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„*Acanthamoeba* spp. as possible host organisms for the pathogen *Burkholderia pseudomallei*"

verfasst von / submitted by

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STATUTORY DECLARATION

I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

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1. Introduction

1.1. *Acanthamoeba* spp.

Acanthamoebae are ubiquitous protozoa having two different life cycle stages (PAGE 1991). On the one hand, there is the motile trophozoite as illustrated in figure 1. These trophozoites have spine-like structures used for movement, attachment and ingestion, referred to as acanthopodia (AP). Furthermore, the formation of food vacuoles (FV) is clearly visible as forked structures. Moreover, the nucleus (NU) with the nucleolus can be seen (PAGE 1991). Trophozoites are characterized by high metabolism rates and the fact that they are able to feed on bacteria and other microorganism. Another nutrition source can be cell debris of lysed mammalian cells extracted during an infection of the human body (PETTIT *et al* 1996). The generation time ranges between 8-24 hours, depending on the nutritional supply and the different species and genotypes, respectively. While the detailed systematics of the genus are still unclear, there are currently 20 18S rDNA sequence types (T1–T20) described. Interestingly, the same sequence types contain several different morphotypes. Furthermore, morphological identical strains can belong to diverse sequence types (FUERST *et al* 2015).

During harsh conditions double walled cysts are produced providing a very resistant stage of life. The formation of immobile but extremely resistant cysts, referred to as encystment and as shown in figure 2, is one of the main and impressive survival techniques of these protozoa. The cyst walls are the important key factor for this characteristic. As BOWERS *et al* in 1969 found, the endocyst is made of fine fibrils; the ectocyst is composed of a laminar, fibrous layer. As described from BOWERS *et al* in 1969 this stage is formed under harsh conditions e.g. desiccation, lack of food or unfavourable pH value (CHAGLA *et al* 1974). The outer ectocyst (ECT) and the inner endocyst (END) show very typical structures. The endo- and ectocyst are linked by a pore with a plug termed opercu-

lum (OP). The opercula are produced in the centre of ostioles, which represent the exit points for the acanthamoeba trophozoite during excystment (BOWERS *et al* 1969).

Furthermore, cysts have a very low metabolism rate which is the reason why they are able to survive over extreme long periods of time. Once the environmental conditions get better again they are able to start excystment and form one trophozoite out of one cyst. Next to the important fact, that cysts are airborne particles, they remain viable over long periods of time while keeping their pathogenicity. Furthermore, cysts are resistant to chlorine, quaternary ammonium compounds, biguanides, chlorine dioxide, and hydrogen peroxide, biocides as well as antibiotics (THOMAS *et al* 2010).
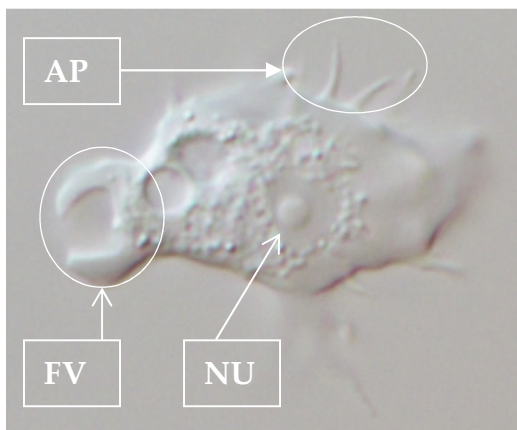
Figure 1. *Acanthamoeba* spp. trophozoite, Orig.
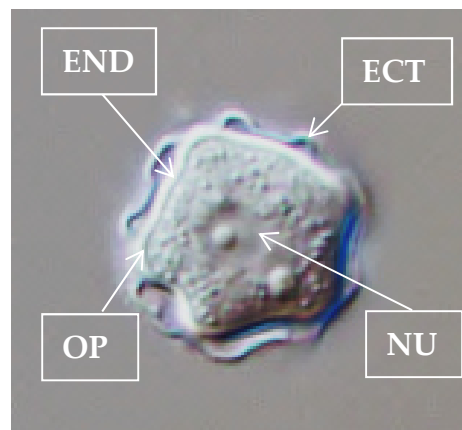
Figure 2. *Acanthamoeba* spp. cyst, Orig.

The size of *Acanthamoeba* spp. trophozoites ranges between 25-40 μm while cysts have diameters between 10-25 μm (BOWERS *et al* 1969).

1.1.1.   Discovery of *Acanthamoeba* species

The first description of amoebae dates back to August Johann Rösel von Rosenhof in 1755 as a part of his work on insects (ROSENHOF 1755). The first isolation of this organism was successful in 1930 by Aldo CASTELLANI. This was also the reason why the organism was first named *Hartmannella castellanii* (DOUGLAS 1930). Already one year later, in 1931 Volkonsky renamed the organism and established the genus acanthamoeba (VOLKONSKY 1931). The de-

scription of three species by PAGE in 1967 was another important step in defining free-living amoebae. The work of PAGE from 1967 was the foundation for the definition of the three morphological groups and the 18 diverse species within them (PUSSARD and PONS 1977).

However, the pathogenic potential was recognized later. The first clearly identified case of granulomatous acanthamoeba encephalitis (GAE) in a human was described in 1972 in a patient with Hodgkin's disease (JAGER and STAMM 1972). Later, in 1986 WILEY *et al* described GAE for the first time in an AIDS patient from the USA. The first case of acanthamoeba keratitis (AK) occurred in a ranger from Texas and was reported in 1973 (JONES *et al* 1975).

1.1.2.   *Acanthamoeba* spp. in the environment

Acanthamoeba spp. are organisms found in any environment including rivers, tap water, lakes, sea water, frozen swim areas, salt water lakes, soil, dust, bottled mineral water, cooling towers, air conditioning and even air (MERGERYAN 1991). TYNDALL *et al* showed in 1987 that *Acanthamoeba* spp. can also be detected in clinical settings such as eye wash stations and dialysis units. Furthermore, several studies showed that *Acanthamoeba* spp. can be found in throat and nasal swabs of healthy individuals (DE JONCKHEERE and MICHEL 1988, MICHEL *et al*. 1982). As described by MERGERYAN in 1991, also around 90% of all collected dust samples were tested positive for acanthamoebae. Overall, these examples demonstrate their ubiquitous appearance in the environment and give an idea on the excellent adaptability of these organisms.

Moreover, *Acanthamoeba* spp. are abundant in soil and there they are of major importance for the nutrient cycle (SINCLAIR *et al*. 1981, RONN *et al*. 2002*)*. These protists are of great significance for the bacterial biomass (SINCLAIR 1981) controlling the abundance, turnover as well as the diversity of bacterial communities.

1.1.3. Life cycle

As shown in figure 3, trophozoites divide by asexual binary fission. They produce two identical daughter cells which again are able to divide (PAGE 1967, BYERS 1979).
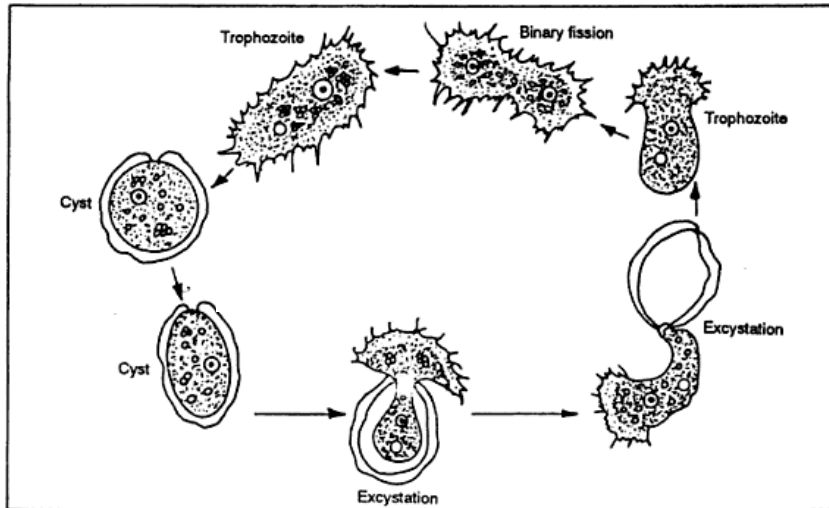
Figure 3. Life cycle of *Acanthamoeba* spp.; modified from the webpage of Tuskegee University.

1.1.3.1. Encystment

As mentioned before, encystment occurs during unfavourable conditions. This stage is characterized by morphological and cellular changes, leading to declining levels of RNA, proteins, glycogen and moreover to a reduced cellular volume. During encystment the cell becomes dehydrated and the cytoplasm has a higher density. While the trophozoite is forming the cyst it removes all food particles and water not needed from the cell. This results in an 80% decrease of the cell volume. Furthermore, the infolding of the outer membrane is also the reason for the smaller size of the cyst in comparison to the trophozoite (WEISMAN 1976). Of course also the nucleus is affected and its volume is decreased to 75% of its former size (VOLKONSKY 1931). However, the nucleic proteins are fully functional as they are of major importance to guide the organisms through encystment. As described by ROTI ROTI and STEVENS in 1975, trophozoites bisected mechanically show two different reactions. On the one hand,

the nucleated halves are able to form cysts, on the other hand, the non-nucleated lost the ability to encyst.

The overall RNA level is decreased during this stage of the life cycle resulting in 50% of the levels measured during the exponential phase. Especially the rRNA levels are declining by 70-80% (SCHULZE and JANTZEN 1982). However, the RNA polymerase I, II, III amounts remain constant during encystation. This ensures that a response to an environmental change can follow immediately and protein production can be adapted to the conditions.

