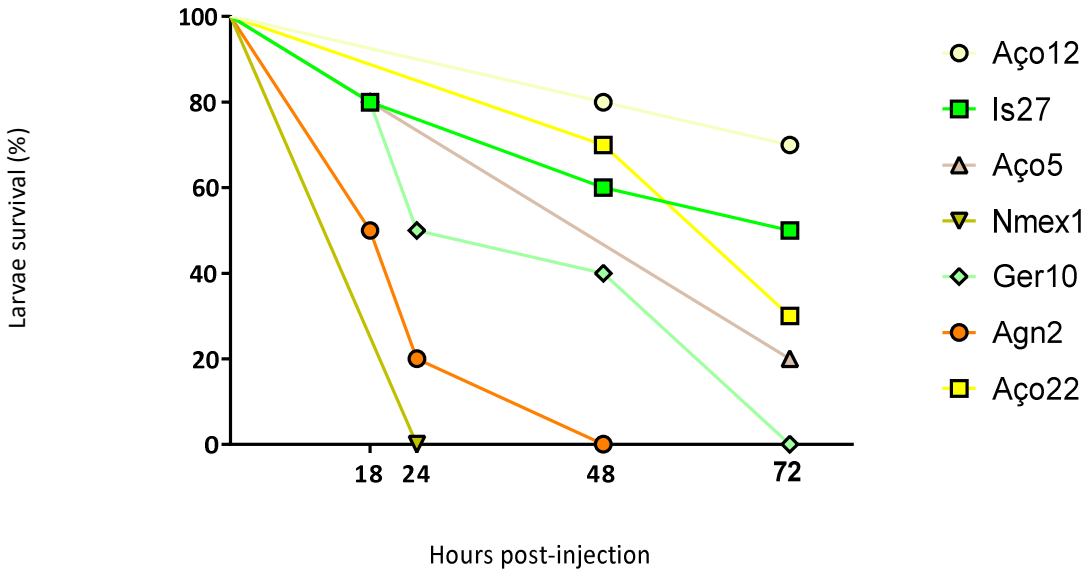


Figure 30. Survival (%) of *L. pneumophila* – infected *G. mellonella* with strains isolated from natural environments over time. Ten larvae were injected with 10^6 CFU for each strain. *G. mellonella* were observed at 18, 24, 48 and 72 hours post-infection. A negative control with only D-PBS was added. Results are the mean of at least three separate experiments, ± standard deviation.

Similarly, *p*-value of 0,0002 (Mantel-Cox test) was obtained when strains from man-made environments were compared, evidencing significant differences on the

ability to kill larvae among them. Namely, strain HUC1 was capable of inducing 100% of larvae mortality while Por3 only induced 50% of larvae mortality (Fig.31).

