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Establishment of a reference database for *Acanthamoeba*, *Legionella* and non-tuberculous mycobacteria using MALDI TOF MS (Matrix Assisted Laser Desorption Ionisation Time of Flight Mass Spectrometry)

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1 ABBREVIATIONS

AIDS: acquired immunodeficiency syndrome

AK: *Acanthamoeba*- keratitis

AN: Acetonitrile

A.U: arbitrary unit

bp: base pair

BBTS: Bruker Bacterial test standard

CNS: central nervous system

DHCA: 3,3-dihydroxycinnamic acid

DNA: deoxyribonucleic acid

FLA: free-living amoebae

GAE: granulomatous amoebic encephalitis

gyrA: gyrase A gene

HCCA: alpha-Cyano-4-hydroxy-cinnamic acid

HPA: Health Protection Agency

NN: non-nutrient

NTM: non-tuberculous mycobacteria

MALDI TOF: matrix-assisted laser desorption ionisation time of flight

mip gene: macrophage infectivity potentiator

MOTT: *Mycobacteria* other than tuberculosis

PBS: Phosphate Buffered Saline

PYG: Proteose-Peptone - Yeast-Glucose

PCR: polymerase chain reaction

rDNA: ribosomal DNA

RNA: ribonucleic acid

rpoB: subunit of DNA-dependent RNA polymerase

SA: sinapinic acid

sg: serogroup

spp: species

supsp.: subspecies

SSU: small sub-unit

2 INTRODUCTION

2.1 *Acanthamoeba*

2.1.1 Classification

2.1.1.1 Phylogeny of *Acanthamoeba*

Many scientists such as Haeckel (1866), Whittaker (1969) Woese, (1977, 1990), Cavalier-Smith (1998), Adl *et al*. (2005) and Smirnov *et al*. (2005 and 2008) modified the biological systematics of amoebae and of protozoa. The current tree of life and the knowledge on the relationships among the eukaryotes is mainly based on sequencing of the SSU (small-subunit) ribosomal RNA (Embley and Martin, 2006). It is permanently changed and expanded. As shown in Figure 1 the domain Eukaryota is currently divided into two monophyla, Unikonta and Bikonta, which are further divided into six supergroups.

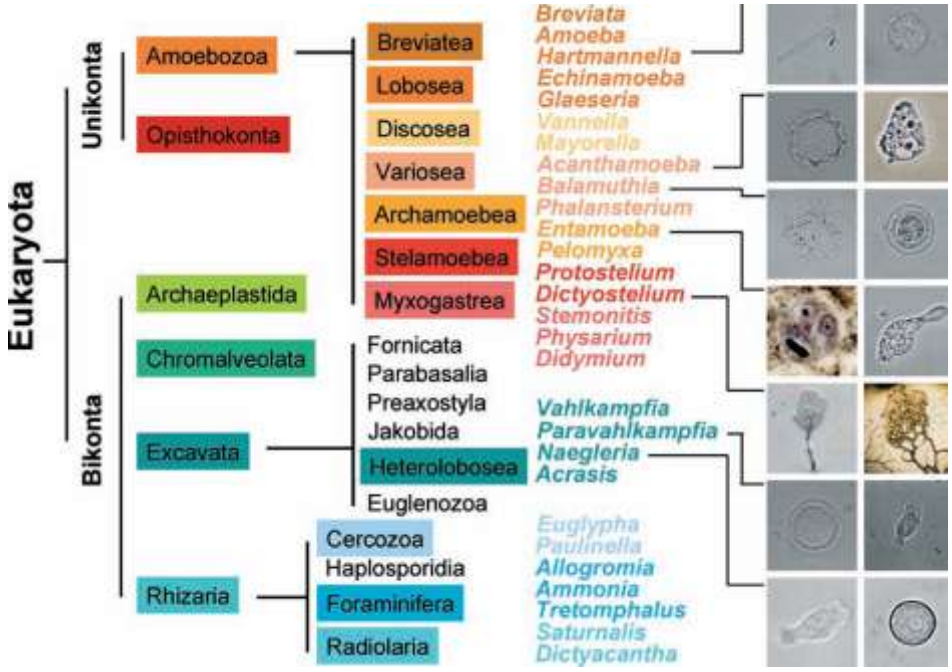


Figure 1: Phylogenetic tree of the eukaryotes (Walochnik and Aspöck 2007).

The monophylum Bikonta contains the Archaeplastida, Chromalveolata, Excavata and Rhizaria. The monophylum Unikonta contains the sister phyla

Opisthokonta and Amoebozoa (Hwan Su Yoon 2008, Cavalier-Smith 2004). Cavalier-Smith (1998) emended the taxon Amoebozoa. First, he divided the phylum Amoebozoa into the subphylum Lobosa and the subphylum Conosa, which include the Archaezoa and Mycetozoa. In 2004, Cavalier-Smith *et al* grouped the naked lobose amoebae into two classes, the Lobosea and the Flabellinea. The clade Tubulinea contains the Hartmanellidae, Amoebidae, Arcellinida, *Echinamoeba* and Leptomyxida. The Flabellinea include the clades Paramoebidae, Vannelidae and Vexilliferidae. To accommodate the Acanthopodida and the genera *Filamoeba*, *Phalansterium* and *Gephyramoeba*, a third class, the Variosea was created (Smirnov *et al*. 2005). After morphological analysis, Smirnov *et al*., (2005) divided the Amoebozoa (Figure 2) into three classes: the Tubulinea (Leptomyxida, Tubulinea, Arcellinida and the incertaesedis *Hartmannella vermiformis* and *Echinamoeba*), the Flabellinea (Vannellida and Dactylopodia) and the Conosea with the Archamoebae and Mycetozoa (Dictyostelia + Myxogastria). The Conosea partially correspond to the Variosea. Cavalier-Smith had included the Acanthopodida (*Balamuthia* and *Acanthamoeba)* into the class Variosea. However, this turned out to be incorrect, because there is no support in any SSU rDNA tree for such a relationship. Now, Amoebozoa *incertae sedis* contains the order Acanthopodida with the genera *Acanthamoeba* and *Balamuthia*. Furthermore there is no support for the position of Thecamoebida, *Vermistella antartica*, *Mayorella*, *Dermamoeba* and *Trichosphaerium* spp. (Smirnov *et al*. 2005, Pawlowski and Burki 2009). In conclusion, six major clades can be currently distinguished: Tubulinea, Flabellinea, Conosea, Variosea, Thecamoebida and Acanthopodida.

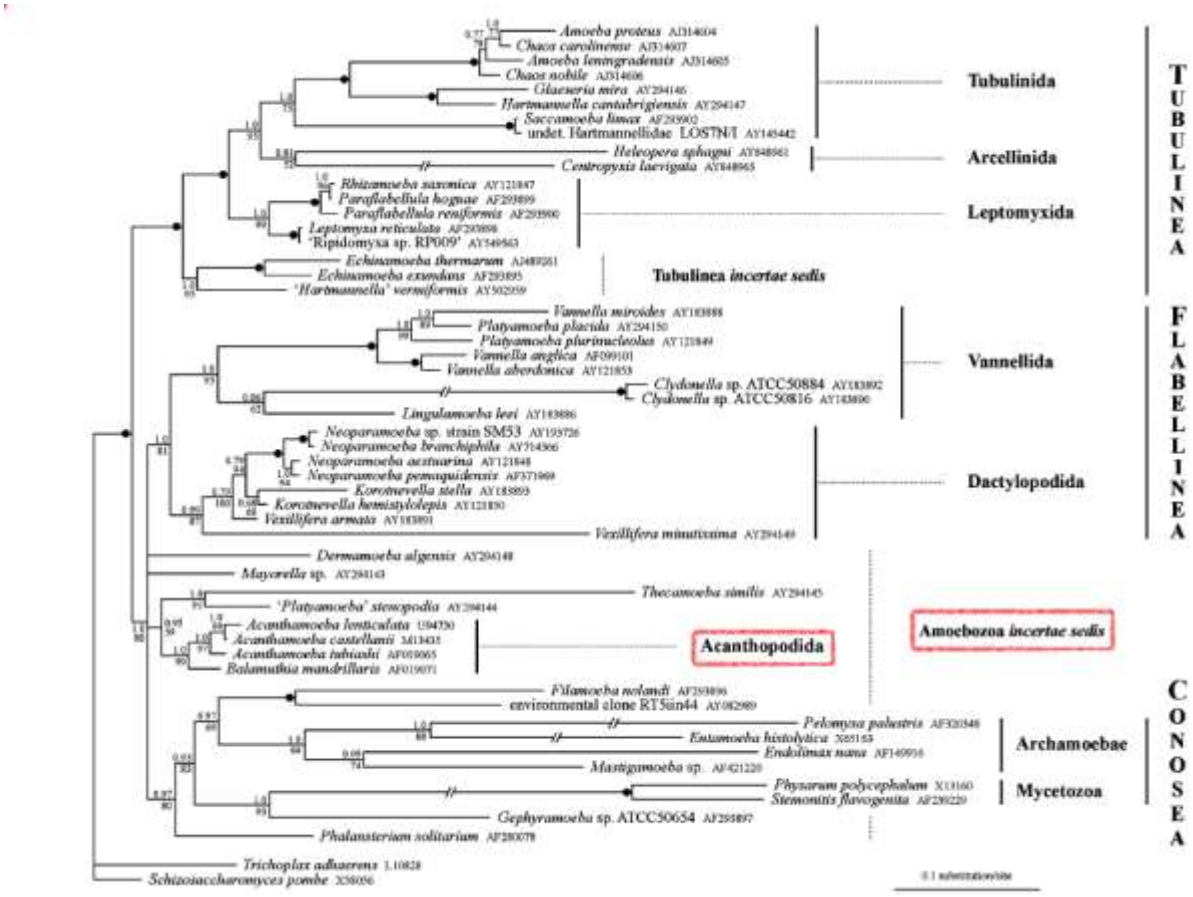


Figure 2: Molecular phylogeny of lobose amoebae modified according to Smirnov *et al*., (2005).

2.1.1.2 Methods of classification

The differentiation and identification of acanthamoebae depend on morphologic and physiologic characteristics, such as cyst size, locomotion, number of pores or growth behaviour.

Morphological characteristics

In the year 1977, Pussard and Pons subdivided the genus into three groups based on morphological structure and dimension of the cyst (Table 1) (Visvesvara *et al*., 2007, Booton *et al*., 2004). Today, the genus *Acanthamoeba* is a group of more than 25 named species that have been identified according to morphological criteria such as cyst morphology and trophozoite size and shape.

Table 1: Classification of *Acanthamoeba* spp. according to Pussard& Pons (1977) (modified according to Marciano-Cabral& Cabral, 2003 and Booton *et al*., 2005**).**

Group I (T7, T8, T9)	Group II (T1,T3,T4,T11)	Group III (T2,T5,T6,T10,T12,T14,T15)
A. *astronyxis* T7	A. *castellanii* T4	A. *culbertsoni* T10
A. *comandoni* T9	A. *divionensis*	A. *healyi* T12
A. *echinulata*	A. *griffini* T3	A. *jacobsi*
A. *pearcei* T3	A. *hatchetti* T11	A. *lenticulata* T5
A. *tubiashi* T8	A. *lugdunensis* T4	A. *palestinensis T2*
	A. *mauritaniensis* T4	*A. pustulosa T2*
	A. *polyphaga* T4	A. *royeba* T4
	A. *quina*	
	A. rhysodes T4	
	A. *stevensoni* T11	
	A. *triangularis* T4	

Table 2: Known *Acanthamoeba* genotypes and their associations with human diseases, i.e., keratitis and granulomatous encephalitis (modified according to Siddiqui and Khan 2012)**.**

Acanthamoeba genotypes	Human disease association
T1	Encephalitis
^T2a	Keratitis, Encephalitis
^T2b - ccap1501/3c-alike sequences	NA
T3	Keratitis
T4*	Keratitis, Encephalitis
T5	Keratitis, Encephalitis
T6	Keratitis
T7	NA
T8	NA
T9	NA
T10	Keratitis, Encephalitis
T11	Keratitis
T12	Encephalitis
T13	NA
T14	NA
T15	Keratitis
T16	NA
T17	NA

*this genotype has been most associated with both diseases

^basis of T2 division into T2a and T2b has been proposed by Maghsood *et al.*, (2005)

NA - no disease association has been found yet

Molecular analyses

Another alternative to identify and to differentiate acanthamoebae is the amplification and sequencing of certain DNA fragments, such as the nuclear and mitochondrial small-subunit (SSU) rRNA genes. The currently most widely used method for molecular taxonomy of *Acanthamoeba* is the sequencing of the 18S rDNA. This allows genotyping into the 17 described genotypes (Table 2).
The genotypes are defined to exhibit more than 5% sequence divergence to all other genotypes. The recently described genotype T17 is considered non-pathogenic (Nuprasert *et al*. 2010). The sequence types T7, T8 and T9 correspond to morphological group one. Sequence types T1, T3, T4 and T11 correspond to group two and sequence types, T2, T5, T6, T10, T12, T14 and T15 to group three. However, several isolates seem to be in conflict with this agreement, such as *A. pearcei* T3 (5043S), *A. polyphaga* T2 (1501/3c), *A. culbertsoni* T4 (Diamond) and *A. castellanii* T1 (V006) (Stothard *et al*. 1998, Marciano-Cabral and Cabral 2003, Schuster *et al*. 2004). The 18S rDNA dissimilarity between group II and III is 12-18% and between group I and the other both 35-37% (Walochnik and Aspöck, 2007). Furthermore the sequencing of the 18S rDNA is used to understand the phylogeny of *Acanthamoeba* (Kong, 2009) and for diagnostics. With the internal transcribed spacers ITS1 and ITS2 specific *Acanthamoeba* genotypes can be distinguished (Visvesvara *et al*., 2007). Qvarnstrom *et al*. (2006) described the simultaneous detection of *Acanthamoeba* spp., *Balamuthia mandrillaris* and *Naegleria fowleri* with a multiplex RT-PCR. Furthermore, a simultaneous detection of pathogenic FLA in the same sample is possible (Siddiqui and Khan, 2012). The advantage of a real-time PCR to conventional PCR is the rapid analysis and the minor risk of amplicon contamination (Qvarnstrom *et al*., 2006).
Protein and isoenzyme profiles have been developed for specific identification and are non-morphological criteria to differentiate acanthamoebae (Visvesvara *et al*., 2007).

2.1.2 Ecology and geographical distribution

Acanthamoebae are worldwide widespread, aerobic, eukaryotic facultative pathogen, which may provoke various infections in humans and animals. Due to their ability to live as “free-living amoebae” in the environment, the amoebae only accidentally infect a host. This is the reason, why they also have been called amphizoic amoebae (Visvesvara *et al*., 2007). The amoebae are ubiquitous. They have been found in a wide range of environments all over the world. Acanthamoebae have been isolated from air, dust, soil, fresh and tap water, ocean sediment, air-conditioning, contact lenses and contact lens cases, hospitals, dental treatment units and dialysis machines. Additionally, they have been isolated from human nasal mucosa, brain, skin, lung, corneal tissue and from healthy individuals (Marciano-Cabral, 2003; Visvesvara *et al*., 2007; Kong, 2009). Moreover, they also have been isolated from different animals, like amphibians, reptiles (Walochnik *et al*., 1999), mammals (Bauer *et al*., 1993; Westmoreland *et al*., 2004; Lorenzo- Morales *et al*., 2007) and birds (Visvesvara *et al*., 2007).

2.1.2.1 Life cycle

The genus *Acanthamoeba* has two life cycle stages, a trophozoite and a cyst (Figure 3). The trophozoite or vegetative stage (Figure 4) is the active stage in which the amoebas multiply by binary fission and feed actively on bacteria from the environment. The life cycle depends on environmental conditions. Under ineligible conditions, e.g. food shortage or desiccation the amoebae round up and encyst to a metabolically inactive resistant dormant cyst (Figure 5). If the conditions change for the better, the cysts excyst and change to the trophozoite stage. The trophozoite as well as the cyst stage can enter the body of the host through micro-lesions of the eye or through the nose and broken skin (Figure 3) and cause various diseases depending on the entry site of the microorganism and the immune status of the patient (Marciano- Cabral and Cabral, 2003, Dudley *et al*., 2005, Visvesvara *et al*., 2007).

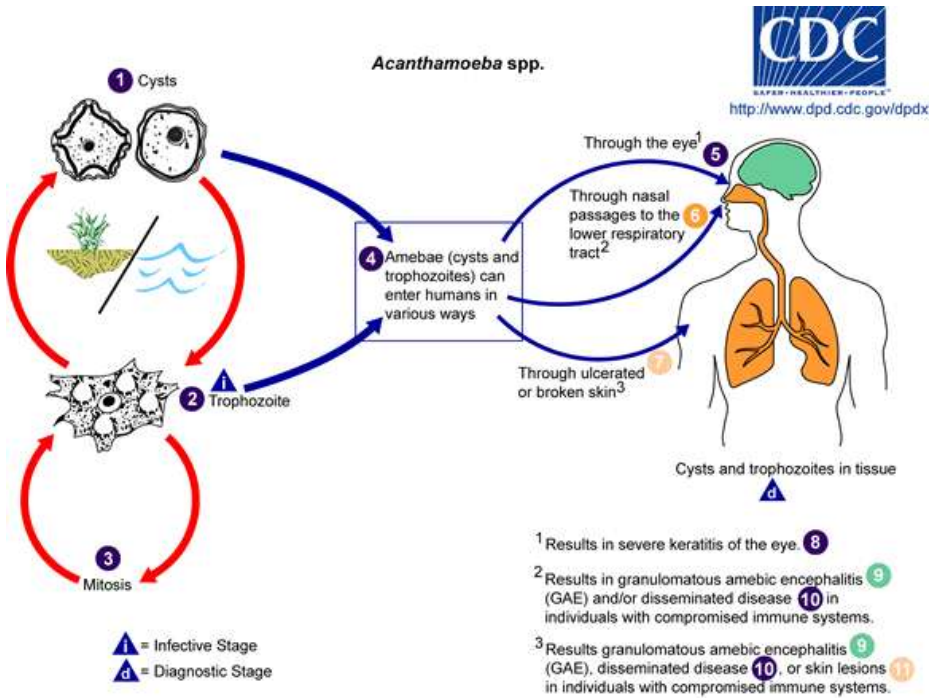


Figure 3: Life cycle of *Acanthamoeba* spp. The trophozoites replicate by mitosis. They encyst under adverse environmental conditions. The trophozoites as well the cysts can enter the body in various ways (eye, nose and skin).
http://www.cdc.gov/parasites/acanthamoeba/biology.html

2.1.2.2 Trophozoites

The size of the flat trophozoites without clearly defined shape depends on the species and varies from 25 to 40 µm (Figure 4). The unique and significant attributes of the trophozoites are the sub-pseudopodia (acanthopodia) that arise from the surface of the amoeba's cell body (Walochnik and Aspöck, 2005). A further feature of the trophozoite is the large number of mitochondria. The genome size of mitochondrial DNA of *Acanthamoeba castellanii* is 41,591 bp (Siddiqui and Khan 2012). The structure of the hyaline pseudopods is the reason for the slow locomotion of *Acanthamoeba*. With the help of the acanthopodia, the amoebae can attach to surfaces, move on and catch prey like yeast, algae or bacteria (phagocytosis). Pinocytosis is an ability of the amoebae to take up liquid nutrients. The development of food cups located on the surface of the trophozoite allows the uptake of particulate matter (Visvesvara *et al*., 2007).

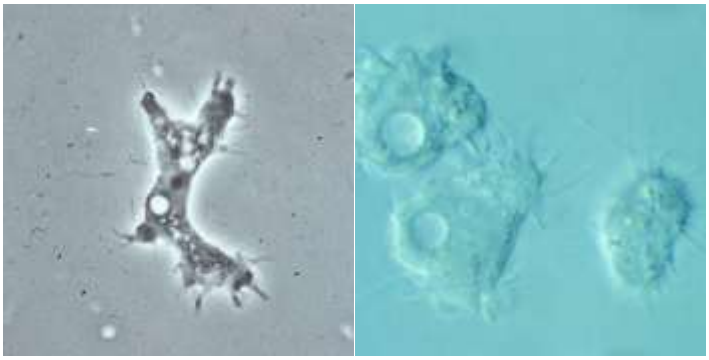


Figure 4: Trophozoites of *Acanthamoeba* spp.: nucleus and acanthopodia.
Modified after: http://dpd.cdc.gov/dpdx/HTML/ImageLibrary/FreeLivingAmebic_il.htm

As demonstrated in Figure 4 the trophozoites have only one nucleus (uninucleated). The nucleus, encased by cytoplasm, is in a central position within the cell. The cytoplasm contains numerous organelles, like ribosomes, mitochondria, a Golgi complex, endoplasmatic reticulum and a contractile and food vacuoles. Summarized characteristic attributes of the trophozoites are a trilaminar membrane, a nucleus with a large nucleolus, the spine-like acanthopodia on the surface and one or more contractile vacuoles. The function of the contractile vacuoles is the osmotic regulation. The cytoplasm includes also digestive vacuoles, lysosomes and glycogen-containing vacuoles (Marciano-Cabral and Cabral, 2003; Siddiqui and Khan 2012).

2.1.2.3 Cysts

The size of the cyst varies from species to species, between 13-20 µm. The formation of the cyst takes place under unfavourable environmental conditions such as lack of food, desiccation or variations in temperature. The cysts are resistant to various chemicals like chlorine and biocides as well as antibiotics and survive low temperature. The double-walled cysts consist of an ectoplasm (outside) and endoplasm (inside) with actin and myosin filaments in between. The wrinkled ectocyst consist of lipids and proteins, while the inner wall (endocyst) consists of cellulose, and therefore is periodic acid-Schiff (PAS)-

positive. The PAS method is a staining method to prove the presence of glycogen (Marciano- Cabral and Cabral, 2003).

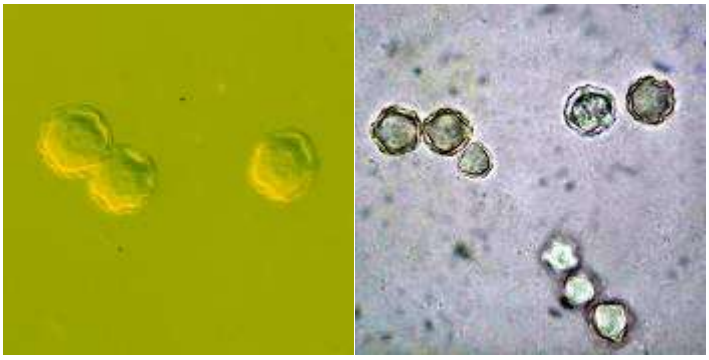


Figure 5: Double-walled cysts of *Acanthamoeba* spp.
Modified after: http://dpd.cdc.gov/dpdx/HTML/ImageLibrary/FreeLivingAmebic_il.htm

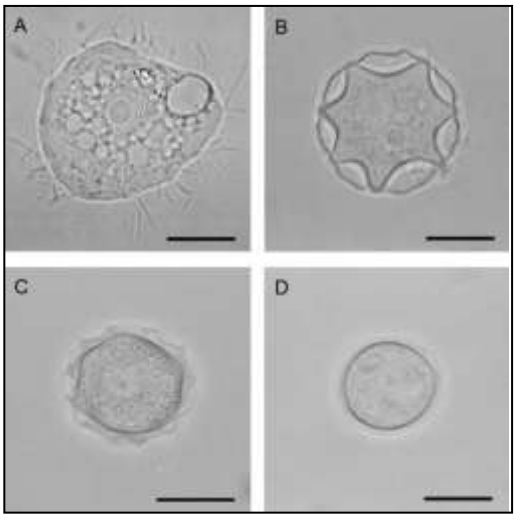


Figure 6: *Acanthamoeba* spp., trophozoite (A) and cysts representing Morphological group I (B), II (C) and III (D).
(Walochnik and Aspöck, 2005 scale bar= 10µm)

The cysts as well as the trophozoites of *Acanthamoeba* group I (Figure 6 B) are the largest. A rounded outer wall and a separated inner wall give the form of the cyst. The appearance of the inner wall results from the association between the outer and inner wall without immersed opercula. The cyst are usually >18 µm in size. Group II (Figure 6 C) contains the largest number of species. The size of the cysts is usually around 18 µm or smaller. The mostly wrinkled ectocyst is

thin or thick, the form can be polygonal, triangular, round or oval. Compared to group I, the operculum of group II is immersed into the ectocyst and clear radiations are normally not visible. The size of the cysts of group III (Figure 6 D) is less than 18 µm. The frequently ripped ectocyst is thin and smooth and the endocyst rather round, rectangular or triangular. If the environmental conditions change, the dormant amoebae leave the cysts by removing the operculum (Page 1991, Visvesvara *et al*., 2007).

