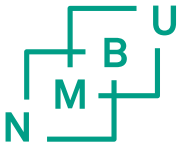
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FFI Kjeller, May 15 2015
Sofia Elise Larsen

Abstract

Different strains of *Legionella* are found ubiquitous in natural environments. Certain strains, such as *Legionella pneumophila* sero- group 1 are known as human pathogens, causing severe pneumonia. *Legionella* is naturally a parasite to protozoa, but shows similar intracellular growth in human alveolar macrophages. Prevention of growth of the bacteria and spread of the disease caused by pathogenic strains of *Legionella* is in the interest of the Norwegian Defense Research Institute (FFI) as a model organism for air borne disseminated bacteria, and for pathogens with an intracellular life cycle in eukaryotic cells.

Not all *Legionella* species are able to replicate in amoeba, and co-culture with amoeba is considered to be selective to pathogenic strains of Legionellae. To investigate pathogenic traits of different strains of *Legionella,* several experiments were carried out in this study. Co-cultures of *Legionella* with *Acanthamoeba castellanii* and human macrophage THP1 cells were cultivated to investigate the ability of intracellular growth. Ten isolates of *Legionella,* including different sero- groups of *L. pneumophila,* along with *L. micdadei,* and an unknown *Legionella* species was used in co-culture experiments. Real-Time PCR analyses with SYBR Green were performed on the different strains with different primer sets in order to map the presence of the virulence genes known as *rtx, lvh, dot, mip* and *hsp* in each strain. Eventually, the registered presence of virulence genes was compared to each strains' ability to replicate intracellularly in eukaryotes. The strains are originally obtained both from clinical isolates after outbreaks, and from environmental samples, as well as common reference strains.

Several of the *L. pneumophila* strains in addition to *L. micdadei* commited intracellular growth in amoeba, by a growth factor of approximately 3 log units CFU/ml. Intracellular growth in macrophages was only accomplished by two strains in one of two repetitions of the experiment. The two strains had a growth by 3 log units. Evaluations of PCR analyses mapped the presence or absence of the 5 virulence genes in the different strains. Some of the strains with all five virulence genes detected were not able to replicate in eukaryotes in these experiments (*L. pneumophila* Colitax and Philadelphia), while one strain with only one virulence gene did replicate in amoebae (*L. micdadei*).

No correlation was found in the ability of the tested *Legionella* strains to multiply in co-culture experiments with amoeba and macrophages. Also, for some strains the infectivity of amoeba and macrophages did not correspond to the presence of selected virulence genes identified using real-time PCR. The findings in this study that only two strains were able to multiply in macrophages indicate methodological problems, and the method needs to be improved in future studies. The results of PCR amplification of selected virulence genes were difficult to interpret concerning whether or not the gene was present. Using sequence specific probes would highly improve the results. The discoveries made in this thesis can be used further in establishing improved detection methods for pathogenic strains of *Legionella*.

Sammendrag

Ulike *Legionella*- stammer er allestedsnærværende i naturen. Enkelte stammer, slik som *Legionella pneumophila* sero- gruppe 1 er kjent som patogen for mennesker, og kan gi alvorlig lungebetennelse. I naturen er *Legionella* hovedsakelig en parasitt for protozoer, men kan utføre liknende intracellulær vekst i alveolære menneske- makrofager. Å hindre vekst av bakterien og spredning av sykdommen forårsaket av patogene stammer av *Legionella* er av interesse for Forsvarets Forskningsinstitutt (FFI) som en modellorganisme for luftbåren bakterie- spredning, og for patogene mikroorganismer med en intracellulær livssyklus i eukaryote celler.

Ikke alle *Legionella*- arter kan replikere i amøber. Derfor blir sam-kultur med amøber regnet for å være selektiv for patogene stammer av legioneller. For å undersøke patogene trekk ved ulike stammer av *Legionella* ble ulike eksperimenter utført i denne studien. Sam-kulturer av *Legionella* med *Acanthamoeba castellanii* og menneske- makrofag THP1 celler ble dyrket for å undersøke stammenes evne til å vokse intracellulært. Ti individuelle isolater av *Legionella,* inkludert ulike sero- grupper av *L. pneumophila*, sammen med *L. micdadei*, og en ukjent *Legionella*- art ble brukt i sam-kulturer. Sanntids PCR analyser med SYBR Green ble utført på de ulike stammene med ulike primer- sett for å kartlegge tilstedeværelsen av virulensgenene kjent som *rtx, lvh, dot, mip* og *hsp* i hver stamme. Til slutt ble den registrerte tilstedeværelsen av virulensgener sammenliknet med hver stammes' evne til å replikere intracellulært i eukaryoter. Stammene kommer opprinnelig fra kliniske isolater, fra miljøprøver og fra ofte brukt referansestammer.

Flere av *L. pneumophila*- stammene, i tillegg til *L. micdadei* foretok intracellulær vekst i amøber, med en vekst- faktor på omtrent 3 log- enheter koloniformende enheter per ml. Intracellulær vekst i makrofager ble kun oppnådd av to stammer, og kun i ett av to forsøk. De to stammene hadde en vekst på 3 log enheter. PCR analysene ble evaluert til tilstedeværelse eller fravær av de 5 ulike genene i de ulike stammene, og varierte fra 1 til 5 gener. Noen av stammene med alle fem gener detektert replikerte ikke i eukaryoter (*L. pneumophila* Colitax og Philadelphia), mens en stamme med kun ett av virulensgenene replikerte i amøber (*L. micdadei*).

Det ble ikke funnet noen sammenheng i infektiviteten til *Legionella*- stammene mellom amøber og makrofager. Infektiviteten korresponderte heller ikke med tilstedeværelsen av flest mulig virulensgener. Resultatene i denne studien indikerer en utilstrekkelig metode for å infisere makrofager med *Legionella*. PCR- resultatene var vanskelige å evaluere i forhold til om genet var tilstede eller ikke. Bruk av sekvens- spesifikke prober ville forbedre resultatene betydelig. Oppdagelsene gjort i denne studien kan bli brukt videre i å etablere bedre egnet deteksjonsmetoder for patogene stammer av *Legionella.*

Table of Contents

Abbreviations

BCYE – buffered charcoal yeast extract

CFU – colony-forming units

COA – Cycles of amplification

CP – Crossing point (site of excessive replication of gene)

DNA – Deoxyribonucleic acid

dNTP – deoxyribose Nucleoside triphosphate

FFI – Forsvarets forskningsinstitutt/Norwegian Defense Research Establishment

FLA – free- living amoeba

GC – Guanine and Cytocine

GVPC – glycine, vancomycin, polymyxin B, cycloheximide

ISO – International Organization for Standardization

LCV – *Legionella*- containing vacuole

LD – Legionnaires' disease

MOI – multiplicity of infection

PCR – Polymerase chain reaction

RNA – Ribonucleic acid

SG – sero- group

Spp – species pluralis

T_M – Melting Point

VBNC – viable but not culturable

1. Introduction

Detecting and preventing outbreaks and sporadic cases of pneumonia due to *Legionella* species is an increasing concern as the casualties increase (Pettersen 2012). Knowledge of growth and resistance of these bacteria is needed to aid preventive measures in battling the disease. The spread of infective *Legionella* bacteria through bio-aerosols in air is an increasing problem due to modern man-made water installation technologies (Bartram et al. 2007). Aerosols are tiny water particles spread through air, and can contain pathogens such as bacteria and viruses present in the water source. Distribution of bio-aerosols into air causes exposure and risk of infection onto residents and passersby. Environmental organisms such as *Legionella pneumophila* become opportunistic pathogens most likely due to their protozoa-dependent parasitic lifespan, which make them able to invade macrophages in the lungs of humans after inhalation. Prevention of disease requires prevention of growth and spread of these bacteria in technical water installations.

1.1 Background

1.1.1 History of disease

Legionella was first discovered in Philadelphia in 1976 when 182 out of 3000 legionnaires at a conference suddenly developed pneumonia. The illness had a high mortality rate, and 34 out of the 182 infected legionnaires died. (Pettersen 2012) The disease became known as Legionnaires' disease (LD) and the bacterium responsible for the outbreak was named *Legionella pneumophila*. LD is a serious lung infection that can have fatal outcome for those who are infected due to respiratory failure (Mekkour et al. 2013). The *Legionella* bacteria have a high infection dose, and the disease usually affects elderly, smokers, and immuno-compromised patients. LD does not spread between human (Hilbi et al. 2010).

Not only LD can be caused by Legionellae. A milder form of disease called Pontiac fever (proven caused by *L. pneumophila* sero-group 1 and 6) gives only mild flu-like symptoms without pneumonia (Bartram et al. 2007). A common name for diseases caused by *Legionella* is "legionellosis". More than 50 species of *Legionella* has been identified (Hilbi et al. 2010). Some of these are known as pathogenic strains, while others are environmental strains not proven to cause disease (Kwaik 1998). *L. pneumophila* sero-group (SG) 1 is the most common pathogen to cause legionellosis in human, and approximately 84 % of the cases are due to this (see table 1.1), but other Legionella species have caused disease occasionally, like

L. longbeache, L. bozemanii, L. micdadei, L. dumoffii, L. feeleii, L. gormanii, L. jordanis, L. oakridgensis L. wadsworthii and *L.anisa* (Mekkour et al. 2013; Muder & Yu 2002; Yu et al. 2002; Brooks et al. 1991). In Australia and New Zealand, *L. longbeache* is almost as common as *L. pneumophila* as a causative pathogen, with 30.4% and 45.7% infection rate, respectively.

Table 1.1, taken from Yu et al. (2002), displays distribution of community-acquired *Legionella* strains responsible for disease in 508 world spread patients. Numbers show distribution in percentage and number of individuals.

***Legionella* species, sero-group**	**Species**	**Sero group**
L. pneumophila	91.5% (465)	
1		84.2% (428)
6		1.7% (9)
5		1.3% (7)
4		1.0% (5)
3		1.0% (5)
7		0.6% (3)
2		0.4% (2)
8		0.4% (2)
13		0.4% (2)
Unknown (not SG 1)		0.6% (3)
L. longbeachae	3.9% (20)	
Unknown		2.6% (13)
1		1.2% (6)
2		0.1% (1)
L. bozemanii	2.4% (12)	
Unknown		2.0% (10)
1		0.2% (1)
2		0.2% (1)
L. micdadei	0.6% (3)	
l. dumoffii	0.6% (3)	
L. feeleii	0.4% (2)	
L. wadsworthii	0.2% (1)	
L. anisa	0.2% (1)	
Unknown species	0.2% (1)	
Total	100% (508)	

There have been a few outbreaks in Norway. One of them was in Stavanger in 2001, where 28 got sick and 7 died. The contamination source was a cooling tower (Pettersen 2012). In the Sarpsborg/ Fredrikstad area, a wood and pulp factory caused an outbreak in 2005, where 56 got sick, and 10 died (Nygård et al.2008; Blatny et al. 2008; Fykse et al. 2014). From the outbreak and up to 2008, 47 more people were diagnosed with LD in the same area. *Legionella pneumophila* ST-15 was responsible for the first outbreak (2005), and 5 cases in 2008 were due to *L. pn.* ST-462. Air scrubbers at the environmental facility in question were initially the suspected source of spread (Blatny et al. 2008). In later experiments, *L. pneumophila* ST-462 was identified at concentrations up to 10^7 CFU/mL in the aeration ponds of the biological treatment plant, and 3300 CFU/L in air samples as far as 200 meters away from the aeration ponds. The outbreak strains were also detected in the river Glomma (Blatny et al. 2008; Olsen et al. 2010). The aeration ponds were later shut down by the Norwegian Climate and Pollution Agency to prevent growth of Legionellae. The two outbreak strains, ST-15 and ST-462 are used in this survey, as well as some other *Legionella* strains collected from the biological treatment plant in follow-up investigations after the outbreaks.

1.1.2 Pathogenic traits

Legionella is an omnipresent Gram negative, heterotroph, aerobe, rod- shaped bacteria with a natural habitat in surface water and soil (Mekkour et al. 2013; Brooks et al. 1991). This environmental bacterium often uses free-living protozoa, such as amoeba, for replication and protection, and has a parasitic lifestyle due to co-evolution with protozoan hosts. The ability to replicate in protozoa possibly makes *Legionella* an opportunistic pathogen to humans (Tao et al. 2013; Rolando & Buchrieser 2012). *Legionella* alternates between a non-motile replicative form, and a flagellated infectious form (Hilbi et al. 2010). In the infectious form, *Legionella* adhere to the amoeba, and gets ingested into the cell, where it avoids phagocytosis by the lysozyme, and remains in a vacuole for replication inside the host. The theory is that replication in free living amoeba (FLA) prepares *Legionella* for replication in other eukaryotic cells, such as human macrophages (Fykse et al. 2014; Willey et al. 2009). It's occurrence in all natural sources of fresh water makes its entry into man-made water regulations such as water tanks and heated water systems inevitable. This allows aerosols containing *Legionella* to spread through common contamination sources (fountains, hot tubs, communal and private showers and air conditioning systems), via air, and to be inhaled by potential hosts of the infective bacteria. (Pettersen 2012).

The spread of *Legionella* in bio-aerosols through air is an increasing risk. Measures have to be made to inhibit growth of *Legionella* species in water installations. Hospitals are especially at risk for holding debilitated patients with decreased immunity, especially prone to get infected. The presence of different strains of the *Legionella* species could also give mixed infections leading to more serious cases of the disease (Hilbi et al. 2010).

1.1.3 Amoeba

Amoebae are free living, protozoan organisms that prey on other single cellular organisms such as bacteria, fungi, algae and other protozoa, as well as free nutrients in water and soil environments. (Cateau et al. 2014) These organisms use pseudopods ("false feet" that stretches far away from the cell) for movement and feeding (Willey et al. 2009). *Acanthamoebidae* used in this study can form cysts with double cell wall which make them more resistant. *Legionella* has been observed in the amoeba's cyst cell wall, indicating that amoeba cysts could protect *Legionella* under harsh conditions (Fykse et al. 2014). Some amoebae are known to give infections in human, such as infection of the brain or eye (due to contact lenses) by *Acanthamoeba* (Khan 2001). FLA can also be the cause of human disease by harboring potentially pathogenic bacteria such as *L. pneumophila* (Allombert et al. 2014).

These amoeba resistant bacteria have suppressing or lethal effect on the amoeba (Zeybek & Binal 2014). *Legionella* spp, after entering a FLA can resist digestion by the amoeba by remaining in a vacuole (membrane-enclosed intracellular compartment) while secreting proteins that interfere with the hosts cell components. (Rolando & Buchrieser 2012). By resisting consumption by the amoeba, and even being able to replicate inside of it, *Legionella* spp. gain protection from non-hostile environments, nutrients for growth, and preparation for survival and replication in macrophages (Cateau et al. 2014; Cateau et al. 2011).

1.1.4 Macrophages

Macrophages are suggested to be derived from circulating monocytes from bone marrow or blood, but are specialized in tissues, larger in size, and have more organelles than monocytes (Willey et al. 2009). Macrophages are immune cells which perform phagocytosis, and are an important agent in the human immune- system (Brooks et al. 1991). Macrophages track bacteria by amoeboid movement. Receptors on the surface of the macrophage recognize microorganisms by binding to common patterns, perceiving composites of surface proteins, antibodies or foreign DNA. Macrophages are also able to send out opsonins such as immunoglobulins, complement components or fibronectin, which are chemical substances that are complementary to the phagocytes' receptors. These opsonins coat foreign cells or material, and the macrophage devours them upon attachment.

Entering into the lungs, microorganisms first have to get through the filtration system in the respiratory tract, where large and small microbes attach to either hairs or mucosal surfaces, which lead the microorganisms out through the mouth. (Willey et al. 2009) If the microorganisms were to reach the alveoli in the lungs nevertheless, it is the alveolar macrophage's job to kill them by phagocytosis. Failing to do so would result in an infection. *Legionella* infecting alveolar macrophages could cause a chronic inflammation and tissue damage, given their resistance to phagocytosis, and the rupture of macrophages subsequent to multiplication (Willey et al. 2009).

1.1.5 Detection methods

The first detection method used for pathogenic *Legionella* was inoculation in guinea pigs (Bartie et al. 2003). This method came across as selective, but expensive and elaborate. The follow-up methods used were cultivation on specific agar media with specific supplements and pre-treatment for selection of *Legionella* species in mixed samples. Typical treatments to these samples are acid- and temperature treatments, and supplement of the amino acid *L*-

cysteine, for favoring survival of *Legionella* only. These methods are not overly elaborate or expensive, and are commonly used today in laboratories. Many modern methods such as the International Standard Method (presented by ISO) used for detection of *Legionella* in Europe are based on this technique. The agars currently used are non-selective buffered charcoal yeast extract agar (BCYE), and the more expensive *Legionella*-selective GVPC medium (glycine, vancomycin, polymyxin B, cycloheximide). The disadvantage of the non-selective agar method is the possible overgrowth and inhibition of Legionellae growth by other organisms. *Legionella* is slow-growing compared to other bacteria and might be outcompeted on BCYE. Cultivating methods might also give mistakenly negative results due to lack of detection of *Legionella* spp. presently in the viable, but not culturable (VBNC) state of the bacteria in solution. Another method is to detect species of *Legionella* in the co-culture method with protozoa (Barbaree et al. 1986). Several studies have shown that pathogenic strains of *Legionella* can be detected using co-culture in amoebae (Cirillo et al. 1994; Schalk et al al. 2012). An assumption drawn from this is that non-invasive, environmental strains of *Legionella* will not be able to invade and replicate in amoebae (Snelling et al. 2006). As a detection method for diagnosing patients possibly infected with *Legionella,* a urine antigen test is applied. This test only determines if the species is *L. pneumophila* sero- group 1. PCR identification or culturing of sputum or blood samples from the patient is often used for detection of other species and sero- groups of *Legionella* (Pettersen 2012).

1.1.6 Prevention

Amoebae are essential for growth and protection of many *Legionella* species in natural water systems (Taylor et al. 2009; Rowbotham 1980; Fykse et al. 2014). By failing to digest ingested *Legionella*, the amoeba protects the bacteria from harmful substances such as chlorine, as well as providing them with necessary nutrients for growth (Willey et al. 2009). If amoebae are absent, *Legionella* has a small chance of survival and multiplication in unhostile environments. Knowledge on how to prevent survival of amoeba would assist in the prevention of proliferation of *Legionella*. Other, simpler methods of preventing *Legionella* from growing, is to keep the water temperature in the container/heating system above 65ºC (Pettersen 2012). *Legionella* is also quite pH resistant, so the pH has to be below 3 or above 10 to prevent growth and survival. Prevention of biofilms would also be crucial, as *Legionella* is able to live in biofilms and conduct active growth. Biofilms are communities of microorganisms on surfaces constantly or alternately exposed to water (Willey et al. 2009; Madsen 2008). It is composed of molecules from the environment, polysaccharides, proteins

and DNA from the microorganisms present, and is surrounded by a layer of slime for protection. The microorganisms in biofilms have a form of communication called "quorum sensing", which is excretion of signaling chemicals into the biofilm, for organizing e.g. antibiotics production, plasmid DNA transfer and other defense mechanisms (Madsen 2008). Release of metabolic waste from one bacterium into the biofilm might be used as an energy-source for other bacteria. Biofilms are omnipresent in nature and in the industry, such as manufacturing, processing etc., sometimes beneficial, other times a substantial problem. When in a biofilm, *Legionella* gains protection against disinfectants and antimicrobial agents such as antibiotics, and at the same time is provided with nutrients and the right conditions for growth (Thomas et al. 2004; Taylor et al. 2009; Coulon et al. 2010).