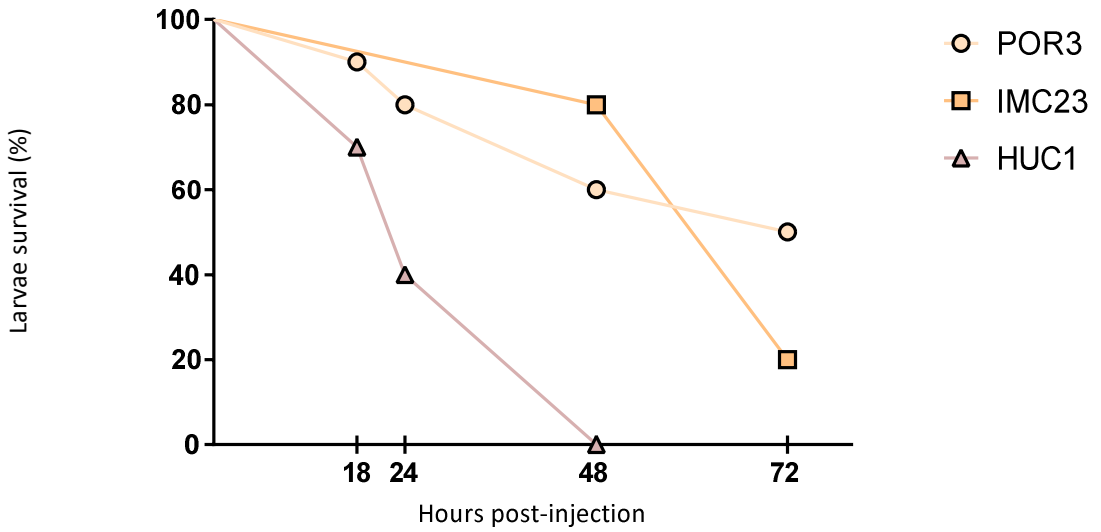


Figure 31. Survival (%) of *L. pneumophila* – infected *G. mellonella* with strains isolated from man-made environments over time. Ten larvae were injected with 10^6 CFU for each strain. *G. mellonella* were observed at 18, 24, 48 and 72 hours post-infection. A negative control with only D-PBS was added. Results are the mean of at least three separate experiments, ± standard deviation.

Regarding strains from disease-related environments, a *p*-value <0,0001 (Mantel-Cox test) reveals significant differences among strains. Namely, MICU B and HRD2 induced 100% of larvae mortality, 18 and 24 hours after injection, respectively. In contrast, Los Angeles1 was capable of causing only 40 % of larvae mortality, and this result was obtained only 72 hours after injection (Fig.32).

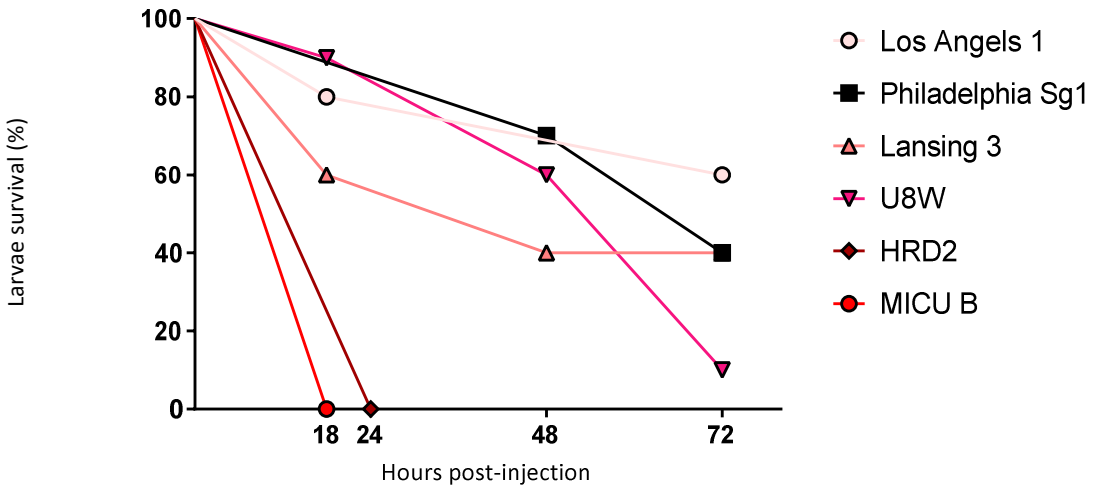


Figure 32. Survival (%) of *L. pneumophila* – infected *G. mellonella* with disease-related strains over time. Ten larvae were injected with 10^6 CFU for each strain. *G. mellonella* were observed at 18, 24, 48 and 72 hours post-infection. A negative control with only D-PBS was added. Results are the mean of at least three separate experiments, ± standard deviation.

These results argue that the ability of *L. pneumophila* strains to induce larvae death is not related with the origin of the strains. In fact, we were able to identify strains capable of inducing 100% of mortality in *G.mellonella* from all the environmental types. Likewise, strains with a reduced ability to cause death were also identified in all environmental types (Fig.30 to Fig.32).

These results contradict the theory that isolates of *L. pneumophila* recovered from clinical cases and man-made environments are a limited, non-random subset of all genotypes existing in nature, representing a group of clones especially adapted to these niches (Coscollá & González-Candelas, 2009).