2.1.3 Medical relevance

Acanthamoeba has the ability to cause serious infections in humans such as sinus infections, cutaneous lesions, *Acanthamoeba* keratitis or rare but fatal granulomatous amoebic encephalitis (GAE). Mainly, the genotypes T2 to T4 and T11 have been reported to cause *Acanthamoeba* keratitis and T1, T4, T10 and T12 to cause GAE (Nuprasert *et al*., 2010). Infections with *Acanthamoeba* spp. are rare, about 1,500 cases of *Acanthamoeba* keratitis and about 200 GAE cases have been recorded worldwide (Walochnik and Aspöck 2002 and 2002a).

2.1.3.1 Comparative characteristics

The distinctions between the two different diseases caused by the genus *Acanthamoeba* are listed in Table 3.

Table 3: Comparable characteristics (modified from Visvesvara *et al*., 2007).

	***Acanthamoeba* granulomatous encephalitis**	***Acanthamoeba* keratitis**
antimicrobial therapy	pentamidine, azole compounds, flucytosine, sulfadiazine	PHMB, chlorhexidine, brolene
clinical course	idolent subacute course; acute stage fatal in weeks	penetration of amoebae into cornea; stromal ring due to PMN infiltrate
disease	Granulomatous amoebic encephalitis; cutaneous lesions; sinus infections	Amoebic keratitis
disease at presentation	stiff neck, headache, behavioural changes, coma	intense pain, photophobia, tearing
distinctive morphological	vesicular nucleus; finger-like	vesicular nucleus; finger- like

features	pseudopodia projecting from surface; cyst wall with two layers and with covered pores (opercula)	pseudopodia projecting from surface; cyst wall with two layers and with covered pores
epidemiology	infection from soil, water, and air; in hospital environment (water taps, hydrotherapy pools, air conditioning cooling towers)	contaminated contact lenses; corneal trauma
groups at risk	typically immunocompromised individuals	mainly contact-lens wearers; low secretory IgA may contribute
humoral reaction	usually strong; protective value uncertain	IgA at corneal surface may be protective
in vitro cultivation	axenic, bacterized, and defined media; tissue culture cells; growth at 37°C (CNS isolates) or 30°C (keratitis isolates)	axenic, bacterized, and defined media; tissue culture cells; growth at 37°C (CNS isolates) or 30°C (keratitis
laboratory diagnostic methods	cysts seen in brain tissue; IFA, IIF and PCR	corneal scrapings or biopsy; confocal microscopy
life cycle	two stages: amoeba and cyst	two stages: amoeba and cyst
neuroimaging (CT and/ or MRI)	presence of space-occupying or ring-enhancing lesions	not relevant
prevention	widespread in soil and water; in hospital setting, monitoring of water supply, ventilators, air conditioning units	use of sterile lens solution; maintaining clean lens case; avoiding swimming with contact lenses
prodromal period	weeks to months	few days

Acanthamoeba Keratitis

The worldwide first case of AK was described at the beginning of the 1970s (Hammersmith, 2006). After the 1980s, the number of *Acanthamoeba* keratitis cases increased because more people used soft contact lenses (Hammersmith, 2006). The number of unreported cases is unknown. The highest number of observed cases is in the United States of America (USA) and Europe with Great Britain as the most affected country. Nevertheless, also several (>100) cases have been reported in Austria (Walochnik and Aspöck 2002).

Independently from the immune status of a person, *Acanthamoeba* spp. can provoke inflammations of the cornea known as *Acanthamoeba* keratitis (AK).

The preferred entry of *Acanthamoeba* spp. into the host is through microlesions in the cornea. The incubation time, defined as the time between exposure to the agent and the appearance of the first symptoms and signs, can be a few days up to several weeks (Walochnik and Aspöck, 2005). The main symptoms of AK are tearing, lid oedema, photophobia and redness of the eye. However, the most characteristic symptom of AK is a ring forming stromal infiltration caused by penetration of inflammatory cells (Claerhout *et al*., 2004). AK can be caused by various *Acanthamoeba* species and genotypes. Principally genotype T4 is responsible for most AK cases (Booton *et al*., 2005). In addition, T3, T5, T6 and T11 are associated with AK (Walochnik *et al.,* 2000, Seal *et al*., 2003, Spanakos *et al*., 2006). Pre-disposed are people wearing contact lenses and having insufficient contact lens hygiene. Hiti *et al*., (2005) found that homemade contact lens solutions are not the only risk factor. Also commercially, available lense solutions bear a certain risk because most of them are not sufficiently effective against acanthamoebae.

Diagnosis

The clinical picture of AK is difficult to differentiate from a bacterial or herpes simplex keratitis infection. The first signs of AK are high light sensitivity, pain, lacrimation, reddening and ocular swelling. The beginning of the infection is confined to the corneal epithelium but in the course of the disease, the amoebae invade the underlying stroma. The penetration of the amoebae induces an inflammation of the eye. For diagnostics, a clinical sample is taken by biopsy or by corneal scraps. Then the sample is cultured on non-nutrient agar plates using *Escherichia coli* as a food source for the amoebae (Walochnik and Aspöck, 2005, Shi *et al*., 2009). The plates are incubated at 28°C to 35°C for one week and then evaluated by phase contrast microscopy. Today, the standard technique is the PCR method with a solid proof of the genotype (Schroeder *et al*., 2001).

Treatment

The mainstay of treatment for *Acanthamoeba* keratitis is the topical application of biguanides with or without diamidines. These agents are presently the most effective in the fight against AK. Biguanides that are in use are chlorhexidine 0.02% to 0.2% and polyhexamethylene biguanide (PHMB) 0.02% to 0.06% and the available diamidines are hexamidine 0.1% and propamidine isethionate 0.1%. For the success of the treatment, it is essential to apply PHMB and hexamidine every hour, day and night for 48 hours and then for a further 72 hours by day. The treatment of AK at the beginning is very important otherwise the trophozoites can penetrate unimpeded into the deeper layers of the eye leading to a longer therapy (Dart *et al*., 2009).

2.2 Bacterial endocytobionts in free-living amoeba (FLA)

To survive unfavourable environmental conditions and toxic stress, bacteria can use cysts as a 'Trojan horse' (Primm *et al*., 2004). Amoebae are known as a carrier for several pathogenic bacteria, including *Legionella* spp. or *Mycobacteria* spp*.,* but also *Vibrio cholerae* and *Listeria monocytogenes*. The amoeba cysts represent a kind of shelter for the bacteria against unfavourable environmental conditions. In 1973, Jadin detected acid-fast stained bacteria within acanthamoebae and assumed that the amoebae act as hosts for *Mycobacteria* spp. Similar to the intracellular pathway of *Legionella* spp*.*, *Mycobacteria* spp*.* can multiply inside the *Acanthamoeba* cyst. Co-culturing of *Acanthamoeba* spp*.* with *Legionella pneumophila* and *Mycobacterium avium* showed increasing virulence in the bacteria (YU Hak Sun *et al*., 2007).

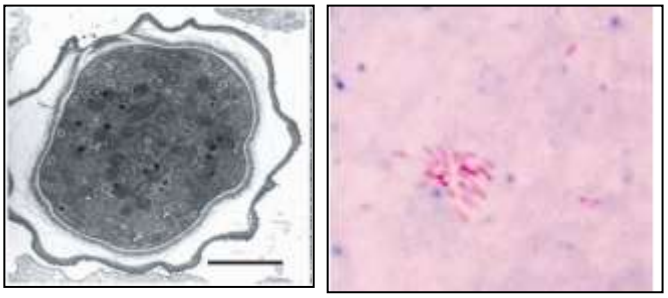


Figure 7: Electron micrographs of *Acanthamoeba* cyst and Ziehl-Neelsen stain of mycobacteria within *Acanthamoeba* (YU Hak Sun 2007).

Interaction between nontuberculous mycobacteria and protozoans are common in the environment. For example, *M. marinum, M. fortuitum* and *M. avium* can replicate and survive within *Acanthamoeba* spp*. M. avium* inhibit the lysosomal function (Visvesvara, 2007). It has been proven that *M. avium* is more virulent after amoebal passage (Primm *et al*., 2004).Also *Legionella* spp. or other bacteria (amoeba-resistant bacteria) (Table 4) can survive intercellular within free-living protozoa especially amoeba in aquatic environments and moist soil.

Virulence profiles of the *Legionella* spp. such as high resistance to low temperatures, antibiotic resistance (Barker *et al*., 1992), motility (Rowbotham, 1986) or the invasive phenotype (Cirillo *et al*., 1994) are not needed any longer within host cells. In the absence of protozoa, the bacteria can only grow under laboratory conditions (Fields *et al*., 2002).

Table 4: Amoeba-resistant bacteria (ARB) modified fromBarker & Brown (1994), Greub & Raoult (2004), Horn & Wagner (2004), Snelling *et al*. (2006)

Amoeba-resistant bacteria (ARB)	protozoal host	life-style	pathogenicity
Escherichia coli O157	*Acanthamoeba*	extracellular	pathogenic
Burkholdereria pseudomallei	*Acanthamoeba*	extracellular	established
Francisella tularenis	*Acanthamoeba*	facultative intracellular	established
Helicobacter pylori	*Acanthamoeba*	facultative intracellular	established
Legionella pneumophila	Many species	facultative intracellular	established
Legionella anisa	*Acanthamoeba*	facultative intracellular	potential
Legionella fallonii	*Acanthamoeba*	facultative intracellular	potential
Legionella drozanskii	*Acanthamoeba*	facultative intracellular	potential
Mycobacterium avium	*Acanthamoeba*	facultative intracellular	established
Mycobacterium leprae	*Acanthamoeba*	obligate intracellular	established
Mycobacterium marinum	*Acanthamoeba*	facultative intracellular	established
Mycobacterium ulcerans	*Acanthamoeba*	facultative intracellular	established
Mycobacterium simiae	*Acanthamoeba*	facultative intracellular	potential
Mycobacterium fortuitum	*Acanthamoeba*	facultative intracellular	potential
Mycobacterium smegmatis	*Acanthamoeba*	facultative intracellular	potential
Rickettsia-like	*Acanthamoeba*	obligate intracellular	potential
Vibrio cholerae	*Acanthamoeba*	extracellular	established

2.3 *Legionella*

2.3.1 History

The first strains of *Legionella* were isolated from guinea pigs in 1943 by Tatlock and 1947 by Jackson *et al*. In the year 1954, Drozanski detected a free-living amoeba in soil (Poland) and isolated a bacterium from the infected amoeba. This bacterium was added to the genus *Legionella* in the year 1996. After a large outbreak of pneumonia among veterans of the American Legion (1976 in Philadelphia), the causative agent of the disease (Legionnaires´ disease) was identified 1979 as a species of *Legionella* (Brown *et al*., 1985, Fields. *et al*., 2002, Greenwood *et al*., 2003, Neumeister B. *et al.,* 2009).

2.3.2 Phylogeny

The genus *Legionella* belongs to the family *Legionellaceae*, to the order *Legionellales* and to the phylum Proteobacteria (class *Gammaproteobacteria).* 16S rRNA analyses confirm the *Legionellaceae* as a single monophyletic subgroup. The DNA relationship between the strains of the genus *Legionella* is at least 70%, between one species to another is the relationship less than 70% (Fields et al., 2002). The genus *Legionella* comprises currently 55 species, 3 subspecies and 73 serogroups (Lo Presti *et al*., 1998; Neumeister *et al*., 2009; http://old.dsmz.de/). The species *Legionella pneumophila* is subdivided into three subspecies: *Legionella pneumophila* subspecies pneumophila, *Legionella pneumophila* subspecies *fraseri* (described in human illness) and *Legionella pneumophila* subspecies *pascullei* (isolated only from the environment) (Greenwood *et al*., 2003). The strain *Legionella pneumophila* contains currently 15 serogroups, (Fields *et al*., 2002, Tronel 2009). Legionellae, which do not grow on standard *Legionella* culture media are termed as *Legionella*-like amoebal pathogens (LLAPs) (Adeleke *et al*., 1996).

2.3.3 Physiology, morphology and distribution of *Legionella*

Legionellae are classified as obligate aerobic, gram-negative, rod-shaped, non-spore-forming, ubiquitous bacteria. The bacteria are approximately 2-20 µm long and 0.3 -1 µm wide and have a monotrichous flagellum. The genus *Legionella* can proliferate intracellularly in monocytes and macrophages but can also exist and proliferate as endocytobionts in free-living amoebae (Horwitz 1983). Due to the ubiquitous occurrence of *Legionella* spp., the bacteria have been isolated from various habitats, natural as well as artificial habitats. Beside the major reservoir, the fresh water the *Legionella* also have been found in air, soil, ground and surface water, air condition units, water lines, soil, swimming pools and rarely from breathing apparatus or ice cream makers (Neumeister B. *et al*., 2009, Fields *et al*., 2002). Environmental legionellae are sensitive against dehydration and disinfectants. Optimal growth temperature is between 35°C and 37°C but the organisms can also multiple between 25°C and 50°C (Katz *et al*., 1987, Fields et al., 2002). *Legionella* spp. cannot survive at temperatures above 60°C. The cultivation of legionellae on standard media for bacteria is impossible because they need L-cysteine and ferric pyrophosphate for the growth. Therefore, special „buffered charcoal-yeast extract“- (BCYE) agar are used (Martson *et al*., 1994).

2.3.4 Pathomechanism

2.3.4.1 Cycle of infection in amoebae and macrophages

Usually, *Legionella* spp. live as intracellular parasites of freshwater protozoa e.g. *Acanthamoeba* spp. and use for the reproduction a similar procedure as in mammalian macrophages. Infection occurs through inhalation of aerosols (<5 µm) or cysts containing bacteria (Fields *et al*., 2002). Legionellae within aerosolized water or protozoa can cause Legionnaires´ diseases in immunocompromised hosts (Fields *et al*., 2002). *Legionella* spp. or *Legionella*-like bacteria are able to penetrate the host cell actively. After the bacteria get in touch with the host cell, a receptor-mediated endocytosis is triggered. A specific form of endocytosis, called "coiling phagocytosis” (Figure 8) is the mechanism

for the uptake of bacteria by *Acanthamoeba* and *Hartmannella* (Bozue & Johnson 1996). The initiation of phagocytosis occurs by binding of the C3, CR1 and CR3 receptor with the *Legionella* surface protein, called major outer membrane protein (MOMP) (Payne &Horwitz 1987, Bellinger-Kawahara &Horwitz 1990). The bacteria are engulfed by phagosomes after the entry of the into the host cell. The fusion of the phagosomes and lysosomes is suppressed by a specific secretion system of *Legionella* (Albert-Weissenberger *et al*., 2007). They are able to inhibit the acidification of the phagosomes as well as the fusion of lysosomes with the phagosomes (Bozue & Johnson 1996). Then the bacteria are in a replicative, acid resistant and non-peritrichous phase. Subsequently the legionellae leave the phagosomes, enter the cytoplasm and lyse the host cell. Within alveolar macrophages and blood monocytes the bacteria can survive, multiply, and consequently infect new cells (Helbig JH *et al*., 2003 Neumeister B. *et al*., 2009, Rowbotham 1980).

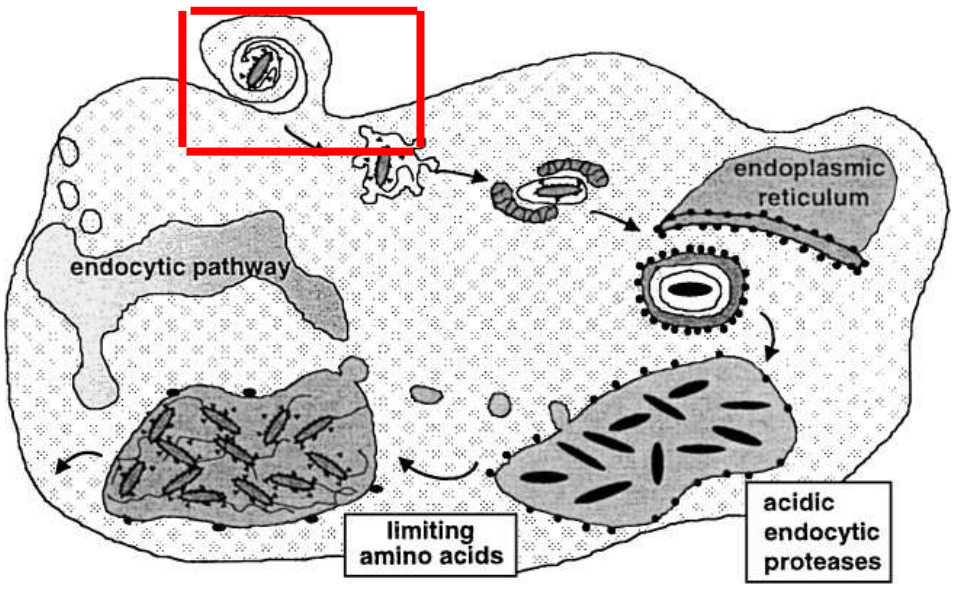


Figure 8: Pathomechanism of *Legionella* (Swanson and Hammer, 2000).

2.3.5 Epidemiology and medical relevance

After the first described outbreak (1976) of legionnaire's disease, similar incidents but also sporadic infections in hospitals and resorts have been reported worldwide (Fraser *et al*., 1977). About 18 *Legionella* species (Table 5) have been associated with legionellosis in humans. Nevertheless, only the *Legionella pneumophila* serogroup one causes almost in 90% of all cases an infection in humans. Person-to-person transmission has not been demonstrated (Greenwood *et al*., 2003, Tronel, 2009, Neumeister *et al*., 2009).

Table 5: *Legionella* species associated with human diseases (Neumeister *et al*., 2009).

***Legionella* species associated with human diseases**
Legionella anisa
Legionella birminghamensis
Legionella bozemanae
Legionella cincinnatiensis
Legionella dumoffi
Legionella feeleii
Legionella gormanii
Legionella hackeliae
Legionella jordanis
Legionella lansingensis
Legionella longbechae
Legionella maceachernii
Legionella micdadei
Legionella parisiensis
Legionella pneumpohila
Legionella sainthelensis
Legionella tusconensis
Legionella wadsworthii

The legionellae give two different clinical pictures (Table 6) the legionnaire's disease and the Pontiac fever. The legionellosis is characterised by an incubation period of 2-10 days, high fever, cough, fever, headache, diarrhoea, dyspnoea, non-productive cough, myalgia's, rigors, respiratory distress and delirium (Fields. *et al*., 2002). Patients with a weakened immune system e.g. AIDS patients are much more predisposed for the disease. Predisposing factors are beside the immune status of a person, smoking and the age (Greenwood D.

et al., 2003). A much lighter and self-limiting flu-like illness represents the so-called Pontiac fever. The symptoms mimic influenza with fever, headache, cough or fatigue but the infection never results in pneumonia. After two to five days, patients also recover without medical treatment (Tossa *et al*., 2006).

Table 6: Clinical characteristics of Legionnaires' disease and Pontiac fever.

	Legionnaires' disease	Pontiac fever
incidence	1-5% (till 30%) of all pneumonia	ten times more than pneumonia
manifestation	1-5%	90-98%
incubation period	2-10 (-14) days	1-2 days
duration of the ilness	2-3 weeks or longer	2-5 days
symptomatic	generally infection with pneumonia, CNS and other extrapulmonary symptomatic	influenza-like illness
lethality	2-10% (till 801%)	0

The clinical differentiation between a pneumonia and legionnaires disease is very difficult. The diagnostic of legionellosis and other types of pneumonia with chest X-ray is very difficult or impossible. The only way to obtain a reliable result is a microbiological investigation of the patient sample (Fields *et al*., 2002).

The largest outbreak of legionellosis was 2001 in Spain with approximately 650 infected individuals (Garcia-Fulgueiras *et al*., 2003). The source of infection was a cooling tower of the local hospital. The source of a *Legionella* outbreak in France in the year 2003 was a cooling tower of a petrochemistry factory. During this outbreak, 86 people suffered from legionellosis and 18 of them died (Nguyen *et al*., 2006). In Norway 2005, ten of 56 sufferers died. The source of infection was an industrial scrubber of a ligning production (Nygard 2005 *et al.*, Nygard *et al*., 2008). In Sweden, two of 32 sick people died after the outbreak 2007 (Hjoth *et al*., 2007). In Vorarlberg 2011, 13 people suffered from legionellosis after they were in a wellness hotel (AGES annual report). The Austrian Agency for Health and Food Safety (AGES) registered an increase of legionellosis cases in Austria since 2004 (Table 7).

Table 7: Legionnaires `disease cases in Austria (AGES annual report 2004 - 2011**).**

Year	Legionellosis cases	cases of death
2004	59	8
2005	65	7
2006	69	9
2007	108	11
2008	100	10
2009	92	8
2010	80	18
2011	100	14

Whereas in the year 2004 about 59 registered cases, in 2011, the number had risen to 100 cases. But also the number of deaths has risen from eight (2004) to 14 (2011).Cases of legionellosis were associated with water supply facilities of working places, hospitals, campsites and hotels, cooling towers or potting soil (AGES annual report 2004-2010).

2.4 *Mycobacteria*

2.4.1 History

In the year 1882, Robert Koch, a German physician and microbiologist, identified the tubercle bacillus as the etiologic agent of tuberculosis in a lecture to the Physiological Society of Berlin. In 1896, Karl Lehmann and Rudolf Neumann established the genus *Mycobacterium* and published a bacteriological manual, which contains the first description of only four different mycobacterial species. In 1907, Clemens von Pirquet developed the tuberculin skin test and demonstated three years later in children (Daniel TM., 2006). Today are more than 100 species described (Devulder *et al.*, 2005, Dostal *et al*., 2003).

2.4.2 Phylogeny and morphology

2.4.2.1 Phylogeny

The genus *Mycobacterium* that are responsible for the largest number of deaths from bacterial infections, belongs to the phylum Actinobacteria to the order *Corynebacteriales* and to the family *Mycobacteriaceae* (Gao B. and Gupta RS., 2012). The genus *Mycobacterium* with more than 100 described species is subdivided into three groups: *Mycobacterium tuberculosis* complex that cause tuberculosis, Mycobacterium leprae, which is not in vitro cultivable and the non-tuberculosis mycobacteria (NTM) which can cause diseases in human, animals and birds (Neumeister *et al*., 2009, Greenwood *et al*., 2003, Primm *et al*., 2004). Characteristic for mycobacteria is the higher interspecies similarity compared to other bacteria. This represents a problem to differentiate the bacteria (Devulder *et al*., 2005).

2.4.2.2 Physiology and morphology

Mycobacteria are gram-positive, 0.2-0.6 x 1-10 µm in size, slightly curved or straight rods, aerobic, non-motile, ubiquitous organisms. They have been isolated from hospitals, water, humid rooms, soil and from the mucosa of humans and animals. The coloration after gram staining is not intensively coloured although the mycobacteria are classified as gram-positive.
Mycobacteria are acid-alcohol fast due to their cell wall (waxy materials like chloroform-soluble mycolic acids with long branched chains within) (Dostal. *et al*., 2003 and Neumeister *et al*., 2009) and do not form any endospores, spurs or capsules (Neumeister *et al*., 2009). The optimum temperature varies according to the species and ranges from 25°C and 50°C (Dostal *et al*., 2003). Molecular biological techniques such as sequencing of the 5´ side of the 16S rRNA submit a sufficient accuracy because the verification or the distinction between NTM and TB bacteria by microscopy is not possible (Neumeister *et al*., 2009).The cell wall is thick and consists of a covalently connected complex of peptidoglycan, arabinogalactan and mycolic acids. This structure gives the bacteria the acid resistance. Characteristic for the mycobacteria are the N-glycosylation of the muramic acid of the peptidoglycan and the high content of mycolic acids and lipids. Based on the structure of the cell wall, the permeability is very low (Neumeister B. *et al*., 2009).