According to studies of *Legionella* on naval vessels, there is a high presence of *L. pneumophila* in water installations (Ahlén et al. 2013). Parts of the water supply system could be old and layered with biofilms containing *Legionella*, even if the cleaning system might be up to date on *Legionella* preventing methods. Positively charged ions of copper and silver are known for their anti-microbial effects, and have proven to eliminate microbial growth of such as *Legionella*, and even defeats biofilm over time in hospital water systems (Braathen 2010).

1.1.7 Toxicity/Virulence

Knowledge of *Legionella's* genome has given insight into pathogenesis of the bacteria and the interactions with their host (Hilbi et al. 2010). A comparison of 5 studies from 2008/2009 shows, according to Hilbi et al. (2010), that some environmental strains of *Legionella* are more pathogenic than others. Looking further into what distinguished these strains from others could elucidate the traits that separates pathogenic strains from non-pathogenic strains of *Legionella*. Genomics has given insight into genes that make these bacteria pathogenic, classifying these genes as virulence factors. According to Isberg et al. (2009), the "Molecular Koch's Postulate" defined "a virulence factor" as the protein missing in a mutant that is "demonstrated to be defective for a process critical in pathogenesis".

Pathogenic strains of *Legionella* has the ability of remaining in a *Legionella*-containing vacuole (LCV) inside the host, where it manipulates the host cells own trafficking pathways, and transport protein substrates out of the vacuole, which protects them from phagocytosis (Isberg et al. 2009). These are considered fundamental pathogenic traits in intra-vacuolar Legionellae. To camouflage the pathogen-containing vacuole, *Legionella* secretes imitating proteins resembling those excreted by the host cells' own organelles, and is recognized as the cells own material. Therefore, the vacuole is not destroyed, nor is the bacteria lysed.

Pathogenic *Legionella* also manipulate mechanisms in the host cell by interfering with its molecular metabolism, inhibiting protein synthesis, and kills it. Looking at figure 1.1, presented by Isberg et al. in 2009, one can see how *Legionella* enters the host cell, recruits mitochondria from cytosol, and ribosomes from endoplasmic reticulum (ER), to appear as endoplasmic reticulum to the cell. The cell is then surrounded by rough ER. The LCV seize vesicles filled with cell material transmitted from the ER directed towards the Golgi apparatus, and fuse with it (Kagan & Roy 2002). When recognized as a compartment of the cell, the vacuole is not lysed, and the Legionellae can multiply in a safe, nutritive environment, before bursting out of the cell.

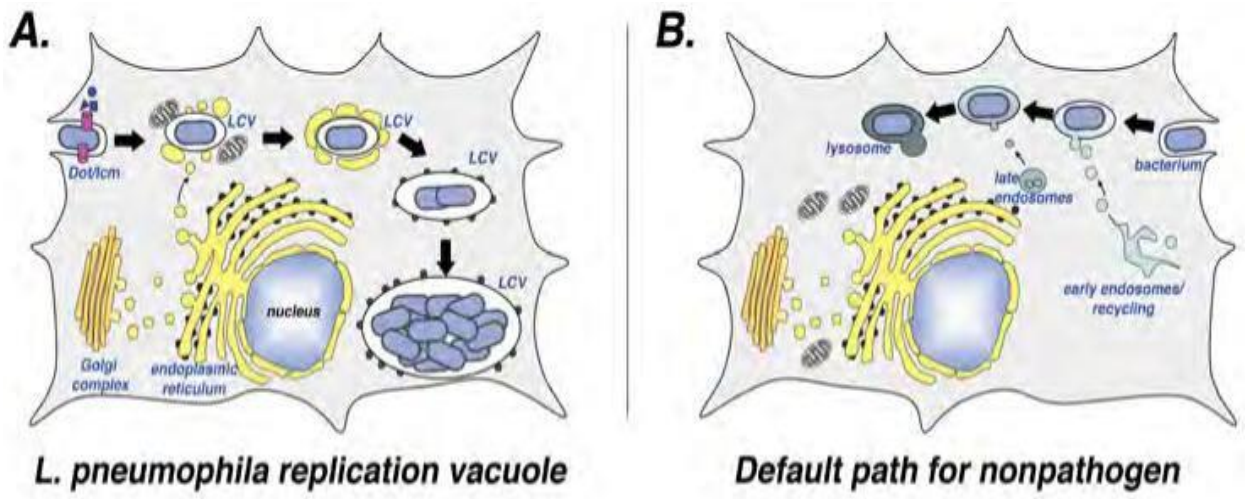

Figure 1.1: Taken from Isberg et al. (2009), shows how *L. pneumophila* survives inside of a eukaryote cell in a LCV, as opposed to a non-pathogenic bacteria entering a eukaryote and being lysed. A: A *Legionella* cell enters a eukaryotic phagocytic cell, remains in a vacuole, attracts mitochondria and ribosomes to resemble ER, get covered with rough ER, and multiply within the vacuole. B: Non-pathogenic bacteria enters the endosomal-lysosomal pathway, gets treated by early endosomes at first, then late endosomes on the pathway from the cell membrane, to the final destruction by the lysosome.

The complex system known as "Defect in Organelle Trafficking; IntraCellular Multiplication" (Isberg et al. 2009), termed Dot/Icm, is composed of 27 genes that are essential for the establishment of the replication vacuole and growth inside the host. This system enables the bacteria to excrete proteins across the host cells/vacuole membrane, to accumulate on the outside of the vacuole.

1.1.8 Genes

Different genes are associated with the virulence of *Legionella* species. Some of them are tested for in this survey. The same primers were used by Tachibana et al. (2013).

- *Dot* gene: "defect in organelle trafficking": a term for mutants of the *dot*- gene, which were unable to replicate intracellularly (Vogel et al. 1998). The *dot* gene is involved with the "Dot/Icm type IV secretion system", which is known as the secretion of proteins into the cytosol of the host cell, preventing fusion of phagosome and lysosome, and hindering acidification of the vacuole (Chien et al. 2004; Zamboni et al. 2003).
- *Mip* gene: "macrophage infectivity potentiator" (Wintermeyer et al. 1995); a species specific primer used for detection of *L. pneumophila* (Wellinghausen et al. 2001; Blatny et al. 2008). The gene encodes a surface protein and is essential for early survival in protozoa. Mutations of this gene has showed decline in lethal effects of *L. pneumophila* in guinea pigs (Cianciotto et al. 1990).
- *Rtx* gene: "repeats in structural toxin", is proven to play a contributory role in *L. pneumophila* adhering to the host cell, pore formation and cytotoxicity, replication and survival intracellularly as well as virulence, in human monocytes and *Acanthamoeba castellanii* (Cirillo et al. 2000; Cirillo et al. 2002).
- *Hsp* gene: member of the family of heat shock proteins (stress protein) that localizes on the cytoplasmic membrane or cell surface of *Legionella*, and are synthesized abundantly, and only when *Legionella* grows intracellularly (Fernandez et al. 1996; Weeratna et al. 1994)
- *Lvh* gene: is also involved in a type IV secretion system, separate from the Dot/Icm system, and is significant for intracellular growth (Tachibana et al. 2013; Chien et al. 2004; Segal & Shuman 1999)
- *JFP/JRP*: *Legionella* 16S ribosomal RNA gene section (Cloud et al. 2000; Parthuisot et al. 2010). 16S rRNA refers to the bacterial small ribosomal subunit used for classification of prokaryotes (Madsen 2008). 16S rRNA is a region that is highly conserved, with an exceptionally low rate of mutations because of the ribosome's high importance to the protein synthesis of the cell (Madsen 2008). This gene is present within all prokaryotes, but differs significantly to determine how closely related species are to each other. This primer is selective for *Legionella* species.

1.2 Methods

1.2.1 Cultivation

According to the International Organization for Standardization (ISO), BCYE agar added *L*-cysteine and iron(III), is commonly the best medium for detecting *Legionella*, but some species of *Legionella* does not grow well on BCYE. (Lee et al. 1993; Brooks et al. 1991). *Legionella* is slow-growing. Some strains grow slower than others, for example, *L. micdadei* grows slow whereas *L. pneumophila* grows faster than other strains (illustrated in figure 1.2). Colonies of different strains of *Legionella* might appear quite different from each other, but generally have a transparent to white color, a clear outer line and glossy surface.

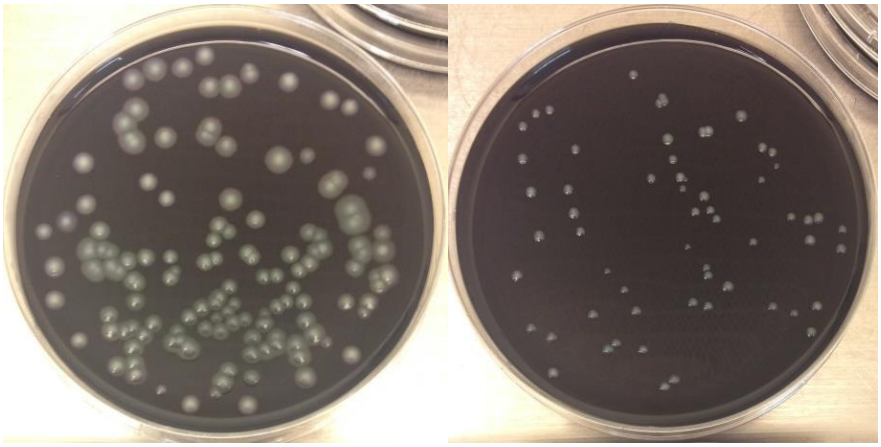

Figure 1.2: shows *L. pneumophila* SG 1 (left), and *L. micdadei* (right) after 4 days of incubation on BCYE. The morphology of the colonies and the rate of growth are different for many strains.

1.2.2 Co-culture w/amoeba

Co-cultivation methods of *Legionella* with amoeba are considered to be selective for pathogenic strains of the genus, because of the similarity in its methods of infecting protozoa and macrophages (Gao et al. 1997). As described by Fykse et al. (2014); Jacquier et al. (2013); Zeybek & Binay (2014), co-culture can be used if *Legionella* cannot grow on agar, perhaps because they are in a viable-but-non-culturable (VBNC) state (Oliver 2000; Epalle et al. 2014), or they are not in a reproductive state, but in an infective state. Co-culture with *A. castellanii* could revive VBNC *Legionella* cells to make them able to grow on agar. It is also preferable to use amoebal co-culture on an environmental sample, due to the fact that *Legionella* are slow-growing, easily out- competed bacteria. However, not all *Legionella* are able to infect and reproduce in amoeba. Co-culture with amoeba has also proven that *Legionella* infect macrophages at a higher rate after co-culture with amoeba. By adding *Legionella* to amoeba in culture, and measuring the amount of *Legionella* prior to and after

co-cultivation, the eventual growth or decline is measured. Amoeba is lysed 2-3 days after infection with *Legionella*.

1.2.3 Co-culture w/macrophages

Being able to demonstrate intracellular growth of a strain of *Legionella* in macrophages or monocytes is regarded as an indicator of pathogenicity of the strain to humans. The articles referred to in this section had varying test results regarding intracellular growth in macrophages/monocytes. As described by Tao et al. (2013), environmental *Legionella* strains growing effectively in amoeba, did not always grow in macrophages, even though the macrophages had given growth to previous laboratory strains. Tachibana et al. (2013) showed that strains harboring all toxicity genes tested for in this survey (*mip, lvhB3, rtxA, dotA* and *hsp60*) were able to grow in mouse macrophages. Cirillo et al. (2000) also proved the importance of the toxicity gene *rtxA*, in *Legionella'*s ability to infect human monocyte cells. Garcia-Nunez et al. (2008) had 22 environmental strains of *L. pneumophila* growing in human macrophages.

1.3 Aim of study

In general, it is difficult to decide if environmental isolates of *Legionella* is pathogenic to humans if clinical isolates of the strain are not identified. Different assays can be performed to investigate the cytotoxicity or infection rate of the strains, and such information can be used to evaluate the potential pathogenicity of the strains. The purpose of this study is to examine the growth of ten different strains of *Legionella*, both environmental isolates and proven pathogenic strains, inside free living amoeba and in human macrophages by co-cultivation. Their ability to grow in amoeba/macrophages will be compared to the presence of different virulence genes of the strains detected by real time PCR. The results will map which of these ten isolates of *Legionella* that will infect and grow inside of amoeba and/or in human macrophages, and compare this information to whether the toxicity genes in question are present in the isolates. This kind of information can be used to evaluate the potential risk to humans if they are exposed to similar strains.

The toxicity of *Legionella* is well covered, its genome is sequenced, and a lot of genes connected to pathogenesis are identified. *Legionella* is also well studied growing in amoeba and macrophages. The underlying issue is how to detect and prevent spread of disease, using PCR and co-culture for detection of pathogenic strains. The question in interest is how to separate the virulent strains of *Legionella* from the non-pathogenic. The aim of the study is to

classify which strains that invade amoeba and macrophages and which genes they possess. An overview of the pathogenic traits in different species is beneficial in the work of detecting potential pathogens.

1.4 Significance

This study sheds light upon challenges regarding today's most commonly used methods of detecting pathogenic *Legionella*. *Legionella* is a complex organism which is difficult to predict in relation to its behavior in natural and engineered water systems. *Legionella* poses an increasing health risk in the modern world, but it is also important as a model organism for other pathogenic bacteria able to survive within protozoa (Cateau et al. 2011). Parasitic pathogens' survival and growth strategies, living inside complex organisms, pose a challenge on purification of all water distribution systems. Knowledge on these organisms' life cycles, and their strengths and weaknesses leads us on to the knowledge on preventing these pathogens from survival and proliferation in our water sources.

2 Materials and Methods

Pathogenic and non-pathogenic strains of Legionellae were used in analyzes. Environmentally free-living amoeba and cancerous human macrophage cells were used as hosts. There were used 10 different *Legionella* strains in the co-cultivation research. DNA from the same strains, and from 9 additional strains, 8 of them from the American Type Culture Collection (ATCC) were used in the PCR assays, testing for the different virulence genes.

2.1 Bacterial strains

The following strains of bacteria were used in this study:

2 *Legionella* strains that originate from outbreaks in Sarpsborg, Norway (Blatny et al. 2008; Olsen et al. 2010; Fykse et al. 2014):

- *L. pn.* ST-15 sero- group (SG) 1(outbreak in 2005)
- *L. pn.* ST-462 SG1(outbreak in 2008)

5 Environmental strains from periodical sampling in Sarpsborg, Norway:

- *L. pn.* SG 1
- *L. pn.* SG 2-14
- *L. micdadei* K1
- *L. gormani* 7 (pathogenic)
- *L.* spp.(non-identified *Legionella*: not *L. pneumophila, L. longbeachae, L.bozemanii, L.dumoffii, L.gormanii, L.jordanis, L.micdadei* or *L.anisa*)

And 3 ATCC strains:

- *L. pn.* Bloomington (SG 3) (CIP 103857, ATCC 33155)
- *L. pn.* Colitax (SG 2-14) (reference strain, from Unilabs Telelab AS)
- *L. pn.* Philadelphia (SG 1) (CIP 103854, CCUG 9568, ATCC 33152)

9 Additional strains used in PCR: SG2 ATCC “103856”, SG4 ATCC “103858”, SG5 ATCC “103859”, SG6 ATCC “103860”, SG7 ATCC “103861”, SG8 ATCC “103862”, SG9 “103863”, SG10 “103864” and *L.pn.* “B11-A3” (Blatny et al. 2007).

2.1.1 Pre-culture

The bacterial strains used in this study were frozen samples, kept at approximately -70°C. Prior to the experiments, the frozen strains were plated out on BCYE agar, selective for gram-negative bacteria, containing iron salts and L-cysteine necessary for Legionellae growth (Bartram 2007) on laboratory media (ready to use from Oxoid, Cambridge, UK) and incubated at 37°C for 3-6 days. The bacteria were used in experiments at least 48 hours after plating. All the strains used in these experiments are known to grow well on BCYE media.

2.2 Co-culture

The two strains from outbreaks, the five environmental strains from periodical sampling and three ATCC reference strains were added separately to layers of *Acanthamoeba castellanii* and human macrophages in 24 well cell plates. After incubation for 4 days in the macrophage co-culture and 6 days in the amoeba co-culture, dilutions of the cells were plated out in duplicate on BCYE agar, and the growth or decline of the bacteria were counted and graphed.

2.2.1 Amoeba growth

Amoeba-*Legionella* co-culture was conducted primarily as described by Fykse et al. (2014), Steinert et al. (1997) and Moffat & Tompkins (1992). Moffat &Tompkins (1992) added gentamicin to the co-culture after infection, in order to kill extracellular bacteria, before removing it again. This procedure was not used by Fykse et al. (2014), Steinert et al. (1997), or in this survey.

The amoeba used in this research was an axenic strain of *Acanthamoeba castellanii* retrieved from ATCC (30234) (Fykse et al. 2014). *A. castellanii* was grown in PYG medium (ATCC 712: 2% proteose peptone, 0.1% yeast extract, 0.1M glucose, 4mM $MgSO_4$, 0.4M $CaCl_2$, 0.1% sodium citrate dehydrate, 0.05mM $Fe(NH_4)_2(SO_4)_2$ * $6H_2O$, 2.5mM NaH_2PO_3, 2.5 mM K_2HPO_3 (ATCC, VA, USA) (Steinert et al. 1997). See appendix A for recipe and preparation of medium. Culture was kept in 25 cm^2 cell culture flask (Sarstedt), in the dark at room temperature.

Amoeba culture was split 4 days prior to the experiment. 250 µl from previous culture was transferred to 5 ml fresh PYG medium in a cultivation flask. Amoeba was also plated out on BCYE and blood agar to test for contamination. After 4 days, the flask was stirred to release the cells from the bottom, into the medium. The cells were counted using the Burker haemcytometer. A dilution of approximately $2x10^5$ amoeba/ml was made by adding PYG medium to the culture. This formula was used for the counting chamber:

$$density\ of\ amoeba\ (\frac{cells}{\mu l}) = \frac{number\ of\ cells}{area(mm^2)(0{,}04mm^2) * depth(mm)(0{,}1mm) * dilution}$$

1 ml of the diluted amoeba cell culture was added to each well in the 24-well cell culture plate (Costar, Corning, NY, USA). It was incubated overnight (24 hours) in room temperature, to let the amoeba sink down, coating the bottom of the wells. The plate was then centrifuged, PYG medium was adsorbed with a pipette, and the amoeba cells in each well were washed in 1 ml Page's amoeba saline (PAS) buffer (PYG medium excluding 2% proteose peptone, 0.1% yeast extract and 0.1M glucose) and centrifuged at 1700 rpm , twice.