3.6 Comparison between *L. pneumophila* type strain Philadelphia1 and other tested strains.

Since the ability of *L. pneumophila* Philadelphia1 strain to infect *G. mellonella* (Harding *et al.,* 2012; Harding *et al.,* 2013) has already been tested by other authors, we decide to use it as a reference strain for comparison purposes. This strategy allowed us to ascertain if there were, or not, significant differences on the ability to kill larvae between the reference strain and the other strains.

The Mantel-Cox test to determine *p*-values was performed using GraphPad Prism 7.01 software. Differences were considered to be statistically significant if the *p*-value was lower than 0.05.

Indeed, strains MICU B, HRD2, HUC1, Agn2, NMex1 and Ger10 revealed significant differences on their ability to induce larvae mortality when compared to Philadelphia1. All other strains exhibited a similar behavior to reference strain (Fig.33). These results confirm that there are strain-specific features related with the ability to cause larvae infection and death.

Since Philadelphia1 has been responsible for several outbreaks of legionellosis (Yu *et al.*, 2002), the identification of other strains with high ability to induce mortality on larvae suggests that they could have a higher potential to cause disease.

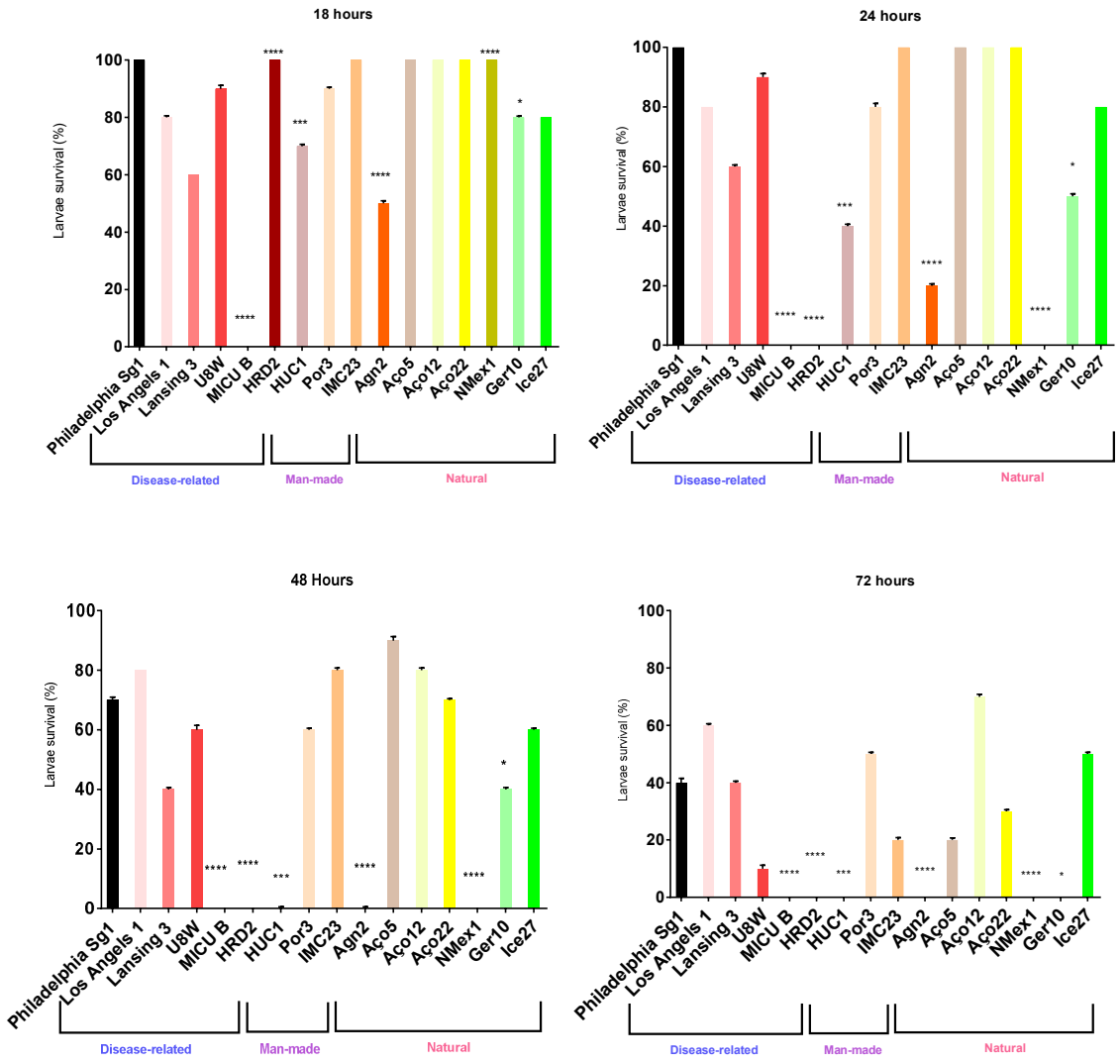


Figure 33. Comparison between the survival of infected *G.mellonella* with *L. pneumophila* type strain Philadelphia1 and the other tested strains. Ten larvae were injected with 10^6 CFU for each strain. *G. mellonella* were observed at 18, 24, 48 and 72 hours post-infection. A negative control with only D-PBS was added. Strains are grouped according to the environmental type of their isolation site. Each strain was statistically compared with *L. pneumophila* Philadelphia1 and differences are considered significant if $p < 0.05$ (*), highly significant if $p < 0.001$(***) and extremely significant if $p < 0.0001$ (****). Results are the mean of at least three separate experiments, ± standard deviation.