During encystation a rounded trophozoite produces a pre-cyst/immature cyst surrounded by a cell wall which later changes into a double-walled cyst. The cyst walls are built during exocytosis of contents (CHÁVEZ-MUNGUÍA *et al* 2013).
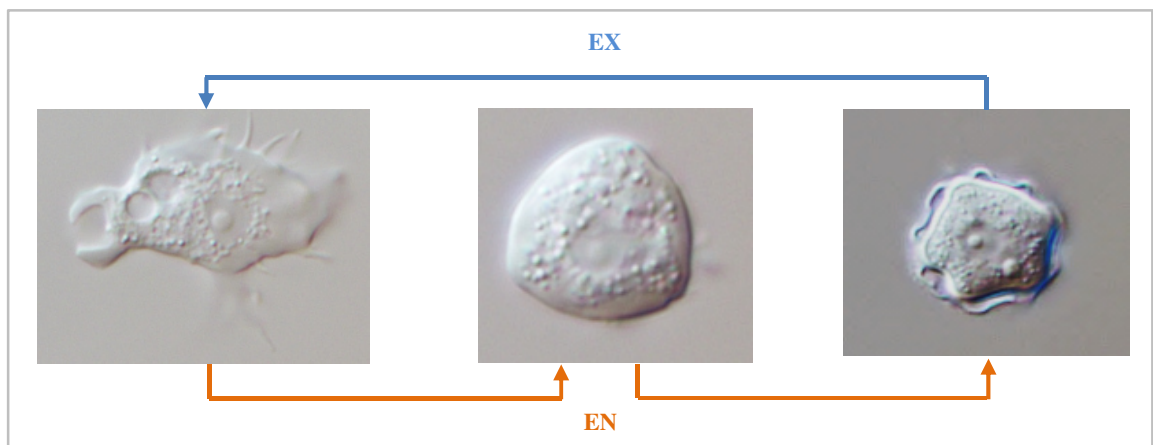
Figure 4. Life cycle stages between trophozoite (left), immature cyst (middle) and cyst (right); encystment (EN) and excystment (EX). Orig.

At the beginning of encystment, vesicles containing fibrillar material are transported to the immature cyst surface. Typically, the cyst wall is made of up to two layers of fibrillary material. While the outer ectocyst is composed of a laminar, fibrous layer, the inner endocyst is made of fine fibrils. Interestingly, cellulose is a major component of the endocyst that cannot be found in trophozoites (CHAMBERS *et al.* 1972, TOMLINSON *et al* 1962). The ectocyst has exclusively acid-soluble protein-containing material. This cyst-specífic protein-21 is hydro-

philic and its mRNA is only produced during early stages of encystation (HIRUKAWA *et al* 1998).

1.1.3.2.   Excystment

As already mentioned, cysts have pores in their cell walls referred to as ostioles (BOWERS *et al* 1969). Presumably these zones are used to monitor changes in the environment. Before the start of excystment a contractile vacuole moves to the cyst wall and the trophozoite detaches from the endocyst. Furthermore, small dense granules can be seen during the early stages of excystment in *in vitro* cultures. Interestingly, these granules are typically present at the plasma membrane near the ostioles. Subsequently, a cytoplasmic bud is formed which pushes through the ostioles (CHAMBERS *et al* 1972). Thus, trophozoites do not digest their cyst but leave the intact, empty cyst walls behind, though parts of these cyst walls can be ingested by young trophozoites (DEICHMANN *et al* 1977). In the end the trophozoite can restart the life cycle as mentioned above.

1.1.3.3.   Organization of life cycle

The control of en- and excystment is crucial and is regulated by various macromolecules. First of all a serine protease is obviously relevant in coordinating the two life stages. An inhibition of this protein leads to a lower ability to start en- as well as excystment. As this protein is essential for the full functionality of the organism, it might be an adequate target for therapy. Additionally, several other molecules e.g. signal transduction histidine kinases, protein kinase C, heat-shock protein, have been identified as being important for encystation (MOON *et al* 2008). Furthermore, several polyamines are found in trophozoites while 1,3-diaminopropane is the one with the highest density amongst them. In general, these molecules are known to be growth factors. Especially interesting is the fact, that polyamines are decreased in trophozoites when they start to form cysts. The important role of polyamines during encystation was confirmed

by ZHU *et al* in 1989, when this compound had been added to growing cultures of acanthamoebae with the result that formation of cysts was blocked.

1.1.4. Cellular structure

Acanthamoeba spp. are moving and feeding with the help of their acanthopodia. Inclusion-bodies and food-vacuoles are visible inside of their transparent cytoplasm. Just as typical for eukaryotic cells, acanthamoebae harvest the important membrane systems as smooth and rough endoplasmatic reticulum, Golgi apparatus, free ribosomes, mitochondria, microtubules and a nucleus (BOWERS and KORN 1968).

1.1.4.1. Nucleus

In general, acanthamoebae have one centrally located nucleus harbouring one nucleolus. However, as shown in figure 5, trophozoites with multiple nuclei can be detected frequently in cultures. As typical in eukaryotic cells, the nuclear envelope is build up from two membranes surrounding the nucleus. These membranes separate the content of the nucleus from the cytoplasm and are dissolved during cell division (PAGE 1967, SINGH 1951).

This important barrier is made of two lipid bilayers enclosing the perinuclear space between them. Their major task is to prevent macromolecules e.g. RNA and proteins from diffusing from and to the nucleus without a very specific transport system. Therefore, nucleus pores are found in the nuclear envelope. They provide a transport system which is free to pass for small

Figure 5. Trophozoite with 2 nuclei (NU); most probably short before cell division, Orig.

molecules like water but is highly regulated for big molecules e.g. proteins. Inside of the nucleus a nucleolus is located without membranes enclosing it. The

major function of the nucleus is to enclose the DNA as linear molecules in association with proteins e.g. histones helping to build up chromosomes. Furthermore, the nucleus helps to control the activity of the cell and to regulate the gene expression with the help of certain transcription factors (KHAN 2009).

1.1.4.2.   Cytoplasm

The cytoplasm is of major importance because it contains, next to the cellular organelles, also the cytoskeleton. It helps the cell to move and defines its shape (HAUSMANN and HÜLSMANN 1996). Moreover, a contractile vacuole is also located in the central part of trophozoites. It is very prominent in actively growing cultures and is needed to expel water.

The main part of the cytoplasm is the cytosol, mainly made of water and salts. This transparent fluid contains numerous vacuoles and organelles. While the ribosomes are used for translation of the mRNA and production of proteins, the Golgi apparatus is a big membrane system and a basic transport system of the cell (BOWERS and KORN 1968).

With the help of inclusions *Acanthamoebae* spp. can store nutrients and also secretory products. Especially polysaccharides e.g. glycogen can be found in the granules, which makes up to 10% of the dry weight of the amoebae (BOWERS and KORN 1968).

1.1.4.3.   Ribosomal RNA

The ribosomes are highly important in the cell and crucial for protein synthesis. Therefore, rRNA (ribosomal RNA) is the most abundant one in the cell accounting for about 80% of the RNA in dividing cells (SCHULTZE and JANTZEN 1982). Acanthamoebae are member of the eukaryotes and therefore have 80S ribosomes. These molecules are constructed out of 4 rRNAs (18S, 5.8S, 28S and 5S), all of them present in one copy per molecule. While 5.8S, 28S and 5S build up the large subunit (50S) the 18S rRNA forms the small subunit (30S). Fur-

thermore, there are about 65 proteins needed (25 proteins for the small, 40 proteins for the large subunit) to bind to the ribosome to make it a fully functional translational machinery (MOORE 1996).

For synthesis of ribosomes the RNA-Polymerase I is needed. This enzyme is exclusively producing precursor-rRNAs in the nucleolus. Moreover, a specific property of exponentially growing acanthamoebae is that they contain a 45 kDa phosphorylated 30S ribosomal subunit protein absent in the ribosomes of the cysts (JANTZEN 1981).

The ribosomes, especially the 18S rRNA, can be described as a "molecular clock" (ZUCKERKANDL and PAULING 1965). According to ZUCKERKANDL and PAULING (1965) certain gene sequences mutate in a nearly constant rate. This clock runs especially slow for sequences coding for genes playing a dominant role in the cellular maintenance. These genes are highly conserved and are subject for purifying selection. Therefore the 18S rDNA gene is commonly used for comparing species and building phylogenetic trees based on the differences of the sequences. The fundamental advantage of this gene and also the reason for its ubiquitous use of their sequences is that every cell needs ribosomes and they always conduct the same function.

1.1.4.4. Surface receptors

Acanthamoebae trophozoites produce and release growth inhibitor factors which are neutralized by unknown factors under favourable conditions e.g. high food supply, low cell density. As soon as the conditions change e.g. high cell density, the growth inhibitory factors start working and lead to encystation (PIGON 1981).

Trophozoites as well as cysts possess receptors on their surface which help them to start ex- and encystation. It was shown that a monoclonal antibody on the surface of trophozoites is specifically bound to a 40 kDa protein responsible for encystation. The same monoclonal antibody can prevent excystment when

bound to the cyst. After washing away these antibodies the trophozoites and cysts, respectively, could proceed with en- and excystment. This finding suggests that the surface receptors react on external signals which are crucial for the change in life style of acanthamoebae (YANG and VILLEMEZ 1994).

1.1.5.   Ingestion

According to BARKER *et al* in 1999 *Acanthamoeba* spp. cause various microorganisms presented on surfaces in various environments as a food source. As described by BOWERS in 1977 and WEEKERS *et al* in 1993, acanthamoebae trophozoites use either pinocytosis ("cellular drinking") for ingesting fluids and small particles or phagocytosis ("cellular eating") for ingesting big particles as mainly bacteria (figure 6). Moreover also
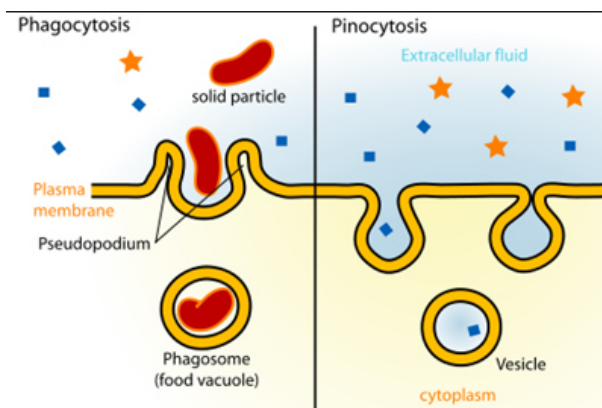
Figure 6. Difference between phago- and pinocytosis (VILLARREAL 2007), modified.

yeast (BUNTING *et al.* 1979; NERO *et al.* 1964), algae and other protozoan organisms can be used as a food source. During feeding many large food vacuoles (FV) occur in the cytoplasm of the amoeba. Therefore acanthopodia (AP) are used as tools for picking up food particles.