Washing assures a pure amoeba culture, and is very important in environmental samples with various organisms, to remove unwanted organisms. The experiments in this study were performed using an axenic strain of *A.castellanii*, so the washing was primarily done to remove dead cell material that could be nutritive to Legionellae, and to make sure there are only amoebae present. Legionellae was diluted in PAS buffer for co- culture with amoeba. Dilutions of *Legionella* strains were added to the amoeba (as described in section 2.2.3) simultaneously as the PAS from the last washing was removed. The co-culture samples were incubated for 6 days. Dilutions of *Legionella* were plated out and counted before and after 6 days of co-culture with amoebae. Negative controls of amoebae without Legionellae, and of Legionellae without amoebae were run in parallel and treated similarly. *L. pneumophila* SG3 Bloomington is previously proven infectious to amoebae, and was used as a positive control in every round.

2.2.2 Macrophage growth

Macrophage-*Legionella* co-culture was performed as described by Steinert et al. (1997), Moffat & Tompkins (1992), and Tao et al. (2013). Previous experiments were performed with monocytes instead of macrophages (Steinert et al. 1997), a higher infection rate (Tachibana et al. 2013), and with gentamicin to kill extracellular bacteria (Moffat & Tompkins 1992), as opposed to this survey.

Monocytic leukemic cell line from ATCC (THP1 (ATCC® TIB-202™)) cultured in RPMI 1640 medium (PAA, Pasching, Austria), added 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin (PS) was used. Four days prior to co-culture, monocytes were spun down at 1000rpm for 3 min, and old medium was replaced with new RPMI with FBS and PS. Cells were counted using Countess (Invitrogen), by mixing 10 µl trypan blue with 10 µl cell culture.

The monocytes were differentiated into macrophages by adding PMA (phorbol 12-myristate 13-acetate) diluted with DMSO (Dimethyl Sulfoxide) as demonstrated by Takashiba et al. (1999). PMA was diluted to 100µM, and 100µL was added to 50 ml monocytic cell line culture with $2x10^5$ cells/ml. The culture was then divided into 2 24-well plates, 1 ml in each well, and incubated at 37°C for 4 days to allow cells to stick to the bottom of the wells. Then, the cell culture plate was washed in RPMI without FBS and PS. The PS would kill Legionellae, so it had to be removed. FBS could give nutrition to undesirable growth, and is not necessary for survival of the cells. The wells were washed 3 times, and centrifuged at 800 rpm between each washing.

Legionella strains were diluted with RPMI without PS and FBS (as described in section 2.2.3), and were added to macrophages after the washing. There were also control wells of macrophages without Legionellae, to test for contamination. Wells of Legionellae without macrophages were incubated in parallel, to compare for growth or decline caused by the media. *Legionella* from co-culture were plated out first after 24 hours, and again after 4 days.

RPMI medium is applied for growth of a variety of mammal cells, and contains essential vitamins and amino-acids. It contains no proteins, lipids or growth factors. These are added by supplement of FBS, which is easily contaminated. The growth on the BCYE plates after 4 days was run on PCR with Legionella specific primers, to verify the species as *Legionella*.

2.2.3 Aliquots of Legionellae for co-culture

Dilutions of the *Legionella* strains were made by filling the hole in a 1 µl inoculation loop with pre-culture bacteria from the BCYE plate and dissolving it in 1 ml media. Experience shows that this gives a concentration of about $1x10^9$ colony-forming units/ml on average. A 6x dilution series was made from this sample, giving a final concentration of $1x10^3$ CFU/ml. Concentrations of presumably $1x10^3$ and $1x10^4$ CFU/ml of the *Legionella* strains were added to approximately $2x10^5$ cells/ml of amoeba/macrophage cultures, distributed as displayed in figure 2.1. Multiplicity of infection (MOI) was at approximately 0.005 and 0.05, respectively. *Legionella* dilutions were plated out in duplicate on BCYE to register the number of CFU's before co-cultivation. The same *Legionella* dilutions were added to the control wells without amoeba/macrophages. Both co-cultured and control strains were plated out in duplicate after 6 (for amoebae) or 1 and 4 (for macrophages) days of incubation, to calculate growth.

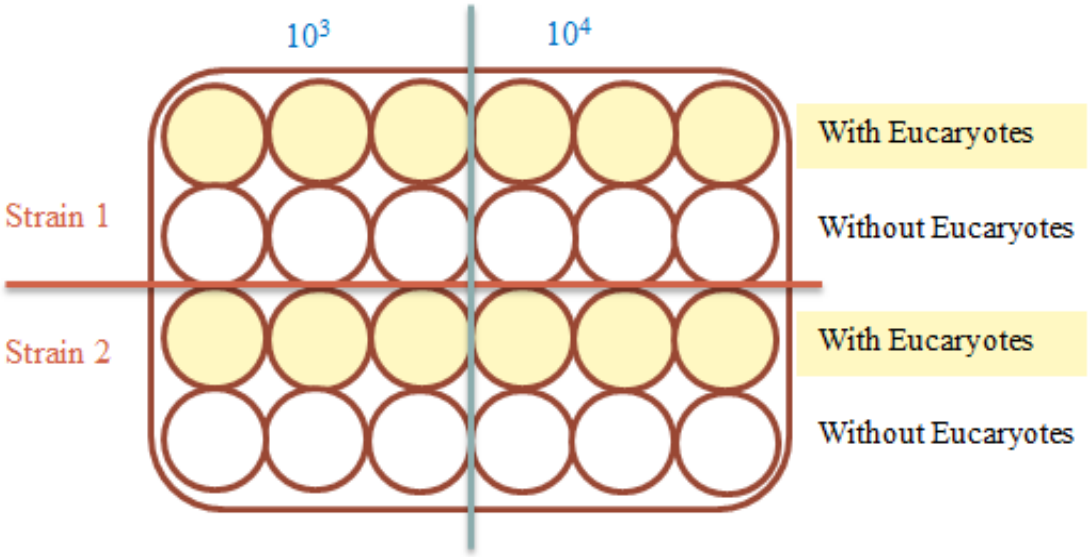

Figure 2.1: Set up of the experiment in a 24 well cell culture plate (Costar, Corning, NY, USA). 2 different strains were cultured on each plate; dilutions of $1x10^3$ and $1x10^4$ CFU/ml, and with and without eukaryotes (amoebae/macrophages). The two top rows had one strain of Legionella, while the two bottom rows had another strain. The first and third (from the top) row had eukaryotic cells in addition to Legionellae, while the second and fourth row had only Legionellae. The first three columns (from left) had dilutions of 10^3 cells of legionella, while the three last columns had 10^4 cells.

2.2.4 Plating and counting

Initial aliquots of *Legionella* used in co-culture were presumed to be $1x10^3$ and $1x10^4$ (from section 2.2.3). To count the actual number of CFU/ml added to the co-culture, these concentrations were measured on the day of the inoculation using plate count. Both of the aliquots added to co-culture ($1x10^3$ and $1x10^4$) were diluted 10 and 10^2 times, respectively, before plating out. When plating, 10µl from each dilution ($1x10^4$ - $1x10^2$) was transferred to BCYE agar plates in duplicate, making the presumed count to be 10- 1000 CFUs on each plate. The number of colonies were counted 5 days after plating, and controlled again after 10 days. Samples taken from co-culture after 1 day, , was diluted 10, 10^2, and 10^3 times, and plated out in duplicate, while samples taken after 4-6 days were diluted up to 10^6 before plating dilutions 10-10^6.

2.3 DNA extraction

DNA from the following strains were isolated using DNeasy® blood and tissue kit (Qiagen), according to Qiagens protocol (described in appendix C): *L. pn.* SG 1 and SG 2-14, *L. micdadei, L. gormani, L. spp, L. pn.* Bloomington, *L. pn.* ST-15 (2005), *L. pn.* ST-462 (2008), *L. pn.* Colitax and *L. pn.* Philadelphia. Frozen isolates of the *Legionella* strains were plated on BCYE agar and incubated for 48 hours before applying the DNA extraction kit protocol.

DNA from ATCC strains 103856, 103858, 103859, 103860, 103861, 103862, 103863 and 103864, along with B11-A3 from a biological treatment plant, was received already isolated (by boiling) from lab personnel.

For cells harvested after co-culture, boiling isolates were made. A small scoop of cells from growth in macrophages was added to 500 µl distilled H_2O (Life Technologies AS, Oslo, Norway), heated and stirred on an eppendorf thermomixer® comfort (at 99°C for 10 minutes at 1400 rpm) to extract DNA. Then, samples were cooled down in room temperature and centrifuged at 10,000 rpm to spin down cell debris. Supernatant was used in PCR analyses.

2.4 Real-time PCR

DNA isolates of all strains used in this survey were run on Real-time PCR in triplicates, with primers specific to various virulence genes in the *Legionella* genome.

Real-time PCR gives a view of the amplification *in situ* by measuring melting temperature or the binding of probes to the amplicon (amplification product), using fluorescence signals (Edwards et al. 2004). It also gives quantification of the accumulation of product between each of the amplification cycles.

Real-time PCR was performed by LightCycler 480 (Roche) real-time thermal cycler PCR machine (figure 2.2), with its compliant SYBR Green I fluorescent Master Mix dye. SYBR Green I become fluorescent when binding to double stranded (ds) DNA in the minor groove on the DNA double helix (Edwards et al. 2004).

Figure 2.2 LightCycler 480 machine and the multiwell plate 96 © 1996-2015 Roche Diagnostics (lifescience.roche.com).

2.4.1 PCR setup

PCR reaction mixtures were made using SYBR Green I Master mix, 6 different primer sets (given in chapter 2.4.3), DNA template and distilled water. The composition of the reaction mixture is presented in table 2.1. The tests were run in triplicates. Control tests were made with the reaction mixture, without the addition of DNA template, to also test for contamination of the primer sets. Added in each well on the PCR multi well plate 96 (Roche) plate was 20 µl, made from the recipe in the following table. The negative control samples contained 19 µl.

Table 2.1: PCR reaction mix for real-time PCR on LightCycler 480 (Roche)

Reagent	Volume (µl)
SYBR Green I Master Mix (Roche) [a]	10
Primer 5µM [b]	4
dH_2O	5
DNA template	1

a: SYBR Green I master mix contains Taq DNA polymerase, reaction buffer, dNTP mix, SYBR Green 1 fluorescent and $MgCl_2$

b: 5µM: made from 100µM 20µl forward + 20µl reverse primer, with 360µl H_2O

2.4.2 PCR amplification

Strains were controlled using *Legionella* spp. specific primers in qPCR after co-culture. PCR amplification of the DNA in the reaction mixtures were performed by LightCycler480® according to the program given in table 2.2. The PCR program used was identical for all primers and *Legionella* strains. Denaturing was set to 95°C for 5 min, followed by 35 cycles of the amplification program in table 2.1.

Table 2.2: PCR amplification program for *Legionella* genome on LightCycler 480 (Roche)

Denaturing		**Primer annealing**		**Extension**	
Temp (°C)	Time (s)	Temp (°C)	Time (s)	Temp (°C)	Time (s)
95	**10**	**58**	**15**	**72**	**30**

Amplification of a gene was analyzed by collecting data on melting curves and crossing point/threshold cycle, and comparing the results. Melting curves and crossing point were examined on LightCycler 480 software 1.5.1. Fluorescence was measured between 483-533 nm of wavelengths.

CP (crossing point) is a measure of quantification and refers to the point where the fluorescence signal from the PCR product exceeds the background fluorescence, and the amplified product first gets visible in the data (MacKay 2007). A low CP indicates a positive result, and shows the cycle where the exponential growth starts. If it starts after, or close to 35 cycles, it could be a contamination in the reaction mixture, and the PCR should be regarded as negative.

Melting point is usually measured at the end of PCR amplification, and represents that temperature at which 50% of the double stranded DNA has melted apart (when using dsDNA specific dye) (Edwards et al. 2004; MacKay 2007). Diverging melting points are mainly due to GC (guanine-cytocine) -content and –distribution, and partly to product length and mismatches. A very wide melting point curve indicates a blend of different products. These factors reflect divergence in the sequences, and results were counted as negative. All melting point curves were compared one by one. This is shown in figure 2.3. The positive results in figure 2.3 has a melting point temperature/peak around 81-82 °C (marked by the yellow rings). The rest of the samples were negative in this experiment.

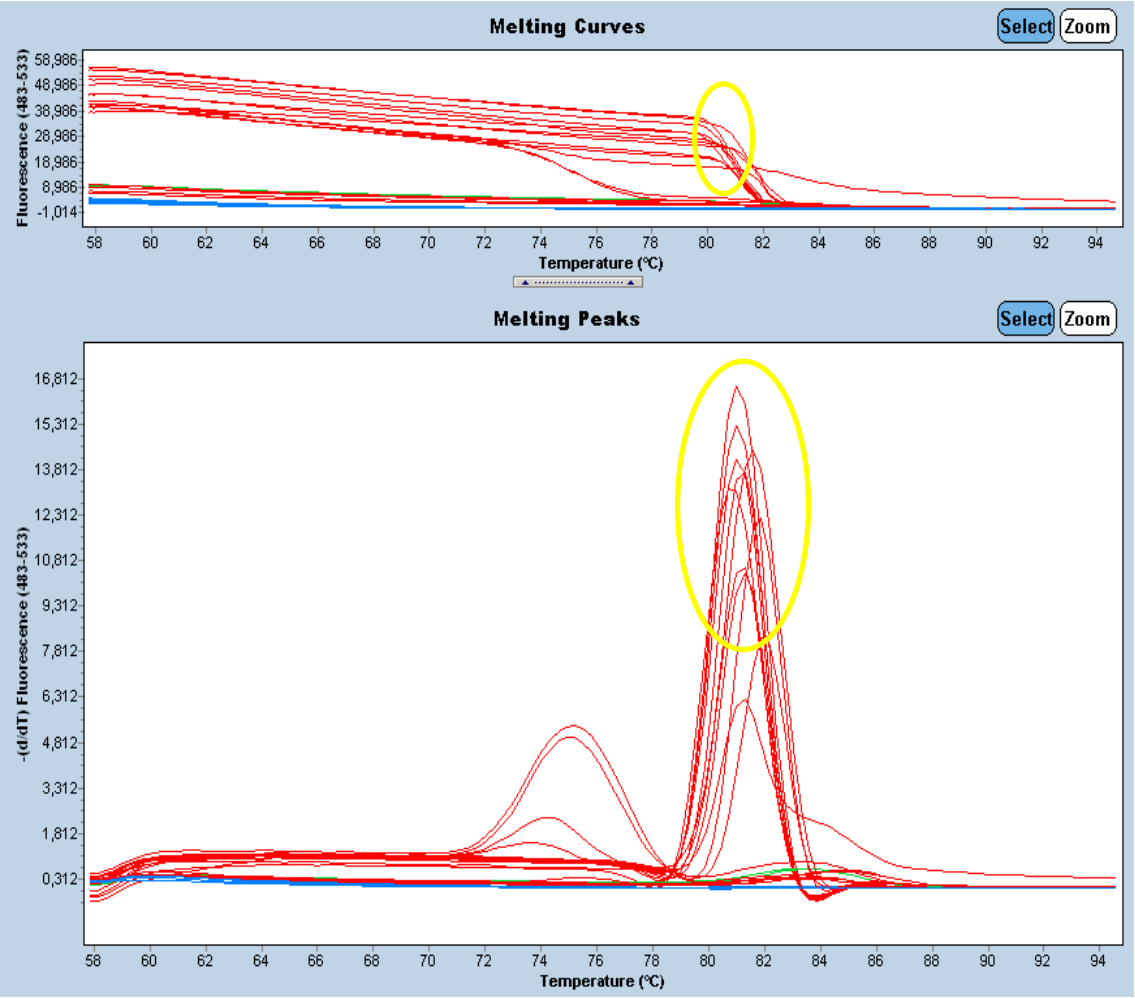

Figure 2.3: T_M calling on LightCycler 480® software 1.5.1 presented as graphs of melting temperature and melting peaks of the ds DNA. In this figure, the lvh1 and lvh2 primers are used on the different strains of *Legionella*. Yellow outlines mark the positive results of the test.

During melting point analyses, the temperature was raised from 55°C to 95°C by 0.1 °C/s. CP data using the 2nd derivative method values were calculated by Light Cycler 480® software.

2.4.3 Primers

The 6 sets of forward and reverse primers of JFP/JRP, rtx, lvh, dot, mip and hsp, (Invitrogen™) listed in table 2.3 were run on all DNA samples in triplicates. Negative samples were included in each run for control.

Table 2.3: List of the primers used in this survey, its target gene and nucleotide sequence.

Primer	Target gene	Sequence (5'-3')	Reference
JFP	16S rRNA	CCAACAGCTAGTTGACATCG	Jonas et al. 1995
JRP	16S rRNA	AGGGTTGATAGGTTAAGAGC	Jonas et al. 1995
lvh1	*lvhB3*	ATTGGGAGCTTCTGGCAATA	Tachibana et al. 2013
lvh2	*lvhB3*	GCTGGGGTGACCTTTGAATA	Tachibana et al. 2013
rtx1	*rtxA*	GCTGCAACCACCTCTTTGAT	Tachibana et al. 2013
rtx2	*rtxA*	CAGGGGCTGGTTATGTTGAT	Tachibana et al. 2013
dot1	*dotA*	CAAATCCGGCATTCAAAATC	Tachibana et al. 2013
dot2	*dotA*	CTATTGTCGCCTTGGGTGTT	Tachibana et al. 2013
hsp1	*hsp60*	GCGAATCGTTGTTACCAAAGAAAAC	Huang et al. 2006
hsp2	*hsp60*	CAATTTGACGCATTGGAGATTCAATAG	Huang et al. 2006
mip1	*mip*	GGTGACTGCGGCTGTTATGG	Jaulhac et al. 1992
mip2	*mip*	GGCCAATAGGTCCGCCAACG	Jaulhac et al. 1992

2.5 Microscopy

Samples from co-culture experiments were studied in microscope after 24 hrs, 48 hrs, 72 hrs, and 4 days of incubation. A Carl Zeiss axioskop 2 plus (Carl Zeiss Microscopy, Thornwood, NY, USA) was used to examine samples at 40x and 100x magnification. Amoeba and macrophages with and without Legionella- containing vacuoles were searched for, and captured on camera. Software used for photographing was Axio Vision SE64 Rel. 4.9.1 (Carl Zeiss).