3.7 *Legionella pneumophila* Sequence-Based Typing

Multilocus sequence typing is a tool used to discriminate clonal groups within several bacterial species (Harb & Kwaik 1998). European Society for Clinical Microbiology Study Group on *Legionella* Infections (ESGLI) maintains an online data base that allows researchers to inquire sequence data and obtain an allelic profile (SBT profile) and a final combined sequence type for each isolate (Mercante & Winchell, 2015). An SBT profile includes a set of numbers referred to the number of individual alleles for each gene separated by commas (Ratzow *et al.,* 2007). This method was used to determine the phylogenetic relationship between the strains and to ascertain whether there was a correlation between this relation and the survival of *L. pneumophila* - infected *G.mellonella.* In order to do that SBT protocol was performed as previously described on Chapter II, based on the partial sequence of seven genes (*flaA, pilE, asd, mip, mompS* and *proA* (Gaia *et al.,* 2005),*NeuA* (Ratzow *et al*., 2007) and *NeuAh* (Mentasti *et al.,* 2012) in order to type the *L. pneumophila* strains used in this project.

All the sequences were individually submitted to the online database (http://www.hpa.org.uk/cfi/bioinformatics/dbases.htm#EWGLI) and the correspondent obtained allelic number is shown on Table XXVIII. Some sequences had no match in the database and we are currently waiting for a new number to be assigned to those alleles.

Table XXVIII. Summary of allelic numbers and SBT profiles of the 16 unrelated *L. pneumophila* strains used in this study.