Phagocytosis and the unspecific pinocytosis are energy-dependent processes. All particles with diameters greater than 1 μm are taken up by phagocytosis and after budding of into the cytoplasm forming a phagosome (KORN and WEISMAN 1967). The phagosome fuses with different vacuoles called lysosomes, containing hydrolytic enzymes. Subsequently, the food particles are digested in the phagolysosomes (BALDWIN and BOWERS 1995). Interestingly, *Acanthamoeba* spp. can distinguish between vacuoles containing indigestible and digestible particles. Food vacuoles with nutrients *Acanthamoeba* spp. cannot

process are guided to the cell surface and the particles are removed via exocytosis (BOWERS and OLSZEWSKI 1983).

1.1.5.1. Metabolism

Acanthamoeba spp. are heterotrophic and therefore need organic substrates as carbon source to maintain the synthesis of carbon-based compounds. Moreover, they are able to occupy extremely challenging environments. Therefore, they have a metabolism which can change rapidly and is able to adapt to the predominating conditions. For this reason, acanthamoebae contain a large number of mitochondria enabling the organisms to produce the necessary energy to fulfil that function (KLEIN and NEFF 1960). Furthermore, they also need a wide range of different enzymes. First of all, they have glycosidases for the degradation of complex sugars. Next to that, acanthamoebae have phospholipases to metabolize lipids and proteases for degrading proteins. Moreover, using acetate allows them to synthese fatty acids (DOLPHIN 1976, INGALLS and BRENT 1983). The Krebs cycle with its enzymes take place inside of the numerous mitochondria to the end, that the universal energy source ATP is produced. *Acanthamoeba* spp. also have glyoxylate and glxcolytic enzymes. Overall, their broad biosynthetic capabilities enable them to be ubiquitous in the environment.

1.1.6. Morphological Groups

The endocyst is typically very thick while the ectocyst is rather thin. These two cell walls conjoin and build up pores which are closed by one operculum each. Besides, the cysts are traditionally used to divide acanthamoebae into three morphological groups I-III (PUSSARD and PONS 1977). In this context, the size and the morphology of the endo- and ectocyst are used to categorize the amoebae. Still the cyst morphology can also fluctuate within clonal populations due to conditions of the culture.

Specific staining methods can be required and beneficial to illustrate certain characteristics of the cysts. One possibility is the warm impregnation method. Besides, also non-morphological methods are used for a more accurate identification of species. The temperature needed for optimal multiplication rate is easy to measure. However, the isoenzyme electrophoresis was the method widely used for a long time (PAGE 1991).

1.1.7. Genotypes

With the development and establishment of molecular techniques, next to morphotyping, also genotyping of *Acanthamoeba* spp. became more and more important resulting in a total number of 20 different genotypes known today. The most common amplified gene is the 18S rDNA because it is highly conserved. The amplicon is compared to reference strains and has to have a dissimilarity ≤5% within one genotype (GAST *et al* 1996, HORN *et al* 1999).

1.1.8. Medical Relevance

Acanthamoeba spp. cause different diseases in humans but neither genotypes nor morphological groups are indicators of pathogenicity. Throughout all genotypes, T4 is most frequently associated with human infections (figure 7). Overall, around 90% of all keratitis cases are caused by T4 (LEDEE *et al* 2009). Furthermore, also this fatal disease granulomatous encephalitis and cutaneous infections are mainly linked to this genotype. The reason for this could be that T4 represents the most abundant genotype. Therefore, the chance of infection is higher. Another explanation is that T4 has a greater virulence as well as a decreased susceptibility to medications than the other strains (MAGHSOOD *et al* 2005).

The majority of pathogenic *Acanthamoeba* species are members of morphological group II, namely *A. hatchetti, A. lugundensis, A. castellanii, A. polyphaga, A. rhysodes, A. qunia* and *A. griffini* (CDC 1986, YU *et al* 2004, SIMITZIS-LE *et al* 1989, LEDEE *et al* 1996)*. A. culbertsoni* is a member of morphological group III and also pathogenic (PUSSARD and PONS 1977). Other species as *A. comandoni, A. divionensis, A. jacobsi, A. lenticulata, A. mauritaniensis, A.royreba, A. triangularis, A. tubashi* have not been linked to infections yet (CDC).

Figure 7. Different ways of *Acanthamoeba* spp. entering the body (©CDC), modified.

Acanthamoeba spp. infections occur very infrequent but worldwide. Due to the fatal outcome of the caused diseases physicians and also the population should be aware of the infection risk.

1.1.8.1.   Amoebic keratitis

This disease was first described in 1973 and on the contrary to the chronic granulomatous amoebic encephalitis (GAE), the amoebic keratitis (AK) is an extremely painful and often acute infection. A keratitis is typically characterized by an inflammation of the eye's cornea. Especially contact lens wearers and patients with previous corneal trauma can develop AK (JONES 1986). Studies by SIMMONS *et al* showed in 1996 that *Acanthamoeba* trophozoites bind to almost all types of contact lenses but stronger interaction could be detected with soft contact lenses with high water content.
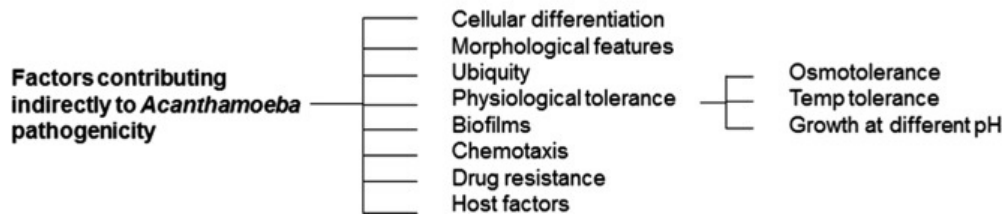
Figure 8. Indirect factors important for the pathogenicity of *Acanthamoeba* spp. (LORENZO-MORALES *et al* 2015).

Furthermore, the binding of *Acanthamoeba* to already worn contact lenses is also significantly higher. Still this disease has been also diagnosed in non-contact lens wearers. Generally, the infection is a multifactorial process involving wearing contact lenses over long periods amoebic keratitis, lack of contact lens hygiene, formation of biofilm in the contact lens cases as a result of that and also exposure to contaminated water or soil (HOLDEN *et al* 2003). One decisive factor might be the mannose-binding protein on the amoebal surface binding to the mannose residues left behind on the worn lens (JAISON *et al* 1998). Also formation of biofilms on the lenses or in the storing devise may play a crucial role for developing AK as the bacterial biofilm can serve as an attachment point and food source for the trophozoites (KELLY *et al* 1996). Adequate and prompt treatment is necessary to prevent the need of corneal transplants due to stromal necrosis. Untreated patients suffer under loss of visual acuity but also blindness may arise (CLARKE and NIEDERKORN 2006).

Figure 9. Ring filtrate typically occurring during *Acanthamoeba* keratitis (CDC).

The pathogenicity of *Acanthamoeba* spp. is based on a variety of different factors. On the one hand there are the direct factors of the amoebae e.g. adhesins, phagocytosis, proteases. On the other hand there are indirect factors as shown in figure 8 (LORENZO-MORALES *et al* 2015).

Diagnosis can be especially difficult due to the unspecific nature of the symptoms shown in table 1 (MARTINEZ and JANITSCHKE 1985b). Furthermore, the onset of the symptoms can vary significantly from days to even several weeks. Interestingly, patients usually only develop AK in one eye. The ring-like stromal infiltrate, as shown in figure 9, is seen in only about 50% of the patients and can be detected in the infected eye by using a slit-lamp. The infiltrate typically has a radial perneural distribution.

Due to similar clinical symptoms AK, is often misdiagnosed in the early stages of the disease as a viral keratitis caused by e.g. *Herpes simplex*. In the later stages of the disease AK resembles a fungal keratitis (BACON *et al* 1993).

As shown in figure 10, the trophozoites bind to the epithelial surface or enter the eye through lesions of the cornea (LORENZO-MORALES *et al* 2015) Cysts seem to not bind to the epithelial cells of the human cornea and have to transform themselves to trophozoites before being able to infect the eye.

Table 1. Symptoms typical for AK (MARTINEZ and JANITSCHKE 1985b).

Acanthamoeba keratitis (AK)
Conjunctivitis
Corneal ulceration
Conjunctival hyperaemia
Lid oedema
Epithelial defects
Considerable productions of tears
Photophobia
Severe pain
Stromal abscesses
Corneal ring stromal infiltrate
Increased intraocular pressures
Hypopyon
Formation of cataract

Figure 10. Interaction of *Acanthamoeba* on the epithelial surface (LORENZO-MORALES *et al* 2015).

1.1.8.1.1.   Diagnosis

As mentioned above, misidentification of AK is frequent. Detection of the amoebae in the cornea can be especially hard when the patient had been treated with antibiotics beforehand because this lowers the Acanthamoeba density significantly. However, a corneal scrape, stored in 200 µL of sterile saline, used for enrichment on culturing plates and PCR is the gold standard for detecting acanthamoebae in clinical samples (BENNETT *et al* 1998).

1.1.8.1.2.   Therapy

Adequate treatment for AK is crucial and yet challenging because there is no specific therapy available. Still currently there is not one single drug and/or therapy available to eliminate both trophozoites and cysts at the same time (LORENZO-MORALES *et al* 2013). The treatment of choice consists of topical antimicrobial agents. Generally, combining a variation of agents is preferred to decrease the probability of the resistant cysts being not affected by the therapy and starting to outgrow again as soon as the therapy is finished (LORENZO-MORALES *et al* 2015).