2.6 Statistical analysis

Data representing means of duplicates from observations of replication in amoebae and macrophages were presented in line charts, with the increase or decline of CFUs displayed with logarithmic values. Statistical analysis using unpaired, two-tailed Student t-tests specified the significance of the data in co-culture experiments. Differences were considered significant when $p<0.05$. PCR results are expressed as column charts of means of triplicates. Error bars represent standard deviations.

3 Results

3.1 Intracellular replication in eukaryotes

The connection between *Legionella*'s infectivity towards amoebae and macrophages is unclear. To investigate the ability of ten different strains of *Legionella* to replicate inside eukaryotes, they were grown separately in co-cultures with amoebae and macrophages. Equal concentrations of bacteria were added at a low MOI to similar concentrations of amoebae and macrophages and incubated. In order to compare the number of CFUs in the wells before and after incubation, dilutions of the co-cultures were plated out for colony counts before and after incubation in co-culture. When counting the number of *Legionella* colonies on the plates, dilutions with 10-100 CFUs were selected for counting. The counts were multiplied with number of dilutions to calculate the number of CFUs in the sample.

3.1.1 Replication in amoeba

The following strains of Legionellae were tested separately for their ability to grow in co-culture with amoebae in PAS buffer:

- *L. gormani, L.spp, L. pn.*Colitax, *L. pn* Philadelphia, *L. micdadei, L. pn.* Sg1, *L. pn.* Sg 2-14, *L. pn.* Bloomington, ST-462 and ST-15.

Comparing the number of CFUs of Legionella from before inoculation, and 6 days post inoculation with amoebae showed that some strains had an increasing number of CFUs during the 6 days of incubation, while others had a decline. Number of CFUs of the *Legionella* strains in co-culture with amoeba, during 6 days of incubation, is displayed in figure 3.1. As seen in the figure, *L. gormani, L.spp, L. pn.*Colitax and *L. pn* Philadelphia showed no sign of growth in amoeba (data available in appendix B). *L. gormani* had a decreased number of CFUs/ml after 6 days, while the three other no-growth- strains did not show any CFUs/ml at all after 6 days of incubation, indicating poor survival in co-culture with amoebae.

L. micdadei, L. pn. Sg1, *L. pn.* Sg 2-14, *L. pn.* Bloomington, ST-462 and ST-15 all showed growth during co-culture, and after 6 days the number of CFU/ml had increased by approximately 4 log-units. *L. pn* Bloomington is well known for its infectivity on these amoebae, and was used as control in all experiments, always showing growth in co-culture. When the same experiment was repeated later *L. pn.* Colitax did show growth in co-culture with amoeba, number of CFUs presented in Appendix B.

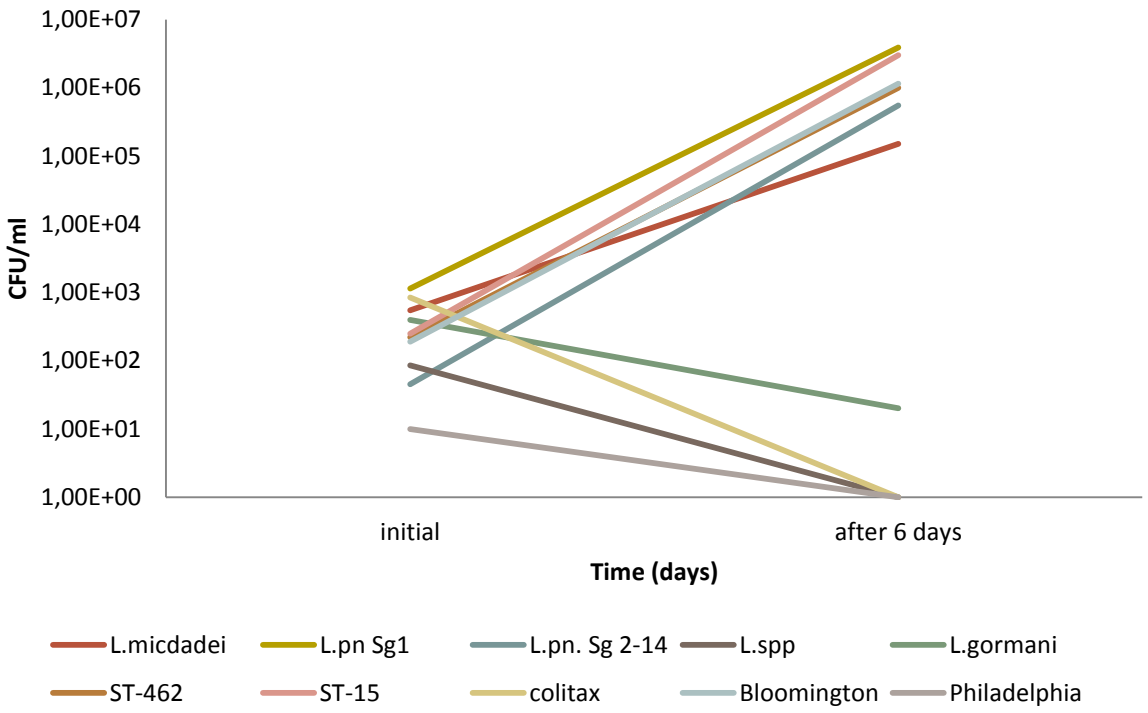

Figure 3.1: Diagram of bacteria concentration pre and post co-culture with amoeba. Y-axis shows logarithmic scale of number of CFUs/ml in solution. X-axis shows time-lapse from the day of co-cultivating to 6 days later. Labels of isolates of *Legionella* are listed at the bottom of the figure. The values represent the average of duplicate sets of data from one set of experiment. Experiments were repeated later for validation of results (not presented in diagram). The two sets of experiments were run separated in time but identical in method. See appendix B for data and standard deviations.

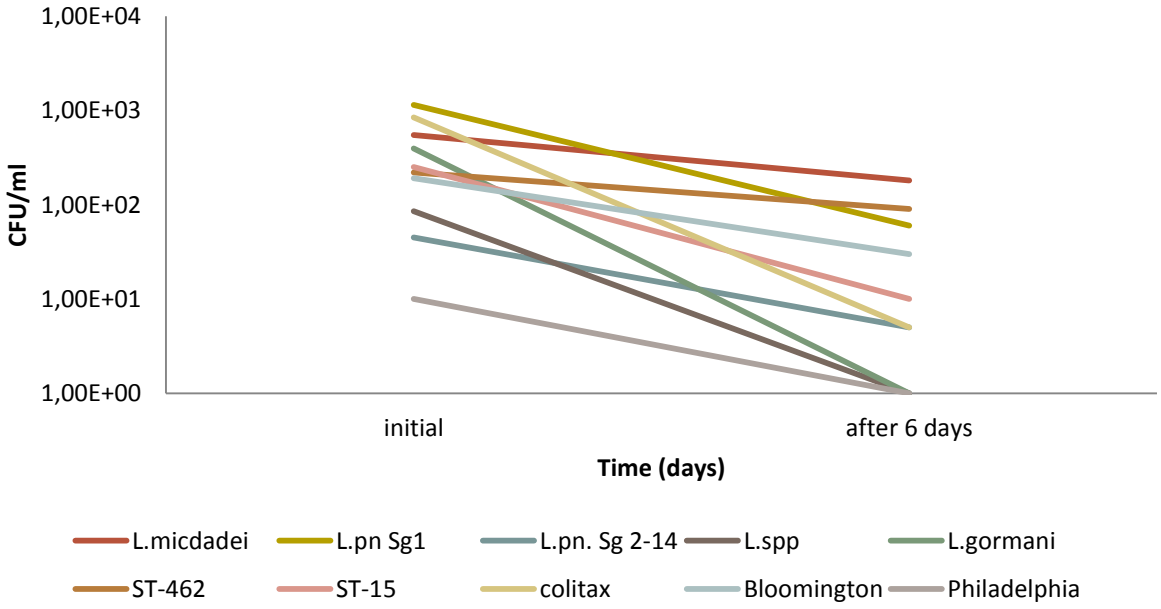

Figure 3.2: Control experiments for co-cultivation studies. No amoebae were added in cultivation wells, only *Legionella* strains in PAS buffer. Y-axis shows logarithmic scale of number of CFUs/ml in solution. X-axis shows time lapse from pre incubation to 6 days post incubation. Labels of isolates of *Legionella* are listed at the bottom of the chart. See appendix C for data and standard deviation.

Parallel control analyses of *Legionella* strains in PAS buffer without amoebae showed the opposite tendency; all isolates had a decreasing number of CFU/ml during incubation for 6 days. Figure 3.2 illustrate the bacteria's poor sustenance in PAS buffer, and shows how all ten strains had a decline in number of CFU's during the incubation time. The strains that infected and multiplied in amoeba also showed better survival in PAS buffer without amoeba than the non-infective strains.

The difference between cell counts before and after incubation for the strains showing replication in amoebae, was counted as significant (p=0.023). Between the cell counts before and after incubation of strains not replicating in amoeba, the difference was insignificant (p=0.18). The difference between the strains that did show growth in co-culture and the ones that did not replicate in amoeba, was significant (p=0.023). For the strains showing growth in amoeba, the difference between growth in co-culture, and only PAS buffer was also significant (p=0.023).

To combine and compare the data in figure 3.1 and 3.2 in one chart, figure 3.3 was made. Displayed in figure 3.3 is both co-cultivated Legionellae with amoeba, and control measurements of *Legionella* strains in PAS buffer before and after 6 days of incubation. The initial concentration in each *Legionella* strain is identical for the co-culture and control samples, so this figure illustrates difference in growth or decline in number of CFUs post incubation. Strains incubated both with and without amoeba in PAS buffer are represented, and placed next to each other in the chart, labeled w/wo amoeba. The difference in height between the red and yellow bars next to each other shows difference in number of CFUs pre and post incubation. The strains with the highest yellow bars, *L. micdadei, L. pn.* SG 1 and SG 2-14, ST-462, ST-15 and Bloomington grew well in amoeba, while the strains with yellow bars lower than the red bars, *L.* spp., *L. gormani,* Colitax and Philadelphia had a decline in number of colony-forming cells during the co-culture incubation period. The samples with no yellow bars had no CFUs 6 days post incubation. The trend in these data is that the more invasive strains were also better at survival in PAS buffer alone (except for *L. pn.* SG 2-14).

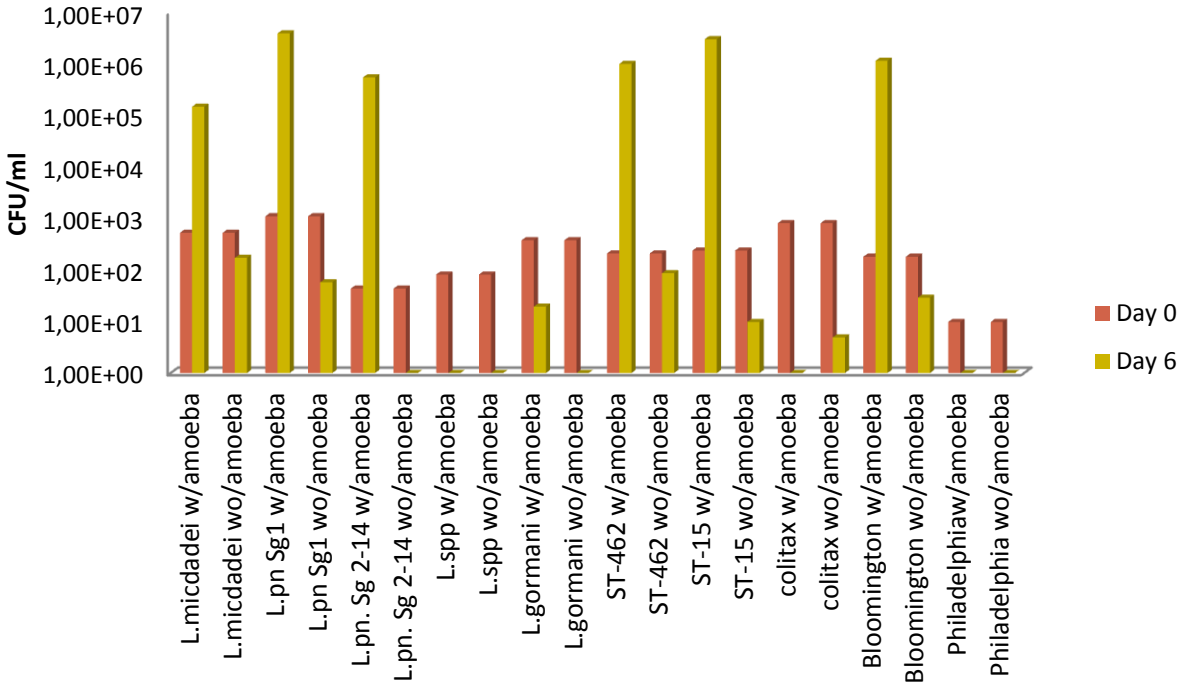

Figure 3.3: Columns indicate number of CFU/ml before and after 6 days of co-culture of Legionellae with amoebae. Data includes all strains, incubated with and without amoeba. Y-axis show logarithmic scale of number of CFU/ml. Red bars represent number of CFUs before incubation, and yellow bars represent number of CFUs after co-culture. The different strains, with and without amoebae, are listed on the x-axis.

Samples were examined under microscope during the incubation period. "*Legionella*-containing-vacuoles" were searched for, to support the theory that *Legionella* grows in vacuoles inside the amoeba, and not from the nutrients excreted by lysed amoeba cells. Several of the strains that showed growth in co-culture with amoeba could be seen in LCVs in the amoeba after 2-3 days of incubation. Amoebae were found in different shapes and sizes in the microscope, shown in figure 3.4. Amoebae with LCVs are presented in figure 3.5. There were no LCVs found in the amoeba cultured with the non-invasive strains.

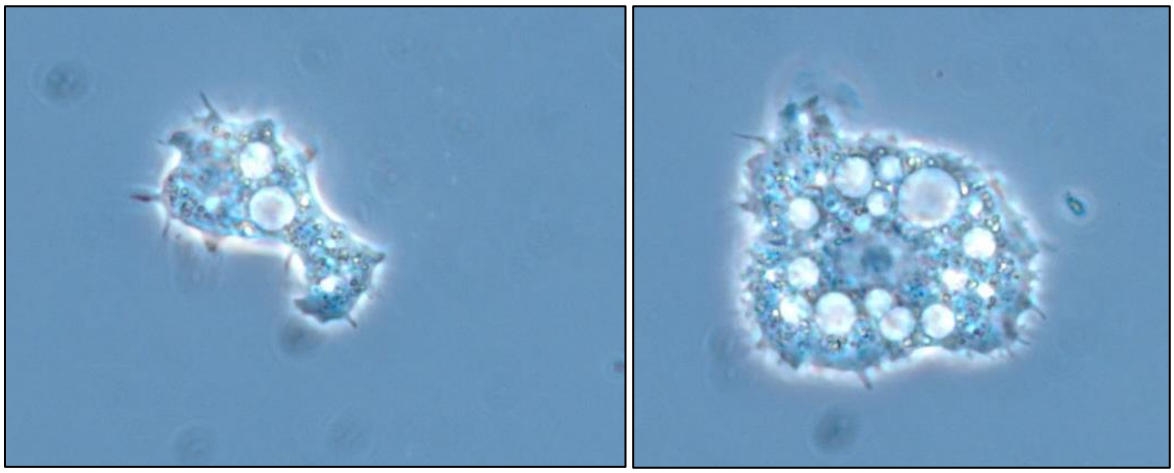

Figure 3.4: Pictures of free living amoeba viewed in microscope at 100x magnification, taken 1 day post co-culture.

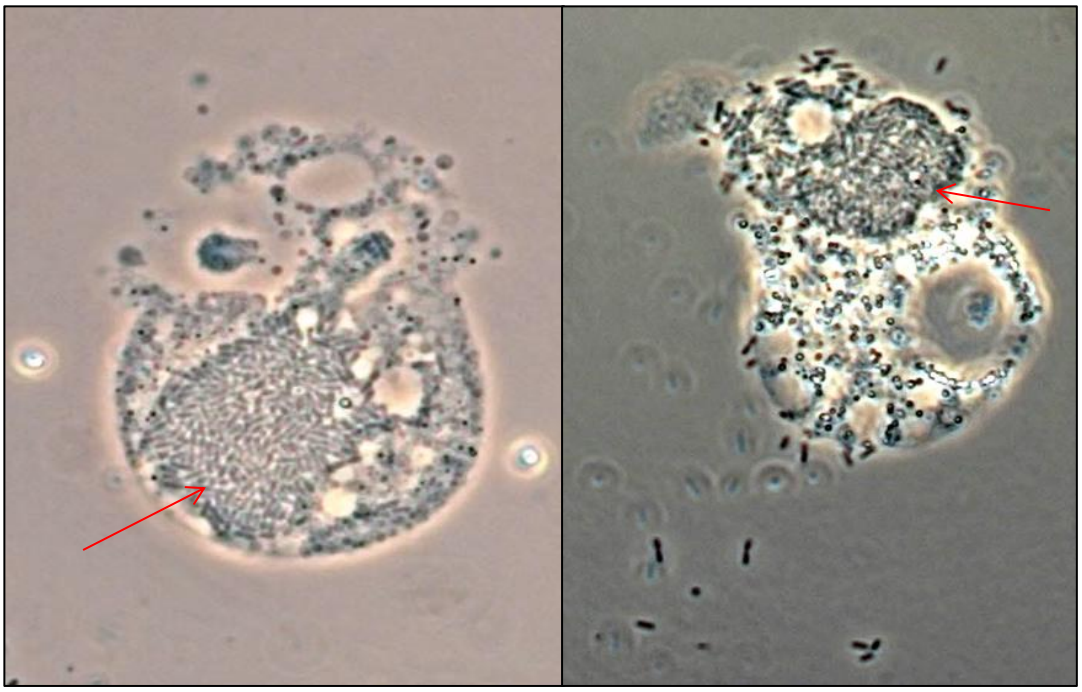

Figure 3.5: Pictures of amoeba with Legionellae in vacuole, taken by Tone Aarskaug, FFI. The *Legionella*-containing vacuole is the big compartment with many grey rod-shaped structures (pointed at by the red arrows). The amoeba on the left is ruptured by the bacteria, and cell material is starting to leak out.

3.1.2 Replication in macrophages

The *Legionella* strains examined for replication in amoebae were examined for their ability to replicate in macrophages also. All ten *Legionella* strains were added separately to cultures of macrophages in RPMI medium. Number of CFUs was measured before co-culture, 1 day post-, and 4 days post incubation. Each dilution was plated out in duplicate and counted, to calculate concentration of CFU/ml. Results of the cell counts are presented in figure 3.6. Negative controls of *Legionella* cultured in RPMI medium without macrophages were treated in parallel, and is presented in figure 3.7.