<u>Classification of the non-tuberculous mycobacteria</u>

The non-tuberculous mycobacteria (NTM) also called atypical mycobacteria or mycobacteria other than TB (MOTT), currently represent more than 130 species and are typical environmental organisms. Nevertheless, the most common name that is used for this group is ‘non-tuberculous mycobacteria’. The NTM are divided in rapid and slow growers. The bacteria which develop visible colonies within 7 days are rapid growers, those which require longer incubation times are slow growers (Tortoli 2009). In the year 1959, Runyon grouped the NTM into four groups based on pigment production and growth rate. Photochromogenic, scotochromogenic and non-chromogenic NTM are slow

growers (groups I to III) and the group IV NTM belong to the rapid growers. According to growth rate, colonial characteristics and pigment production the non-tuberculous mycobacteria are classified as follows (Rogall T., 1990, Stahl D., 1990, Schlossberg, 2005, Jarzembowski *et al*., 2008):

Photochromogens

If the photochromogens are incubated in the dark, they stay colourless. However, if young cultures are exposed to a natural or artificial light source for only a brief time they develop colouration (yellow, orange)**.** The following species belong to the group: *Mycobacterium kansasii* and *Mycobacterium simiae* (pulmonary disease), *Mycobacterium marinum*-swimming pool granuloma (skin infection) and *Mycobacterium asiaticum* (Greenwood *et al*., 2003).

Scotochromogens

Scotochromogens produce pigmented colonies even in the dark. The following bacteria belong to the scotochromogens: *Mycobacterium gordonae* (pulmonary disease), *Mycobacterium scrofulaceum (*cervical or scrofula lymphadenitis and pulmonary disease) and *Mycobacterium szulgai* (Greenwood *et al*., 2003).

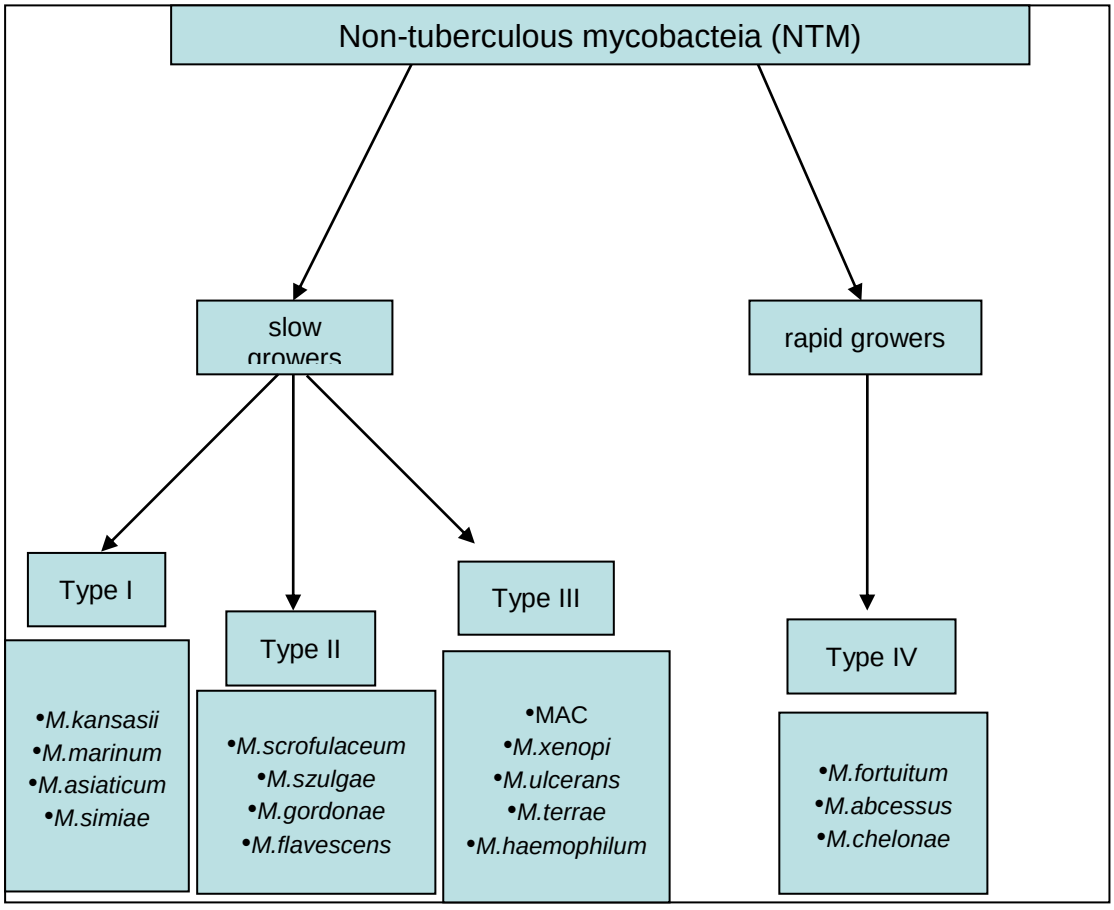
Non-chromogens

Non-chromogens non-tuberculous mycobacteria are unpigmented. *Mycobacterium intracellulare* and *Mycobacterium avium* are the most prevalent and important opportunistic agents in humans and are combined to the *Mycobacterium avium* complex (MAC). MAC cause pulmonary lesions, lymphadenitis and many other disseminated diseases in patients with AIDS. Other non-chromogens are *Mycobacterium ulcerans, Mycobacterium paratuberculosis, Mycobacterium xenopi, Mycobacterium malmoense* and *Mycobacterium haemophilum.* They can cause lymphadenitis in pigs, tuberculosis in birds and in immunosuppressed humans (Greenwood *et al*., 2003).

Rapid growers

Within one week, rapid growing bacteria produce visible colonies on the medium. These bacteria may be scotochromogens, non-chromogens or even photochromogens. *Mycobacterium chelonae* and *Mycobacterium fortuitum* can be human pathogens. These mycobacteria can cause pulmonary and disseminated diseases, post-injection abscesses and wound infection (Greenwood *et al*., 2003).

Table 8: Classification of NTM according to Runyon 1956.



2.4.3 Distribution of non-tuberculous mycobacteria

Non-tuberculous mycobacteria or environmental opportunistic bacteria can be found as commensals, symbionts and saprophytes. They are generally free-living, ubiquitous not obligate pathogens that have been frequently isolated from soil, mains water, stagnant water, swimming pool, water house dust, animals such as fish and birds, in immunocompromised individuals, eukaryotes plants and also in medical equipment such as endoscopes and surgical solutions(Primm *et al*., 2004, Schlossberg, 2005, Jarzembowski *et al*., 2008).

2.4.4 Pathomechanism of non-tuberculous mycobacteria

The most likely possibility of being infected with NTM is the contact with contaminated environments. A transmission from human to human is currently not documented (Tortoli 2009). The route of entry of non-tuberculous mycobacteria is through inhalation or through inoculation of the bacteria. Most of NTM are non-pathogenic for humans, but for all that, all of them can behave as opportunists. Non-tuberculous mycobacteria occur in most cases in persons with a weakened immune system, for example with chronic obstructive pulmonary disease (COPD) or previously pulmonary disease, tuberculosis, AIDS or histoplasmosis. During the 1980´s, the growth of disseminated NTM was noted as well as the explosive increase of the AIDS and HIV rate (Schlossberg D., 2005; Tortoli 2009).

2.4.4.1 Medical relevance

From more than 100 described species, only a few are obligatory pathogens. *M. tuberculosis complex* includes the closely related species *M. tuberculosis, M. bovis, M. africanum, M. caprae, M. microti*, *M. pinnipedii*, *M.canetti* and the Bacillus Calmette-Guérin, (BCG) strain. All of them can cause tuberculosis in man and animals. *M.leprae* is pathogen for human and some animals and most of NTM are non-pathogenic for humans (Neumeister *et al*., 2009).

Tuberculosis

M. tuberculosis complex is the causative agent of tuberculosis. People with active tuberculosis (TB) transmit the bacteria from person to person by droplet infection (Neumeister et al., 2009) or by zoonotic transmission (e.g. *M. bovis* from cattel via unpasteurized dairy products, inhalation of aerosols or close contact to the animals) (Mandal *et al*., 2011). The infection occurs through inhalation of aerosolized bacilli. The World Health Organization (WHO) reported more than nine million new TB cases and about two million deaths every year (Pawlowski *et al*., 2012). Figure 9 shows the highest number of tuberculosis cases in Asia, Russia and parts of Africa in the year 2006.*M. tuberculosis* and HIV, potentiate one another. According to the WHO occur 2.6 million new cases of HIV infections and 1.8 million AIDS-related deaths every year. Approximately about 14 million individuals are dually infected (Pawlowski, 2012). Tuberculosis can present in various forms. Most common is the infection of the lungs, bones and gastro-intestinal tract (Neumeister et al., 2009, Smith 2003).

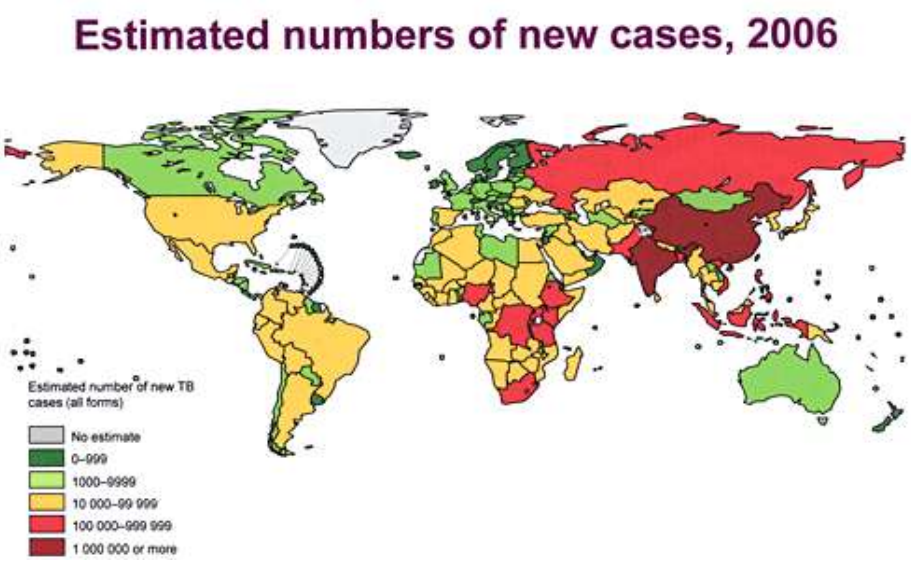


Figure 9: Estimated numbers of new cases of tuberculosis worldwide (2006).
http://www.fz-borstel.de/cms/forschungszentrum/nationales-referenzzentrum-fuer-mykobakterien.html

Leprosy

Until 2008, *Mycobacterium leprae* was the only known causative agent of leprosy. The newly discovered species *Mycobacterium lepromatosis* causes the diffuse lepromatous leprosy (DLL). Han *et al*., (2008) have established the existence of *Mycobacterium lepromatosis*. This unique form is only endemic in Caribbean and Mexico. Rare cases have been reported in France, USA, Brazil, Tunisia, India, Iran, Malaysia and Singapore. Analyses revealed a 9.1% differences between the two species (Han *et al*, 2009 and 2012, Vera-Cabrera 2011). The leprosy caused by *M. leprae*, is a chronic granulomatous disease of the skin and the peripheral nerves. The clinical features of leprosy are muscle paralysis, anaesthesia and visible lesions of the skin (Greenwood *et al*., 2003). Until now is the cultivation on artificial cell culture media of *M.leprae* impossible. The bacterium can only be cultivated in model organism such in mouse footpads and nine-banded armadillos. The analysis of the genome revealed that many genes are missing fail for the independent survival, e.g. for the ex vivo replication. The symptoms of the disease are multifaceted they show a wide range of pathological and immunological forms (Neumeister *et al*., 2009). The WHO noticed the highest number of infected individuals in areas (Figure 10) south of the equator, except Australia.

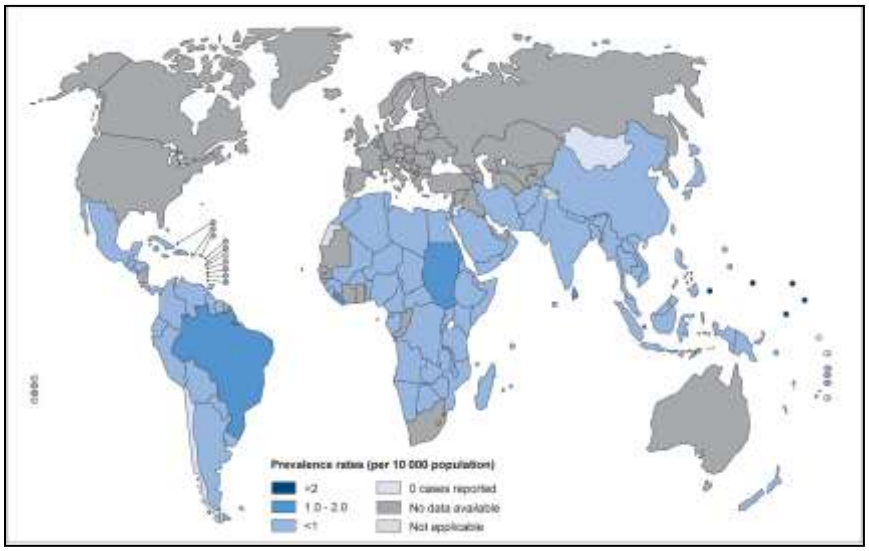


Figure 10: Global prevalence of Leprosy in 2011.
(http://www.who.int/lep/situation/Leprosy_PR_2010.pdf)

Non-tuberculous mycobacteria

Non-tuberculous mycobacteria are in contrast to *M. tuberculosis* and *M. leprae* of low virulence. Following infections have been described in man: lymphonodal infections, skin lesions (swimming pool granuloma, Buruli ulcer, post injection abscesses), infections of joints and bones, pulmonary infections and disseminated infections mostly in immunocompromised people (Greenwood *et al*. 2003: Tortoli 2009). Mycobacterial opportunistic infections (M. *avium intracellulare*, *M. scrofulaceum, M. xenopi and M. kansasii*) are a major cause of morbidity and mortality in patients with HIV and AIDS (Sampada S. Karne *et al*., 2012).The following non-tuberculous mycobacteria in Table 9 have clinical significance.

Table 9: Clinical significance of non-tuberculous mycobacteria. (Neumeister *et al*., 2009).

Species	Clinical symptoms
M. avium complex (MAC)	pulmonary disease; disseminated disease in immunocompromised patient (AIDS) or in children (lymphadenitis)
M. kansasii	based on predisposing pulmonary disease, pulmonary infections
M. marinum	swimming pool granuloma of the extremities
M. scrofulaceum	pulmonary infections rarely
M. simiae	pulmonary infections rarely
M. ulcerans	buruli ulcer; necrotic infections of the skin and soft tissue
M. xenopi	often no clinical significance; pulmonary infections rarely

The transmission of NTM from person to person is until today not reported. It is more likely that the germs are transmitted through water, by absorbing from aerosols. An obligation to notify the authorities about the illness with non-tuberculous mycobacteria is not essential as in contrast to tuberculosis (Neumeister *et al*., 2009).

2.5 Maldi TOF MS

2.5.1 Introduction

The matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) is a new, rapid and cost-effective method for species identification in e.g. bacteria, viruses or fungi. It is a technique to detect the protein composition of a bacterium, which is specific for each bacterium (Moliner *et al*., 2010). The size of the peptides and proteins is characteristic for each organism and is used for the identification of bacteria (Mellmann *et al*., 2008). Since a few years, microbiology laboratories use the method also in routine diagnostics (Bizzini *et al*., 2011). MALDI TOF has different possible applications for the identification of an organism for example the identification and characterization of proteins and the detection of biomarkers or SNP (Single Nucleotide Polymorphism) genotyping. The analysis are completed in the range of 2,000 – 20, 000 m/z (masses to charge ratio) in a positive linear mode with a MALDI TOF (Moliner *et al*., 2010).

Figure 11: MALDI TOF microflex.
(http://www.bdal.com/products/maldi/microflex-series/overview.html)

2.5.2 Principle of the method

According to the molecular mass of an organism, the MALDI TOF technique creates mass spectra and consequently identifies the organism. Figure 12demonstrates a schematic description and an overview of the working principle. The construction consist of an ion source, a mass analyser that dissociates the ions in accordance to their individual mass-charge-ratio (m/z-ratio) and a detector to provide a mass spectrum of the sample (MALDI Theory; Mass Spectrometry , Bruker Daltonics 2004).

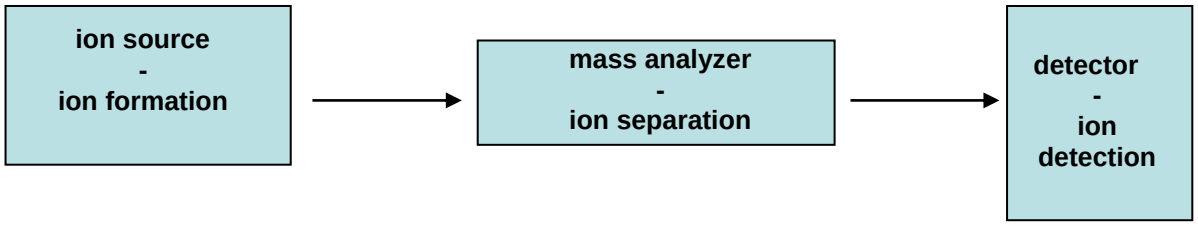


Figure 12: A simple construction of the device.
(MALDI Theory Mass Spectrometry; Bruker Daltonics 2004)

Generally, 10^5 - 10^6 of the cells are enough for the MALDI TOF analysis (Neumeister *et al*., 2009). By applying the matrix on the sample, the matrix co-crystallizes with the sample after evaporation of the solvent.

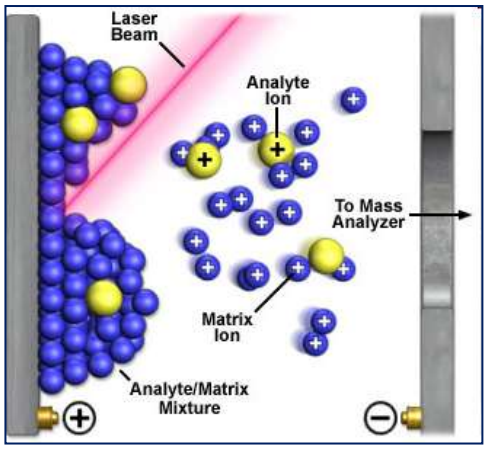


Figure 13: Sample overlaid with the matrix- bombardment with laser.
(http://www.magnet.fsu.edu/education/tutorials/tools/ionization_maldi.html)

The sample explosively evaporates (Figure 13) through bombarding the co-crystallized sample with a class III B-laser emitting 337 nm UV-light (microflex

user manual; Bruker Daltonics version 1.2; 2008).The proteins of the “unknown” sample carry off with the matrix and fly through the drift region, also called flight tube, to the detector. The detector (microflex User manual; Bruker Daltonics 2008) calculates the flight time, after ionisation, the proteins are accelerating in an electric field (10-30kV).The MALDI TOF exactly calculates the time of flight for each protein (Neumeister *et al*., 2009).

Table 10: MALDI Biotyper classification results report: meaning of score value.

Range	Description	Symbols	Colour
2.300 ... 3.000	highly probable species identification	(+++)	green
2.000 ... 2.299	secure genus identification, probable species identification	(++)	green
1.700 ... 1.999	probable genus identification	(+)	yellow
0.000 ... 1.699	not reliable identification	(-)	red

Table 10 shows the classification results report of the concluding analyte. Log score values between 2.0 ≤ x <3.0 show highly probable species identification. Values between 2.0 ≤ x < 2.299 identify an organism to the genus level. Values between 1.7 ≤ x <1.999 identify the organisms to the genus level and values under x<1.699 are not identifiable.

Cross identification with the Bruker taxonomy database

If the microorganism is unknown, a comparison with the Bruker taxonomy database (Figure 14) is possible.

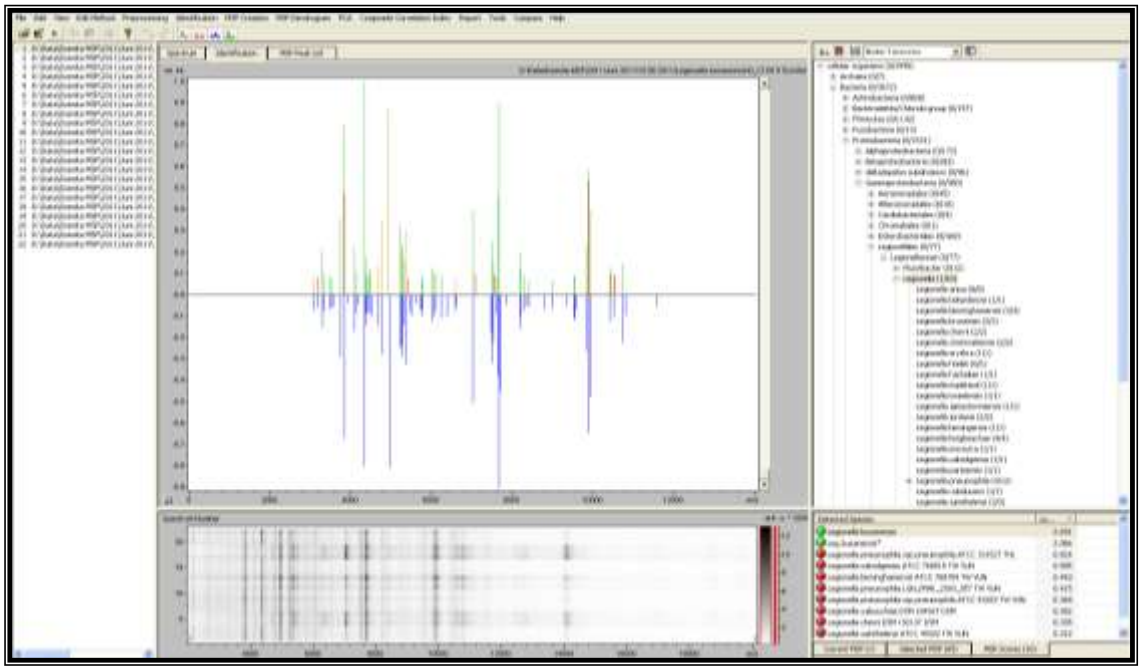


Figure 14: Cross identification of samples with the Bruker taxonomy database.

As seen in the Figure 14 the file view is on the left side, the spectrum views is in the middle, underneath is the gel view, on the top right is the taxonomy tree and on the bottom right the MSP score table. The x-axis of the spectrum view illustrates the m/z (mass-to-chargo ratio) value and the y-axis the intensity of the peak in arbitrary units. The spectrum shows green, yellow, red and blue peaks. The green peaks show perfect matching, the yellow the match in broader boarders and the red peaks do not match within the reference spectrum (Bruker Daltonics MALDI Biotyper 2.0-Software for Microorganism identification and classification).

2.5.3 Application in the routine clinical analysis

The investigation with the MALDI TOF is independent from the cultivation medium on which the organism grows. Only a small sample is required for the identification of a microorganism. The sample should not be older than 48 hours because the ribosomal proteins degrade which leads to substantial uncertainties in the results. Routine investigations use the smear method for identification because it is easier and faster than the extraction method (Neumeister *et al*., 2009).