Medication includes PHMB which can only be used in very low concentrations (0.02%) due to its toxicity to corneal cells. Chlorhexidine is a potent alternative with the advantage that low concentrations (0.02%) are not toxic to human corneal cells (ROBERTS and HENRIQUEZ 2010). These agents are mostly used in an efficient combination therapy with 0.1% propamidine isethionate Brolene ® (Sanofi, England), 0.15% dibromopropamidine, hexamidine 0.1% Desomedine © (Chauvin, France) and neomycine. Unfortunately, hexamidine and propamidine are not obtainable everywhere.

The very time-consuming therapy requests to apply these agents hourly during the first days of treatment. Afterwards the patient has to administer the medication every three hours. However, the first results can be seen not before two weeks of therapy. Overall, the recommended duration of treatment should be at

least three to four weeks; some authors even recommend therapies lasting for 6-12 months. Close observations of the patients are fundamental to avoid renascent infections (LORENZO-MORALES *et al* 2015).

As described by BLACKMAN *et al* in 1984, keratoplasty was the therapy of choice before medication became the first method of choice. Today, it is used in cases of corneal perforation or treatment failure. However, the optimal time point for surgery is not well defined. Furthermore, medical therapy should be sustained to ensure the removal of a potential remaining acanthamoebae reservoir (COHEN *et al* 1987).

1.1.8.2. Cutaneous acanthamoebiasis

This disease particularly occurs in immunosuppressed people and the first described case occurred in 1892 in a patient with an amoebic liver abscess. After drainage the patient suffered from ulceration as well as necrosis of the abdominal skin (NASSE 1892).

1.1.8.2.1. Disease pattern

Acanthamoebae are the causative agents of nasopharyngeal and cutaneous infections. As shown in figure 11 this disease is characterized by skin ulcerations and nodules. As described by CHANDRASEKAR *et al* in 1997, in immunocompetent people cutaneous acanthamoebiasis is usually self-limiting. However, in patients with underlying diseases it can cause major problems (DELUOL *et al* 1996). This infection can be the route of entry into the blood, followed by haematogenous spread to
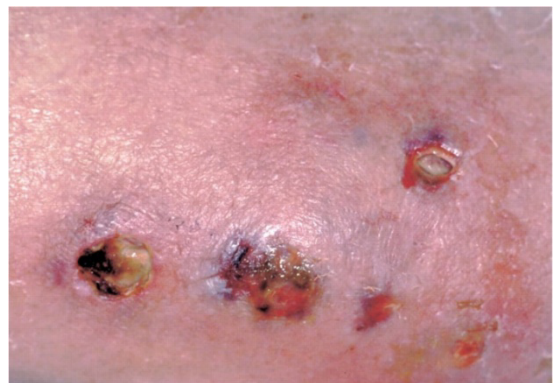
Figure11. Cutaneous acanthamoebiasis in an immunocompromised patient (STEINBERG *et al* 2002).

different organs, including the central nervous system. In this case the mortality rate is extremely high (TORNO *et al* 2000).

1.1.8.2.2. Therapy

Until today, there is no recommended standard treatment but the topical application of pentamidine, isethionate, ketoconazole, itraconazole and chlorhexidine gluconate have been proven to help (SLATER *et al* 1994).

1.1.8.3. Granulomatous amoebic encephalitis (GAE)

GAE is a rare and progressive infection of the central nervous system (CNS). In comparison to other parasites a special feature of free-living amoebae (FLA) is that they can finish their life cycle outside of the host. Next to *Balamuthia mandrillari* and *Sappinia pedata,* also *Acanthamoeba* spp. can be the source of GAE, chronic and fatal infection of the CNS. The overall mortality rate lies over 50% (MEERSSEMAN *et al* 2007). Especially, immunosuppressed patients suffering from e.g. HIV-AIDS, diabetes or tuberculosis are at risk of developing GAE. Moreover, also patients treated with chemotherapy or undergoing organ transplantation have higher risk of infection (MARTINEZ and JANITSCHKE 1985b). Until 2004, several hundred GAE cases were reported showing a mortality rate of 85% (CDC, SCHUSTER and VISVESVARA 2004, ZAMORA *et al* 2014). Since 2000, 61% of the patients suffering from GAE had an underlying immunosuppressive disease. However, GAE has also been described in immunocompetent people (STIDD *et al* 2012). Typically, the diagnosis is made post-mortem and only a few patients could be treated successfully (DIAZ 2010).

Many of the described cases had preceding skin lesions and lung infections before the CNS infection occurred. The typical way of the amoebae to enter the human body is through skin lesions or via the nasal route (VISVESVARA & MAGUIRE 2006). Afterwards the amoebae are spread via the blood through the

body and finally also to the brain. Nonspecific ring-enhancing lesions as shown in figure 12 are commonly found during diagnostic radiology. Under histo-pathological aspects this process is mainly characterized by granulomas and parenchymal necrosis (ZAMORA *et al* 2014).
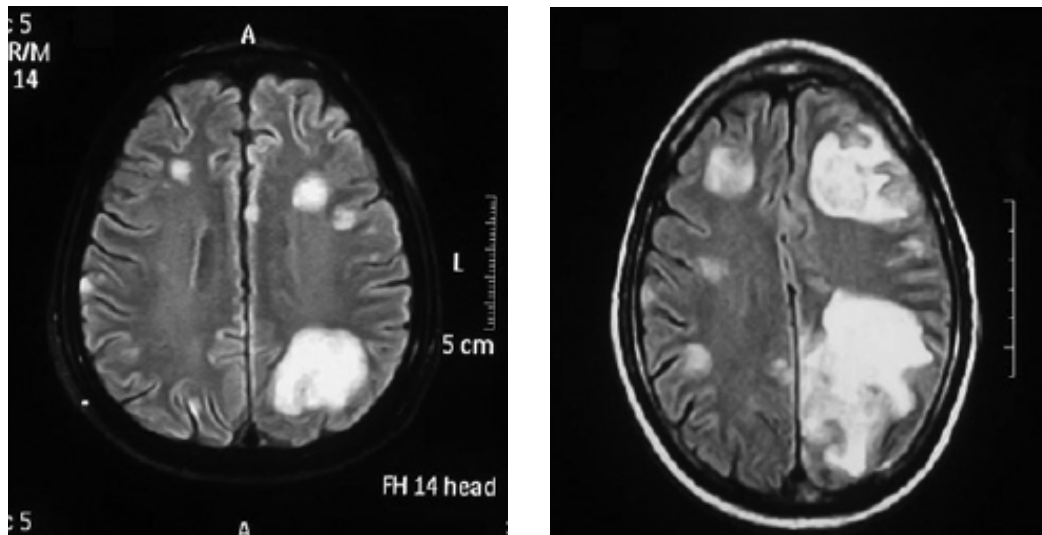
Figure 12. Lesions (white areas) in a human brain due to GAE infection. caused by *Acanthamoeba* spp. (THAMTAM *et al* 2016).

Cysts and trophozoites are typically located around blood vessels. Due to the fact that cysts are highly resistant treatment is very challenging.

Several *Acanthamoeba* species have been described to cause GAE. The symptoms for GAE, as shown in table 2, are typical for localized encephalopathy. Generally, the symptoms are unspecific (MARTINEZ and JANITSCHKE 1985b).

Misdiagnosis e.g. as tuberculous meningitis, viral encephalitis or toxoplasmosis is frequent. A late diagnosis is typical because of the slow progression and very unspecific nature of this rare disease. Due to the unspecific

Table 2. Symptoms typical for GAE (MARTINEZ and JANITSCHKE 1985b).

Acanthamoeba **Granulomatous encephalitis (GAE)**
Headache
Fever
Sleep disturbances
Hemiparesis
Change of mental status
Visual disturbances
Ataxia
Nausea and vomiting
Hallucinations
Change in personality
Photophobia
Seizures
Coma

symptoms and the lack of awareness among clinicians this often remains undiagnosed (LIU 2012).

1.1.8.3.1.   Diagnosis

In the case of suspected GAE the definite diagnosis is made by direct detection of the amoebae in using cerebrospinal fluid (CSF). Furthermore, a diagnosis can be made by immunofluorescence in using tissue samples. Serology is inappropriate because due to the ubiquitous occurrence of acanthamoebae in the environment positive results are to be expected (ZAMORA *et al* 2014).

1.1.8.3.2.   Therapy

Unfortunately, no recommended standard therapy for GAE is available. Therefore, clinicians have to decide on the therapeutic regime individually. As recommended from the Infectious Diseases Society of America (IDSA) granulomatous amoebic encephalitis should either be treated with a combination of fluconazole, sulfadiazine and pyrimethamine or of trimethoprim-sulfamethoxazole, rifapin and ketoconazole. Furthermore, the CDC recommends miltefosine according to AICHELBURG *et al* 2008 should be included in any GAE therapy. However, the number of cases is way too small to give reliable conclusions about the efficacy of therapy. Moreover, the treatment of patients surviving this infection and dying of it showed big overlaps.

1.2. *Burkholderia pseudomallei*

The first description of *Pseudomonas pseudomallei* was made by WHITMORE and KRISHNASWAMI in 1912. In 1996, JONES *et al* renamed the bacterium as *Burkholderia pseudomallei*. As shown in figure 13 it is rod-shaped and it is a natural resident of soil habitats. It is a gram negative, aerobic, motile and facultative intracellular bacterium. Moreover, it is the causative agent of an infectious disease called melioidosis. According to the CDC it is a pathogen belonging to the category B bioterrorism agents as it is mod-

Figure 13. *B. pseudomallei.* Scanning electron microscopy image (colour-enhanced) (YATHISH et al *2015).*

erately easy to disseminate, it has a moderate morbidity rate and low mortality rate and it requires specific increase of CDC´s diagnostic capacity and disease supervision. As indicated by CHAOWAGUL *et al* in 1989, *B. pseudomallei* is a natural resident of water and soil in sub- and tropical regions. The most important key factor of *B. pseudomallei* is its ability to survive intracellularly in non-phagocytic, e.g. HeLa, CHO (JONES *et al* 1996, HARLEY *et al* 1998), as well as in phagocytic cells, e.g. macrophages (PRUKSACHARTVUTHI *et al* 1990, JONES *et al* 1996) and polymorphonuclear leukocytes (PRUKSACHARTVUTHI *et al* 1990). This is the reason why treatment is so complex and long lasting. Furthermore, also recrudescence and latency are common in infections with this pathogen (NGAUY *et al* 2005).