ST-15 and ST-462 showed increased growth in the first set-up of the experiment. LCV's were also observed in the microscope during the incubation period. PCR analyses of the growth on BCYE plates after 4 days confirmed the species as *Legionella*. However, when used as positive controls in later experiments, ST-15 and ST-462 did not show any growth. This observation is interesting, since the same method was used in every set up in the experiment. Another observation is that the strains incubated in co-culture had a better rate of survival (higher number of CFUs) after 4 days of incubation than the same strains incubated in RPMI medium alone. The strains growing intracellularly in the first experiment (ST-15(1) and ST-462(1)) had a much better survival in RPMI medium alone, than the same strains in the next experiment (ST-15 and ST-462) where they were unable of intracellular replication.

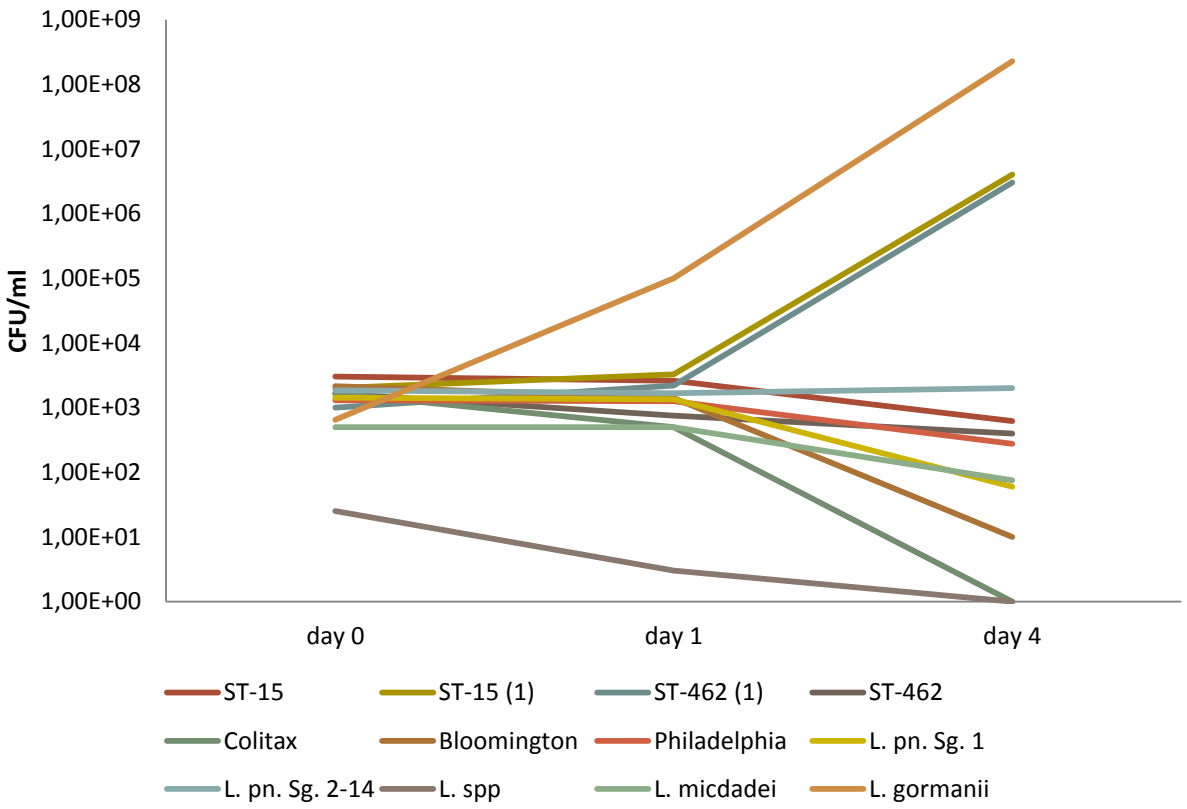

Figure 3.6: Logarithmic scale of number of CFUs (y-axis) after 1 and 4 days in time (x-axis) in co-culture of *Legionella* with macrophages in RPMI medium. Experiments were run two times each, and plated out in duplicate both times. ST-15 and ST-462 reported contradictory results in the two runs, therefore presented twice. Presented in the graphs are the mean of duplicate values of cell counts. Data, including standard deviation is given in Appendix C.

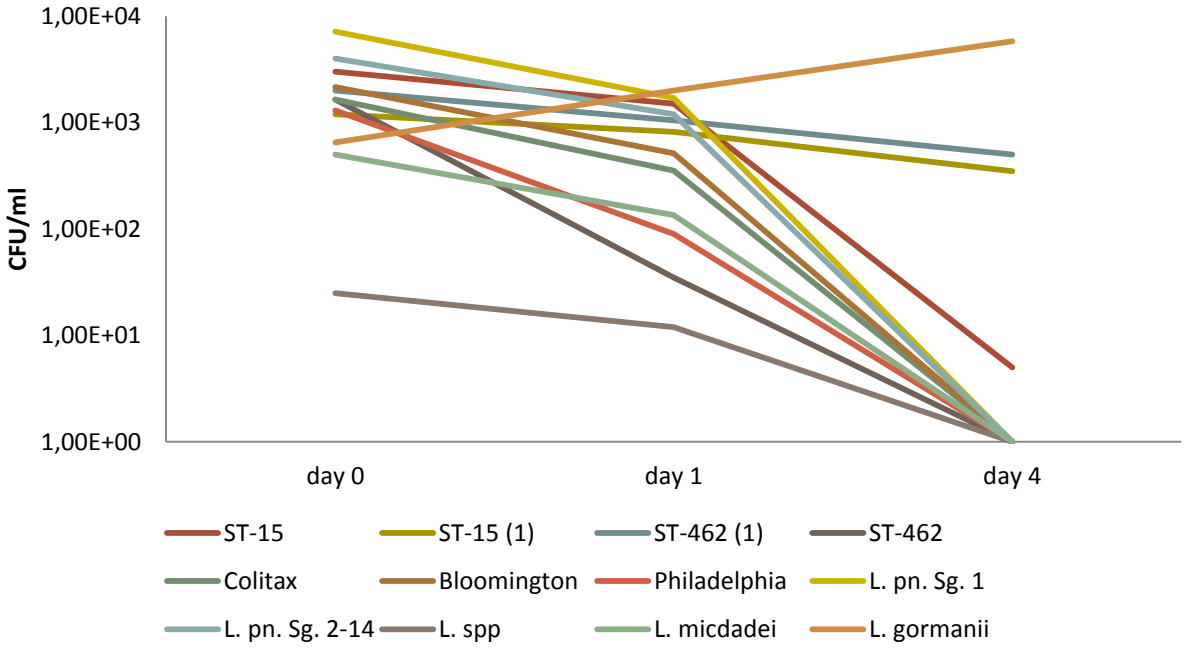

Figure 3.7: Logarithmic scale of number of CFUs (y-axis) after 1 and 4 days in time (x-axis) in negative control with *Legionella* in RPMI medium, in absence of macrophages. Experiments were run two times each, and plated out in duplicate both times. ST-15 and ST-462 reported contradictory results in the two runs, therefore presented twice.

The strain labeled *L. gormanii* used in this survey had an early exponential growth in macrophages. In microscope investigations, no LCV's were found. What was observed, was a rapid growth of the bacteria, a visibly opaque solution in the wells, and lysed macrophages, after only one day of incubation in co-culture. When controlled with JFP/JRP primer set in PCR, it was clear that this strain was not a *Legionella* species, as it gave no signal in real-time PCR analyses with the *Legionella*-specific primers. Samples were then run on MALDI-TOF MS (matrix-assisted laser desorption/ionization – time-of-flight - mass spectrometry, described in appendix E), and identified as *Staphylococcus cohnii*, with a score value of up to 1.76.

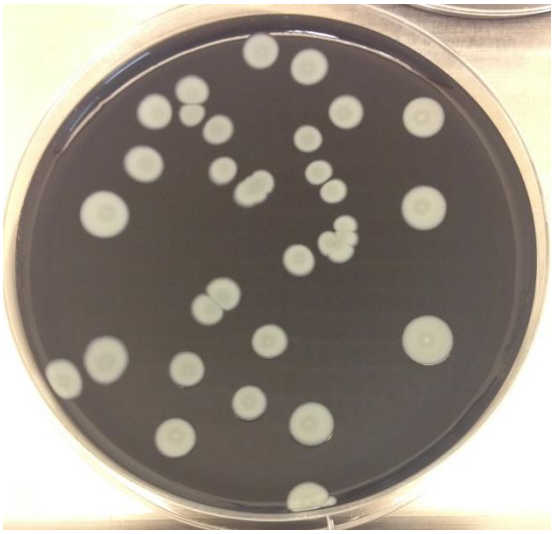

Figure 3.8: Growth of *S. cohnii* on BCYE after co-culture with macrophages, mistaken for *L. gormanii*. The colonies are photographed after 4 days of incubation.

3.1.3 Comparing replication in amoebae and macrophages

The strains that were able to grow inside the amoebae did not necessarily grow in macrophages as well. All strains were tested twice for growth in macrophages. Only two of the strains (ST-15 and ST-462) were able to grow in macrophages. However, the experiments could not be repeated, as the strains did not grow in the second experiment. *L. gormanii* isolates were contaminated with *S. cohnii*, and can therefore not be determined due to these test results. Table 3.1 shows an overview of which strains that were able to conduct growth in each of the two eucarya, according to these experiments.

Table 3.1: Analysis of ten different strains of *Legionella* tested for their ability to infect and grow inside of amoebae and macrophages in co-cultivation.

Strain	Growth in amoeba	Growth in macrophages
***L. pneumophila* serogroup 1**	Yes	No
***L. pneumophila* serogroup 2-14**	Yes	No
K1 *Legionella micdadei*	Yes	No
Legionella gormani* 7 pathogenic	(No)	(No)
***Legionella* spp**	No	No
***L. pn.* Bloomington**	Yes	No
***L. pn.* ST-15**	Yes	Yes/No
***L. pn.* ST-462**	Yes	Yes/No
***L. pn.* Colitax**	No	No
***L. pn.* Philadelphia**	No	No

strain later identified as *Staphylococcus cohnii.

3.2 Real- Time PCR detection of virulence genes

Several virulence genes are defined as important in *Legionella*'s ability to survive and replicate in eukaryotes. Detection of five such genes was performed by polymerase chain reaction analyses on all the ten strains used in co-culture experiments. DNA isolates from 9 additional strains were also tested. Table 3.2 lists a summary of the findings after detection of toxicity genes connected to *Legionella*'s infective traits. The presence of each gene in each strain is highly variable. For example, Bloomington, Philadelphia and Colitax tested positive for every gene, while *L. micdadei* only tested positive for one of the genes.

Table 3.2: Presence (+) or absence (-) of different genes connected to toxicity in each strain of *Legionella*, after quantitative PCR analyses. Results in parentheses are uncertain.

SG	Strain	JFP/JRP	mip	LvhB3	dotA	rtxA	hsp60
1	ST-15	+	+	-	+	(+)	+
1	ST-462	+	+	-	+	(+)	+
1	*L. pn.*	+	+	-	+	+	+
1	Philadelphia	+	+	+	+	+	+
2	103856	+	+	-	+	+	(+)
3	Bloomington	+	+	+	+	+	+
4	103858	+	+	+	+	-	-
5	103859	+	-	-	+	-	-
6	103860	+	+	+	+	+	(+)
7	103861	+	+	+	+	+	(+)
8	103862	+	+	(+)	+	+	(+)
9	103863	+	+	+	+	+	(+)
10	103864	+	+	+	+	+	(+)
2-14	*L. pn.*	+	+	-	+	+	+
2-14	Colitax	+	+	+	+	+	+
n/a	*L. pn.* B11-A3	+	+	-	+	+	(+)
	L. spp.	+	-	(+)	(-)	-	-
	L. gormanii	+	-	-	(+)	(+)	(+)
	L. micdadei	+	-	+	-	-	-
	Total	**19/19**	**15/19**	**11/19**	**17/19**	**15/19**	**15/19**

Presented in figures 3.9-3.14 are the quantitative CP and qualitative T_M results from the PCR analyses. The findings in the following sections 3.2.1 through 3.2.6 are summarized and presented in table 3.2.

In some occasions, the results from CP and T_M do not correspond well to one another, and the assessment of possession of the gene is less reliable. The results will then be counted as negative. Melting point curves are complex, and were compared to each other one by one on LightCycler 480 software. Because of intricate displaying, the curves are not presented here. Presented in the following sections are the temperatures at which the melting point curves peaked.

3.2.1 *Legionella*- specific 16*S* rRNA gene

To lay the foundations for the PCR investigations, all DNA isolates of the different strains were run in PCR with the JFP/JRP primers targeting a section of *Legionella*'s 16S rRNA gene. This gene is common for all strains of Legionellae, and the JRF/JRP primer ought to differentiate between *Legionella* and non- *Legionella* DNA. Looking at figure 3.9, it is necessary to assume that all results below 35 cycles of amplification (COA) indicate positive results, although a few strains were amplified at a later cycle. Because this specific gene should be present in equal amount in all the strains, the site of exponential growth (CP) of the different strains in this chart was used as reference regarding the DNA concentration in each isolate. The strains showing an early CP value had a high concentration of DNA, while a late CP value indicates a low concentration of DNA isolate. ATCC strains 103859 and 103862, Bloomington and *L.* spp. had a lower DNA concentration than the rest of the strains. The negative control deviated from the rest in T_M measurements, being significantly lower, and was counted as the only negative result.

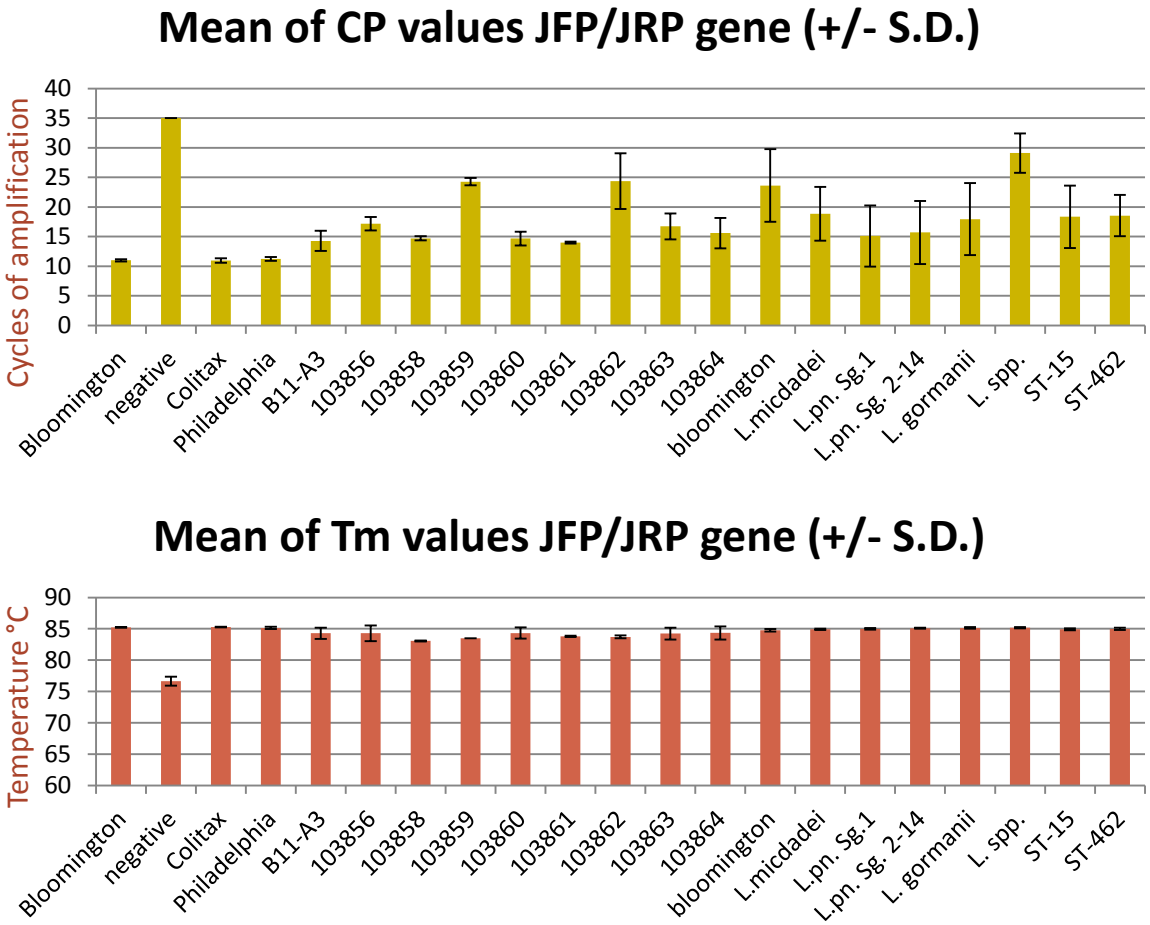

Figure 3.9: Cycle of exponential growth (CP) and melting temperature (T_M) results from PCR of all strains with JFP/JRP Legionella specific primer. The bars show mean values of results from experiments run in triplicate, with the standard deviation presented in error bars.

3.2.2 *Legionella pneumophila* specific gene *mip*

The *mip* gene distinguishes *L. pneumophila* from other *Legionella* species, but is also important for the species' survival in eukaryotic cells. Amplification in PCR with the *L. pneumophila*- specific *mip* primers was performed on all strains, and results are presented in figure 3.10. The average T_M value of the 103859 ATCC strain was greatly divergent from the norm, and had to be considered negative for the *mip* gene. According to CP values, *L. micdadei*, *L. gormanii*, *L.* spp and ATCC 103859 had a crossing point too late in the PCR cycles, and were therefore negative for this gene. Some of the strains had a much later exponential growth than the others, but was counted as positive. Bloomington, Colitax, Philadelphia, *L. pn* SG 1 and SG 2-14, ST-15, ST-462, B11-A3, and ATCC strains 103856, 103858, 103860, 103861, 103862, 103863 and 103864 were regarded as positives.