2.6 Aims

The aim of the present study was the establishment of a new reference database using the MALDI TOF MS and the MALDI Biotyper software, for three *Legionella pneumophila* species, 36 non-pneumophila species, 45 non-tuberculous mycobacteria and 10 *Acanthamoeba* species. To investigate also the potential of MALDI TOF technology to detect bacterial endocytobionts within acanthamoebae, axenic cultures of *Acanthamoeba* spp. were artificially infected with *L. pneumophila* and *L. longbeachae*.

3 MATERIAL AND METHODS

3.1 Samples

All *Legionella* spp. and nontuberculous mycobacteria (NTM) strains (Table 12 and Table 13) investigated during this study were ordered from the German Collection of Microorganisms and Cell Cultures (DSMZ). The strains of *Acanthamoeba* spp.are from the collection of the Institute of Specific Prophylaxis and Tropical Medicine, Medical University Vienna, Kinderspitalgasse 15, A-1090 Vienna.

3.2 Cultivation

3.2.1 *Acanthamoeba* spp.

During the study, investigated amoeba strains (Table 11) are from the collection of the Institute, which were obtained from contact lenses cases, corneal epithelium, cornea smear or froma a *Boa constrictor* and an *Eunectes murinus*.

Table 11: *Acanthamoeba* strains used in this study.

Strain	Species	Sequence type	Sample	NN-agar	PYG
11DS	*A.hatchetti*	T6	corneal epithelium		x
1BU	*A.castellanii*	T4	corneal epithelium		x
2HH	*A. hatchetti*	T4	corneal epithelium		x
3ST	*A.hatchetti*	T4	corneal epithelium	x	
4CL	*A. polyphaga*	T4	contact lenses case		x
4RE	*A. hatchetti*	T11	contact lenses case	x	
ADS09	*A.*spp.	T4	cornea smear	x	
Am23	*A. rhysodes (A.astronyxis)*	T9	physiotherapy-Michel	x	
Bud9	*A.*spp.	T12	Bük/Hungary		x
Pb30/40	*A. comandoni*	T7	physiotherapy-Michel		x
Pb40	*A. comandoni*	T9	physiotherapy-Michel	x	
PJ	*A.*spp.	T9	PJ-soil Turkey	x	
PSH	*A.*spp.	T9	PS-human	x	
Zoo11	*A.*spp.	T11	mouth- *Boa constrictor*	x	
Zoo9	*A.* spp.	T11	mouth- *Eunectes murinus*		x

ATCC 50494	*A. castellanii*	T1	ATCC		x
ATCC 30171	*A. culbetsoni*	T10	Berlin	x	

Culture plates

For the preparation of culture plates, 5 g NN-agar (Agar Bacteriogical (Agar NO.1) Oxoid) were dissolved in 500 ml ddH_2O and subsequently autoclaved (CertoClav$_R$) for 5 min at 140°C. After the agar had cooled down, 15 ml per dish were poured into sterile petri dishes and stored at 4°C.

Cultivation on agar plates

Acanthamoebae were cultivated on NN (non-nutrient) agar plates (Figure 15) in the presence of living bacteria (*Escherichia coli*) as a food source. Approximately 50 µl of a 48 h old *Escherichia coli* culture were spread with a Drigalski over the entire plate. Depending on the growth rate, the subcultivation of acanthamoebae was performed every few weeks, by cutting out a piece from an agar plate covered with cysts or trophozoites which was subsequently transferred and placed upside down onto a new culture plate in presence of *E. coli* as a food source. The agar plates were sealed with parafilm and incubated at room temperature.

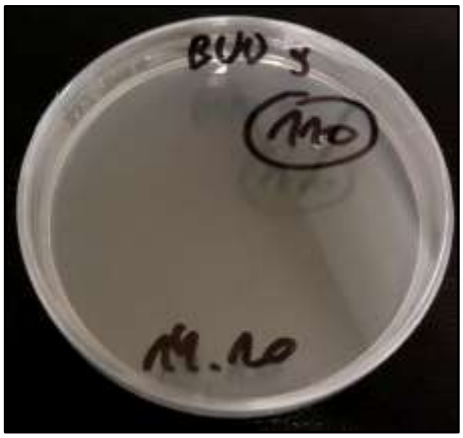


Figure 15: NN-agar plate photo: Hasanacevic D.

Preparation of PYG medium

Additionally to the cultivation on NN agar plates, strains were cultivated in Proteose-peptone-Yeast extract-Glucose medium (PYG medium).
The components (10 g proteose peptone(Merck), 10 g yeast extract granulates (Merck), 5 g sodium chloride –NaCl (Merck), 5 g D(+)-Glucose (Merck), 0.7 g di-sodium hydrogen phosphate –Na_2HPO_4 (Merck), 0.7 g potassium phosphate (Sigma), 10 ml antibiotic-antimycotic) were dissolved in 990 ml ddH_2O using a magnetic stirrer (IKA ^{R} RCT basic) until the medium became clear. In the next step, the medium was filter-sterilized (Thermo Scientific Nalgene Filter Units – 1,000 ml Capacity, MF75™ Series) under the laminar flow. Finally, the medium was stored at 4°C.

Cultivation of *Acanthamoeba* spp. in axenic culture

The medium was changed once a weak. For axenisation, the cultures of *Acanthamoeba* spp. were harvested with sterile cotton tipped applicator from the NN agar plates into falcon-tubes (Sarstedt or BD) containing 7 ml of sodium chloride (NaCl) and the samples were centrifuged at 2,500 g for 10 min. After centrifugation the supernatant was discarded, 5 ml 3% hydrogen chloride (HCl) was added and the samples were incubated overnight at room temperature. On the next day the samples were centrifuged for 10 min at 500 g, the supernatant was discarded and the pellet was resuspended in 7 ml PYG-medium. Finally, the suspension was transferred to a sterile culture flask filled with 7 ml PYG medium and stored at room temperature. The acanthamoebae were cultivated in 25 ml culture flasks (Falcon), (Figure 16) filled with 7 ml PYG medium (Visvesvara & Balamuth 1975).

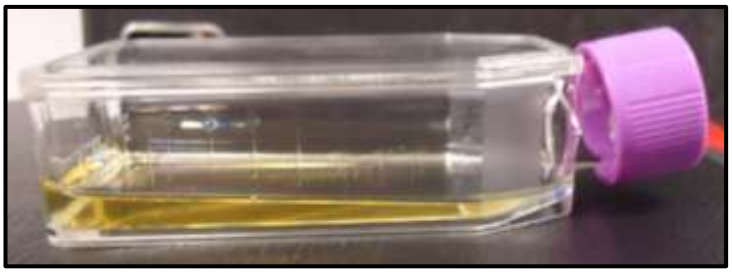


Figure 16: Culture flask photo: Hasanacevic D.

Harvesting of *Acanthamoeba* spp.

Amoebae were harvested by shaking the culture flask very hard to detach the trophozoites from the bottom of the culture flask. The suspension was transferred into 15 ml sterile Sarstedt tubes (Sigma) and centrifuged for 15 min at 1,500 g. To wash the samples the following steps were performed for three times. The supernatant was discarded and the pellet was resuspended in 400 µl ddH_20 and transferred to a sterile microcentrifuge tube. The samples were centrifuged for 15 min at 1,500 g and the supernatant was discarded. After the last washing step, the pellet was resuspended in 400 µl ddH_20. For subsequent DNA isolation, 100 µl of the suspension were transferred to a sterile microcentrifuge tube. The samples were stored over night at -20°C to break up the cell membrane of the amoebae. The remaining 300 µl of the sample were washed for two more times before they were used for the extraction method, which is necessary for the creation of a MALDI TOF main spectrum as described in chapter MALDI TOF (3.4.1 Sample preparation).

3.2.2 *Legionella* spp.

All *Legionella* strains (Table 12) were cultivated on BCYE-medium plates (Buffered Charcoal Yeast medium; composed of Legionella-C.Y.E agar basis-Merck and Legionella-B.C.Y. a growth supplement-Merck) and on GVPC-medium plates (Buffered Charcoal Yeast Extract medium with GVPC-selective supplement; Biomérieux). 100 µl of *Legionella* suspension was removed and spread on culture plates. The plates were incubated in an anaerobic box (Becton Dickinson) including a CO_2 bag (Becton Dickinson; GasPak^{TM} EZ CO_2 Container System) at 30°C or 37°C for 5 to 7 days under humid conditions. The temperature difference during the incubation time depends on different growth behaviour of the *Legionella* strains.

Table 12: *Legionella* spp. from the "German Collection of Microorganisms and Cell Cultures" (DSMZ). (http://www.dsmz.de/)

Number	DSMZ strain	*Legionella* species
1	19 888	*L.adelaidensis*
2	17 627	*L. anisa*
3	19 152	*L. beliardensis*
4	19 232	*L. birminghamensis*
5	19 236	*L. brunensis*
6	22 853	*L. busanensis*
7	19 213	*L. cherrii*
8	19 233	*L. cincinnatiensis*
9	19 890	*L. drozanskii*
10	19 889	*L. fallonii*
11	21 217	*L. geestiana*
12	21 233	*L. gratiana*
13	21 218	*L. gresilensis*
14	19 214	*L. hackeliae*
15	18 493	*L. impletisoli*
16	19 235	*L. israelensis*
17	19 215	*L. jamestowniensis*
18	19 212	*L. jordanis*
19	19 556	*L. lansingensis*
20	21 234	*L. londiniensis*
21	10 572	*L. longbeachae*
22	19 234	*L. moravica*
23	21 805	*L. nautarum*
24	21 215	*L. oakridgensis*
25	19 216	*L. parisiensis*
26	7514	*L. pneumophila* subspecies *fraseri*
27	7515	*L. pneumophila* subspecies *pascullei*
28	7513	*L. pneumophila* subspecies *pneumophila*
29	11 884	*L. rubrilucens*
30	19 231	*L. sainthelensi*

31	23 075	*L. santicrucis*
32	23 087	*L. shakespearei*
33	19324	*L. spiritensis*
34	23 076	*L. steigerwaltii*
35	21 897	*L. taurinensis*
36	19 246	*L. tucsonensis*
37	21 896	*L. wadsworthii*
38	21 908	*L. waltersii*
39	21 907	*L. worsleiensis*
40	18 492	*L. yabuuchiae*

Cultivation of *Legionella* spp.

Ampoules (Figure 17) containing dried *Legionella* spp. were opened by heating the tip of the ampoule in a flame. Subsequently, a few water drops were placed onto the hot tip of the ampoule and the glass ampoule broke. The inner vial containing the dried *Legionellae* were taken out, 500 µl of the nutrient medium (peptone 5 g, meat extracts 3 g, 1,000 ml ddH2O) was added to dissolve the dried bacteria and finally incubated for 30 min at room temperature for rehydration.

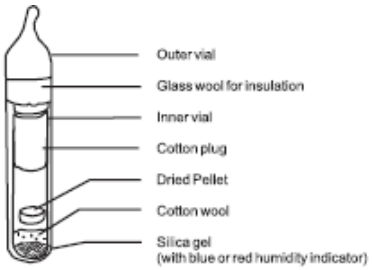


Figure 17 : Opening of ampoules and rehydration of dried cultures.
http://www.dsmz.de/microorganisms/main.php?contentleft_id=11

3.2.3 *Mycobacterium* spp.

The ampoules containing dried cultures of *Mycobacterium* spp. (Table 15) were opened and handled as previously described for *Legionella* spp. However, the glass ampoules with nontuberculous mycobacteria were opened at the high security laboratory (L3) where 100 µl of the mycobacteria suspension was inoculated onto a solid Löwenstein-Jensen medium (Heipha). The culture tubes were incubated at 30°C or 37°C for a few days or up to weeks depending on the different growth behaviour of the bacteria. Periodically, the cultures were checked for bacterial growth and *Mycobacteria* spp. were harvested by using a cotton tipped applicator for subsequent MALDI TOF analysis and DNA isolation as previously described.

Table 13: *Mycobacteria* spp. from the "German Collection of Microorganisms and Cell Cultures" (DSMZ) (http://www.dsmz.de/).

Number	DSMZ	Species
1	44 196	*M. abscessus*
2	44 176	*M. alvei*
3	44 156	*M. avium* ssp. *avium*
4	44 133	*M. avium* ssp. *paratuberculosis*
5	44 175	*M. avium* ssp. *silvaticum*
6	45 071	*M. bovis*
7	44 243	*M. celatum*
8	44 196	*M. abscessus* *M. chelonae* ssp. *abscessus*
9	43804	*M. chelonae* ssp. *chelonae*
10	44 159	*M. chelonae ssp. niacinogenes*
11	44 623	*M. chimaera*
12	43 991	*M. flavescens*
13	43 075	*M. fortuitum* ssp. *fortuitum*
14	43 505	*M. gastri*
15	44 160	*M. gordonae*
16	44 634	*M. haemophilum*
17	44 764	*M. immunogenum*
18	44 064	*M. interjectum*
19	44 049	*M. intermedium*
20	43 223	*M. intracellulare*
21	44 162	*M. kansasii*
22	44 418	*M. lentiflavum*
23	44 476	*M. mageritense*
24	44 163	*M. malmoense*
25	44 344	*M. marinum*
26	44 625	*M. mucogenicum*
27	44 572	*M. palustre*
28	44 133	*M. avium* ssp. *paratuberculosis*
29	43 271	*M. peregrinum*

30	43 239	*M. phlei*
31	43 992	*M. scrofulaceum*
32	44 393	*M. septicum*
33	45 125	*M. shottsii*
34	44 165	*M. simiae*
35	43 756	*M. smegmatis*
36	44 166	*M. szulgai*
37	43 995	*M. xenopi*
38	44 220	*M. fortuitum* ssp. *acetamidolyticum*
39	43 503	*M. kansasii* ssp. *aurantiacum*
40	43 494	*M. kansasii* ssp. *luciflavum*
41	43 528	*My. parafortuitum*
42	44 648	*M. parascrofulaceum*
43	43 290	*Gordonia alkanivorans formerly: M. rubrum*
44	43 227	*M. terrae*
45	44 191	*M. austroafricanum*

3.2.4 Infection of *Acanthamoeba* spp. with *Legionella* spp.

3.2.4.1 Preparation of *Legionella* spp. and *Acanthamoeba* spp. before infection

The *Legionella pneumophila* serogroups 1, 4, 5 and *Legionella longbeachae* were suspended with an inoculation loop into a microcentrifuge tube filled with 4.5 ml phosphate buffered saline (PBS) and vortexed to a homogenic suspension. One colony was dissolved in 4.5 ml PBS and compared to McFarland test tubes (Biomerieux) to get a McFarland standard 1 (Figure 18).

Figure 18: McFarland standard.
(http://labtubes.equipmentlab.net/2011/04/19/mcfarland-standard-latex-equivalent-2-1-per-pack/)

The McFarland standard from 0.5 until 5 shows the density of bacteria in PBS (Table 14). A McFarland 0.5 means a density of 150 x 10^6 bacteria/ml.

Table 14: McFarland standard and density of bacteria.

McFarland standard	Density of bacteria x 10^6/ml
0.5	150
1	300
2	600
3	900
4	1200
5	1500

On the day before infection of the acanthamoebae, the medium of *A.hatchetti* T6 (11DS) and *A.hatchetti*T4 (2HH) was changed. On the following day, the trophozoites and cysts of the acanthamoebae were counted with a Fuchs-Rosenthal counting chamber. The culture flask with the axenic culture was shaken very hard and vortexed to detach the trophozoites from the bottom of the flask. Approximately 260 µL of the suspension was transferred to the counting chamber, which was covered with a cover glass. The cells were counted by screening diagonally four squares (one square contains 16 smaller squares, Figure 19). After counting, the value was multiplied with four and divided by three. The result was multiplied with 1,000 and the resulted number was multiplied again with the amount of liquid culture contained in the culture flask. Subsequently the measured amoebae were infected with bacteria.

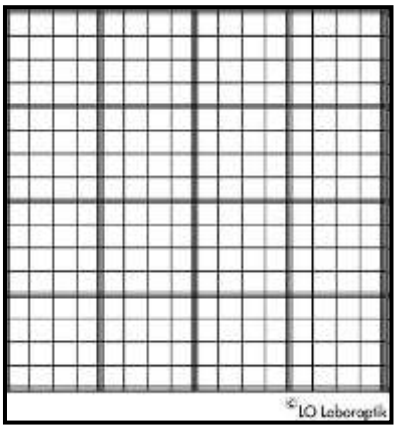


Figure 19: Details of the Fuchs- Rosenthal counting chamber.
(http://www.zaehlkammer.de/gfx/fuchs_rosenthal.jpg)

A. hatchetti T6 and *A. Hatchetti* T4were infected with *L*. *pneumophila* serogroup 1, 4, 5 and *L. longbeachae,* dissolved in 140 µl PBS (to get a density of 150 x 10^6/ml). On the next day, the infected amoebae were harvested and handled as described. Amoebae cultures were generally infected in triplets and harvested three days consecutively. Finally, the samples were measured with the MALDI TOF.

3.3 Molecular biology

3.3.1 DNA Isolation MagNa Pure Compact

The *Acanthamoeba* samples (100 µl) were stored in microcentrifuge tubes overnight at -20°C. After thawing of the samples, 550 µl ddH_2O were added. A few colonies of each *Legionella* strains were collected from agar plates by using cotton tipped applicator and were thoroughly suspended in different sterile microcentrifuge tubes containing 650 µl ddH_20. Non-tuberculous mycobacteria were transferred from Löwenstein medium into a micro centrifuge tube filled with 700 µl ddH_2O. The suspension was incubated for 30 min at 95°C in an Eppendorf thermomixer compact 3030 to inactivate the bacteria.

The genomic DNA was extracted using the commercial MagNa Pure Compact (Roche Diagnostics, Germany) according to the manufacturers' manual. The Reagent cartridges (well one contain Proteinase K, well two Guanidiniumthiocyanat(e)/TritonX-100, well three Isopropanol, well four and five Guanidinium HCl, ethanol and well six ethanol) and the tip trays (containing two large reaction tips, one small reaction tip, and one piercing tool) were inserted into the rack of the MagNa Pure. The tubes with the suspended samples in 650 ddH_2O µl (Sigma) were inserted into the MagNa Pure. Inserted the elution tube (with barcode), closed the door, pressed confirm data, drop catcher present and then on start. The procedure takes about 30 min.

3.3.2 Polymerase chain reaction (PCR)

3.3.2.1 *Acanthamoeba*- PCR

For genotyping of *Acanthamoeba* spp. the primer pairs JDP1 and JDP2 (Schroeder *et al*., 2001) were used. The primer binding sites for the forward primer JDP1 (18s f 5´-GGCCCAGATCGTTTACCGTGAA -3´) and the reverse primer JDP2 (18s r 5´-TCTCACAAGCTGCTAGGGAGTCA-3´) are located in a highly conserved regions of the 18S rRNA gene. The primers have a length of 22bp (JDP1) and 24bp (JDP2) and a balanced GC/ AT ratio. The amplicon has a size of about 423-551 bp, depending on genotype (Schroeder *et al*., 2001). The master mix for PCR contained 25 µl Hotstar MM Qiagen, 5 µl JDP1 (5 pmol/µl $^{-1}$), 5 µl JDP2 (5 pmol/µl $^{-1}$), 14 µl water and 1 µl sample DNA. The PCR was run with the following program:15 min 96°C; 45 cycles: 1 min 95°C, 1 min 60°C, 2 min 72°C; and a final elongation step of 7 min at 72°C. The entire run takes about 3h 50 min.

3.2.2.2 LegMIP PCR

For identification of *Legionella* spp., the MIP (microphage infectivity potentiator) gene was analysed. The forward (Legmip_f: 5´-GGG RAT TVT TTA TGA AGA TGA RAY TGG -3´) and the reverse (Legmip_r: 5´TCR TTN GGD CCD ATN GGN CCD CC-3´) primer were required for the amplification (Ratcliff *et al*., 1998). The Legmip_f and Legmip_r primer targeted the binding site and the peptidyl-prolyl isomerase (PPIase) site of the *mip* gene was intended to amplify a 661 to 715 bp long DNA fragment. The master mix for the PCR contained 10 µl Amplitaq Gold^{R} PCR Mastermix (Applied Biosystems), 1 µl Legmip_f (20pmol/µl $^{-1}$), 1 µl Legmip_r (20 pmol/µl $^{-1}$), 4 µl Q-Solution (Qiagen), 3 µl water and 1 µl sample DNA. The amplification PCR run under the following conditions: 3 min 96°C; 35 cycles: 1 min 94°C, 2 min 58°C, 2 min 72°C; and finally the elongation step 5 min 72°C. The procedure takes about 3 h 24 min.

3.2.2.3 16S PCR

For identification of gram-negative or gram-positive bacteria, the 16S forward primer 5´-TCCTACGGGAGGCAGCAGT -3´ and the 16S reverse primer 5´-GGACTACCAGGGTATCTAATCCTGTT -3´ were used (Nadkarni *et al*., 2002).
The master mix for PCR contained 25 µl Hotstar MM (Qiagen), 0.5 µl 16S forward primer, 0.5 µl 16S reverse primer (10 pmol/µl $^{-1}$), 19 µl water and 5 µl sample DNA. PCR was performed with the following PCR program: 15 min 95°C; 35 cycles: 30 sec 94°C, 1 min 60°C and a final elongation step for 1 min at 72°C. The procedure takes about 2 h 02 min.

3.3.3 Protocol for PCR product clean up

For PCR-clean up with Shrimp Alkaline Phosphatase (SAP) and Exonuclease I, the master mix contained 1 µl Exonuclease I and 2 µl SAP and 10 µl sample DNA. The clean up step run with the following program: 15 min 37°C and 15 min 85°C.

3.3.4 Sequencing PCR

To perform the sequencing PCR only one primer for each sample was used. The master mix for sequencing PCR contained 2 µl big dye mix (Applied Biosystems), 1 µl sequencing buffer, 4 µl water, 1 µl of the primer (JDP1 or JDP2 for acanthamoebae, Legmip_f_s or Legmip_r for *Legionella* and 16S forward or 16S reverse for NTM) and 2 µl sample DNA.
The PCR was run with the following program:1 min 96°C followed by 30 cycle of: 20 sec 96°C, 20 sec 50°C and final elongation step of 4 min at 60°C. The procedure takes about 2 h 43 min.

3.3.5 Cleanup with the Centri-Sep 8 strips

The samples were cleaned up with the Centri-Sep 8 strips (Princeton Separations). The strip was spun for 2 min at 750 g (Eppendorf centrifuge 5804R) to remove the liquid. The whole volume of the sample was added to the centre of the centri-sep well, placed into an 8-well PCR strip (Nerbe Plus) and centrifuged for 2 min at 750 g.

3.3.4 DNA-Sequencing

Three µl of the cleaned PCR product and 10 µl of Hi-di ™ Formamide Genetic analysis grade AB (Applied Biosystems) were pipetted into a MicroAmpTM optical 96 well reaction plate (AB). The plate was closed with a septa mat and centrifuged for 1 min at 200 g. Subsequently, the plate was placed into the 96-well plate base / retainer. The samples were sequenced with different instrument protocols by using a 4-capillary sequencer (Applied Biosystems 3130 Genetic Analyzer), a POP 7 Gel and a 36 cm capillary.

3.3.4.1 Data analysis

The sequenced *Legionella* spp., non-tuberculous mycobacteria and acanthamoebae samples were analysed by using the CLC DNA Workbench 5 and subsequent compared to the reference sequences from the NCBI GenBank database, the *mip* sequence database from the Health Protection Agency and to the reference sequences at Ridom framework 16S rDNA.