B. pseudomallei has two chromosomes. The pathogenic strain K96243 from Thailand was sequenced showing a comparably large genome with a total size of 7.2 Mbp (HOLDEN *et al* 2004). While the small chromosome (3.17 Mbp) is required for adapting to various niches, the large chromosome (4.1 MbP) is used for the core functions e.g. growth and metabolism (HOLDEN *et al* 2004). Especially

interesting is the fact that *Burkholderia mallei,* the causative agent of glanders, has a genome which is significantly smaller than the one of *B. pseudomallei.* However, it is strictly parasitic, infecting typically horses and donkeys (HOWE 1950), and lost its ability to survive in the environment.

1.2.1.   Melioidosis

The mode of infection is through skin lesions, by bacteria-containing aerosols from contaminated soil and surface water and/or also by ingestion (LIMMA-THUROTSAKUL *et al* 2013b, INGLIS *et al* 2000a, CURRIE *et al* 2001).

Looking on certain areas of northeast Thailand, one of the melioidosis hotspots, about 50% of the population are seropositive. Seroconversion typically occurs in children between the age of 6 month and 4 years (WUTHIKANUN *et al* 2006). Interestingly seropositive rate in the endemic regions of Australia is below 5% although the confirmed cases of melioidosis are similar to Thailand. This under-lines the need for more studies to fully understand the epidemiology of this infection. It is still un-certain how many peo-ple are asymptomatic while being seroposi-tive. The individuals may harbour and de-velop disease in case of immunosuppression. This can be observed with the US veterans
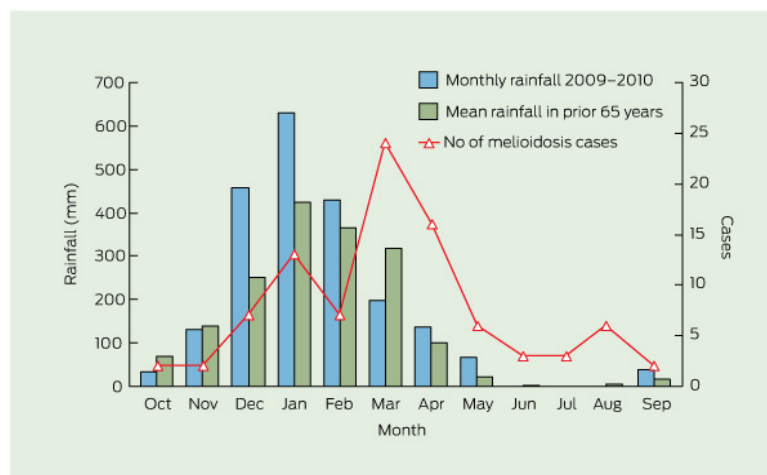
Figure 14. Correlation of rain fall and cases of melioidosis in Darwin, Australia (PARAMESWARAN *et al* 2012).

from the war in Vietnam. Potentially 225.000 people could be infected with *B. pseudomallei.* This is also why melioidosis is frequently called the "Vietnamese time bomb" (CLAYTON *et al* 1973). However, the actual cases of melioidosis concerning the veterans had been lower than anticipated (CURRIE *et al* 2010).

The case with the longest latent period was found in a soldier, who had been a prisoner in Thailand, Burma and Malaysia and developed melioidosis after 62 years (NGAUY *et al* 2005).

SUPUTTAMONGKOL *et al* described in 1999 that members of poor rice farming families in Southeast Asia are at an especially high risk to acquire an infection. Due to the fact that the farmers mostly do not wear protective footwear they are extremely prone to infect themselves.

The sepsis-related mortality of melioidosis in Thailand lies at 40%. Typically this disease affects people who have an altered immune response due to other underlying diseases as diabetes (23-60% of patients), alcoholism (12-39% of patients), chronical lung diseases (12-27% of patients) and chronical renal diseases (10-27%) of patients (CURRIE *et al* 2010). While healthy children are not the typical melioidosis patients, only 5-15% of the cases occur in this cohort, children with one or more risk factors, as described above, represent 16% of the patients (MCLEOD *et al* 2014). Also tuberculosis is a disease leading to severe melioidosis. Interestingly, the peak of infections can typically be observed during the monsoonal wet season as shown in figure 14. Overall studies revealed that about 75% of the cases in Thailand and 81% in Australia are resulting from severe weather phenomena (CURRIE *et al* 2010. SUPUTTAMONGKOL *et al* 1994). Correct diagnosis can be a major challenge in non-endemic regions due to the wide range of symptoms. The clinical spectrum, as shown in figure 15, ranges from
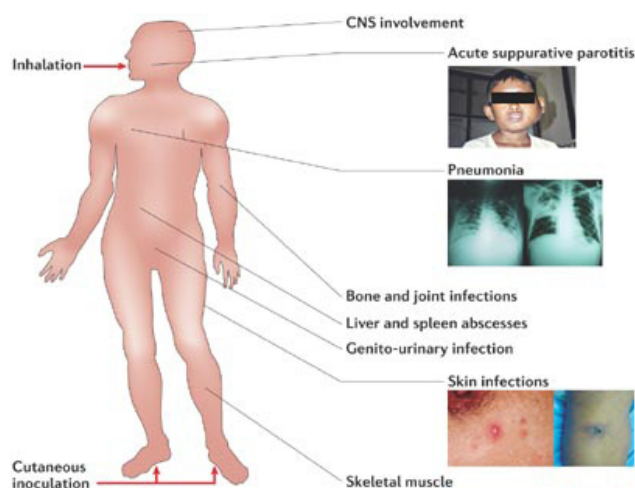
Figure 15. Mode of infection and the diverse symptoms caused by melioidosis (WIERSINGA *et al* 2006).

no manifestation to cutaneous infections to sepsis and death. Next to the bacterial load, also the mode of infection plays an important role. The most severe

clinical picture includes septic shock in infections of the lung, liver and spleen (WIERSINGA *et al* 2006). Overall it is reported that the respiratory tract is the major site of infection in adults. The infection can also be chronic and then it is hard to be distinguished from pulmonary tuberculosis (MEUMANN *et al* 2012). Patients suffering from pneumonia due to *B. pseudomallei* infection have mortality due to septic shock of up to 90%. However, mortality rates are significantly different between Thailand and Australia. As described by LIMMATHU-ROTSAKUL *et al* in 2010a, 10% of the patients in Australia die of melioidosis while 40% of the cases in Thailand have a fatal outcome. Overall, it can be assumed that the virulence of the Thai strains is higher.

Furthermore, skin infections, liver and spleen abscesses as well as bone and joint infections are common. *B. pseudomallei* can also cause acute suppurative parotitis and also involvement of the central nervous system (CNS) is frequent-ly reported (DANCE *et al* 1989).

To sum up, the clinical manifestations are extremely variable and therefore a reliable diagnosis mainly depends on molecular biological methods e.g. real-time PCR using the type III system of *B. pseudomallei* as a target (NOWAK *et al* 2006).

Figure 16. Melioidosis is the third most common reason of infectious acquired death in Thailand (LIMMATHROTSAKUL *et al* 2010a).

Interestingly, in Thailand melioido-sis is the third most common infectious disease resulting in death (LIMMA-THUROTSAKUL *et al* 2010a). As shown in figure 16 melioidosis ranges right after AIDS and tuberculosis.

The bacterium infects humans but also animal species ranging from rodents, pigs, sheep, goats, horses, birds to reptiles and even plants can be affected. With the detection of *B. pseudomallei* in the rhizosphere, as well as in aerial parts of

specific grasses the question arises if the dung of grazing animals could also play a major role in the dispersion of this pathogen (DUNAEV *et al* 1995).

1.2.1.1. Treatment

B. pseudomallei is well known to be highly resistant to various antibiotics. Especially, the common drugs for gram-negative bacteria, e.g. ampi-/amoxicillin, aminoglycosides and ceftriaxone, are not efficient. Nevertheless, due to the fact that this disease can have a fatal outcome, treatment has to be initiated as soon as possible. Overall, current treatment schemes rely on three tracks as visible in table 3 (INGLIS 2010).

The therapeutic regime for melioidosis is very complex due to the necessity of long-term treatment and the different possibilities of antibiotics. Therefore, patients should have constant access to medical attendance to check their health status. This, of course, can be a big issue in several countries, where medical care and medication are not available at any time or because of financial reasons. Therefore, people living in highly endemic regions with low health care standards are at an especially high risk to die of melioidosis (LIMMATHU-ROTSAKUL *et al* 2013a).

While phase 1 treatment is directed against acute septicaemia, phase 2 therapy is initiated to eradicate the pathogen from the patient (INGLIS 2010).

The first part mainly relies on ceftazidime or meropenem. Generally, ceftazidime does develop resistances, while carbapenems show good intracellular penetration (SIMPSON *et al* 1999a). Serendipitously, *B. pseudomallei* does not acquire resistance to any of the antibiotics used in phase 1.

While diseased people without relevant underlying diseases are treated for around two weeks, persons with an altered immune system due to diabetes, renal failure and alcoholic liver diseases or also slow response to therapy, are treated for about three to four weeks in the first phase. In these cases intravenous medication and close monitoring is crucial for the clinical progress. Fur-

thermore, also longer treatment times can be necessary in the first phase, when the patient is in need of intensive care due to e.g. multiple organ system failure (INGLIS 2010).

As described by CHAOWAGUL *et al* in 1993, especially phase 2 is important because of the fact that some patients are under a higher risk of septicaemia relapse. Therefore, the therapy has to be planed carefully and the progress has to be observed. Intravenous treatment (phase 1) and the oral medication (phase 2) can overlap a certain time to assess the tolerance for the compounds. This step is not necessary when co-trimoxazole is used in the first phase of therapy. As shown by LIMMATHUROTSAKUL *et al* in 2008, therapy is successful when the duration is rather long with 12 to 20 weeks. However, the exact time depends on the patient's health status. Even longer periods of eradication therapy can be realized. Nevertheless, patients with underlying co-morbidities are likely to never be totally cured even with prolonged phase 2 treatment.