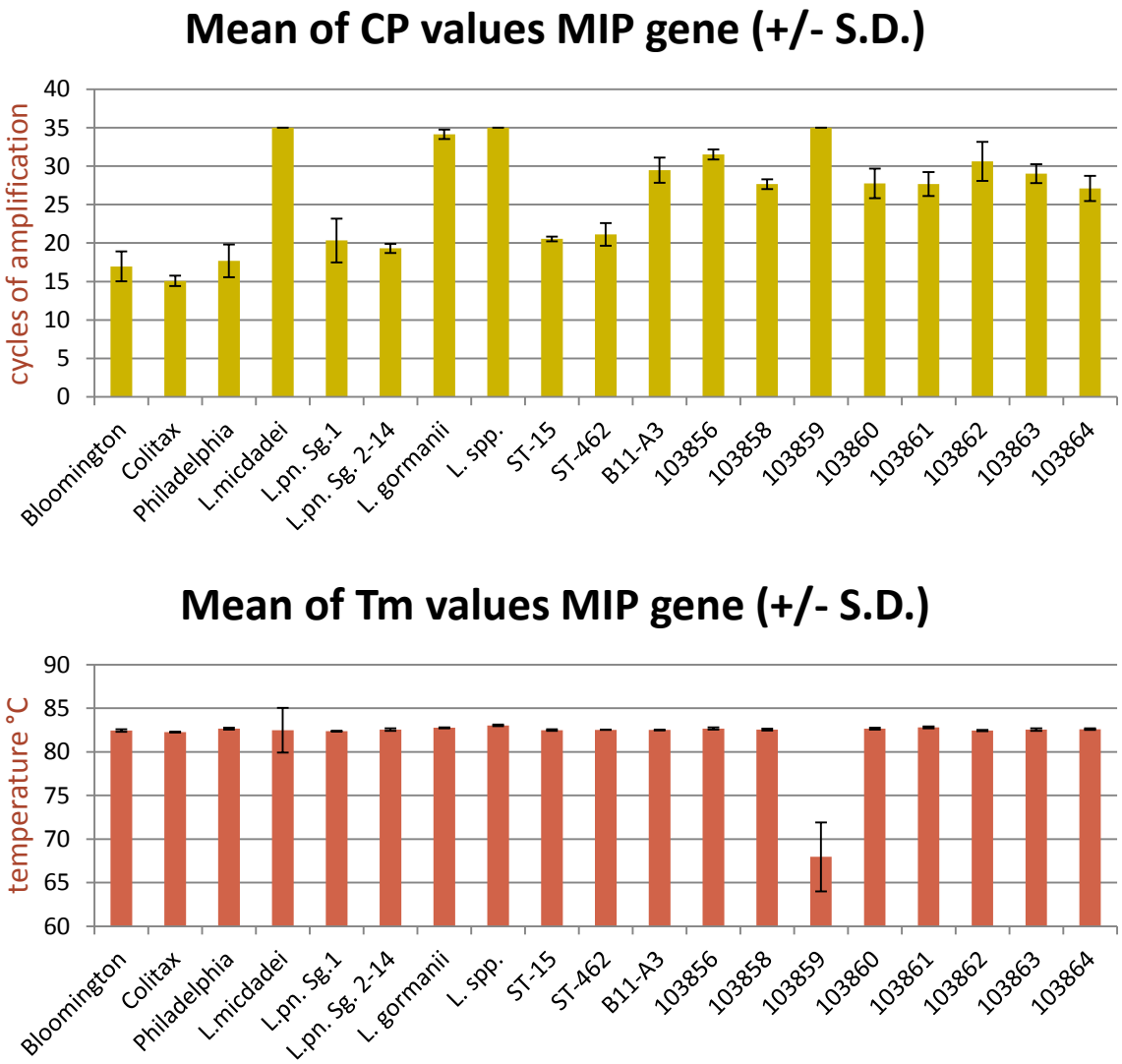

Figure 3.10: Cycle of exponential growth (CP) and melting temperature (T_M) results from PCR of all strains with *mip* *L. pneumophila* specific primer. The bars show mean values of results from experiments run in triplicate, with the standard deviation presented in error bars.

3.2.3 Virulence gene *lvh*

The *lvh* gene is involved in *Legionella*'s type IV secretion system, and is regarded as a toxigenic trait. Figure 3.11 displays CP and T_M values of PCR results of the amplification of the *lvh* gene. CP measurements indicated that *L. pn.* Sg. 1, *L. pn.*sg. 2-14, ST-15, ST-462, B11-A3, ATCC 103856 and 103859 were negative for this gene. *L. gormani* could be considered positive, as its CP value was close to 35. The doubt made it count as a negative for this gene. The CP signals for *L.* spp. and ATCC 103862 were late, but when seeing their late signal from the JFP/JRP amplification in section 3.2.1 also, it is possible that it was due to low concentration of DNA in the sample. T_M measurements supported the negativity of *L. pn.* Sg. 1, ST-15, ST-462 and 103856, as their values were above or below the average melting point. Bloomington, Colitax, Philadelphia, *L. micdadei*, *L.* spp, 103858, 103860, 103861, 103862, 103863 and 103864 were counted as positives.

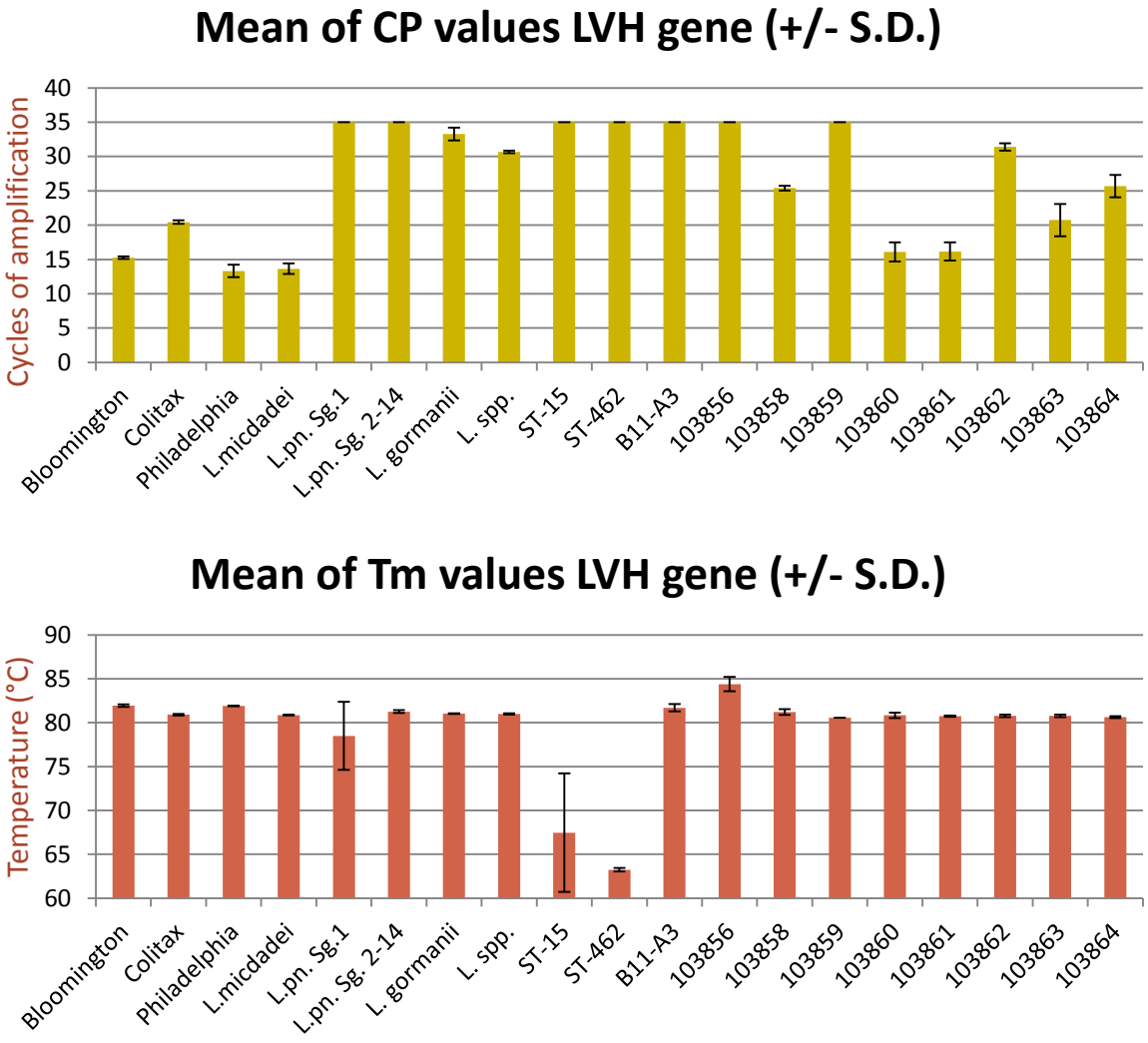

Figure 3.11: Cycle of exponential growth (CP) and melting temperature (T_M) results from PCR of all strains with lvh primers. The bars show mean values of results from experiments run in triplicate, with the standard deviation presented in error bars.

3.2.4 Virulence gene *dot*

The role of the *dot* gene is involved in *Legionella*'s type IV secretion system, and was explained in section 1.1.8. This gene is also related to virulence in *Legionella* strains. The PCR results are depicted in figure 3.12. According to the measured CP values of the *dot* gene, all strains except *L. micdadei* and *L.* spp. were presumably positive for the gene. *L.* spp. could be considered positive, because of late amplification, but possibly low concentrations of DNA, presumed in section 3.2.1. It is therefore an uncertain negative result. *L. gormani* could be considered negative, as it had a very late amplification crossing point compared to concentration of DNA, and it is considered an uncertain positive. Judging from the T_M measurements, all strains had similar melting point properties.

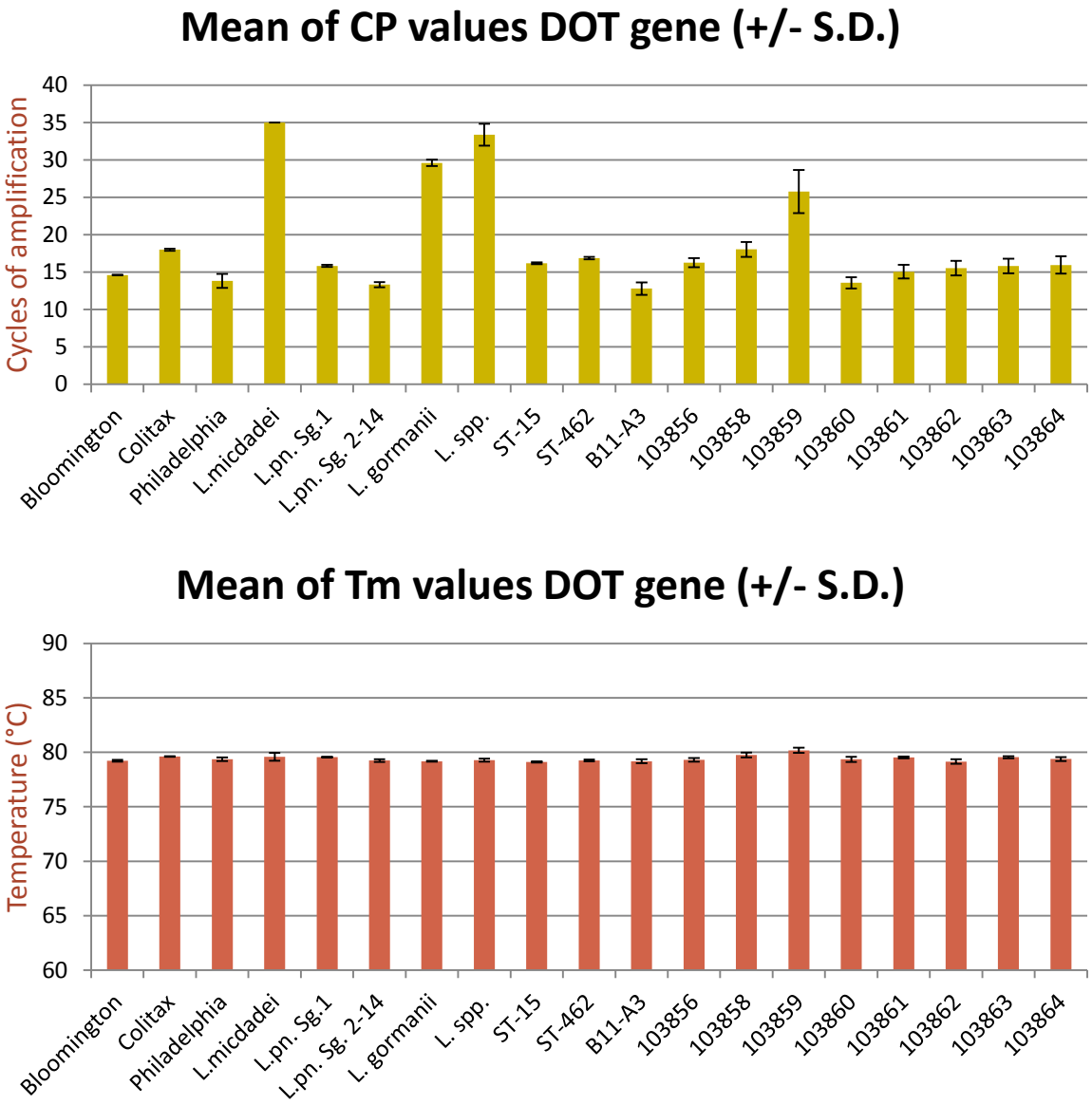

Figure 3.12: Cycle of exponential growth (CP) and melting temperature (T_M) results from PCR of all strains with dot primers. The bars show mean values of results from experiments run in triplicate, with the standard deviation presented in error bars.

3.2.5 Virulence gene *rtx*

The *rtx* gene is connected to the pathogen's ability to adhere to the host cell, as well as other virulence traits in *Legionella*'s parasitic life cycle, explained in section 1.1.8. Referring to figure 3.13, which shows CP values pointing out the absence of the gene in the ATCC strains 103858 and 103859, as well as *L. micdadei*, and *L.* spp. *L. gormani*, ST-15 and ST-462 had a very late CP, which cannot be explained by low DNA concentrations. They were accordingly counted as uncertain positives. The mean values of T_M measurements vaguely supported negativity of gene presence in *L. micdadei*. All of the remaining strains were regarded as positives.

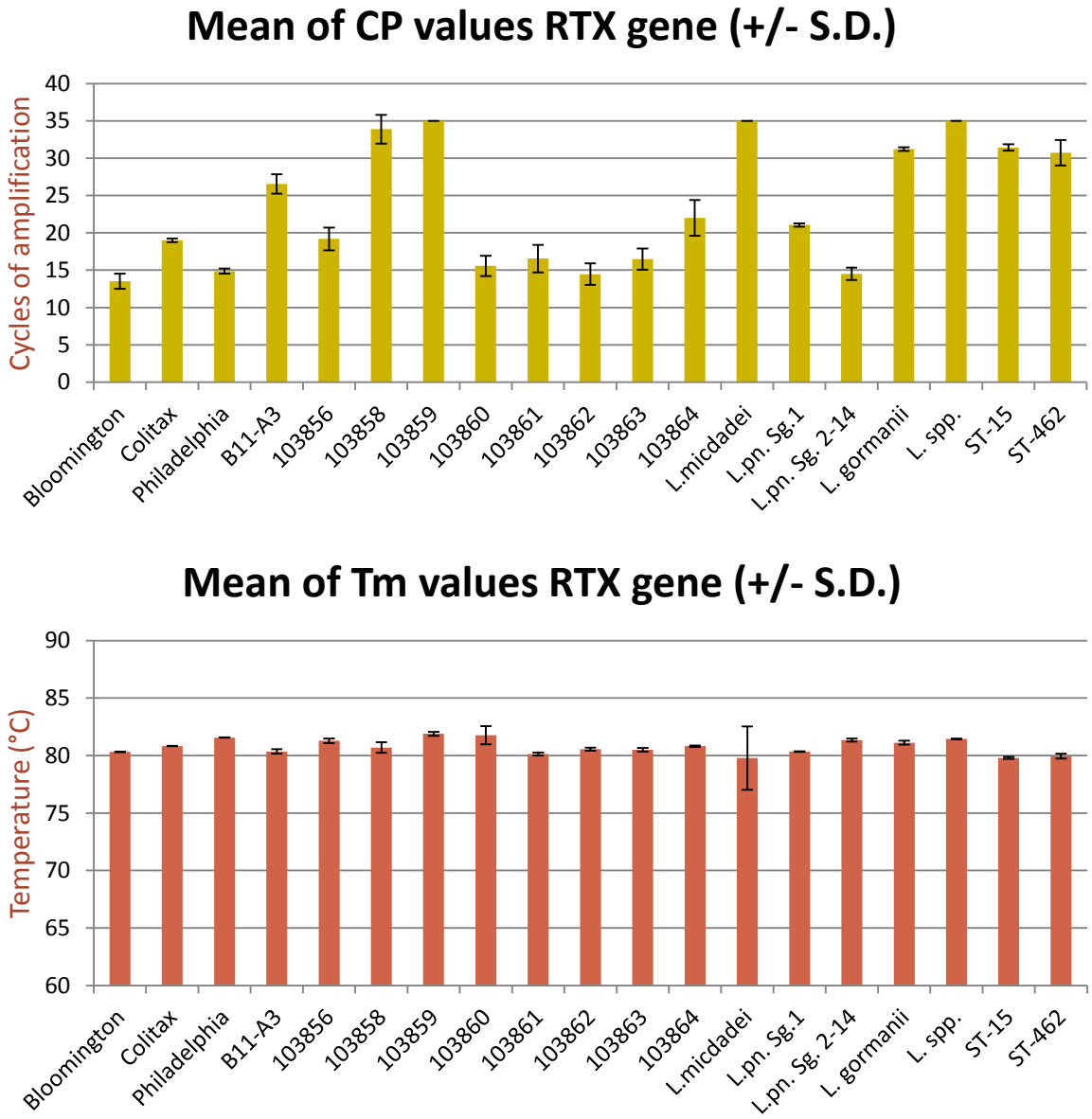

Figure 3.13: Cycle of exponential growth (CP) and melting temperature (T_M) results from PCR of all strains with rtx primers. The bars show mean values of results from experiments run in triplicate, with the standard deviation presented in error bars.

3.2.6 Virulence gene *hsp*

The *Hsp* gene produces a stress protein produced in excess when *Legionella* grows intracellularly, and is involved in *Legionella*'s virulence. PCR results from the last toxicity gene tested in this survey, *hsp*, are shown in figure 3.14. When compared to the negative control in T_M, there were a couple clearly negative results, the strains 103858 and 103859, but there were also a lot of uncertain findings. Many of the strains showed exponential growth very late in the cycle. When looking at the uncertain results, which are primarily the same ones as for the *mip* gene, it is all the additional strains used only in the PCR experiments (ATCC strains plus B11-A3). *L. gormani* and *L.* spp. were amplified late as usual. Compared to the negative control in the CP measurements, a lot of the strains could be valued as negative for gene presence. *L. spp* and *L. micdadei* were counted as negative in addition to 103858 and 103859. Bloomington, Colitax, Philadelphia, *L. pn.* Sg. 1 and sg. 2-14, ST-15 and ST-462 were regarded as positives, and the rest as very uncertain positives.