3.4 MALDI TOF

3.4.1 Sample preparation

Extraction method

The samples were collected from culture plates with an inoculation loop and suspended into microcentrifuge tubes filled with 300 µl HPLC water (high-performance liquid chromatography–LiChrosolv®; Merck). Subsequently, 900 µl of 70% ethanol (Uvasol®; Merck) was added and the sample was vortexed. After centrifugation at 16,100 g for 2 min (Eppendorf centrifuge 5115R), the supernatant was removed. The centrifugation step was repeated for two times. The remaining supernatant was discarded with a plastic pasteur pipette. After that, the pellet was dried at 37°C for 15 min on a thermomixer (Eppendorf; thermomixer comfort) to maximize the efficiency of the formic acid treatment in the following step. Moreover, 50 µl of 70% formic acid (Emsure®; Merck) was added to the pellet and mixed well by vortexing. Subsequently, 50 µl of 100% acetonitrile (LiChrosolv®; Merck) was added to the pellet and the components were mixed carefully. Depending on the biomass amount 10-50 µl of acetonitrile and formic acid were added to the pellet. Then, the samples were centrifuged at 16,100 g for 2 min, 1 µl of the supernatant was transferred onto a polished steel Bruker MSP(Micro SCOUT Plates) 96-target and the samples were incubated at room temperature for 5-10 min to dry. Finally, 1 µl of$HCCA_{portioned}$ matrix was added to the samples and the samples were dried and at room temperature for 10- 15 min (Mellmann *et al.* 2008).

Experimental procedure of “Mycobacteria bead preparation”

The protocol for “Bruker Mycobacteria bead preparation” was similar to the extraction method with ethanol and formic acid. As described in the chapter “extraction method”, a 1.5 ml Eppendorf tube was filled with 400 µl of HPLC water and 900 µl ethanol. After centrifugation at maximum speed for 2 min the supernatant was removed, 500 µl HPLC water was added and the pellet was thoroughly suspended. After centrifugation (VWRTM Galaxy 16DH) at

maximum speed (16,100 g) for 2 min, the supernatant was discarded and the remaining pellet was centrifuged again. The residual water was carefully removed by pipetting. 50 µl of 100% acetonitrile were added to the samples and resuspended. To inactivate the mycobacteria the samples were boiled at 99°C (Eppendorf thermomixer compact 3030) for 20 min. Subsequently, the tubes were centrifuged for 50 sec at maximum speed. Right after that, a tip of a small spatula of Silica beads (0.5 mm Zirconia / Silica beads, BioSpec Products, Catalog no. 1107911052) was added to the samples. The microcentrifuge tube was vortexed for 1 min and centrifuged for 1 min at 16,100 g. Subsequently, 50 µl of 70% formic acid were added, mixed by vortexing and centrifuged at maximum speed for 2 min. Finally, 1 µl of the supernatant, containing the bacterial extract, was placed on the polished steel Bruker MSP 96-target and overlaid with matrix after the sample had dried.

3.4.2 Creation of MSPs with Maldi Biotyper 2.0 and the establishment of a new database

Establishment of a new reference database

The MALDI TOF was calibrated by using the Bruker Bacterial Test Standard (BBTS; *Escherichia coli*) according to the manufacturers' manual. The standard was used as a positive control for correct calibration and sample preparation. For each analysis performed with the MALDI TOF one position at the centre of the target plate (D6) was overlaid with BBTS. For the creation of a new database, each investigated organism was spotted on eight positions of the target and measured 3 times per spot. Finally, the 24 created spectra were selected due to their quality for subsequent creation of main spectra. After the measurement, the mass spectra were analyzed with the flex analysis.

Spectra quality control with the Flex Analysis

The spectra set of each species was opened in Flex Analysis. The smoothing and baseline subtraction was performed with the MBT_standard method. The existence of flatlines or spectra with abnormal peaks opposed from the other spectra were verified and if essential deleted. The deviation maximum was than 500 ppm. At least 20 spectra had to be available after the quality control for the MSP creation with the Maldi Biotyper 2.0. The mean time for one isolate for the spectra quality control was ~ 5-10min, depending on the number of organism. All spectra of each strain were imported into the Maldi Biotyper 2.0 software after the quality control in Flex Analysis. All spectra were selected, the button "MSP creation, create MSP" was clicked and a new project was entitled.

4 RESULTS

4.1 Culture

4.1.1 *Acanthamoeba*

4.1.1.1 Culture plates and axenic cultures

All used acanthamoebae grew on NN-agar plates and proliferated in liquid culture. *Acanthamoeba culbertsoni (*ATCC 30171) was only culturable on NN-agar plates and was unable to proliferate in PYG medium. The axenisation of the *Acanthamoeba* strains was successful with *A. hatchetti* T4 (3ST) and *A.* spp. T4 (ADS09).

4.1.2 *Legionella*

Legionella species purchased from the German collection of microorganisms and cell cultures (DSMZ) grew at a temperature between 30°C (*L. drozanskii, L. santicrucis, L. shakespearei, L. steigerwaltii, L. waltersii* and *L. fallonii)* and 37°C (the remaining *Legionella* strains) on GVPC culture plates (Buffered Charcoal Yeast Extract medium with GVPC selective supplement -glycine, vancomycin hydrochloride, polymyxin B, cyclohexemide). Only *Legionella gestiana* did not grow on the culture plates (Figure 20).

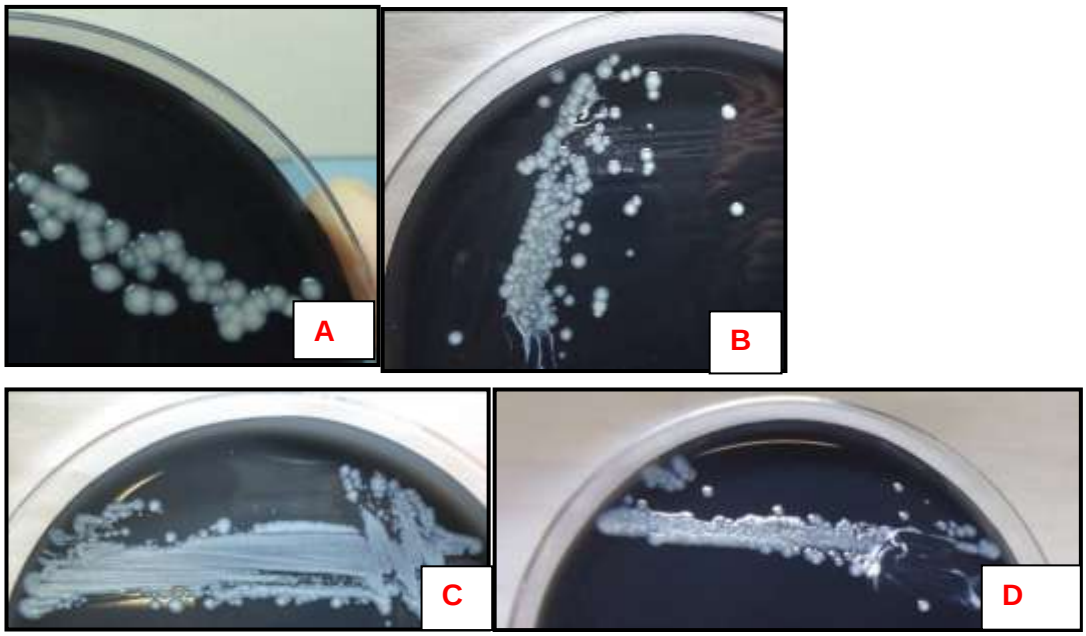


Figure 20: *L. pneumophila* serogroup 1 Olda (A), *L. pneumophila* serogroup 1 Bellingham (B), *L. pneumophila* serogroup 1 Benidorm (C), *L. pneumophila* serogroup 1 Camperdown (D) on a GVPC plates. (Buffered Charcoal Yeast Extract medium with GVPC selective supplement -glycine, vancomycin hydrochloride, polymyxin B, cyclohexemide) photo: Hasanacevic D.

4.1.3 Non-tuberculous mycobacteria

4.1.3.1 Löwenstein medium

Of 45 mycobacteria species obtained from the German collection of microorganisms and cell cultures (DSMZ), only *M. avium* subspecies *silvaticum* and Gordonia alkanivorans (formerly *Mycobacterium rubrum*) did not grow on the Löwenstein medium. *M. abscessus, M. chelonae* subspecies *abscessus, M. chelonae* subspecies *chelonae, M. haemophilum, M. intracellulare and M. marinum* grew at 30°C. *M.shottsii* grows generally at a temperature of 30°C. The strain was removed from the incubator and stored at room temperature (~22°C), since there was no evident growth after an incubation period of a month. However, three months later a significant growth could be detected. The remaining mycobacteria strains grew at a temperature of 37°C (Figure 21).

The growth of non-tuberculous mycobacteria took a few days (e.g. *M.abscessus, M.phlei, M.austroafricanum and M.alvei)* to two months (*M.haemophilum, M.lentiflavum, M.palustre).*

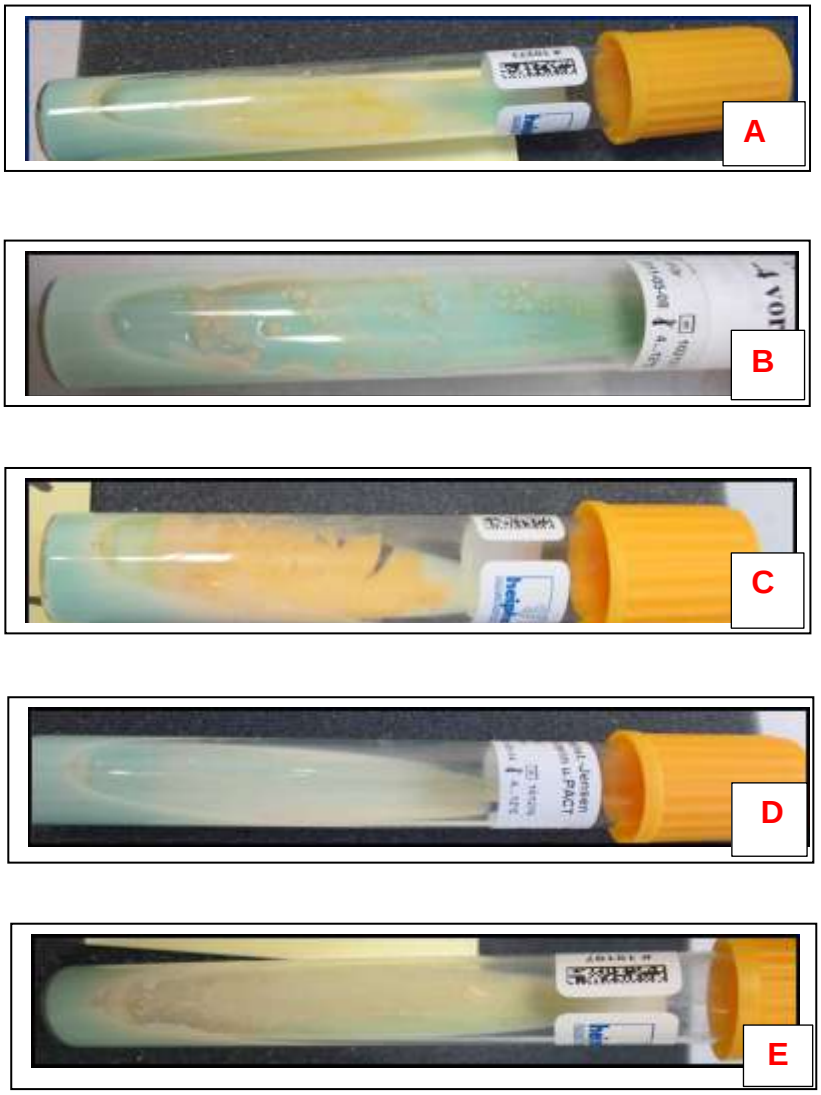


Figure 22: Non-tuberculous mycobacteria on Löwenstein medium: *M. Austroafricanum* (A), *M.fortuitum* subspecies *fortuitum* (B), *M. phlei* (C), *M. abscessus* (D), *M. chelonae* subspecies *chelonae* (E). photo: Hasanacevic D.

4.2 Molecular biology

4.2.1 Sequencing results

4.2.1.1 *Acanthamoeba*

DNA sequencing analysis successfully genotyped ten amoeba species. The sequences were analysed by using CLC DNA Workbench 5 and subsequently compared to a reference sequence obtained from the NCBI GenBank database (Figure 23) (http://www.ncbi.nlm.nih.gov/pmc/).

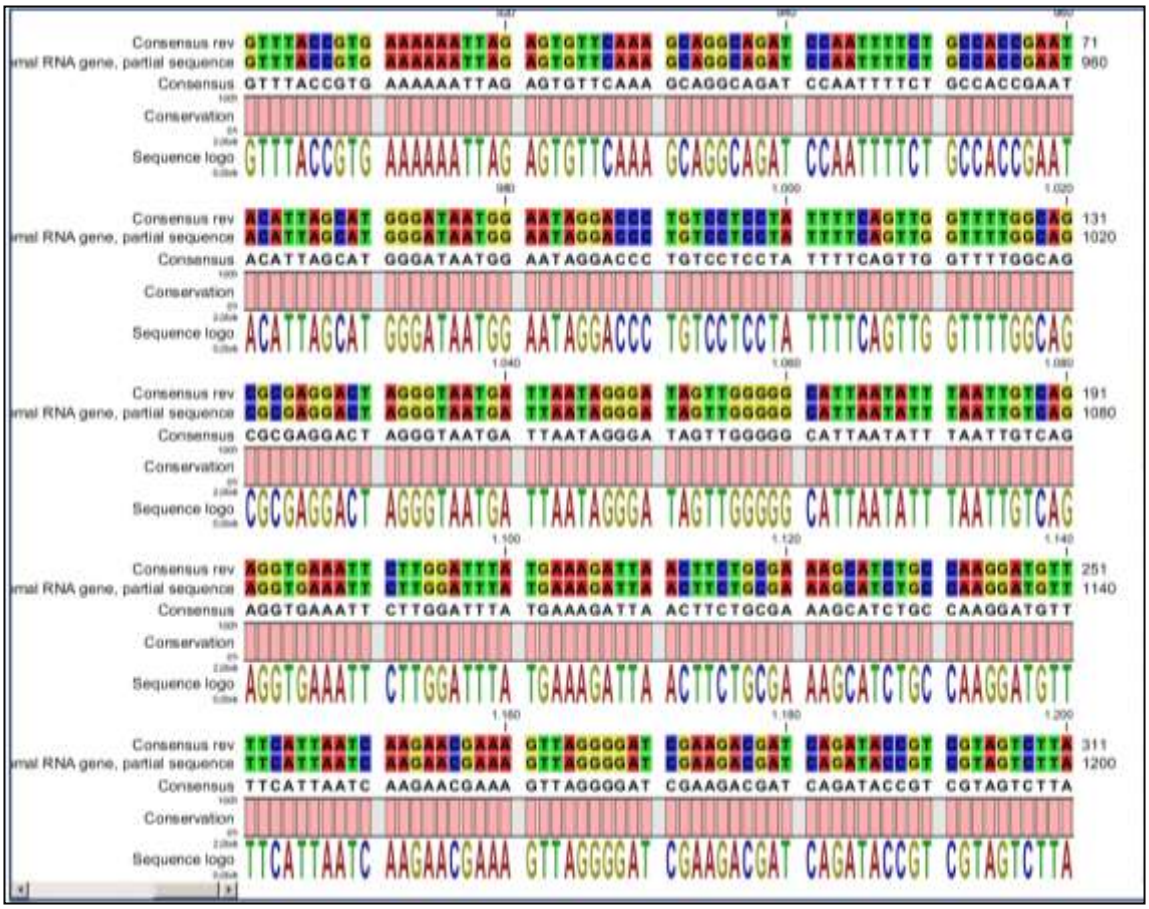


Figure 23: CLC DNA Workbench: Alignment of *Acanthamoeba castellanii* (1BU).

The degree of relationship of the used acanthamoebais is shown in a phylogenetic tree of the CLC DNA Workbench (Figure 24).

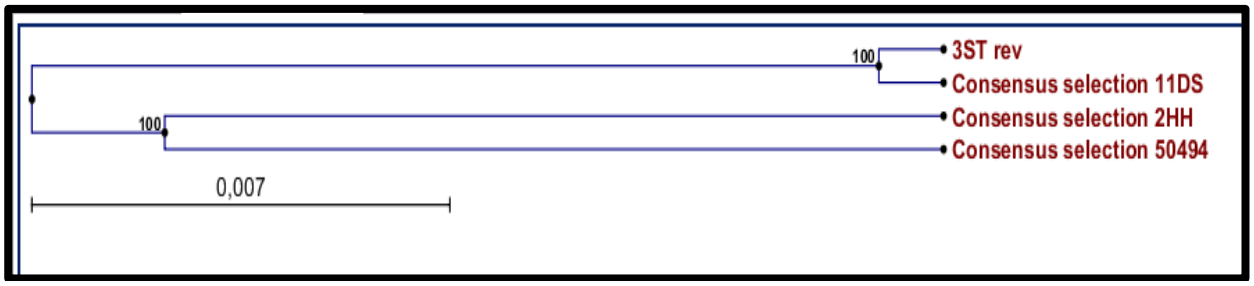


Figure 24: Phylogenetic tree of 18S (CLC DNA Workbench 5) of *Acanthamoeba* spp. (*A. hatchetti* T4 (3ST), *A. hatchetti* T6 (11DS), *A. hatchetti* T4 (2HH), *A. castellani* T1 (ATCC 50494)).

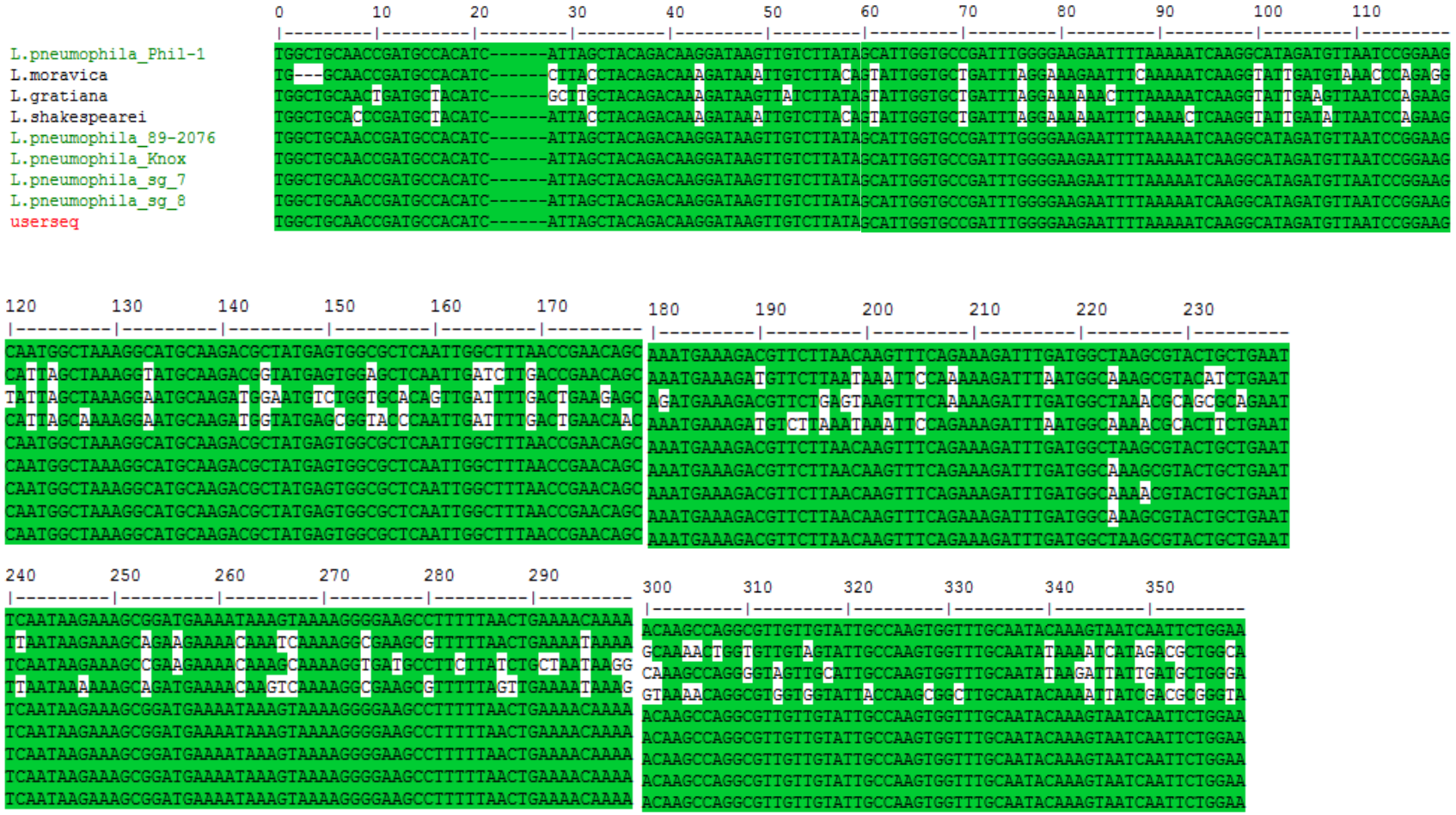
4.2.1.2 *Legionella*

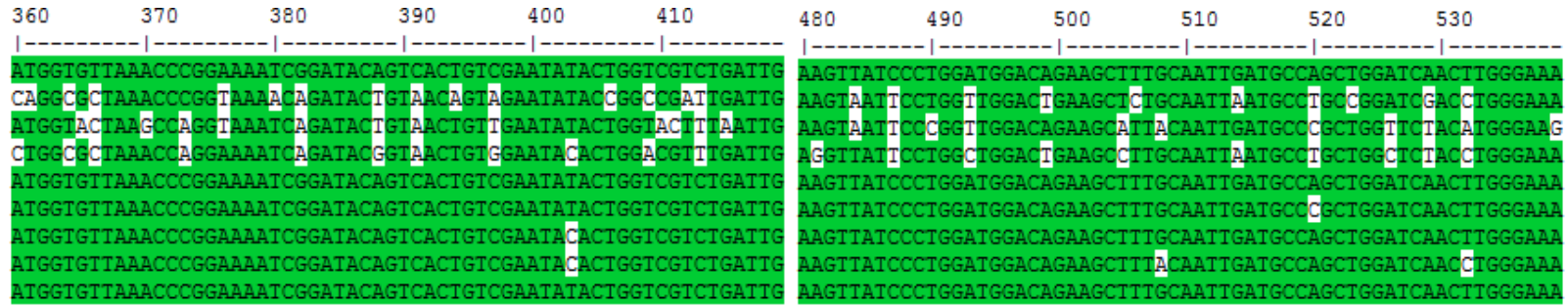
In the present study, *mip* sequencing was ude as reference identification method. All 39 *Legionella* species were successfully identified to the species level. The Table 15 shows the percentage concordance of the *mip* gene compared to the *mip* sequence database of the Health Protection Agency (HPA).Conflicts in some origin sequences led to no absolute correlation (100%) with the reference database. Even after several sequencing repetitions of the *mip* region, the top hit in case of *Legionella impletisoli* remained at 87.29%.

Table 15: Identification of the *Legionella* species and compared with the *mip* sequence database interrogator of the Health Protection Agency.

Legionella spp.	percentage concordance
L. adelaidensis	100.00%
L. anisa	99.83%
L. beliardensis	100.00%
L. birminghamensis	100.00%
L. brunensis	100.00%
L. busanensis	100.00%
L. cherrii	99.83%
L. cincinnatiensis	99.83%
L. drozanskii	99.82%
L. fallonii	99.83%
L. gratiana	100.00%
L. gresilensis	100.00%
L. hackeliae	100.00%
L. impletisoli	87.29%
L. israelensis	96.23%
L. jamestowniensis	100.00%
L. jordanis	100.00%
L. lansingensis	100.00%

L. londiniensis	100.00%
L. longbeachae	100.00%
L. moravica	99.82%
L. nautarum	96.11%
L. oakridgensis	96.80%
L. parisiensis	100.00%
L. pneumophila subsp. *fraseri* →serogroup 4	100.00%
L. pneumophila subsp. *pascullei* → serogroup 5	100.00%
L. pneumophila subsp.*pneumophila* →serogroup 1	100.00%
L. rubrilucens	97.40%
L. sainthelensi	100.00%
L. santicrucis	100.00%
L. shakespearei	100.00%
L. spiritensis	100.00%
L. steigerwaltii	99.66%
L. taurinensis	100.00%
L. tucsonensis	100.00%
L. wadsworthii	99.83%
L. waltersii	100.00%
L. worsleiensis	97.24%
L. yabuuchiae	97.12%


L.pneumophila_Phil-1
L.moravica
L.gratiana
L.shakespearei
L.pneumophila_89-2076
L.pneumophila_Knox
L.pneumophila_sg_7
L.pneumophila_sg_8
userseq



Species	Percent id
L.pneumophila_89-2076	100.00
L.pneumophila_Phil-1	100.00
L.pneumophila_Knox	99.65
L.pneumophila_sg_7	99.48
L.pneumophila_sg_8	98.96
L.moravica	82.47
L.shakespearei	80.90
L.gratiana	80.38

Figure 25: Concordance of the *mip* gene compared to the *mip* sequence database of the Health Protection Agency (HPA)*: Legionella pneumophila.*

Figure 25 shows a complete alignment of the *mip* gene of the *Legionella pneumophila* serogroup 1. The reference *mip* sequence of the *Legionella pneumophila* serogroup 1 was compared to the HPA database showing a 100% match to *L. pneumophila*_89-2076 and *L. pneumophila*_Phil-1.