Also immunomodulatory treatment, e.g. with granulocyte colony stimulating factor (G-CSF) has shown satisfying results in Northern Australia. However, as demonstrated by STEPHENS *et al* in 2002, although some people have increased lifetime expectancy with this treatment it seems to be not the optimal medication for melioidosis cases in Thailand.

Table 3. Treatment and prophylaxis of melioidosis (INGLIS 2010).

Phase 0: Post-exposure prophylaxis

APPLICATION	AGENT	DOSIS	ROUTE	FREQUENCY	DURATION	VARIATIONS
Within 24 h of highly probable exposure	Trime-thoprim-sulpha-methoxa-zole	320:1600 mg	p.o.	12 hourly	3 weeks	Amoxicillin/ clavulanic acid if allergy is existent to recommended antibiotic

Introduction

Phase 1: Acute & severe infections, induction stage

APPLICATION	AGENT	DOSIS	ROUTE	FREQUENCY	DURATION	VARIATIONS
Primary therapy	Ceftazidime	2g	i.v.	8 hourly	≥14 days	4–8 weeks if deep infections occur
	OR Meropenem	1g (2 g if CNS is affected)	i.v.	8 hourly	≥14 days	
	OR Imipenem	1g	i.v.	8 hourly	≥14 days	
Complementary therapy for deep-seated focal infections	AND trimethoprim-sulpha-methoxazole	320:1600 mg	p.o.	12 hourly	≥14 days	For infections in prostate, bone, joint and brain
	AND folic acid	5 mg	p.o.	daily		
	AND G-CSF can be considered	263 µg	s.c.	daily	3 days	Within 72 h of admission
Step-down combination for extended use and outpatients	Ceftazidime	6 g in 240 mL Normal saline	i.v.	24 hour infusion	2-4 weeks	For hospital in the home (HITH)
	AND trimethoprim-sulpha-methoxazole	320:1600 mg	p.o.	12 hourly		

Phase 2: Eradication stage

APPLICATION	AGENT	DOSIS	ROUTE	FREQUENCY	DURATION	VARIATIONS
2 of, the named ones, after phase 1 or in case of a superficial infection used as phase 1 treatment	Trimethoprim-Sulpha-methoxazole	320:1600 mg	p.o.	12 hourly	≥ 3 months	According to antibiotic susceptibility
	Doxycycline	100 mg	p.o.	12 hourly	≥ 3 months	
	Amoxicillin/ clavulanic acid	500/125 mg	p.o.	8 hourly	≥ 3 months	
	Folic acid	5 mg	p.o.	daily	≥ 3 months	With trimethoprimsulph-amethoxyzole

1.2.2.   Distribution worldwide

The worldwide distribution of melioidosis and the corresponding environmental distribution of the bacterium itself are still unknown. It is currently timed to create a worldwide risk map for melioidosis helping to raise the awareness for this disease (CURRIE and KAESTLI 2016). Furthermore, a definition of endemic areas helps the people living in high risk areas. In figure 17 the red areas are the high endemic countries, the colour orange indicates the regions which are most probably endemic but no detailed reports are available yet.
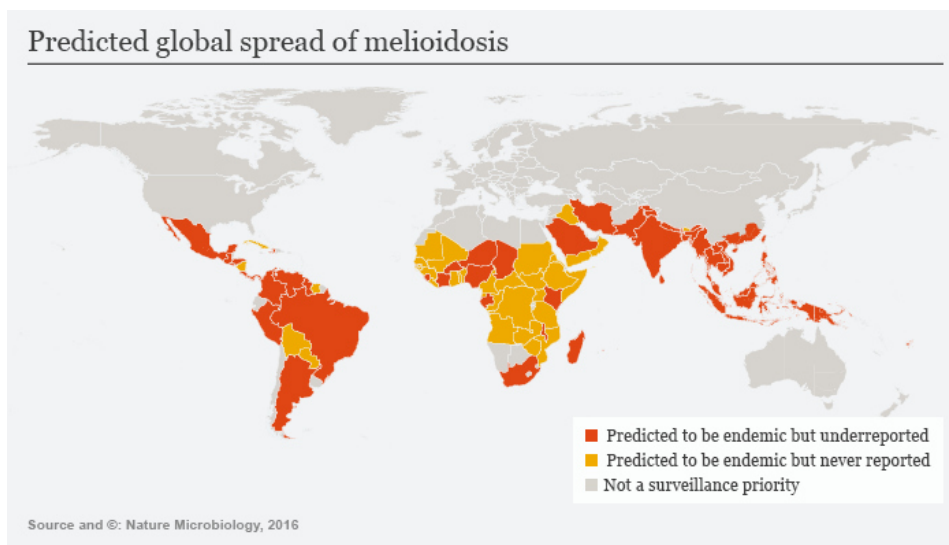
Figure 17. Worldwide distribution of *B. pseudomallei* (CURRIE and KAESTLI 2016).

Next to already known regions in Northern Australia and Southeast Asia also countries in Africa e.g. Burkina Faso, Niger and Madagascar are confirmed endemic regions for melioidosis. Although *B. pseudomallei* is defined as a bacterium which occurs only in sub- and tropical regions, there are also several reported cases from Europe e.g. France (GALIMAND *et al* 1982) and Italy (ZANETTI *et al* 2000). Unfortunately, due to the lack of a rapid test, *B. pseudomallei* is frequently misidentified, particularly in non-endemic regions (INGLIS *et al* 1998).

Overall, the pathogen has been identified in serval countries by using standard culturing methods or a specific PCR instead. Moreover, singular cases of infections with this pathogen have been reported from numerous countries. Definite

countries are Australia, Burkina Faso, Brazil, Cambodia, China, Iran, Lao PDR, Madagascar, Malaysia, Papua New Guinea, Singapore, Sri Lanka, Taiwan, Thailand and Vietnam (LIMMATHUROTSAKUL *et al* 2013b).

1.2.3.   Identification methods

First of all the sampling strategy is crucial for further experiments. Environmental samples have to be protected from direct sunlight during their transportation and storage. As indicated by LIMMATHUROTSAKUL *et al* (2013a), the procession of the soil samples soon after collection is important. It is crucial that the ratio of soil and media is around 1:1. For optimal extraction it is advised to vortex the sample for around 30 seconds. Subsequently, the sample can be further analysed by culture, PCR or animal inoculation of e.g. hamsters or guinea-pigs. Culturing can be done on solid media and these should be incubated for about 48 hours at temperatures between 37 to 42 °C. Subcultures can even be incubated between 24 hours up to 7 days before further use. The identification of *B. pseudomallei* can be achieved with a variety
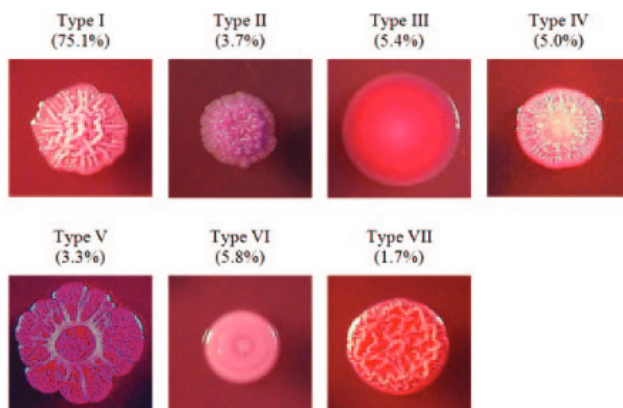
Figure 18. Different colony morphologies of *B. pseudomallei* (CHANTRATITA *et al* 2007).

of microbiological tests. First of all, the bacteria have a typical colony morphology (CHANTRATITA *et al* 2007). However, the identification of this pathogen needs a lot of practice. As indicated in figure 18, *B. pseudomallei* is able to change its colony morphology via phenotype-switching (CHANTRATITA *et al* 2007). Overall 7 phenotypes have been described and therefore misidentifications are frequent.

Figure 19. Colony morphology of one and the same strain of *B. pseudomallei* showing different colony morphologies on Ashdown's agar (WUTHIEKANUN *et al* 2006*).*

As shown in figure 19, especially the fact, that the same strain can show different phenotypes on the same plate poses a challenge for technicians (WUTHIEKANUN *et al* 2006).

Another identification method is gram staining, and a positive oxidase test constitutes the next step to further verify the result. In addition, a disk diffusion test can be performed to perceive the resistance of the bacterium to gentamicin

Figure 20. API NE 20 test showing the various colour reactions indicating biochemical reactions © biomerieux.

and colistin and the susceptibility to co-amoxiclav (SIMPSON and WHITE 1999b). To verify the results at least one confirmatory assay e.g. API 20NE, specific latex agglutination test or PCR assay is recommended. API 20NE, as shown in figure 20, identifies 98% of all *B. pseudomallei* samples although misidentifications are possible. This method has been used since 1999 (LOWE *et al* 2002).

1.2.4. Virulence factors

The virulence factors of this gram negative bacterium are still not entirely known. Putative virulence factors are antibiotic resistance, biofilm formation and quorum sensing, which is part of the communication strategy between bac-

teria. For this latter system gram negative bacteria are in need of autoinducers such as *N*-acyl-homoserine lactones (AHL). Their task is it to regulate gene expression depending on the density of a bacterial population (ULRICH *et al* 2004). On the one hand LuxI proteins are responsible for LuxR representing transcriptional regulators and AHL biosynthesis. As shown by ULRICH *et al* in 2004, these two proteins are responsible for repression and expression of genes.