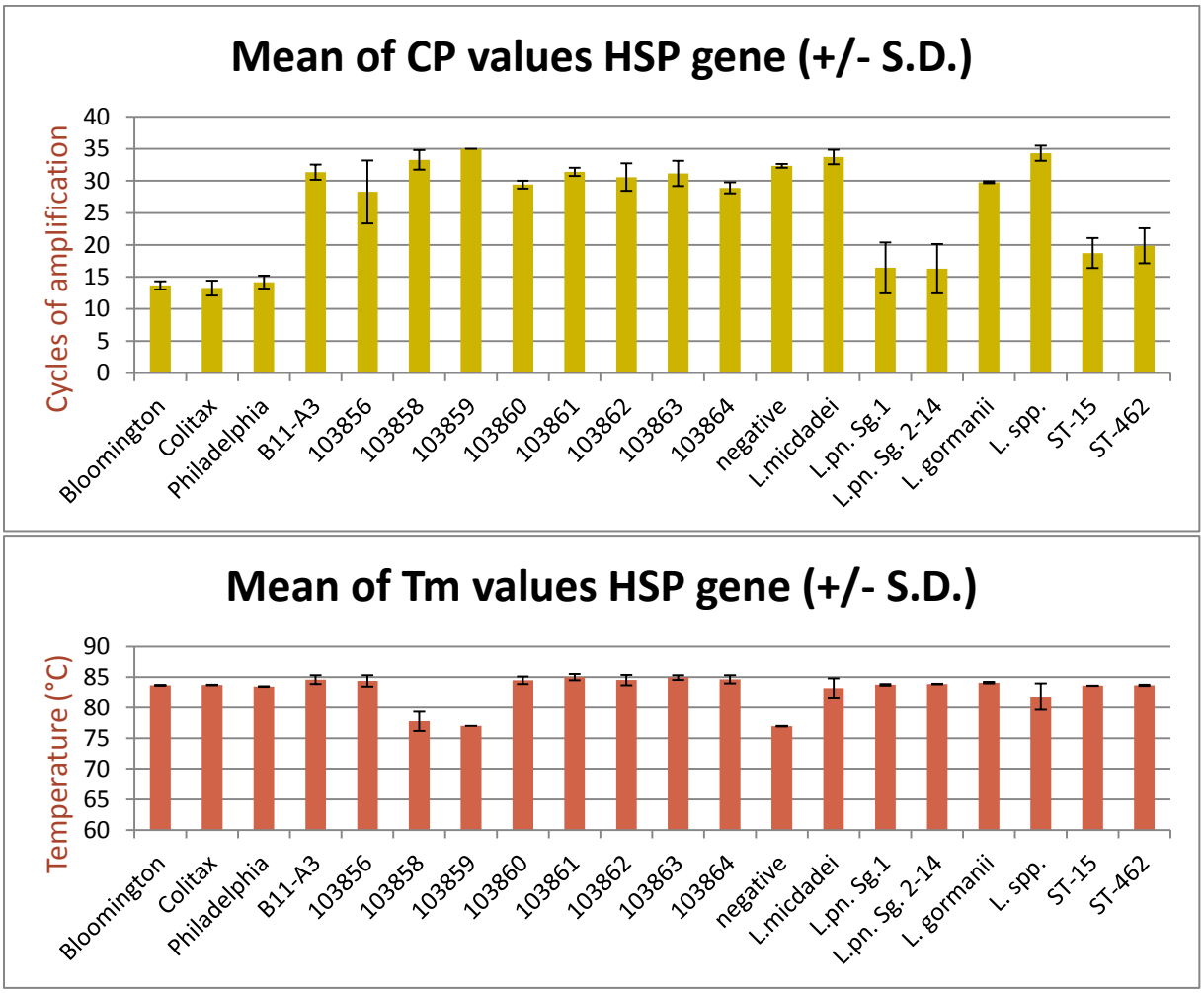

Figure 3.14: Cycle of exponential growth (CP) and melting temperature (T_M) results from PCR of all strains with *hsp60* primer. The bars show mean values of results from experiments run in triplicate, with the standard deviation presented in error bars.

4 Discussion

Legionella is a highly interesting model organism for bacteria infecting eukarya, and a lot of research has been done on this organism. It is called a "model for intravacuolar pathogens" by Isberg et al. (2009). The pathogen's virulence is characterized by the presence of different toxicity genes in its genome, involved in the infection of the cell, and maintenance of the *Legionella*-containing vacuole inside the host cell.

The purpose of this study was to investigate intracellular replication in amoebae and macrophages by ten different strains of *Legionella,* and the presence of five different toxicity genes in nineteen different strains of *Legionella.* The virulence in the different strains of *Legionella* was examined by detecting toxicity genes using quantitative PCR, and by co-culture with *Acanthamoeba castellanii*, and macrophage THP1 cells. In this chapter, the results from this study will be compared to findings from previous studies with similar methods. The possible drawbacks and sources of errors will also be discussed. A question I would like to elaborate on in this thesis is the quality of frequently used detection methods for *Legionella* species, and their reliability in determining a strain's virulence traits. I will also discuss whether there is a clear difference between environmental strains and clinical strains of *Legionella*.

4.1 *Legionella* as a pathogen

The rise of environmental bacteria like *Legionella* as a pathogen to humans will be briefly discussed in this section. The reason for the late discovery (1976) of *Legionella* as a pathogen is debated. The rise in use of containers with water holding optimal temperatures for growth of such pathogens in households and public institutions could be a factor of the increased incidents in later ages. The discovery of the pathogen led to an increased focus on retrieving more information on it, and detecting many more species during the past 40 years. Pneumonia has always been a common lung disease, and legionnaire's disease is often mistaken for pneumonia. The rise in information on detecting *Legionella* has probably led to a higher rate of diagnosing it as the infective agent.

4.2 Prevention and detection

Prevention of growth in circulating and stagnant water systems is basically limited to keeping the water temperature above 65 °C. Different detection methods for *Legionella* species have various drawbacks, and application areas. *Legionella* is demanding in terms of growth conditions, and has a slow growth rate. For culturing on BCYE, the ISO 11731 method (established in 1998, revised in 2004) for water quality standard considering the content of *Legionella* organisms was used in this study (described by Doleans et al. 2004). Some strains of *Legionella* showed a tendency of being better suited for growth on BCYE than others, and gave higher numbers of CFUs compared to the other, less "replicative" strains in the same dilutions. The "replicative" strains grew faster and resulted in higher CFU numbers. The alternation of *Legionella* between an infectious form and a replicative form could have significance when it is added to the media. Cells of Legionellae that are in an infective state could be less prone to enter the replicating state in an instant on agar media (Molofsky & Swanson 2004).

BCYE medium can easily become contaminated, which also applies for the PYG and RPMI media used for dilutions. Therefore, growth of other colonies of the same color, shape and size can easily be mistaken for *Legionella*. This happened with the strain labeled *L. gormanii*, which turned out to be *Staphylococcus cohnii* instead, a common human skin bacterium discovered in 1975 (Schleifer & Kloos 1975). Colonies of *S. cohnii* had a similar morphological structure as the *Legionella* strains in the experiments, but had a faster growth rate. The problem causing such errors makes it necessary to verify the growth on BCYE as *Legionella* by PCR. When the species are not verified with PCR, the results may be unreliable. In this survey, the contamination of the *L. gormanii* sample was traced back to the frozen samples kept at FFI for use in all experiments on the species. It is prudent to assume that all experiments carried out on this strain were carried out on *S. cohnii* instead of *L. gormanii*. All experiments performed on this strain are therefore unreliable.

When identified on MALDI-TOF, the *S. cohnii* strain got a score value of less than 2 for *Staphylococcus cohnii*, which is not a completely reliable species identification (see Appendix E). The reason for this can be wrong calibration of the machine, because of long times' un-utilization. It could also be due to the possibility of the analyte containing an organism not present in the software library, but most closely related to *S. cohnii*.

4.2.1 Co-culture

A detection method used for infective strains of *Legionella* is resuscitation and growth in co-culture with amoeba known for hosting *Legionella* in nature, and in human alveolar macrophages. *Legionella* strains that are able to replicate in macrophages in laboratory experiments are considered possible pathogenic to humans. When working with *Legionella* bacteria and eukaryotic hosts such as *A. castellanii* and human macrophages, everything has to be planned ahead and the methods have a very little margin for error. The bacteria and amoeba/macrophage cultures had to be prepared a given number of days before co-cultivation. If something happened to one of the cultures, or the co- culture, the whole set-up had to be started over. All cultures and media were also tested for contamination before and during the experiments. Media containing organisms were thrown away, and cultures contaminated by other organisms were ruled out in further experiments. Experiments were much more time-consuming than expected.

The multiplicity of infection (MOI) was low (0.05-0.005) because those concentrations were the most convenient to work with; they showed exponential growth well, and were not too elaborate to dilute into countable concentrations for plating.

4.2.2 In co-culture with amoebae

The non-invasive strands of *Legionella* were more prone to dying out (or possibly going into a VBNC state) during incubation in co-culture. *Legionella* in co-culture with amoebae did give growth to most of the strains, but not all. These results indicate that this method might be able to separate between virulent strains of *Legionella* and non-virulent strains. Some of the strains were run several times. The majority of these showed the same results, but *L. pn.* Colitax showed growth only the second round in experiments. In previous experiments run at FFI this strain did not grow in co-culture with amoebae. However, not all the strains were tested multiple times. Given more time and resources, one should run the same tests at least 2-3 times in order to increase the value of the results, and retrieve more significant data.

In the original method, presented by (amongst others) Jacquier et al. (2013) and Moffat and Tompkins (1992), extracellular bacteria were removed after infection in co-culture. By killing extracellular bacteria with gentamicin, or wash away extracellular material by replacing the PAS buffer, one ensures that organisms which are non-infective to amoeba are removed from samples. Cateau et al. (2011) proved that pathogenic bacteria can grow both in amoeba and in supernatant derived from amoeba culture. The reason for not washing the cultures after infection in this study was to resemble the conditions provided in natural habitats. It was

therefore important to find amoebae in microscope to check for *Legionella*- containing vacuoles. The microscopy investigations showed growth inside of amoeba in the strains that showed significantly increased number of CFUs during co-culture. The amoebae containing vacuoles of *Legionella* were also lysed at the end of the co- culture period. When viewing the *Legionella* strains that did not show any growth in the microscope, no bacteria was found inside the amoebae; nor were the amoebae lysed at the end of co-cultivation. These findings indicate that the Legionellae undergoing growth in co-culture did replicate inside of the amoebae, and not by the supernatant surrounding the cells. Because of observation of LCVs in the amoebae, the Legionellae cells growing in amoebae were not considered confirmed by Real-time PCR at the time of the experiment. The results from this study resembled those retrieved by other studies (mentioned earlier in this paragraph) using similar methods, but showed some variations between different set-ups.

Reflecting on the methods used in this survey, I see that the counts of colonies on BCYE after co- culture could advantageously be run in PCR for control with *Legionella*- specific primers. This was not done in these experiments; hence there is a small chance of contamination of other organisms. For even more reliable results, this should be conducted. Nevertheless, the observation of the LCVs in amoebae makes the possible contamination limited to other, very similar, intracellular parasitic organisms.

4.2.3 In co-culture with macrophages

The strains that were able to replicate in amoebae were to some extent expected to replicate in macrophages as well. Legionellae in co-culture with macrophages did not give the expected results. It could seem as if the method used for examination of growth in macrophages failed in determining toxicity of *Legionella* strains, since *L. pneumophila* has been proven to infect macrophages in other experiments (Tao et al. 2013, Tachibana et al. 2013, Cirillo et al. 2000, Cateau et al. 2011, Parthuisot et al. 2010).The experiments started promising, with growth of the ST-15 and ST-462 strains, but no growth of *L. pn.* Bloomington or Colitax. LCVs were detected in the infected macrophages in microscope, and the colonies after growth were verified as *Legionella*. In spite of this, when using ST-15 and ST-462 as controls in the next set up, none of the strains showed any growth. My first assumption was that it is very important to spin macrophage cells down and replace the old medium with new before adding PMA. This was performed the first time, but not the second time, where the ongoing macrophage cell lines were simply diluted in fresh media. Otherwise, the macrophage cell count was the same, and the rest of the procedure was identical for each set up. Even so, when

reintroducing the method from the first set- up, co- culture with macrophages did not give growth to any of the strains in any of the following trials. An observation from the macrophage- co- cultures was that the ST-15 and ST-462 dilutions from the first experiment that replicated in macrophages also had better survival in RPMI medium than the same strains in later experiments, where they did not replicate in macrophages. This could indicate that the difference in infectivity is linked to the state of the bacteria rather than the macrophages

Many *Legionella* strains showed better survival in co-culture with macrophages than in RPMI alone. The observation of better survival for the strains when incubated with macrophages rather than in only RPMI medium could indicate that the macrophages does not ingest the Legionellae at all, and that Legionellae feed on excretes from the macrophages in the medium. This possibility could be investigated further.

A study of recently isolated environmental strains grown in bone-marrow deprived macrophages from mice also showed no growth even though the macrophages were proven to host laboratory strains earlier (Tao et al. 2013). The results from different studies are somewhat contradictory, but generally, growth in macrophages or monocytes is used as an indicator of pathogenicity. The inability of infection of macrophages by the strains in this thesis is incomprehensible. Some of the strains were known as pathogenic strains, but all of them were more or less incapable of replication in macrophages. This may indicate a flaw in the method applied in this thesis alone, or show a general unpredictability of the co- culture method with macrophages as an indicator of pathogenicity.

Similar as for the amoeba co-culture, most researchers wash the macrophage cells with medium with or without antibiotics approximately 1 hour after inoculation of co-culture. Kwaik (1998) removed extracellular bacteria by centrifugation and washing the co-culture, and plating the supernatant for bacteria cell count. The procedure of removing extracellular *Legionella* was not performed in this survey, because the possibility of legionella killing macrophages by excretion of toxins was also in interest. Whether *Legionella* replicates inside of macrophages, or by predation of dead macrophages leaking cell material is incompletely investigated. The lack of washing should not have any negative effect on *Legionella*'s ability of intracellular replication. The discovery of *Legionella*-containing vacuoles inside of the macrophages in the first experiment indicates *Legionella*'s ability of replicating inside of macrophages, in the same manner as within amoebae. Failing to reproduce these results indicate the importance of all the right conditions to be present for the chance of *Legionella* to replicate in macrophages, which is not its natural host after all.

The way *S. cohnii* and *Legionella* replicated in co-culture with macrophages unfolded in 2 different ways. *S. cohnii* seemed more likely to kill macrophages instantly, by extracellular stress, followed by lysing of the cells. The *Staphylococcus* may have predated on the dead macrophages, leading to a rapid growth of bacteria. It is unknown whether the strain used in amoeba co-cultures was actually *Legionella gormani* or *S. cohnii*.. In amoebae, *S. cohnii/L. gormani* showed no growth, either intracellular or extracellular.

4.3 PCR detection of virulence genes

PCR is another conventional method of detecting Legionellae in a sample, and is able to calculate quantity, giving the correct proportion of *Legionella* in the sample. The results from qPCR analyses were varying. Some strains were shown to possess all 5 genes, like Philadelphia, Bloomington and Colitax. Other strains only possessed 1 of them, such as ATCC strain 103859, *L.* spp. and *L. micdadei*. The discovery of absence of many of the virulence genes in some of the strains isolated were expected, as many of the strains were environmental. The absence of the genes in the strains that did replicate inside amoebae was less expected, as intracellular replication is expected to rely on the function of these genes (Cianciotto et al. 1990; Cirillo et al. 2000; Fernandez et al. 1996; Segal & Shuman 1999).

Comparison of crossing point and melting point analyses were made in order to determine the presence of a gene. CP depends on the initial concentration of DNA, which is variable. Usually, the negative control sample in PCR analyses has no transcription. In some of these samples, the transcription of the negative control started above 35 COA, which is too late for determining a presence of the product in question. Because the negative control contains only primers, and no DNA, the detected transcription product could be due to primer-dimers, most likely. False negative results or a late amplification signal could be due to mutations in the gene sequence. The primers are specific, and a mutation in the binding site of the primers would have a substantial impact on the rate of gene transcription, giving negative results even when the gene might be present in the DNA. Melting point analyses are often used as a validation of the product specificity, but are not completely certain, because mis-primed products might be similar to the target strand in molecular mass and melting point (Edwards et al. 2004). The CP and T_M measurements from PCR analyses were not always coherent. Melting point peaks were often characterized by the presence of artifacts from mis-primed products and/or primer-dimers. The crossing point measurements often showed very high values, close to or above 35 cycles. This could indicate unspecific PCR products, or low initial concentrations of DNA.

The PCR results from analyses using the JFP/JRP primer were as expected, seeing that it classifies between *Legionella* and non- *Legionella*, and was positive for all the strains in the survey. The results from amplification with JFP/JRP primers also gave an indication of the amount of DNA present in each sample. ATCC strains 103859 and 103862, in addition to Bloomington and especially *L.* spp. were considered having a lower concentration of DNA than the rest of the strains. Late signals of amplification in these strains using the different primers in the survey were therefore considered positive to a greater extent than the strains with higher concentrations of DNA.

The PCR results from amplification of the *mip*- gene appeared as expected, except for ATCC strain 103859, seeing that all the positive results are known as *L. pneumophila* strains. ATCC 103859 is originally identified as *L. pneumophila* SG 5, and was expected to show positive results for this gene. Looking at this strain's signal from the JFP/JRP PCR, it shows an indication of a lower DNA concentration. Even so, the melting temperature of the product is completely separate from the rest of the products. As mentioned earlier, the reason for false negative results may be a mutation in the gene sequence. Regardless of this, the results are counted as negative, as it could as well mean a lack of the gene.

All the additional strains: the ATCC strains and the B11-A3, had a later crossing point during amplification of the *mip*- gene than the other *L. pneumophila* strains. A difference in preparation between the additional strains and the strains used in co-culture was that DNA from the additional strains was isolated by boiling bacteria in water, which might cause damage to some parts of the DNA. The strains used in co-culture were isolated using DNeasy blood and tissue kit, which is based on enzyme activity and is gentler in isolating the DNA. The assumption made from this was that the additional strains might have had damage to some parts of the DNA in greater extent than the co-cultured strains. The same explanation goes for the uncertain positives of the *hsp* gene, where the same tendency was shown. The negative control in *hsp* gene analyses had a CP value below 35 COA, around the same cycle as a lot of the other samples, but a clearly lower T_M. These results were clearly uncertain, and a lot more strains were close to be regarded as negative. Many of the results from the *hsp* gene were therefore uncertain positives. There were no pre-expectations for the rest of the genes, except for a correlation between possession of toxicity genes and infectivity to amoeba.

The strain labeled *L. gormani* had a late CP signal in almost all of the genes tested, except for the JFP/JRP gene. The reason for this could be the sample contamination in *L. gormani*, leading to less isolated DNA from this species. *L. gormani*, later identified as *S. cohnii* did get positive results for 4 of the 5 genes, in addition to the JFP/JRP gene in PCR analyses. This indicates that the DNA isolate did contain *Legionella*, and that some of the co-culture tests with amoebae could have been performed on *L. gormani.* This information is not certain, and cannot be used in conclusions.