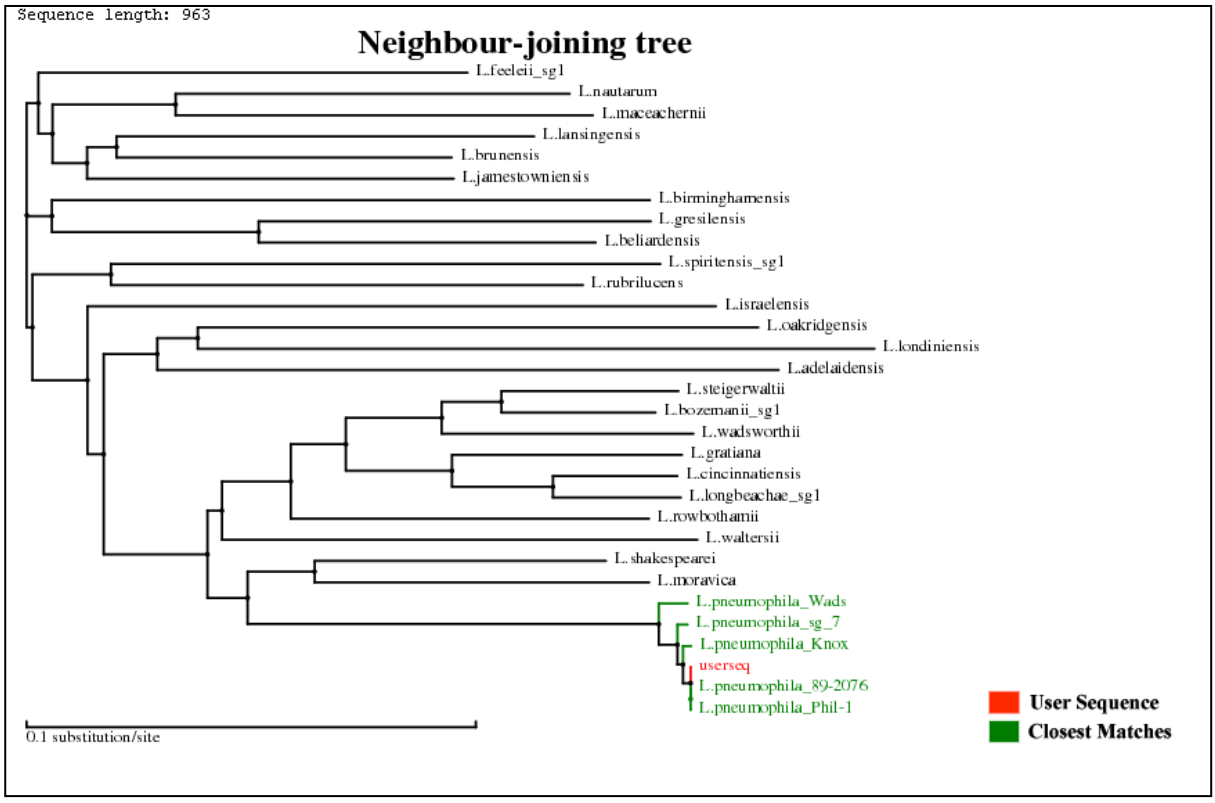


Figure 26: Health Protection Agency- Phylogenetic tree of *Legionella* spp. red depicts the user sequence whereas green shows the matching results.

A neighbour-joining tree was created to show the association between the *Legionella* species as demonstrated in Figure 26.

4.2.1.3 Non-tuberculous mycobacteria

The 40 non-tuberculous mycobacteria species were successfully identified to species level. The ensuing results were compared to the data from the reference database of the Ridom framework16S rDNA (http://www.ridom.de/rdna/) or with BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 16: Identification of the non-tuberculous mycobacteria species, compared with the RIDOM database.

Non-tuberculous mycobacteria	percentage concordance
M. abscessus	99.34%
M. alvei	100.00% (NCBI)
M. avium spp. *avium*	100.00%
M. avium spp. *paratuberculosis*	-
M. avium spp. *silvaticum*	-
M. bovis	99.39%
M. celatum	100.00%
M. abscessus *M. chelonae* spp. *abscessus*	100.00%
M. chelonae spp. *chelonae*	100.00%
M. chelonae spp. *niacinogenes*	100.00%
M. chimaera	100.00% (NCBI)
M. flavescens	100.00%
M. fortuitum spp. *fortuitum*	100.00%
M. gastri	100.00%
M. gordonae	100.00%
M. haemophilum	100.00%
M. immunogenum	100.00%
M. interjectum	100.00%
M. intermedium	99.34%
M. intracellulare	100.00%
M. kansasii	99.39%
M. lentiflavum	97.33%
M. mageritense	99.34%
M. malmoense	100.00%
M. marinum	100.00%
M. mucogenicum	100.00% (NCBI)
M. palustre	100.00% (NCBI)
M. avium spp. *paratuberculosis*	-
M. peregrinum	100.00% (NCBI)
M. phlei	100.00%
M. scrofulaceum	100.00%
M. septicum	99.34%
M. shottsii	-
M. simiae	100.00%
M. smegmatis	100.00%
M. szulgai	100.00% (NCBI)
M. xenopi	100.00%
M. fortuitum spp. *acetamidolyticum*	100.00%
M. kansasii spp. *aurantiacum*	100.00%
M. kansasii spp. *luciflavum*	99.39%
M. parafortuitum	100.00%
M. parascrofulaceum	100.00% (NCBI)
Gordonia alkanivorans formerly: M. rubrum	-
M. terrae	100.00%
M. austroafricanum	100.00%

Similarities in the search results of the partial 16S of nontuberculous mycobacteria were illustrated in form of a legend. Non-tuberculous mycobacteria, which were not present in the RIDOM database, were compared to the reference sequences from BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Table 16 illustrates the top scores of the matching NTM species. Species without a value (*M. avium* subspecies *paratuberculosis, M. avium* subspecies *silvaticum, M. shottsii and Gordonia alkanivorans formerly: M. rubrum)* did not grow on the Löwenstein-Jensen medium and therefore could not be sequenced.

4.2.2 MALDI TOF

4.2.2.1 *Acanthamoeba* spp. - classification results and the mass spectra

To verify the validity of the MALDI TOF results, *Acanthamoeba* strains were remeasured (Table 17) and identified with highly probable species identification. The strains were correctly idendified at the species level with a score of >2 by MALDI TOF MS. Only the strain *Acanthamoeba comandoni* could not be remeasured.

Table 17: MALDI Biotyper classification results: *Acanthamoeba* spp.

Analyte Name	Analyte ID	Organism (best match)	Organism (second best match)	Score value
(++)	*Acanthamoeba castellanii* T1 (ATCC 50494)	*Acanthamoeba* castellanii_T1_ATCC 50494	*Acanthamoeba* hatchetti_T6_11DS	2.027
(+++)	*Acanthamoeba hatchetti* T4 (2HH)	*Acanthamoeba* hatchetti_T4_2HH	*Acanthamoeba* hatchetti_T4_2HH	2.544
(+++)	*Acanthamoeba hatchetti* T4 (3ST)	*Acanthamoeba* hatchetti_T4_3ST	*Acanthamoeba* hatchetti_T4_3ST	2.436
(+++)	*Acanthamoeba hatchetti* T6 (11DS)	*Acanthamoeba* hatchetti_T6_11DS	*Acanthamoeba* hatchetti_T6_11DS	2.594
(-)	*Acanthamoeba comandoni* T7 (Pb30/40)	no peaks found	no peaks found	< 0

As shown in Figure 27 - Figure 31 *Acanthamoeba* strains were correctly identify with the MALDI Biotyper.

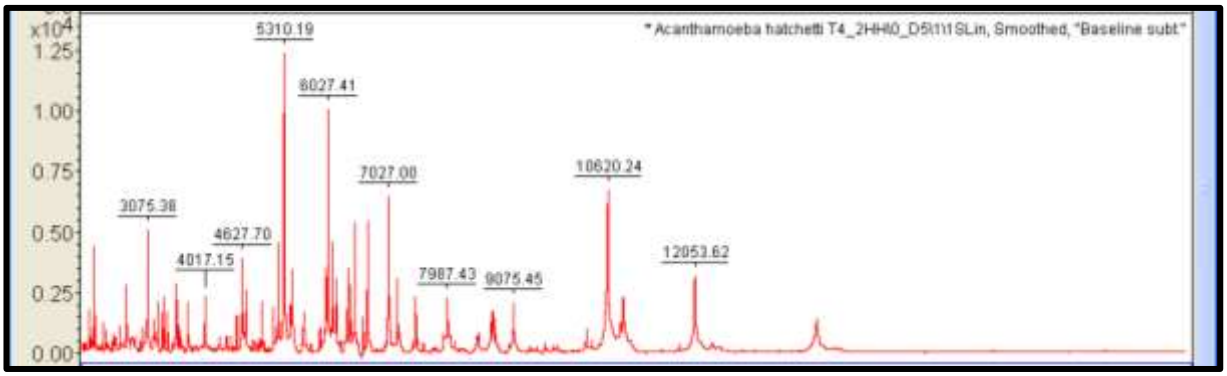


Figure 27: Mass spectrum of *A. hatchetti* T4 (2HH).

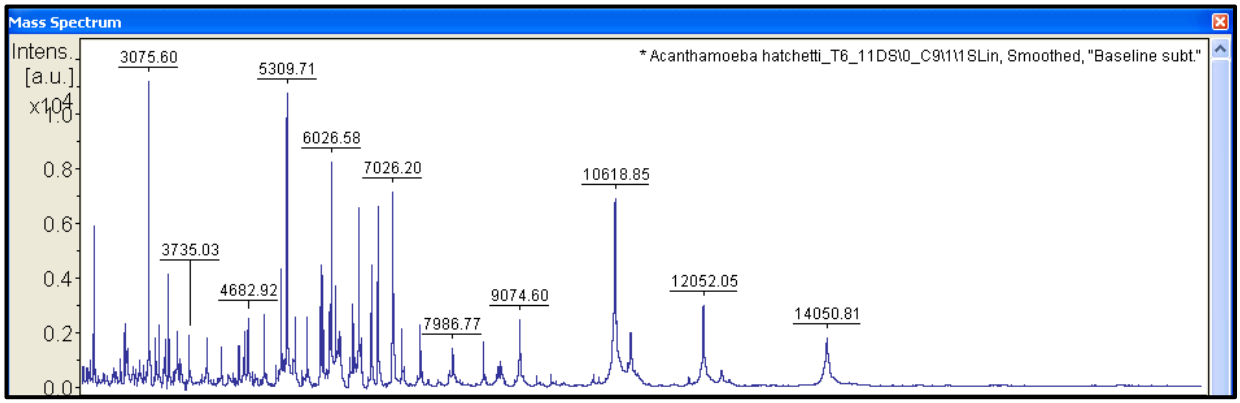


Figure 28: Mass spectrum of *A. hatchetti* T6 (11DS).

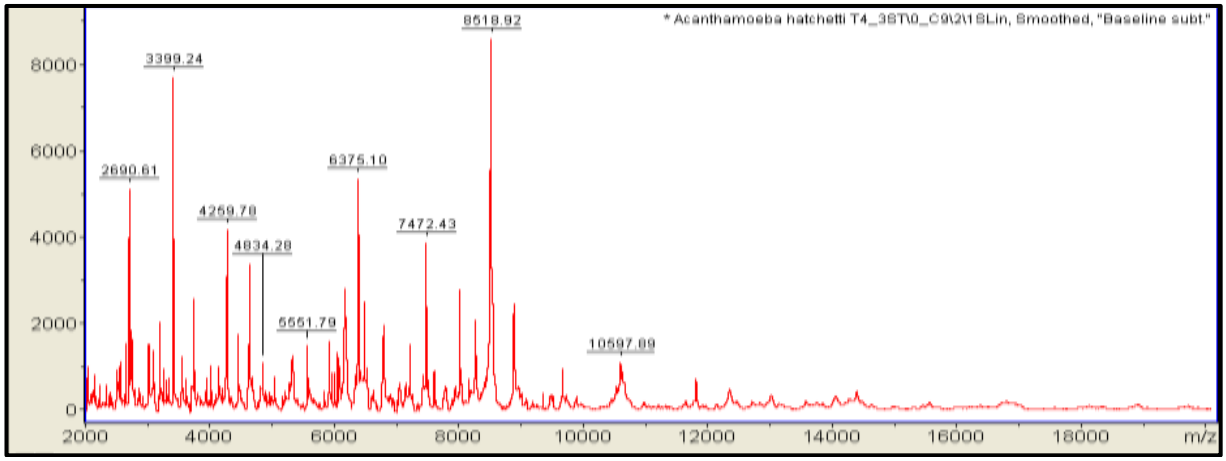


Figure 29: Mass spectrum of *A. hatchetti* T4 (3ST).

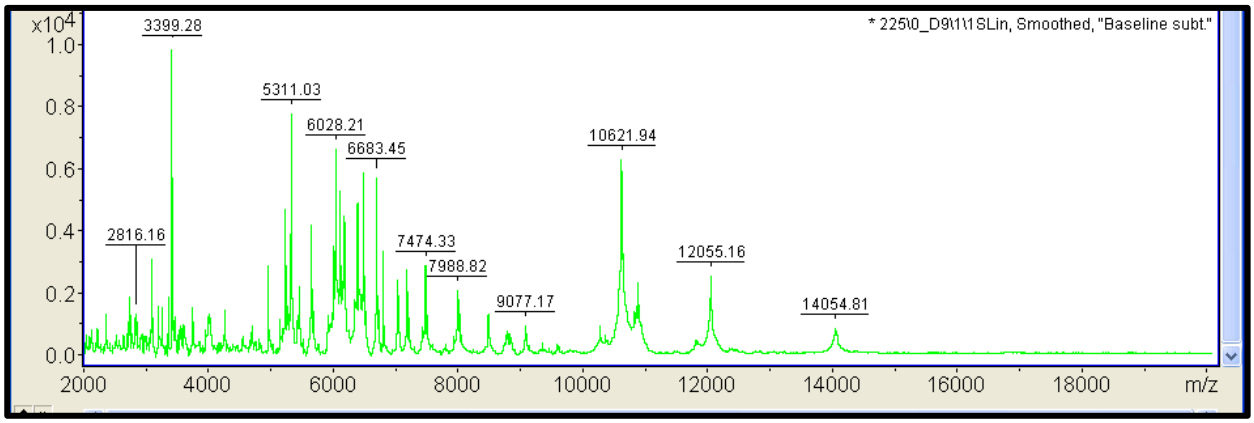


Figure 30: Mass spectrum of *A. castellanii* T1.

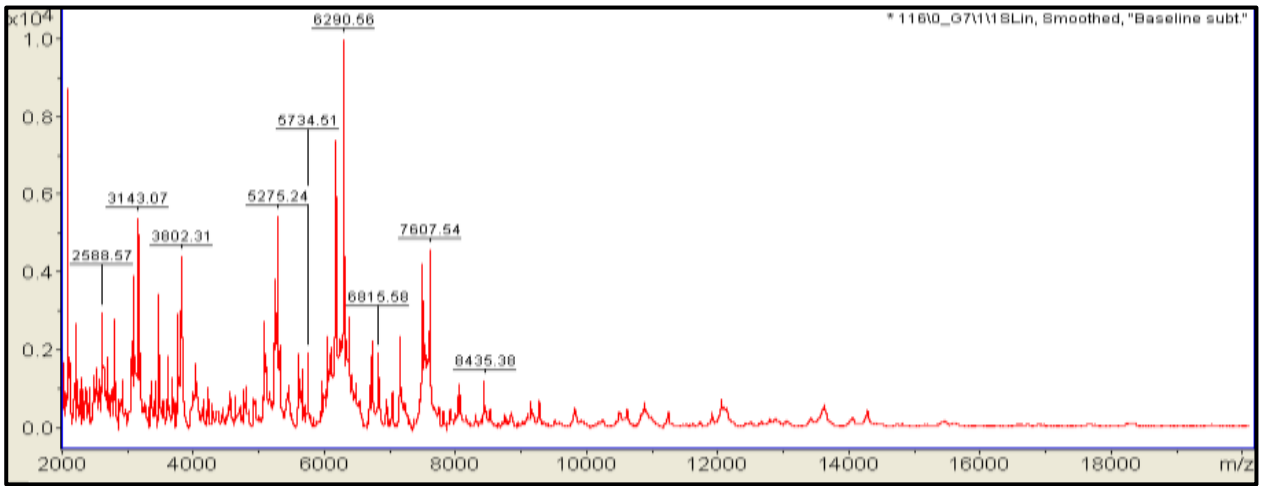


Figure 31: Mass specreum of *A. comandoni* T7.

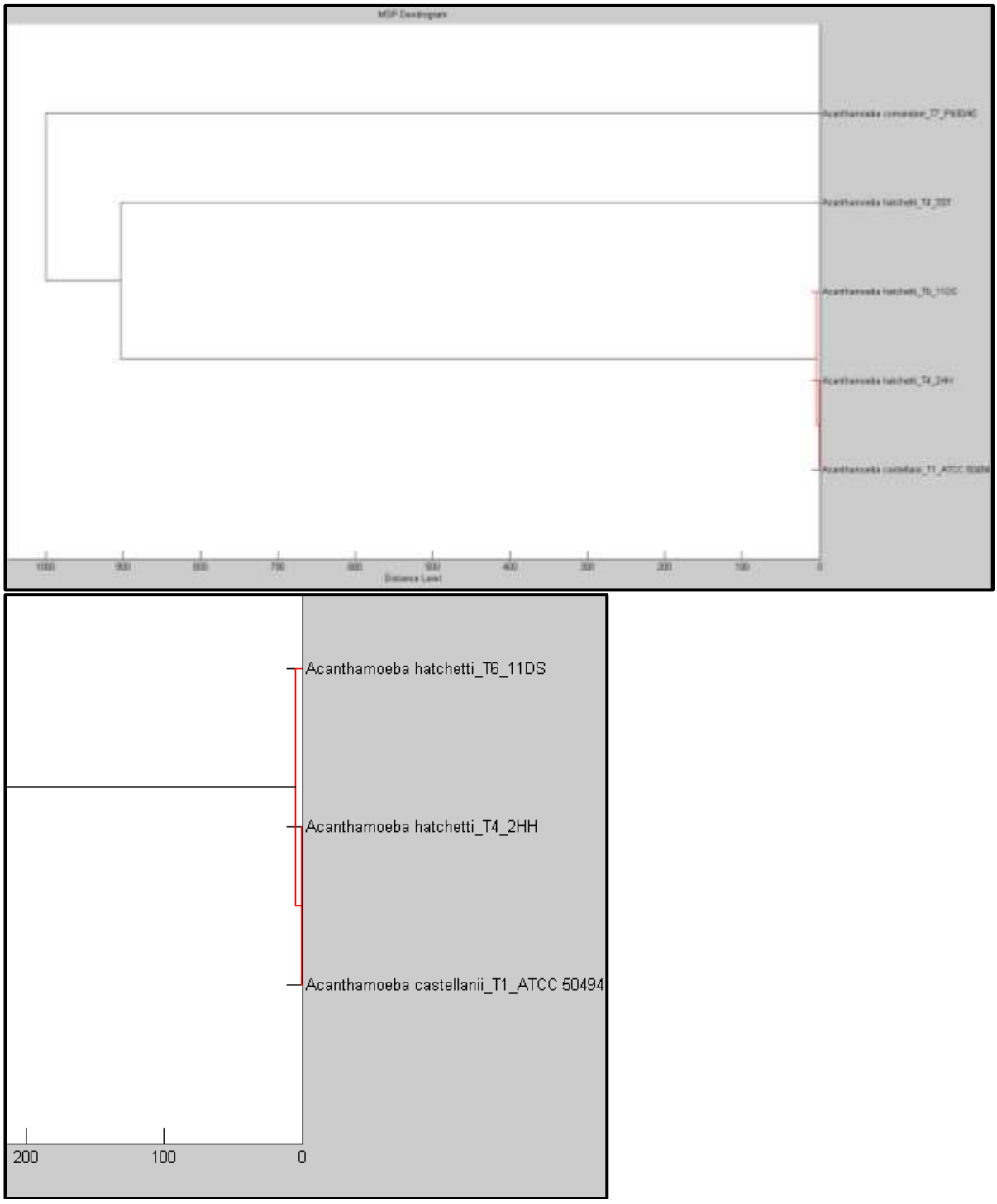


Figure 32: Dendrogram of the five different *Acanthamoeba* strains, established with MALDI TOF software.

As demonstrated in Figure 32, the five different acanthamobae strains (*A.castellani* T1 (ATCC 50494), *A.hatchetti* T4 (2HH), *A.hatchetti* T4 (3ST), *A.hatchetti* T6 (11DS), *A. comandoni* T7 (Pb30/40) can be distinguished with the MALDI Biotyper.

4.2.2.2 *Legionella* spp. – classification results and the mass spectra

All *Legionella* strains investigated during this study were remeasured with the MALDI TOF Biotyper to verify the accuracy of the new reference database. Table 18 shows an overview of the results. The software identified the *Legionella* species with highly probable species identification.

Table 18: MALDI Biotyper classification results report of the analyte.

Analyte Name	Analyte ID	Organism (best match)	Score value
(+++)	*L. adelaidensis*	*Legionella adelaidensis*	2.725
(+++)	*L. anisa*	*Legionella anisa*	2.565
(++)	*L. beliardensis*	*Legionella beliardensis*	2.209
(+++)	*L. birminghamensis*	*Legionella birminghamensis*	2.604
(+++)	*L. brunensis*	*Legionella brunensis*	2.343
(+++)	*L. busanensis*	*Legionella busanensis*	2.47
(+++)	*L. cherrii*	*Legionella cherrii*	2.38
(+++)	*L. cincinnatiensis*	*Legionella cincinnatiensis*	2.322
(+++)	*L. drozanskii*	*Legionella drozanskii*	2.369
(+++)	*L. fallonii*	*Legionella fallonii*	2.046
(+++)	*L. gratiana*	*Legionella gratiana*	2.378
(+++)	*L. gresilensis*	*Legionella gresilensis*	2.428
(+)	*L. hackeliae*	*Legionella hackeliae*	2.498
(+++)	*L. impletisoli*	*Legionella impletisoli*	2.597
(+++)	*L. israelensis*	*Legionella israelensis*	2.682
(+++)	*L. jamestowniensis*	*Legionella jamestowniensis*	2.533
(+++)	*L. jordanis*	*Legionella jordanis*	2.542
(+++)	*L. lansingensis*	*Legionella lansingensis*	2.463
(+++)	*L. londiniensis*	*Legionella londiniensis*	2.579
(++)	*L. longbeachae*	*Legionella longbeachae*	2.094
(+++)	*L. moravica*	*Legionella moravica*	2.397
(+++)	*L. nautarum*	*Legionella nautarum*	2.473
(+++)	*L. oakridgensis*	*Legionella oakridgensis*	2.603
(+++)	*L. parisiensis*	*Legionella parisiensis*	2.361
(++)	*L. pneumophila* sp. *fraseri*	*Legionella pneumophila*	2.348
(++)	*L. pneumophila* sp.*pascullei*	*Legionella pneumophila*	2.059
(++)	*L. pneumophila* sp.*pneumophila*	*Legionella pneumophila*	2.184

(+++)	*L. rubrilucens*	*Legionella rubrilucens*	2.184
(++)	*L. sainthelensi*	*Legionella sainthelensi*	2.162
(++)	*L. santicrucis*	*Legionella santicrucis*	2.028
(+++)	*L. shakespearei*	*Legionella shakespearei*	2.633
(+++)	*L. spiritensis*	*Legionella spiritensis*	2.509
(+++)	*L. taurinensis*	*Legionella taurinensis*	2.405
(++)	*L. tucsonensis*	*Legionella tucsonensis*	2.273
(+++)	*L. wadsworthii*	*Legionella wadsworthii*	2.561
(+++)	*L. waltersii*	*Legionella waltersii*	2.365
(+++)	*L. worsleiensis*	*Legionella worsleiensis*	2.355
(+++)	*L. yabuuchiae*	*Legionella yabuuchiae*	2.427

The MALDI TOF Biotyper is able to identify *Legionella* species and to discriminate *Legionella pneumophila* from non-*pneumophila* species as shown in Figure 33 - Figure 36. All strains were correctly identified to the species level with a score of >2 by MALDI TOF MS. Due to the similarity of *L. pneumophila* serogroups (Figure 33 and Figure 34), no difference was identified with the MALDI Biotyper.