Subspecies	Strain designation	*flaA*	*pilE*	*asd*	*mip*	*mompS*	*proA*	*neuA*	SBT profile	*STB type*
L. pneumophila subsp. *pneumophila*	Philadelphia 1 (ATCC 33152^T)	3	4	1	1	14	9	1	3,4,1,1,14,9,1	36
L. pneumophila subsp. *pneumophila*	Agn2	7	29	2	21	3	20	*	7,29,2,21,3,20,*	*
L. pneumophila subsp. *pneumophila*	IMC23	20	23	14	44	21	4	6	20,23,14,44,21,4,6	*
L. pneumophila subsp. *pneumophila*	NMex1	6	10	14	28	21	14	3	6,10,14,28,21,14,3	1892
L. pneumophila subsp. *pneumophila*	Por3	1	4	3	1	1	1	1	1,4,3,1,1,1,1	1
L. pneumophila subsp. *pneumophila*	Ice27	19	6	17	3	13	11	6	19,6,17,3,13,11,6	*
L. pneumophila subsp. *pneumophila*	aço5	*	6	3	28	9	*	6	*,6,3,28,9,*,6	*
L. pneumophila subsp. *pneumophila*	aço12	*	13	1	*	14	*	*	*,13,1,*,14,*,*	*
L. pneumophila subsp. *pneumophila*	hrd2	*	8	11	10	10	12	*	*,8,11,10,10,12,*	*
L. pneumophila subsp. *pneumophila*	ger10	*	10	*	28	9	4	207	*,10,*,28,9,4,207	*
L. pneumophila subsp. *pneumophila*	huc1	*	3	6	10	1	1	*	*,3,6,10,1,1,*	*
L. pneumophila subsp. *pneumophila*	aço22	17	13	1	28	27	9	3	17,13,1,28,27,9,3	*
L. pneumophila subsp. *fraseri*	Los Angeles 1(ATCC 33156^T)	11	14	16	25	7	13	206	11,14,16,25,7,13,206	1334
L. pneumophila subsp. *fraseri*	Lansing 3 (ATCC 35251)	11	14	16	25	7	13	24	11,14,16,25,7,13,24	336
L. pneumophila subsp. *pascullei*	U8W (ATCC 33737^T)	14	18	8	18	28	19	201	14,18,8,18,28,19,201	1335
L. pneumophila subsp. *pascullei*	MICU B (ATCC 33735)	14	18	8	18	28	19	201	14,18,8,18,28,19,201	1335

Five strains matched SBT types in the database, namely Philadelphia1, Los Angeles1, Lansing3, U8W and MICU B, all clinical isolates. The remaining strains presented new and different SBT profiles which are in accordance with our initial goal of using unrelated strains. Some of these strains show a new SBT type due to their association with a new SBT profile, namely IMC23, Ice27 and Aço22. All the other strains with no SBT type match in the database presents new alleles. The gene associated with the largest number of new alleles is *flaA* followed by *neuA*. Only the strains U8W and MICU B presented equal SBT profiles. Interestingly the clinical-related strains HUC1 and HRD2 showed no match in the database.