Transcription is not activated at low cell density

Transcription is activated at high cell density
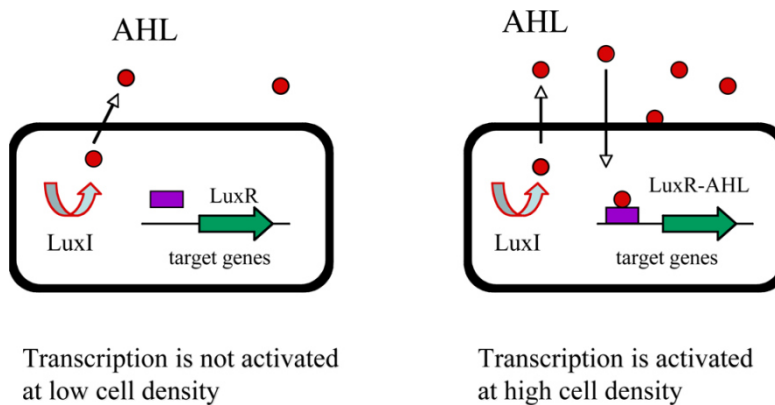
Figure 21. Principle of quorum sensing in gram negative bacteria regulated by AHL (YUNG-HUA *et al* 2012).

In *B. pseudomallei*, five different quorum sensing proteins are found; five LuxR as well as three LuxI homologues. When knockout mutants are produced affecting all 8 genes an extensive increase in the LD$_{50}$ in the model organism of Syrian hamsters is achieved. The mutations result in prolonged survival and reduced organ colonization (ULRICH *et al* in 2004). Another important virulence factor is the type III secretion system (TTSS) which includes 16 to 18 gene clusters (ATTREE *et al* 2001, WARAWA *et al* 2005). As shown in figure 22, a needle-like structure is described in various gram-negative bacteria and used to inject bacterial proteins into eukaryotic cells. In order to infect the host cell the TTSS injects certain proteins. One of the TTSS is similar to *Salmonella enterica serovar Typhimurium* (ATTREE *et al* 2001) and another one is comparable to *Shigella flexneri* (STEVENS *et al* 2002). The expressed TTSS proteins are important for invasion, pathogenesis, and escape from endocytic vacuoles as well as intercellular spread. Particularly the capability to escape from the phagosomes into the cytoplasm of its host cell is of importance considering acanthamoebae as reservoirs for *B. pseudomallei*.

When parts of the type III secretion system gene cluster of *B. pseudomallei* (*bsa*) are knocked out, the bacterium loses the ability to produce actin tails and bulges in the membrane. Additionally, escaping from vacuoles formed during endocytosis is no longer possible. Interestingly WARAWA *et al* (2005) described as an arabinose-assimilation operon with nine genes from the species *B. thailandensis* leading to downregu-

Figure 22. Type III secretion system found in several gram-negative bacteria (DIEPOLD *et al* 2014).

lation of virulence. This gene cluster is non-existent in the species *B. pseudomallei* and *mallei,* which are both pathogens. Cloning the cluster and introducing it into *B. pseudomallei,* Syrian hamsters, frequently used as model organism, had a lower death rate. This was confirmed by microarray analysis and indeed showed a down regulation of the TTSS3 genes.

Furthermore, also the capsular polysaccharide of *Burkholderia pseudomallei*, 2-O-Acetyl-6-deoxy-β-D-*manno*-heptopyranose, is an important virulence factor. Basically it is a characterized type I O-polysaccharide and its importance in the virulence of *B. pseudomallei* had already been described by RECKSEIDLER *et al* in 2001 using animal models. Lipopolysaccharides (LPS) are well-known virulence factors. However, the LPS of *B. pseudomallei* are not needed for their virulence. While the LPS of other gram negative bacteria lead to an activation of the immune system through binding Toll-like receptor 4 (TLR4) and an expression of cytokines, this is different for *Burkholderia* LPS. As BEUTLER *et al* demonstrated in 2003, TLR 4 seems not to play a role in eradicating melioidosis. In several experiments with the active LPS of *B. pseudomallei* and the non-active one of *Burkholderia thailandensis* it was shown that they display similar im-

munoblot profiles. This leads to the idea that the LPS does not play a significant role in the pathogenicity of the bacterium. A different explanation might be that the LPS have different biological activities while they are very similar in their antigens. Thus, they show similar profiles in immunoblotting against sera from melioidosis patients (ANUNTAGOOL *et al* in 2000).

1.2.5.   Activation of the immune system

Pathogen-associated molecular patterns (PAMPs) are responsible for the recognition of the bacterium via receptors. The most important receptors are Toll-like receptors (TLRs) and they help to activate the adaptive immune system and link it with the innate immune system (TAKEDA *et al* in 2003). Until today the whole activation process of the immune system by *B. pseudomallei* is not fully understood.

As shown in figure 23, the recognition of the pathogen via TLR4, CD14 and MD2 has been already described. Together, the receptors seem to identify *B. pseudomallei* LPS. Moreover, TLR5 is needed to recognize flagellin and TLR 9 identifies CpG or bacterial DNA. Especially interesting is the finding that *tlr4*-deficient mice are resistant to high doses of LPS produced by *B. pseudomallei* (MATSUURA *et al* in 1996).

However, an interaction of the host cell with *B. pseudomallei* PAMPs leads to an activation of transcription factor NFκB and results in the release of proinflammatory cytokines. Other factors help the host cell to identify the pathogen but experiments still have to proof this.
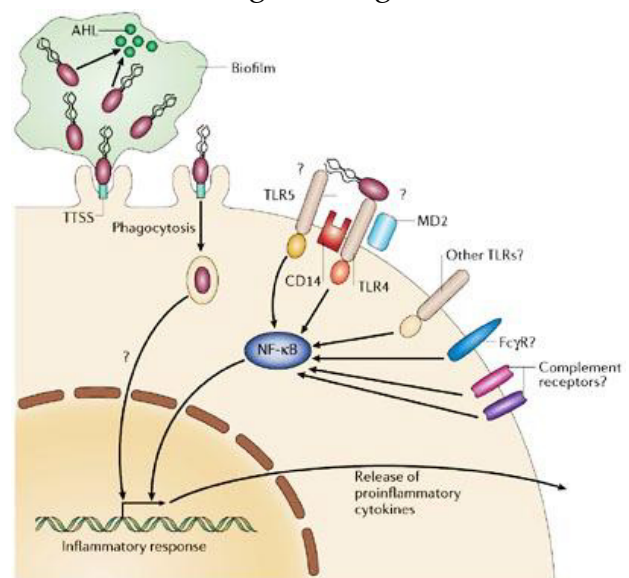
Figure 23. Interaction of the *B. pseudomallei* with its host (WIERSINGA *et al* 2006).

Additionally, IFN-γ, one of the proinflammatory cytokines, is produced and plays an important role in the early resistance against the infection. Furthermore, IFN-γ as well as IL-12 and IL-18 are fundamental factors for the T_H1 cell-mediated immune response. Due to the fact that the concentrations of IL-6, IL-15, IFN-γ-inducible protein (IP-10) as well as IL-10 are higher in patients with melioidosis, multiple inflammatory pathways seem to be activated (LAUW *et al* in 1999).

As shown by EGAN *et al* in 1996, *B. pseudomallei* additionally activates the alternative pathway of the complement system. This results in the sedimentation of C3 on the bacterial surface and is followed by opsonisation. Moreover, it has been shown that a lytic activation of the complement system is not happening. This feature has already been described in connection with other pathogens e.g. *Salmonella* spp., *Borrelia* spp. and *Neisseria* spp. as well as streptococcus (FRANK *et al* in 1987).

In patients with diagnosed melioidosis infection the levels of IgG, IgA and IgM show a positive correlation with disease severity. Furthermore, the disease is associated with human leukocyte antigen class II (HLA) in patients from Thailand (DHARAKUL *et al* in 1998). KETHEESAN *et al* (2002) and BARNES *et al* (2004) demonstrated that patients recovering from melioidosis have a cell-mediated immune reaction being antigen-specific. However, patients without symptoms have a strong cell-mediated immune reaction.

1.3. Interactions between free-living amoebae (FLA), human host cells and *B. pseudomallei*

Several facultative intracellular bacteria e.g. *Mycobacterium avium*, *Listeria monocytogenes* and *Legionella pneumnophila,* have been proven to survive as endosymbionts in FLA (INGLIS *et al* 2000b, CIRILLO *et al* 1997, LY *et al* 1990). The interactions between *L. pneumophila* and *Acanthamoeba*/macrophages have been investigated in greater detail. The bacteria are assumed to benefit by developing mechanisms to adapt to the similar conditions in mammalian phagocytic cells. Furthermore, as described by CIRILLO *et al* (1994, 1997) amoebic endosymbiosis leads to an increased virulence of *M. avium* and *L. pneumophila.* Besides, the interactions of *L. pneumophila* with *Acanthamoeba* spp. and also with macrophages result in coiling phagocytosis. This rare form of phagocytosis is shown in figure 24 and is also used by *Borrelia* spp. (RITTIG *et al* 1998). Moreover, encystment of the amoebae can be outlasted by these bacteria. These mechanisms allow
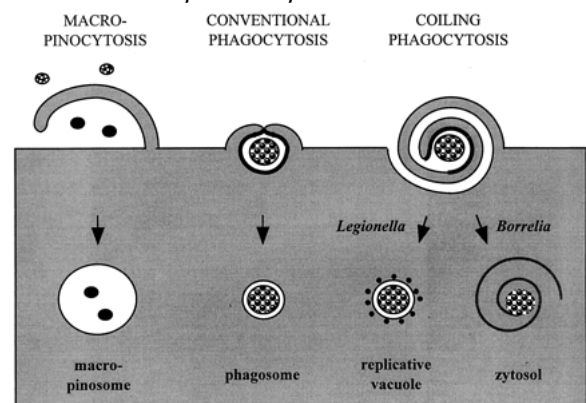
Figure 24. Coiling phagocytosis. Next to the typical phagocytosis in the middle of the figure, coiling phagocytosis on the right side is a rare form (RITTIG *et al* 1998).

several bacteria to exploit FLA as their hosts. As described by MICHEL *et al* in 1982, *Ralstonia* (originally *Burkholderia*), a close relative of *B. pseudomallei,* was found inside of *Acanthamoeba* spp. during screening of samples from hospitals. Furthermore, survival of *Burkholderia cepacia* inside of these amoebae has been shown in *in vitro* experiments (MAROLDA *et al* 1999). About 20% of *Acanthamoeba* spp. isolated from environmental and clinical samples were found to enclose bacterial endosymbionts. These interactions are stable and several of these bacteria cannot be cultured outside of their hosts.