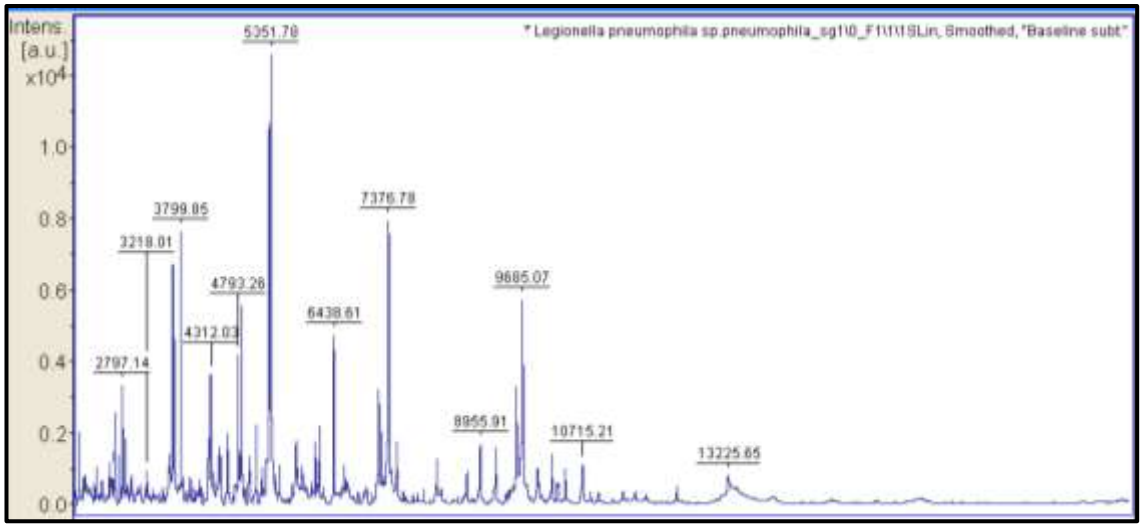


Figure 33: Mass spectrum of *L. pneumophila* sp. *pneumophila*.

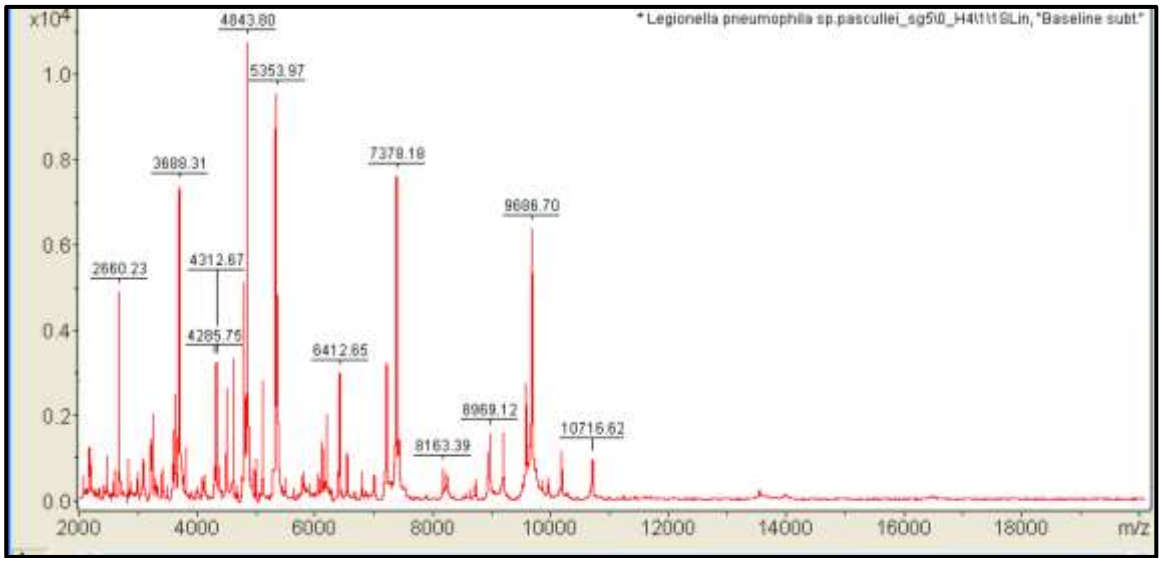


Figure 34: Mass spectrum of L. *pneumophila* sp. *pascullei.*

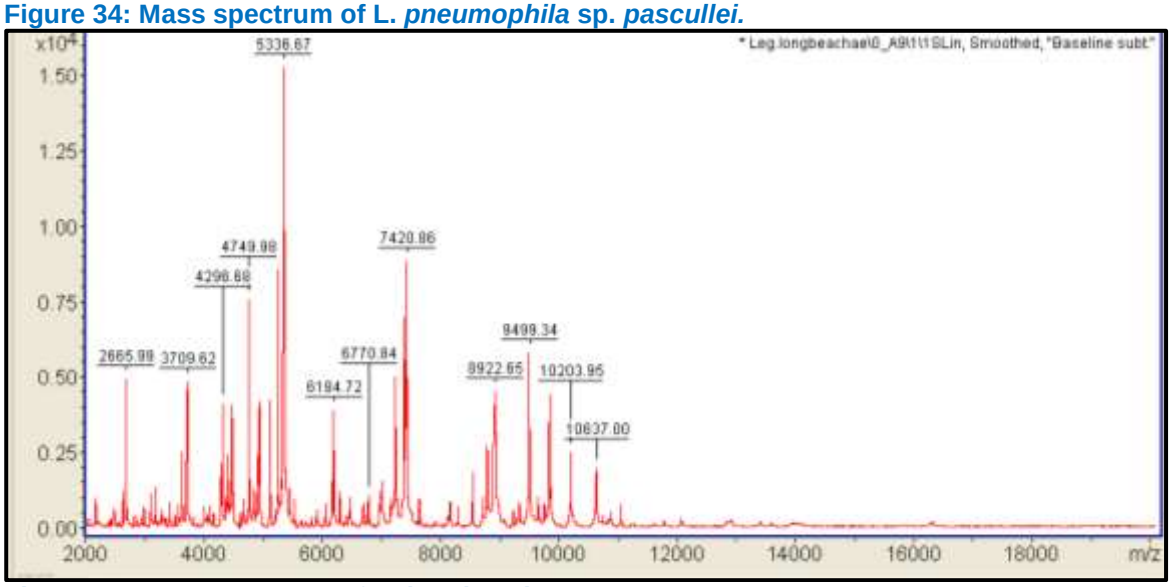


Figure 35: Mass spectrum of *L. longbeachae.*

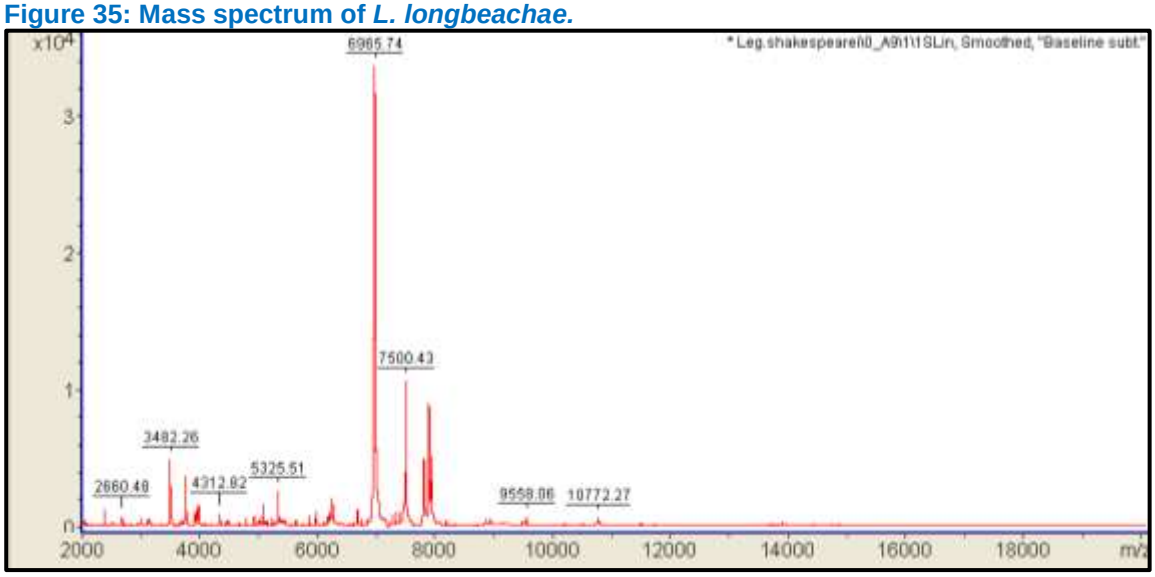


Figure 36: Mass spectrum of *L. shakespearei.*

4.2.2.3 Non-tuberculous mycobacteria – classification results and the mass spectra

A new reference database could be created for eight of 45 (17.7%) non-tuberculous mycobacteria strains. A reference database of following NTM could be established: *M. abscessus, M. avium* subspecies *avium, M. celatum, M. chelonae* subspecies *abscessus, M. chelonae* subspecies *chelonae, M. phlei, M. smegmatis* and *M. parafortuitum*.

Table 19: MALDI Biotyper classification results report: result overview of the analyte.

Analyte Name	Analyte ID	Organism (best match)	Score value
(++)	*M. abscessus*	*Mycobacterium abscessus*	2.122
(+++)	*M.avium* spp. *avium*	*Mycobacterium avium*	2.634
(++)	*M. celatum*	*Mycobacterium celatum*	2.156
(+++)	*M. chelonae* spp. *abscessus*	*Mycobacterium abscessus*	2.267
(++)	*M. chelonae* spp. *chelonae*	*Mycobacterium chelonae*	2.165
(+++)	*M. phlei*	*Mycobacterium phlei*	2.586
(+++)	*M. smegmatis*	*Mycobacterium smegmatis*	2.577
(+++)	*M. parafortuitum*	*Mycobacterium parafortuitum*	2.519

As demonstrated in Table 19 the Biotyper software identified eight NTM with highly probable species identification. The subspecies such as *M.avium* subspecies *avium* (Figure 38) and *M. chelonae* subspecies *abscessus* were not correctly identify. The MALDI TOF software was not able to identify the species at the subspecies level. As shown in Figure 37 and Figure 38 the discrimination of the species is possible with the MALDI Biotyper.

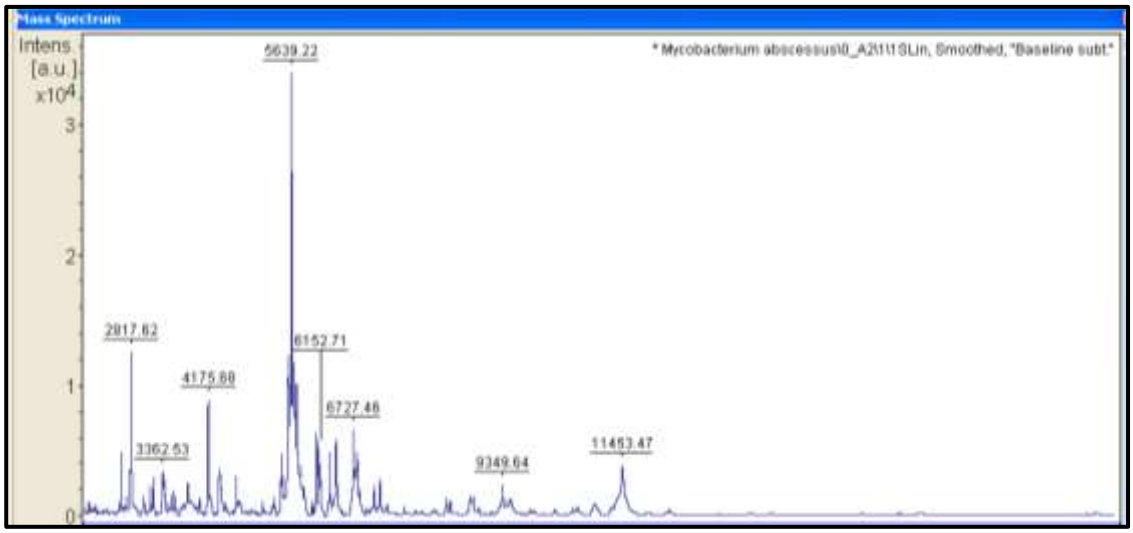


Figure 37: Mass spectrum of *M. abscessus*.

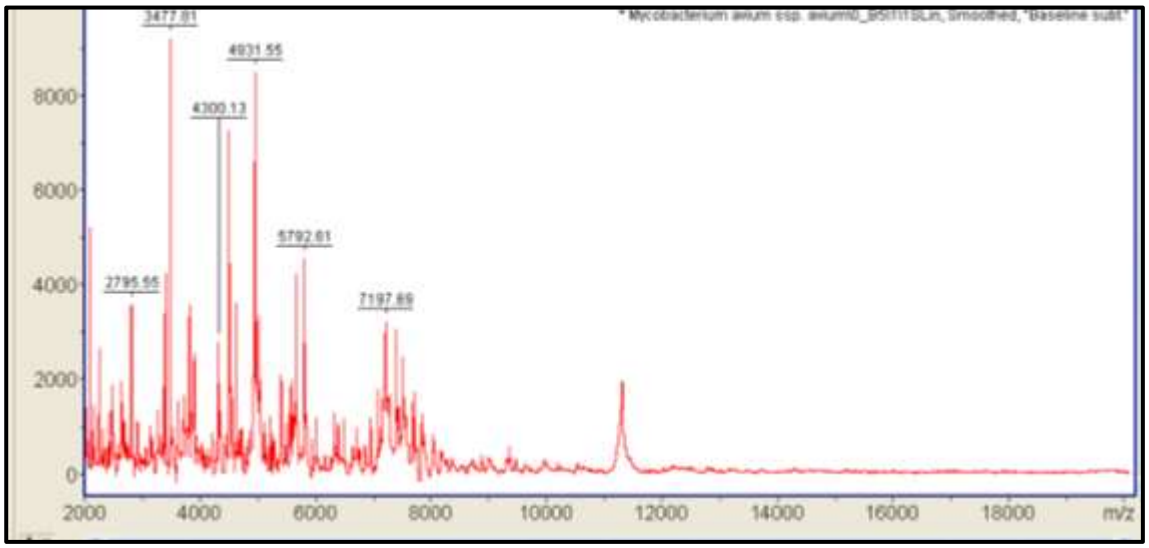


Figure 38: Mass spectrum of *M. avium* sp. *avium*.

4.3 Mass spectra of acanthamoebae, *Legionella* spp. and of the infected acanthamoebae

The Table 20 and Table 21 show the number of uninfected acanthamoebae cells (trophozoite and cysts) in 7 ml PYG broth.

Table 20: Cell density (trophozoites and cysts) of *A. hatchetti T6* (11DS) in 7ml PYG-medium.

A.hatchetti T6 (11DS)	culture flask 1	culture flask 2	culture flask 3	culture flask 4
	634 666 / 7ml	588 000 / 7ml	336 000 / 7ml	420 000 / 7ml
	224 000 / 7ml	149 333 / 7ml	289 333 / 7ml	186 666 / 7ml
	158 666 / 7ml	168 000 / 7ml	205 333 / 7ml	270 666 / 7ml
	121 333 / 7ml	121 333 / 7ml	102 666 / 7ml	102 666 / 7ml

Table 21: Cell density (trophozoites and cysts) of *A. hatchetti T4* (2HH) in 7ml PYG-medium.

A.hatchetti T4 (2 HH)	culture flask 1	culture flask 2	culture flask 3	culture flask 4
	373 333 / 7ml	429 333 / 7ml	401 333 / 7ml	410 666 / 7ml
	134 400 / 7ml	153 066 / 7ml	187 600 / 7ml	141 866 / 7ml
	158 666 / 7ml	155 866 / 7ml	158 666 / 7ml	105 466 / 7ml
	184 800 / 7ml	185 733 / 7ml	166 133 / 7ml	162 400 / 7ml

After the infection of the amoebae, the samples were measured with the MALDI TOF Biotyper. The mass spectra of acanthamoebae, legionellae and the infected amoebae are shown in Figure 39 and Figure 40. As demonstrated in Figure 39 the mass spectrum of the infection experiment of *A. hatchetti* T4 (2HH) with *L. pneumophila* sg1, showed no significant differences to the original amoeba spectrum and no analogy with *L. pneumophila* sg1. The intensity of the peaks was different. The intensity of the peaks of *A. hatchetti T4* was lower (1,500 arbitrary units (a.u)) than of *L.pneumophila* sg 1 and of the infected amoeba ($0.8x10^4$ a.u).

The infection with *L. longbeachae* (Figure 40) showed no significant differences to the original amoeba spectrum or no analogy with the bacterium. The mass spectrum of the infected amoeba demonstrated no significant or characteristic

peaks of the bacteria. The intensity of the peaks was different in all three measurements (6,000 a.u, 1.5x10^4a.u and 1,500 a.u).

The infection of *A. hatchetti* T6 with *L. pneumophila* serogroup 1 or with *L .longbeachae* (Figure 40) showed no significant differences to the original amoeba spectra and no analogy with the bacteria such as the infection of the genotype T4. The intensities of the peaks were different (*A. hatchetti* T6 2,000 a.u, *L. pneumophila* serogroup 1 0.8x10^4a.u, *L. longbeachae* 1.5x10^4a.u and the infected *A. hatchetti*1.5x10^4a.u).

The second infection experiment (data not shown) of the amoebae, *A. hatchetti* T4 was infected with *L. pneumophila* sg 1 (the strain was infected in triplets). The mass spectra of the infected acanthamoebae showed no significant differences to the original spectrum and any analogy or significant characteristic with *L. pneumophila*. The isolation and measurements of the infected amoebae were performed on three consecutive days. The intensity of the peaks of *A. hatchetti* T4 was lower, compared to the peaks of L. pneumophila sg1 and of the infected amoebae. The intensity of the peaks of *A. hatchetti* T4 were 750, 1 000 and 6 000 a.u. In contrast to these were the intensity of the peaks of *L. pneumophila* sg 1 and the infected amoebae 1.0x10^4, except the infected amoeba from the first set of the triple (8,000 a.u). The mass spectra of the amoebae were not as clear as the spectra of the bacteria and the infected amoebae.

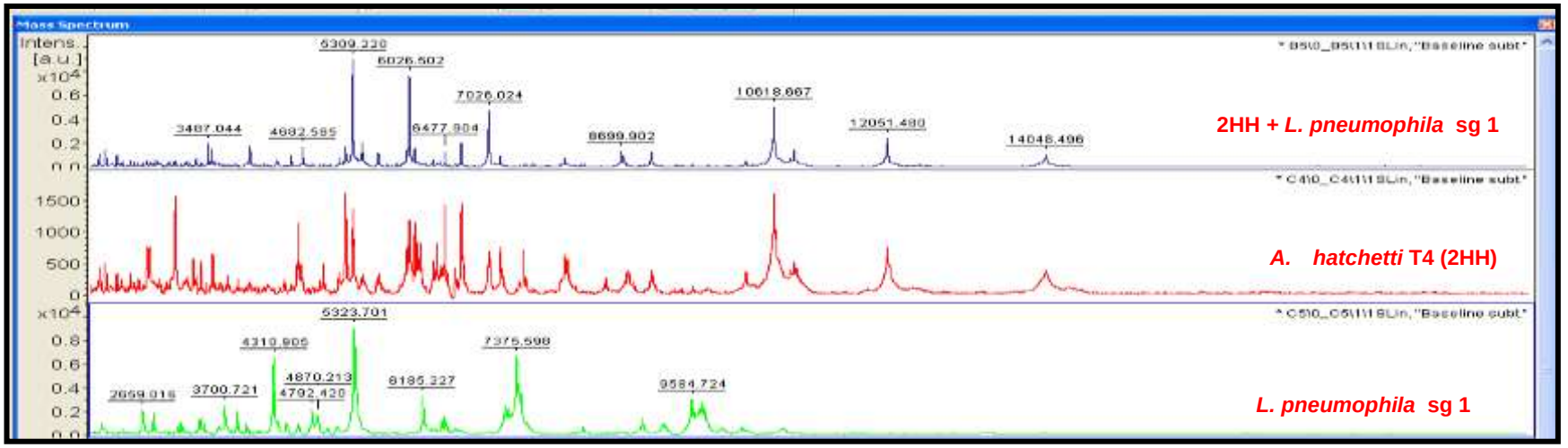


Figure 39: Mass spectrum of the uninfected *A. hatchetti* T4 (2HH), *L. pneumophila* sg 1 and of the infected *A. hatchetti* (2HH).

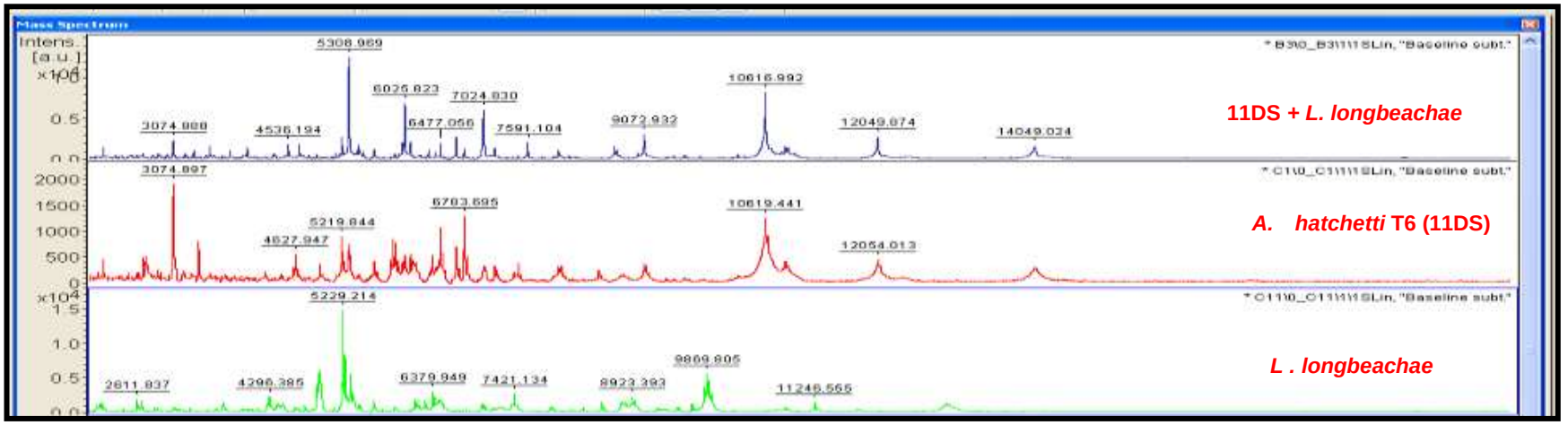


Figure 40: mass spectrum of the uninfected *A. hatchetti*T6 (11DS), *L. longbeachae* and of the infected *A. hatchetti* (11DS).