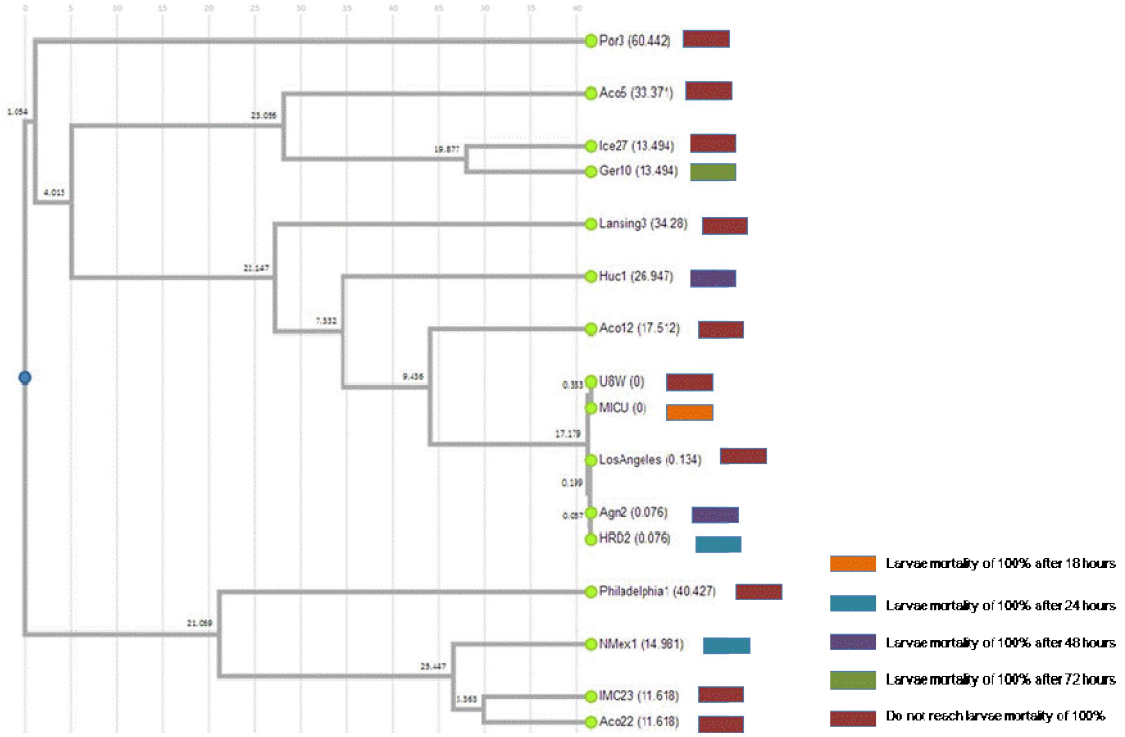


Figure 34. Dendrogram produced by DendroUPGMA using the Pearson correlation coefficient and UPGMA algorithm on the basis of the SBT profiles of *L. pneumophila* strains

In order to capture the phylogenetic relationship between the strains inferred from a set of variables (SBT profiles) an Unweighted Pair Group Method with Arithmetic Averages (UPGMA) was used to build a dendrogram. The method calculates all the Pearson correlation coefficients between pairs of sets of variables, transforms these coefficients into distances and makes a clustering using the UPGMA algorithm (Garcia-Vallvé *et al.,* 1999). These results allow us to conclude that the SBT profiles did not reconstruct the *L. pneumophila* species phylogeny since strains from the same sub-species are in distinct cluster, namely Lansing3 and Los Angeles1 (*L. pneumophila* subsp. *fraseri*). Additionally, no relation could be established between SBT profile and the origin of strains. Indeed, it was possible to identify strains in the same cluster that

IV. Conclusions and future perspectives

From the present study we can conclude that survival of *L. pneumophila*-injected *G. mellonella* was dependent on the bacterial load. Larvae injected with the same strain, in the same conditions, but with different concentrations revealed different survival rates during the experience time frame.

We also could determine that the ability of *L. pneumophila* to induce larvae death was strain-specific, since a different response was obtained for each strain. Nevertheless, and despite the fact that not all strains were able to induce 100% of larvae mortality, all the larvae showed morphological alterations. Thus all the strains tested seems to be pathogenic to *G. mellonella* but clearly different strains had distinct effects on the model used. Our results did not support the existence of a correlation between the environmental origin of the isolates and their ability to induce *G.mellonella* death. Indeed, different degrees of virulence were observed within strains with the same environmental origin, and conversely, strains with distinct origins exhibited similar levels of virulence.

Regarding the SBT profiling of the strains used in this study we observed that this analysis did not reconstructed the phylogeny of the *L. pneumophila* species since strains from the same subspecies were in distinct clusters. Additionally, no relation could be established between the SBT profiles and the origin of strains. Indeed, it was possible to identify strains within the same SBT cluster that were isolated from distinct environments, and some strains from the same environmental type were in different clusters.

Finally, we could not correlate the SB types, or SBT-based clusters, with the degree of virulence since strains capable of inducing similar results regarding larvae mortality were in separate clusters. Likewise, strains with distinct virulence patterns were clustered.

The results clearly shows diversity in the virulence degree of the isolates. That fact may be related with the known genetic diversity verified in *L. pneumophila*. That genetic diversity is one of the key factors that explains the plasticity of the species. The observed variability on the virulence of the strains tested may be a reflection of this plasticity vital for the maintenance of the characteristic life style of *L. pneumophila*.

The *L. pneumophila* strains used in this study were chosen based on their previously determined allelic diversity from crucial virulence-related effectors (Costa *et al*., 2010; Costa *et al.,* 2012; Costa *et al.,* 2014). In this study we have determined that those strains exhibit different levels of virulence. In order to understand the role of each virulence-related effector in *L. pneumophila* virulence we intend to compare these *L. pneumophila* strains with their effector mutant for defective phenotypes in *G. mellonella*. This approach will allows us to correlate some allelic forms with pathogenicity that could be used to design more efficient detection methods and pave the way for new control measures.

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