The SYBR Green dye used in these experiments fluorescence when it is bound to the minor groove of double stranded DNA. This type of dye is more likely to give false positive results than dyes with probes binding to the amplicon, because of for instance primer-dimers, or one DNA strand binding to itself. Other false positives can be due to contamination of small amounts of DNA, which could cause a huge problem in traditional PCR. In real-time PCR, however, such contamination would be amplified late compared to template present in higher concentrations, and would have much fewer copies compared to the target gene. The unspecificity of SYBR Green dye makes it work generically in Real Time PCR, without having any knowledge of the target gene. SYBR Green could therefore also bind to the non-specific amplicons, giving false positive results (Zipper et al. 2004). Melting point analyses can only be done when using SYBR Green I, and are usually performed during real time PCR, to help verify the results from the quantification measurements (Mackay 2007).

Other Real Time PCR methods could also have been applied if time and economy was sufficient. The use of nucleic acid probes is generally more specific than SYBR Green. TaqMan is a fluorescently labeled hydrolysis probe. The method of using fluorescent probes is strand- specific, as the probe is targeted towards a specific sequence on the amplicon. The 5'-3' exonuclease DNA synthesis is performed by Taq polymerase. When moving along the amplicon, Taq polymerase moves across the sequence where the dual-labelled TaqMan probe is bound, and splits it during synthesis of the complimentary strand, causing it to send out fluorescent light. (Edwards et al. 2004).

4.4 Virulence genes versus infectivity

Legionella pneumophila sero-group 1 is by far the most common human pathogen, while not the most prevalent in the environment (Doleans et al. 2004). The detection of virulence genes in the different strains of *Legionella* did not correspond to the infectivity of the strains in amoebae. The strains that were able to replicate in amoebae were not necessarily able to replicate in macrophages. Patrizia et al. (2012) presented, likewise, in their study that different strains of *L. pneumophila* showed different virulence traits in different hosts, indicating a missing similarity between *Legionella'*s infection in protozoa and macrophages.

The expectations towards the PCR analyses were that the strains proven to replicate in amoebae would possess a majority of the virulence genes, and vice versa. The strains showing intracellular growth in amoebae , *L. pn* SG 1 and SG 2-14, *L. micdadei*, *L. pn* Bloomington, ST-15 and ST-462, contained everything from 1 (*L. micdadei*) to 5 (Bloomington) of the virulence genes. At the same time some of the strains containing all 5 of the virulence genes (*L. pn.* Philadelphia) did not replicate in amoebae.

The reference strain *L. pn.* Philadelpha is previously known as uninfective to amoebae by Patrizia et al. (2013). In their study, however, Philadelphia did grow intracellularly in macrophage- like cells. Tachibana et al. showed that all strains in their survey from 2013 of *L. pneumophila* SG 1 and 4 contained all of the virulence genes used in this survey. Especially the absence of *lvh*- detection in this survey in many of *L. pneumophila* SG 1 strains does not correspond to their results.

L. spp. had a late CP signal in all of the genes tested, and also showed poor growth on BCYE, no replication in eukaryotes, and generally poor survival in laboratory media. These observations together indicate a low possibility for this strain being pathogenic.

The results in these experiments indicates that a lack of Dot/Icm substrates or other virulence factors previously known as important in the survival and replication of *Legionella* did not inhibit replication in amoebae. These results contradict those from previous studies, where the virulence factors were proved as important to the toxicity of the strain. The reason behind this might be a misconception in the methods applied, or in the execution of the methods in these experiments. Table 4.1 shows the comparison of the different strains' ability to replicate in amoebae, and in macrophages, as well as the detection of number of virulence genes during PCR. There were no relation between the presence of virulence genes and the infectivity to

amoebae and macrophages. This independence between presence of virulence genes and infectivity has not been shown in any previous studies.

Table 4.1: Revision of table 3.1 and 3.2: shows the ability of different strains of *Legionella* to infect and replicate in amoeba and macrophages, in relation to the presence of different toxicity genes.

Strain	**Growth in amoeba**	**Growth in macrophages**	**Virulence genes present (out of 5)**	***mip***	***lvh***	***dot***	***rtx***	***hsp***
L. pneumophila serogroup 1	Yes	No	4	X		X	X	X
L. pneumophila serogroup 2-14	Yes	No	4	X		X	X	X
K1 *Legionella micdadei*	Yes	No	1		X			
Legionella gormani 7 [a]	(No)	(Yes)	3			(X)	(X)	(X)
Legionella spp	No	No	1		(X)			
L. pn. Bloomington	Yes	No	5	X	X	X	X	X
102/ST-15	Yes	Yes/No[b]	4	X		X	(X)	X
104/ST-462	Yes	Yes/No[b]	4	X		X	(X)	X
L. pn. Colitax	No	No	5	X	X	X	X	X
L. pn. Philadelphia	No	No	5	X	X	X	X	X

a:strain later identified as *Streptococcus cohnii*.
b: replicated in one of two experiments.
Parentheses indicate uncertain results.

4.5 Improvements and further work

To improve the reliability of the results in these experiments, the experiments should be run several times. Some of the methods also have room for improvement. Most of the improvements would be significantly more costly and elaborate. The cultivating method would be more accurate with the use of GVPC agar media, which is selective for *Legionella,* instead of BCYE. Co-culture with amoebae did distinguish between non-infective and infective strains of Legionellae, but the results are not completely reliable. Strains showing growth in one experiment might not show the same in the next one. Therefore, every strain has to be tested several times, in order to increase the significance of the data obtained. In macrophage co-cultures, very few of the experiments gave positive results. This method, or variation of the method might not be fully suited for detection of virulent strains. My suggestion would be to try centrifuging the Legionellae down for a longer time, for an increased chance of contact between *Legionella* and macrophage. Another suggestion would be to use monocytes instead of macrophages, or try with a different cell line, as cell lines kept over time could get increasing amounts of mutations. For Real-Time PCR analyses, the denaturing and annealing temperatures could be set just above and below the melting temperature of the product template, to eliminate most of the pollution fluorescence from the artifacts in solution.

To increase the scale of the investigation, more environmental isolates could be included. The pathogenic strains are usually well covered, but less attention has been given to the environmental strains, which are not (yet) linked to disease in human. It is proposed that intracellular growth of Legionellae in amoebae prepare the bacteria for infection of macrophages. The strains' inability to replicate in macrophages in this thesis was unexpected. An approach to investigate this problem could be to use colonies of *Legionella* strains that has recently replicated in amoeba, and present to the macrophages. By transferring the strains directly after growth in amoeba to macrophages, one should assume the strains to be better suited for infection of macrophages, which is shown by Cirillo et al. (1994). An additional comment to this is that Bartram (2007) presented a thesis concerning the transfer of *Legionella* from one host to another, describing how some *Legionella* species might lose their dependence of *L*-cysteine for growth. The main idea of this is that *Legionella* survives mutations in their genome with less difficulty in co-culture, when some genes are no longer essential for survival.

5 Conclusion

To investigate the potential pathogenicity of the 10 different Legionella strains used in this study I have examined their ability to grow intracellularly in the amoeba *Achantamoebe castellanii* and in macrophages THP1 cells, and compared this information to the presence or absence of several toxicity genes.

In this study, it was found that the co-culture methods of *Legionella* with amoebae worked as a better indicator for virulence than co-culture with macrophages. The co-culture experiments with amoebae gave comparable results, and were reproducible most times, but not all. This method was a good indicator for virulence, but the results were not completely reliable. The right conditions and some coincidence are needed for a pathogenic strain to infect a host. *Legionella*'s replication in amoebae could not be used as an indication of pathogenicity towards human macrophage cells, as all the strains were ineffective at replication in macrophages in most experiments. The co-culture method with macrophages did not work well in these experiments, and the procedure needs improvement and modifications.

Real-Time PCR detections of virulence genes in *Legionella* strains were somewhat unclear, and could be improved by changing the amplification program to match the melting point of the gene, or by replacing SYBR Green dye with TaqMan probes. Comparison of the detected virulence genes to the infectivity of eukaryotic cells showed that there were no connections between those results, which did not match with the expectations. Experiments were much more time- consuming than expected, and could have been planned better in advance, in order to be more streamlined and accurate.

There were no clear difference between environmental strains and outbreak strains in these experiments, hence, the methods used were not suited for determining pathogenicity of different strains of *Legionella*.

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Pictures from Roche Life Science in figure 2.2:

- http://lifescience.roche.com/shop/en/no/products/lightcycler14301-480-multiwell-plate-96
- http://lifescience.roche.com/shop/ProductDisplay?catalogId=10001&tab=&langId=1&partNumber=3.8.1.4.1.3&storeId=15012

7 Appendixes

Appendix A

PAS buffer – non-nutritive medium

1. NaCl – 1.20 g / 100 ml H_2O
2. $MgSO_4$ – 0.04 g /100 ml H_2O
3. $CaCl_2$ – 0.04 g /100 ml H_2O
4. Na_2HPO_4 – 1.42 g /100 ml H_2O
5. KH_2PO_4 – 1.36 g /100 ml H_2O

From each of these solutions, 10 ml were mixed together and added 950 ml distilled H_2O.

PYG medium(ATCC Medium 712)

Basal medium:

1. 20.0g Proteose Peptone
2. 1.0g Yeast extract
3. 900.0ml Distilled water.

Inorganic Stock Solutions:

1. 0.4M $MgSO_4 * 7H_2O$
2. 0.05M $CaCl_2$
3. 0.005 $Fe(NH_4)_2(SO_4)_2 * 6H_2O$
4. 0.25M $Na_2HPO_4 * 7H_2O$
5. 0.25M KH_2PO_4

2M Glucose Stock Solution:

1. 18.0g Glucose
2. 1.0g Sodium Citrate * $2H_2O$
3. 50.0ml Distilled water

Preparation: All ingredients in basal medium were added to 1L glass flask and autoclaved. Inorganic stock solutions were prepared separately and autoclaved. After they were cooled to room temperature, Inorganic Stock Solutions were added aseptically in the right order, according to the list above, to the basal medium. Solutions were mixed on magnetic stirrer. 2M Glucose solution was prepared by adding all ingredients and stirred, and subsequently filter sterilized and eventually added to the medium. Distilled water was added to obtain a total of 1 litre completed PYG medium.

Appendix B

Listed in the table below is data from counting of colonies (CFUs) after growth on BCYE, before and 6 days post co-culture with amoebae. Means and standard deviation is given in addition to the data from the duplicate data.

	day 0				day 6			
strain	**data 1**	**data 2**	**Mean**	**SD**	**data 1**	**data 2**	**Mean**	**SD**
ST-15	200	300	250	50	3000000	3000000	3000000	0
ST-462	200	240	220	20	1100000	900000	1000000	100000
Colitax	800	890	845	45	3	3	3	0
Colitax (2)	450	550	500	50	600000	500000	550000	50000
Bloomington	16	19	17,5	1,5	2900000	2500000	2700000	200000
Philadelphia	10	10	10	0	2	3	2,5	0,5
L. pn. Sg. 1	1100	1190	1145	45	3700000	4100000	3900000	200000
L. pn. Sg. 2-14	20	70	45	25	600000	500000	550000	50000
L. spp	70	100	85	15	0	0	0	0
L. micdadei	500	600	550	50	130000	170000	150000	20000
L. gormanii	390	400	395	5	10	30	20	10

Parallell control experiments for growth of *Legionella* in PAS buffer without amoeba added:

	day 0				day 6			
strain	**data 1**	**data 2**	**Mean**	**SD**	**data 1**	**data 2**	**Mean**	**SD**
ST-15	200	300	250	50	0	20	10	10
ST-462	200	240	220	20	80	100	90	10
Colitax	800	890	845	45	7	5	6	1
Colitax (2)	450	550	500	50	90	150	120	30
Bloomington	16	19	17,5	1,5	0	0	0	0
Philadelphia	10	10	10	0	0	0	0	0
L. pn. Sg. 1	1100	1190	1145	45	50	70	60	10
L. pn. Sg. 2-14	20	70	45	25	14	9	11,5	2,5
L. spp	70	100	85	15	0	0	0	0
L. micdadei	500	600	550	50	190	170	180	10
L. gormanii	390	400	395	5	0	0	0	0

The results from the measurements before co-cultivation (day 0) are equal, because it is taken from the same sample prior to the experiment. “6 days” shows the number of CFU plated from the co-cultures 6 days after the *Legionella* was added to a thin layer of amoeba in PAS buffer. *L. pn.* Colitax did not show growth in the first experiment, but in the second. Therefore, two results of Colitax are presented. The first results (no growth) is included in the line chart in the results’ section.

Appendix C

Cell counts from co-culture with macrophages. Included are the duplicate cell counts, the means and standard deviations from the day of incubation, the 1st day after incubation, and the 4th day after incubation in co-culture with macrophages.

With macrophages

10^3	**day 0**				**day 1**				**day 4**			
strain	**data 1**	**data 2**	**Mean**	**SD**	**data 1**	**data 2**	**Mean**	**SD**	**data 1**	**data 2**	**Mean**	**SD**
ST-15	3000	3000	3000	0	2600	2600	2600	0	680	560	620	60
ST-15	2000	2000	2000	0	3400	3100	3250	150	4000000	4000000	4000000	0
ST-462	2000	2000	2000	0	3600	2800	3200	400	4000000	4000000	4000000	0
ST-462	1400	1900	1650	250	600	900	750	150	370	420	395	25
Colitax	2100	1200	1650	450	700	300	500	200	0	0	0	0
Bloomington	1700	2600	2150	450	1100	1700	1400	300	10	10	10	0
Philadelphia	1300	1300	1300	0	1200	1300	1250	50	300	250	275	25
L. pn. Sg. 1	1400	1440	1420	20	1240	1450	1345	105	30	90	60	30
L. pn. Sg. 2-14	1800	1900	1850	50	1620	1710	1665	45	2000	2000	2000	0
L. spp	10	40	25	15	2	4	3	1	0	0	0	0
L. micdadei	500	500	500	0	400	600	500	100	80	70	75	5
L. gormanii	600	700	650	50	100000	100000	100000	0	220000000	230000000	2,25E+08	5000000

Table lists the cell counts from the control experiments, with *Legionella* incubated without macrophages, parallel to the macrophage co-culture experiments.

Without macrophages

10^3	**day 0**				**day 1**				**day 4**			
strain	**data 1**	**data 2**	**Mean**	**SD**	**data 1**	**data 2**	**Mean**	**SD**	**data 1**	**data 2**	**Mean**	**SD**
ST-15	3000	3000	3000	0	1500	1500	1500	0	10	0	5	5
ST-15	1200	1200	1200	0	750	890	820	70	500	200	350	150
ST-462	2000	2000	2000	0	1130	970	1050	80	200	800	500	300
ST-462	1400	1900	1650	250	40	30	35	5	0	0	0	0
Colitax	2100	1200	1650	450	380	330	355	25	0	0	0	0
Bloomington	1700	2600	2150	450	480	550	515	35	0	0	0	0
Philadelphia	1300	1300	1300	0	80	100	90	10	0	0	0	0
L. pn. Sg. 1	7700	6600	7150	550	1600	1800	1700	100	0	0	0	0
L. pn. Sg. 2-14	4100	3900	4000	100	1200	1200	1200	0	0	0	0	0
L. spp	10	40	25	15	0	0	0	0	0	0	0	0
L. micdadei	500	500	500	0	140	130	135	5	0	0	0	0
L. gormanii	600	700	650	50	2000	2000	2000	0	5000	6600	5800	800

Appendix D

Protocol for Qiagen's DNeasy blood and tissue kit:

A few colonies were stirred into 180 µl ATL buffer.

20 µl proteinase K was then added to the tube.

The tube was placed in shaker at 56 degrees for about an hour, and then vortexed.

200 µl Buffer AL was added and mixed on vortex.

200 µl ethanol was added and vortexed.

The mixture was then transferred to a tube with a filter, and the fluid was centrifuged out.

Filter was washed with 500 µl Wash buffer 1 and 500 µl Wash buffer 2.

To elute the DNA from the filter, 200 µl Buffer AE was added directly to the filter, set for 1 min, and centrifuged into a new tube.

Appendix E

MALDI-TOF

As described by Anders Halgunset in his master thesis in 2012, matrix-assisted laser desorption/ionization – time-of-flight (MALDI–TOF) mass spectrometry (MS) is an effective, cheap method of identification of microorganisms. MALDI-TOF MS "fingerprinting" is based on the characteristics of the organisms' preoteome. Reliable identifications of different species of *Legionella* can be made using this method. The sample preparation is minimal, the throughput is high, and so is the speed of the analysis. The purpose of this method is to measure the mass of macro molecules, such as DNA and proteins. The mass/charge is measured by gaseous molecules.

In MALDI-TOF MS, cell material is ionized by a pulsating laser via a light absorbing matrix. The molecules gets ionized with a positive charge and transferred as a cluster of vaporized particles through a time-of-flight tube. This ionization is performed in vacuum, and is accelerated in an electric field towards a detector. The analysis only depends on the mass of the molecules. The ions with the smallest mass will reach the detector first, and the largest ions will hit it last. A mass spectrum will be made with the tops representing the amount of ions registered at each mass/charge, giving a specific picture of the analyzed organism. The mass spectrum is then compared to the other profiles in the database.

When registering a new organism, the mass spectrum is used as a reference spectrum that is added to the library of the database, and represents a fingerprint of the organism. The software MALDI Biotyper 3.0 (Bruker Daltonics) is used in the recognition of the organism in question. A correlation matrix between the tops in the mass spectrum are compared to each other, and given a score. The score is between 0 and 3, where 3 is a perfect match. Scores above 2.3 is considered a most probable species identification, while scores between 2.0 and 2.3 is reckoned to be a reliable identification of family, and a presumable identification of species.

Appendix F

Student t-tests:

Null hypothesis: H0= data is not statistically relevant.

(P-values of significant data is outlined in red).

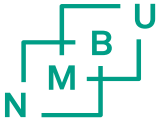
P-values below 0.05 gives rejection of H0.

Amoeba co-culture

Different T-tests	**p-value**
Difference before and after incubation, for the strains showing growth	0,022971338
Difference before and after incubation, for strains not showing growth	0,182833239
After incubation, between those showing growth and not showing growth	0,022951055
Between growth in amoeba and in PAS buffer, for those showing growth	0,022954282
Between co-culture in amoeba and only PAS buffer, for those not showing growth	0,375029922

Macrophage co-culture

Different T-tests	**P-value**
Difference before and after incubation, for the strains showing growth	1,19379E-05
Difference before and after incubation, for strains not showing growth	0,007253468
After incubation, between those showing growth and not showing growth	7,6795E-32
Between growth in macrophages and in RPMI medium, for those showing growth	1,19379E-05
Between co-culture in macrophages and only RPMI medium, for those not showing growth	0,113588621



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