In addition, the infection of *A. hatchetti* T4 with *L. longbeachae* experiment (data not shown) did not provide significant peaks of the bacterium. The intensity of the peaks of *A. hatchetti* T4 (2HH) compared to *L. longbeachae* and to the infected amoebae was lower (750, 1,000 a.u). The mass spectra of the amoebae were not clear. The intensity of the peaks of *L. longbeachae* showed high values and the spectra were clear. The high of the peaks of the infected amoebae showed different intensities (6,000 and 8,000 a.u.).

The mass spectra of *A. hatchetti* T4 were not clear as those of *L. longbeachae* and of the infected amoebae. The intensity and the clearness of the mass spectra of the infected amoebae were higher and clearer than those of non-infected *A.hatchetti* T4.

The experiment was repeated four times but unfortunately, the infection of the amoebae failed.

5 DISCUSSION

5.1 DNA sequencing analysis vs. MALDI TOF

The aim of every identification method is to get rapid and meaningful results. Furthermore, the method should be easy to handle and should be cost effective. Infections with *Legionella* spp., non-tuberculous mycobacteria and *Acanthamoeba* spp. are difficult to diagnose and are often wrongly diagnosed and sometimes too late. The currently used methods for diagnostic and identification are difficult, in some cases not meaningful enough and time-consuming. The PCR amplification and sequencing of the 16S and 18S rDNA and of the *mip* gene became a promising standard method for the routine diagnosis. During this study, 39 different *Legionella* strains, 45 different non-tuberculous mycobacteria and ten different *Acanthamoeba* strains were used to establish a reference database with the MALDI Biotyper. At the end of the study, a reference database of all 39 different *Legionella* species, only eight different non-tuberculous mycobacteria and only five different *Acanthamoeba* species could be established.

Nowadays, a rapid, reliable and inexpensive identification method is important. The matrix-assisted later desorption ionization time of flight, mass spectrometry (MALDI TOF) has already been used for the identification of bacteria for several years.

5.1.1 Composition proceedings of the used application

<u>The potential to identify different organisms</u>

During the study, three *Legionella pneumophila* and 36 non-pneumophila species was successfully identify with the MALDI Biotyper as well with sequencing. The discrimination of *L. pneumophila* serogroups one, four and five was difficult with the MALDI Biotyper. The species were identified only to the

species level. Unfortunately, a meaningful and reliable discrimination is not possible between the serogroups. Also Moliner *et al* (2010) found that it is not possible to identify the bacteria to the species level and to differentiate *L. pneumophila* serogroups. In their study, the MALDI TOF Biotyper was not able to discriminate the strains to the serogroup level (114 strains) because of the similarity. The sensitivity varied between the species. The *Legionella* strains were correctly identified in 99.2% to the genus level (235/237) and in 89.9% to the species level (213/237)).

The level of discrimination was more precise by investigation of a certain gene (*mip*) by sequencing. Both methods (DNA sequencing analysis and MALDI Biotyper) correctly identified the legionellae to the species level. The usage of only one locus (*mip* gene) identified all *Legionella* species and distinguished non-pneumophila from *L. pneumophila* strains and also the serogroups *L. pneumophila* serogroups one, four and five. Sequence based typing identified *Legionella* spp. in 96 - 100%. The macrophage infectivity potentiator (mip) gene has been used for the identification of *L. pneumophila* serogroups and other *Legionella* species. The gene is relatively stable and specific and exhibits 3–31% nucleotide sequence variation among *Legionella* species (Ratcliff *et al*., 1998). Alignments of different *Legionella* species showed that the bacteria have enough base differences to be distinguished. Other studies also showed the heterogeneity of the region. Haroon *et al* 2011 came to the same conclusion as Ratcliff *et al*., that the mip sequencing is a reliable and simple method and that the serogroups can be distinguished from non-pneumophila species. Furthermore they found out that *L. pneumophila* serogroups 2, 3, 5 and 10 had same nucleotide sequences. Nevertheless, the method represents a useful tool for identification of environmental and clinical samples (Haroon *et al* 2011).

Sogawa *et al.,* (2011) described several studies in which bacteria were successfully identified with the Biotyper. Eigner *et al*., Van Veen *et al*., Cherkaoui *et al*. and Bizzini *et al*. showed that MALDI Biotyper could identify

bacterial strains to the species level in 79.9% to 93, 6%. Bessede *et al*., (2010) analyzed 1012 clinical bacteria samples by conventional phenotypic methods and with the Biotyper. The MALDI TOF could correctly identified 986 (97.3%) of the bacteria to the species level.

The sequence typing of the used acanthamoebae revealed about 97 % identity to published strains. All used acanthamoebae (10 different strains) were correctly identified with DNA sequencing (18S) analysis method. The 18S sequencing is one of the standard methods in the routine diagnostic and also in the research. Walochnik *et al* (2000) correctly identified a total of 13 *Acanthamoeba* strains and designated it correctly to one of the published sequenced types. Hewett *et al* (2003) isolated a strain from a marine source in the United States of America that showed accordance to the species *A. jacobsi* after 18S sequencing. The strain was allocated to at that time presently known sequence type T15.

To the best of our knowledge, this is the first study with the aim to establish a MALDI TOF reference database for *Acanthamoeba* spp. At the end of the study a reference database of five different strains was established. It could differentiate between the genotypes however not within one genotype. The development is still in the initial stages because no comparative data are available. But the first step was taken.

All 40 non-tuberculous mycobacteria were successfully identify (100%) with the 16S DNA sequencing analyses method. Unfortunately, a reference database of eight different NTM strains could be established with the MALDI Biotyper. Nevertheless, all eight strains could be identified and differentiate. The MALDI-TOF mass spectrometry represents a relatively new and very powerful method for the characterization of microorganisms.

The difference in time

The main disadvantages of the currently used methods for identification of bacteria and also of acanthamoebae are the enormous amount of time, the often low specificity and sensitivity of the methods and the great impact of interferents. The DNA sequencing analysis of the species took one day or longer. For final species identification is the DNA-based method still preferred. The disadvantage of the sequencing method is the higher expenditure of time and the increased susceptibility.

The preparation time was different for sequencing as for measurement with the MALDI Biotyper. The culture time for acanthamoebae took three to four days, for legionellae about a week and for NTM between one week and three months. DNA isolation with the MagNA Pure took 30 min for legionellae and acanthamoebae. NTM had to be boiled (30-45min). The DNA-sequencing took one to two days. The time from the DNA-isolation step with the Magna Pure until the sequencing step, took over eight hours.

In contrast to sequencing, the measurements with the MALDI Biotyper were faster. The time from the extraction step until the preservation of the results was for one sample about 30 minutes and for the whole target more than three hours.
The “mycobacteria bead preparation” was similar to the extraction protocol with ethanol and formic acid but unfortunately very time-consuming. The preparation of the 31 non-tuberculous mycobacteria with the “mycobacteria bead preparation method” took about three hours. The method appeared to be more complicated and time consuming than the extraction with ethanol and formic acid. Another advantage of the Biotyper is that the data are analyzed and compared to the existing database automatically while after DNA sequencing a blast search has to be performed by hand. The establishment of the reference database was time-consuming and some measurements had to be repeated.

For the establishment of a reference database the measurement took for one organism (eight spots were measured three times) about 15-20 min. After the measurement, the 24 mass spectra were quality-controlled to ensure truly accurate and reproducible mass spectra. Finally the mass spectra were entered into the database. In conclusion for one sample you need 45 min.

Nonetheless, the MALDI Biotyper is a rapid and unambiguous identification method and can be used for the identification of various microorganisms such as mycobacteria, non-fermenting bacteria, *Enterobacteriaceae,* yeasts, gram-positive bacteria and anaerobes (Van Veen *et al*., 2010). It can be used to differentiate organisms, especially bacteria, to genus, species and subspecies levels (De Bruyne *et al*., 2011). The specificity and sensitivity depends on various factors. Different factors, such as the age of the culture, sample preparation, different matrices and organic solvents affect the quality and reproducibility of bacterial mass spectra (Pennanec *et al.,* 2009, De Bruyne *et al*., 2011). The major factor that influences the quality of the mass spectra is the age of the culture.

Immediately after the legionellae samples were removed from the incubator, (after 5-6 days) they were prepared with the extraction method and were measured with the MALDI Biotyper. The storage duration of one week did not have any negative effects on the quality of the mass spectra. Culture plates which were stored at room temperature or in the refrigerator overnight, showed differences in the mass spectra. Therefore *Legionella* strains were immediately prepared after they were taken out from the incubator. This may be the reason why the establishment of a reference database was successful with all legionellae species.

The establishment of the database for non-tuberculous mycobacteria (eight different strains) and acanthamoebae (five different strains) was not as successful as the of *Legionella* strains. The reason for the relatively low number

of the established mass spectra might be that the samples were not immediately extracted after they have grown.

Pennanec *et al*., (2009) investigated the effect of the age of culture of *L. pneumophila* serogroup 1 on the quality of the mass spectra. The bacteria were investigated between 48-102 hours. They concluded that the small differences of the spectra (variation in the intensity) had no influence on bacterial identification. However, Arnold *et al.* (1999) found huge variations of peaks in *Escherichia coli* between 6 and 48 hours. The difference between the two studies was the medium. Pennanec *et al.* used a solid medium and Arnold *et al*. used a liquid growth medium. The difference is that in liquid culture the nutrients are consumed and the organisms discard their waste. Modification of the medium induces the adaptation of the organisms (Pennanec *et al*., 2009). Sogawa *et al*., (2011) analyzed bacteria on day one and day three. They concluded that the culture time had no influence on the identification of the bacteria.

The CHCA (α-Cyano-4-hydroxycinnamic acid) matrix was used during the diploma thesis. All samples were identified to the species level and the mass spectra delivered the best quality of signals.
Drevinek *et al*., 2012 evaluated sample preparation methods for the identification with MALDI TOF. They concluded that the extraction with formic acid and acetonitrile showed the highest extraction efficacy and spectral quality. Most of the studies did not use the same matrix. In addition to the CHCA matrix there are five other matrices. Pennanec *et al*. 2009 compared the matrices with each other. They came to the decision that the CHCA in acetonitrile / 2.5% trifluoroacetic acid (TFA) deliver the best quality of signals. Furthermore they analyzed the differentiation of the concentration of TFA from 1-2.5%. They concluded that using of 2.5% TFA deliver the most informative spectra (Pennanec *et al*., 2009).

Sequence analyses as well as MALDI-TOF MS have the potential to identify *Legionella* spp., NTM and *Acanthamoeba* spp. to the species level. Only sequencing analyses allow subspecies level identification. Compared to the traditional sequencing method, the Biotyper is an adequate alternative because it is a quick, cost-effective and reliable identification method (Wüppenhorst et al., 2012).

The MALDI TOF measures the exact size of peptides and proteins, which is characteristic for each organism (Mellmann *et al*., 2008). Mellmann *et al.* investigated different non-fermenting bacteria to evaluate the MALDI Biotyper in comparison to the 16S rRNA sequencing method. They established a reference database with 248 bacteria. To evaluate the database, 78 unknown bacterial strains were analyzed. The Biotyper identified 67 of the 78 isolates (47 of 57 were identify to the species level and 20 of 21 were identify to the genus level). Compared to, the 16S rRNA sequencing method produce a >99% sequence similarity in case of 57 isolates.

5.1.2 Reproducibility

The sequencing of the *mip* gene is a useful method to identify clinical and environmental strains and is an effective method to differentiate *Legionella pneumophila* from non-pneumophila strains. In the current study, *mip* and 16S rRNA sequencing were used as reference identification method beside the identification with the MALDI Biotyper. During the establishment of the reference database for the *Legionella,* the species were identified to the species level with a score of 2.046 – 2.725, providing a sensitivity of > 90%. *Legionella pneumophila* species were identified with a score of 2.059 – 2.348 only to species level. Compared to, *Legionella pneumophila* and non-pneumophila were correctly identified with the *mip* sequencing to the species respectively serogroup level, therefore for final species identification DNA-based methods are still preferred. Nonetheless, both methods are reliable for the identification

of legionellae. The DNA sequencing is costly and time consuming however the Biotyper is cheap and fast. The standard routine methods identified bacteria within 8-24 h or longer. In contrast to, the MALDI Biotyper needs only few minutes. Maybe with an optimized *Legionella* database, MALDI Biotyper could be able to replace sequencing, at least for routine identification one day (Svarrer and Uldum, 2011).

Nowadays, few laboratories used the MALDI TOF as a reference identification method for legionellae. Nevertheless many studies demonstrated that MALDI Biotyper shows equally high discrimination of *Legionella* non-pneumophila strains as sequencing of the *mip* gene. Gaia *et al*. (2011) and Moliner *et al*. (2010) confirmed in their studies the MALDI Biotyper as a reliable alternative identification method for *Legionella* species. Moliner *et al*. (2010) identified 89.9% of 21 *Legionella* species to the species level with the Biotyper.

The Bruker taxonomy reference database currently contains 3,995 spectra of cellular organisms. Seven belong to the Archaea, 3,672 to the Bacteria and 316 to the Eukaryota. The users themselves can constantly enlarge through updates or the database. To be suitable for use in clinical routine and one day might be able to replace the commonly used identification methods, the focal points of future development are the constant extension of the database, the direct detection of microorganism from patient samples and the differentiation of subspecies.

5.2 Differentiation of *Legionella* species by MALDI Biotyper

L. pneumophila and non-pneumophila species can be successfully identified with the MALDI Biotyper. Furthermore, the non-*pneumophila* species can be distinguished from *L. pneumophila* species. The work in the routine diagnostics has been made easier after the establishment of the reference database of 39 *Legionella* species. The database has been expanded and the identification has thus become faster and more efficient. Unfortunately, the *L. pneumophila*

serogroup one, four and five cannot be identified to the serogroup level and the serogroups cannot be distinguished between each other. The MALDI Biotyper software is currently not sensitive enough to recognize the minimal differences between the different serogroups. Nonetheless, the MALDI Biotyper is a promising rapid and reliable diagnostic tool.
In similar studies, Svarrer and Uldum, Fujinami *et al*., Gaia *et al*., and Moliner *et al*. found that the MALDI Biotyper could be a reliable method for the identification of *Legionella* species (Svarrer and Uldum 2011).
Moliner *et al* demonstrated that the discriminatory power of the MALDI TOF Biotyper is high for identification of *Legionella* strains at the species level, but not at the serogroup level, because the antigen variations (lipopolysaccharide and major-outer membrane protein) of the serogroups are too similar to be detected by MALDI Biotyper. Furthermore they observed that the distinguished power of the Biotyper varied according to the species and by the number of the spectra in the reference database.

Research has shown that the predominant species in Europa (data from 2007-2008) are *L. longbeachae* and *L. bozemanae.* In Austria, about 203 people have been positively tested since 1991. The most commonly species belonged to *L. pneumophila* serogroup 1 (87.2%). Seven isolates (3.4%) belonged to *Legionella* non-pneumophila (L*. micdadei*, *L. longbeachae* and *L. bozemanii*) (AGES annual report). Legionellosis is the most frequently reported disease among water-borne illnesses. For example in the USA, each year 8000 and 18 000 cases are reported (Pennanec *et al*., 2009). In Austria, however, there are between 80 -100 cases reported each year (AGES annual report). Therefore, for quick identification is the use of MALDI-TOF-MS an appropriate method. It is difficult to identify some strains if no reference spectra were present or if the database had one reference spectrum for each of strain. Therefore each database should always be updated and renewed to be suitable for use as a routine method (Svarrer and Uldum 2011).

5.3 The Biotyper as a promising new method for identification of *Acanthamoeba* spp.

Acanthamoeba are ubiquitous organisms and their cysts are resistant against external influences. The acanthamoebae are responsible for two different diseases in humans. The granulomatous amoebic encephalitis occurs predominantly in immunocompromised humans and *Acanthamoeba* keratitis occurs predominantly in non-immunocompromised contact-lens wearers. The gold standard for diagnostics is the culture method and the gold standard for identification is the sequencing of the 18S rRNA gene after PCR amplification with a genus-specific primer. Unfortunately, these methods are time-consuming and costly.

To our knowledge this is the first study with the aim to establish a MALDI TOF reference database for *Acanthamoeba* spp. The MALDI Biotyper is a fast and uncomplicated method to identify various microorganisms. During the study a reference database of five different *Acanthamoeba* strains with four different genotypes (T1, T4, T6 and T7) could be established. The Biotyper could distinguish all the four different genotypes. Today a total of 17 different *Acanthamoeba* genotypes are known. During this study the MALDI Biotyper can distinguish between four different acanthamoebae genotypes (T1, T4, T6 and T7). The next big steps are the enlargement of the reference database with many different acanthamoebae strains and furthermore the expansion of the database by adding several spectra of each stem in order to achieve better results.

The genus A*canthamoeba* has been classified into three distinct morphological groups based on their cyst morphology. *A. hatchetti* T4 strains (2HH and 3ST) show group II morphology, *A. hatchetti* T6 (11DS) strain shows a typical group II morphology but also a T6 sequence type, which corresponds to morphological group III. *A. castellanii* T1 belongs to morphological group II and *A. comandoni* T7 to group I. According to Walochnik *et al*. (2000), *A. hatchetti* T4 strains (2HH

and 3ST) differ in 2.92% from each other and from *A hatchetti* T6 (11DS) in 8.53% (3ST) and 9.01% (2HH). Thus it can be said that the samples of the two genotypes T4 (3ST and 2HH) are closely related to each other (Walochnik *et al*., 2000).

Accordance's are seen in the results of the DNA sequence data and of the MALDI Biotyper. The only difference is *A.hatchetti* T6 (11DS). According to the DNA sequence data of the CLC DNA Workbench, *A. hatchetti* T6 (11DS) is more closely related to *A. hatchetti* T4 (3ST) as to *A.hatchetti* T4 (2HH) and *A. castellanii* T1. Furthermore, the dendrogram of the MALDI Biotyper software revealed that *A.hatchetti* T6, *A.hatchetti* T4 (2HH) and *A. castellanii* T1 are very closely related in difference to *A.hatchetti* T4 (3ST).

In conclusion, it can be said that the method has the potential to be used for routine diagnostics, but can also be used for scientific purposes in research. The next steps are to improve the extraction method to obtain better mass spectra and meaningful results and to enlarge the reference database of acanthamoebae.

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„Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.“

7 APPENDIX

7.1 Abstract

Erstellung einer Referenz-Datenbank für Akanthamöben, Legionellen und nichttuberkulöse Mykobakterien mit dem MALDI-TOF (Matrix-unterstützte Laser-Desorption/Ionisation)

Acanthamoeba spp. gehören zu den häufigsten Protozoen welche in verschiedenen Lebensräumen auf der ganzen Welt gefunden wurden. Neben zahlreichen schweren Infektionen können sie auch die *Acanthamoeba*-Keratitis (AK) und die granulomatöse Amöben-Enzephalitis (GAE) hervorrufen.
Darüber hinaus können sie als Vektoren für Legionellen, Mykobakterien und auch andere wichtige menschliche Krankheitserreger dienen.

Auch die ubiquitär vorkommenden Legionellen, können aus verschiedenen Habitaten isoliert werden.Der Erreger der Legionärskrankheit (Legionellose) ist in den meisten Fällen *L. pneumophila*.

Nicht-tuberkulöse Mykobakterien (NTM), welche neben potenziell tödlicher Infektionen der Lunge und des Weichteilgewebes ebenso eine Lymphadenitis oder eine mykobakterielle Erkrankung hervorrufen können, gehören zur der Gattung *Mycobacterium*, zu der auch die Arten *M. tuberculosis* und *M. leprae* angehören.

Der MALDI Biotyper ist ein neues, schnelles und kostengünstiges Gerät zur Artbestimmung auf der Basis der Proteinzusammensetzung eines Organismus.

Das Ziel der vorliegenden Studie war die Erstellung einer Referenzdatenbank anhand von Protein-Massenspektren für Akanthamöben, Legionellen und nicht-tuberkulöse Mykobakterien mit Hilfe des MALDI-TOF-MS und der MALDI Biotyper Software.

Die Organismen wurden auf verschiedenen Kulturmedien (Akanthamöben auf NN-Agar-Platten, Legionellen auf BCYE Platten und NTM auf dem Löwenstein Medium) kultiviert.

Vor der Erstellung der Referenzdatenbank wurden die Organismen einem Reinigungsschritt unterzogen.Während der Diplomarbeit wurden insgesamt drei Referenz-Datenbanken erfolgreich mit acht verschiedenen Akanthamöbenstämmen, 39 verschiedene Legionellenstämmen und acht verschiedene NTM-Stämme erstellt.

Die neu erstellten Datenbanken mit dem MALDI Biotyper werden der wesentlich schnelleren Identifizierung von Mikroorganismen dienlich sein.

Abstract

Establishment of a reference database for *Acanthamoeba, Legionella* and non-tuberculous mycobacteria using MALDI TOF MS (Matrix Assisted Laser Desorption Ionisation Time of Flight Mass Spectrometry)

Acanthamoeba spp. are among the most common protozoa, found in various habitats all over the world. They can provoke severe infections, including *Acanthamoeba* keratitis (AK) and granulomatous amoebic encephalitis (GAE). Moreover, they can serve as hosts and vehicles for legionellae, mycobacteria and several other important human pathogens.

Also the ubiquitous bacteria *Legionella* spp. can be isolated from various habitats. The causative agent of Legionnaires´ disease (legionellosis) is in most cases *L. pneumophila*.

Non-tuberculous mycobacteria (NTM), which can provoke potentially lethal lung infections, lymphadenitis, soft tissue disease and disseminated mycobacterial diseases, belong to the genus *Mycobacterium* and to the family Mycobacteriaceae, as also *M. tuberculosis* and *M .leprae*.

The MALDI Biotyper is a new, rapid and cost-effective device for species identification based on the analysis of the protein composition of an organism.

The aim of the present study was the establishment of a reference database for protein mass spectra of *Acanthamoeba* spp*, Legionella* spp. and non-tuberculous mycobacteria using the MALDI TOF MS and the MALDI Biotyper software.

The organisms were cultivated on different culture media (*Acanthamoeba* on NN-agar plates, *Legionella* on BCYE plates and NTM on Lowenstein medium).

For the establishment of a reference database the organisms were subjected to a purification process.

During the diploma thesis three reference databases were successfully established with eight different *Acanthamoeba* strains, 39 different *Legionella* strains, and eight different NTM strains, respectively.
The newly established databases will serve as essential tools for the rapid identification of these microorganisms with the MALDI Biotyper.

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7.3 Curriculum vitae

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10/ 2003 - 09/ 2005:	study of Human medicine at the Medical University of Vienna
June 2003:	school-leaving examination

1995 – 2003:	grammar school with focal point on natural sciences, 1040 Vienna
1992- 1995:	primary school, 1100 Vienna
1991 – 1992:	primary school, Zvornik (Bosnia and Herzegovina)

Further Activities:

2012:	Tutor at the Department of Molecular Parasitology, Institute of Specific Prophylaxis and Tropical Medicine, Center for Pathophysiology, Infectiology and Immunology University of Vienna
Since 08/ 2009:	Wiener Sozialdienste
05-06 / 2008:	promotion ÖBB (Austrian Federal Railways)

Attendance at national congresses:

17 – 19. Nov. 2011:	45. Conference of the ÖGTP in Vienna / Austria*
04. July 2011:	Meet the expert: C.difficile in animals: a disaster waiting to happen (Vienna / Austria)
24. March 2011:	World tuberculosis day: Mycobacterium tuberculosis -Undecover. Die Latente Tuberkuloseinfektion
18 – 20. Nov. 2010:	44. Conference of the ÖGTP in Graz / Austria*

* Postersession

2010 Student Travel Grant Award