1.3.1. Strategies of intracellular survival of *B. pseudomallei* in the human body

Since the early 90ies it is well accepted that *B. pseudomallei* can survive inside both, non-phagocytic and phagocytic cells, significantly contributing to its pathogenicity. This brings the major advantage for the bacterium that while establishing the infection it is able to hide from the immune system. To achieve this, *B. pseudomallei* had to acquire several routes for intracellular survival (JONES *et al* 1996).

1.3.1.1. Adhesion to host cells

The molecular mechanisms needed for adhesion of *B. pseudomallei* to the host cell are still not fully understood. Several cell lines have been tested with different results. Human A549 lung epithelial cells adhere strongly to *B. pseudomallei* but not as good to the non-pathogenic *B. thailandensis* (KESPICHAYAWAT-TANA *et al* 2004). Furthermore, it has been shown that an increased ability to adhere to the host cell results in an increased invasion. However, more details are not known yet.

Several studies have found that the lack of the capsule has no impact on cell adhesion but it is needed for full virulence (RECKSEIDLER *et al* 2001).
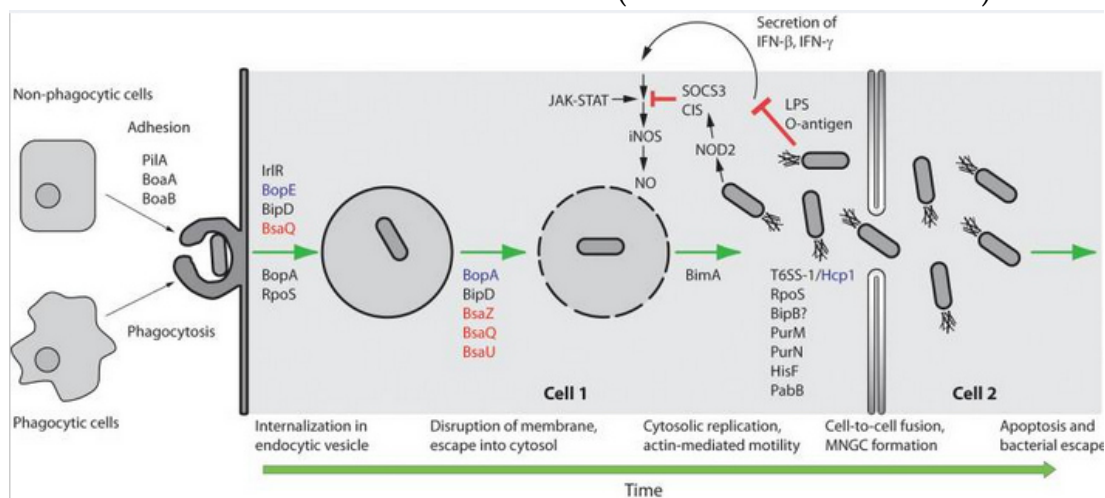
Figure 25. Intracellular life of *Burkholderia pseudomallei* with all important factors and genes (ALLWOOD *et al* 2011).

As described by PELICI *et al* (2008) type 4 fimbriae (pili) are crucial for adherence to the hosts and are the reason they are able to attach to a wide range of different tissues. Important for the function of the pilus, the *pilA* gene is needed for encoding the pilin subunit protein (see figure 25). As a result of this the aforementioned A549 cell line showed almost no adhesion anymore (ESSEX-LOPRESTI *et al* 2005).

Interestingly, two novel adhesins of *B. pseudomallei* had been identified by BALDER *et al* (2010). The BoaA protein is similar to YadA, which is known from *Yersinia enterocolitica* and *Burkholderia mallei* as an autotransporter adhesin. The inactivation of BoaA reduces the adhesion of the bacterium to A549 and Hep2 cells. The second adhesin is BoaB and has not yet been found in any other organism. The inactivation of the *boaB* gene had the same effect as with *boaA*, resulting in a decreased adhesion to the cells. However, still the adhesion was not totally inhibited. Hence, multiple adhesins seem to be involved in the adhesion of the bacterium to eukaryotic cells. Furthermore, cell surface components could be needed during the interaction of *B. pseudomallei* with the various cell lines (BALDER *et al* 2010).

1.3.1.2. Intracellular survival

After adhesion to the cell surface the host cell is invaded playing a crucial role in the pathogenesis of *B. pseudomallei*. The bacterium is engulfed by phagocytic cells such as macrophages and can also invade non-phagocytic cell lines e.g. HeLa and A549. While comparing *B. thailandensis* with *B. pseudomallei* it has been shown that the non-infectious bacteria have a lower capacity of invading the A549 cell line (KESPICHAYAWATTANA *et al* 2004). A study by JONES *et al* in 1997 was able to identify a two-component response regulator *irlR* (invasion related-locus) involved in the invasion of the A549 cell line but not of macrophages. *IrlS* is the sensor and located downstream of the regulator. This *irlRS*

two-competent system does not affect virulence, as knock outs of this system left the bacteria with the ability of infecting different cell lines (JONES *et al* 1997).

The type 3 secretion system (T3SS) is a system of gram negative bacteria which is well understood and highly conserved while being also highly adapted. Around 20 different proteins are involved and arranged in this complex machinery (see figure 22) in a syringe-like structure spanning the outer and inner membrane of bacteria. This enables the bacteria to inject and secrete effector molecules into the cytoplasm of the host cell to interfere with the cellular functions (HUECK 1998, ZHANG *et al* 2006). Despite the high conservation of this system the secreted proteins are highly diverse. In total, 3 clusters of T3SS are encoded by *B. pseudomallei.* While the cluster 1 and 2 are similar to the ones of the plant pathogens *Ralstonia solanacearum* and *Xanthomonas* spp (WINSTAN-LEY *et al* 1999), the third cluster has homologies to the *inv/spa/prg* T3SS of *Shigella flexneri*. Therefore, this cluster is referred to as the *Burkholderia* secretion apparatus (*bsa).*

The T3SS of *B. pseudomallei* definitely is of great importance for the pathogenesis of due to its role in replication, intracellular survival and virulence. There are several factors in this system, but for invasion the effector protein BopE, the translocator BipD and the structural component BsqA are required (STEVENS *et al* 2003). The protein BipD is on the tip of the needed and has similarities to the *Salmonella* SipD. Moreover, BipD is needed for invading non-phagocytic cells (STEVENS *et al* 2003).

Furthermore, BsaQ is an important channel protein of the inner membrane of *B. pseudomallei* and located on the base of the T3SS (MUANGSOMBUT *et al* 2008). Studies by MUANGSOMBUT *et al* (2008) showed, that mutations of the *bsaQ* gene inhibit the secretion of BipD and BopE, respectively. As a result of this the invasion of non-phagocytic cells is not possible anymore. After entering the cells, the bacterium quickly has to adapt to the new and changed environment.

As demonstrated by HARLEY *et al* in 1998, *B. pseudomallei* is able to escape from endocytic vesicles within 15 minutes of engulfment by lysing the endosomal membrane.

As shown by *in vitro* experiments (UTAISINCHAROEN *et al* 2001) *B. pseudomallei* is able to survive and perform replication inside of neutrophils and monocytes. Furthermore, *B. pseudomallei is* able to escape the immune system and the lysis of macrophages, respectively. In macrophages in mouse models, which are infected with the pathogen, no activation of the inducible nitric-oxide synthase (iNOS) takes place. iNOS is an important enzyme and is needed for lysing intracellular bacteria with reactive nitrogen intermediates. Moreover, the bacterium is able to live in membrane compartments, especially in phagolysosomes, due to the fact that they can cope with acidic environments. The T3SS effector protein BopA is of major importance for the escape from phagosomes. In comparison to the wild type *bopA* mutants loose this function and are degraded within the vesicles. Also *bsa*Z gene encodes a component of the T3SS and mutations lead to an inability or delayed ability of escaping into the cytoplasm. Overall, it took the majority of *bsa*Z mutants 6 hours post infection to get out of the vesicles (MUANGSOMBUT *et al* 2008). Several T3SS mutants and also a complete knock-out of this system showed a delayed escape from the phagosomes. Hence, other mechanisms play a role in this step (BURTNICK *et al* 2008).

Of course the ability to survive inside of the cytoplasm is important for *B. pseudomallei* to establish an infection. Several genes crucial for intracellular replication have been identified. It seems that macrophages are the optimal host cells for *B. pseudomallei* due to their relative long half-life and microbiocidal capacity in comparison to neutrophils and monocytes (HARLEY *et al* 1994). The bacterium can also survive encapsulated in biofilms inside of their hosts. Likewise *in vitro* survival is also possible in distilled water and as already shown also in *Acanthamoeba* spp. (PUMPUANG *et al* 2011)

1.3.1.3.   Formation of multinucleated giant cells (MNGCS)

Several intracellular pathogens infecting humans are able to transmigrate the cytoplasm by manipulating actin polymerization. As shown by MONACK *et al* in 2001, this mechanism works in phagocytic and non-phagocytic cells and is important for *B. pseudomallei* for the spread from cell to cell (see figure 25). Interestingly, MNGC has been described in histopathological tissue samples from patients with previous melioidosis (WONG *et al* 1995).

Several *in vitro* cell culture experiments by KESPICHAYAWATTANA *et al* in 2000 indicate that fused and unfused cells infected with *B. pseudomallei* are compelled to apoptosis. However, inside of MNGC both, apoptotic and normal nuclei, can be found. Unfortunately, the mechanism behind the formation of MNGC during *Burkholderia* infections is not fully understood. Nevertheless, as shown in figure 25, the production of these giant cells has been linked with BipB, T6SS, RpoS as well as the two toxin-encoding genes *bpsl0590* and *bpsl591*. In general, the MNGC are produced to serve as a niche for optimal replication and spread but the wide range of different mechanisms are not fully understood (ALLWOOD *et al* 2011).

1.3.1.4.   Inhibition of macrophage activation

The ability of killing pathogens by macrophages is based on the production of nitric oxide (NO) by induction nictric oxide synthetase (iNOS). Several cytokines e.g. IFN-β, IFN-γ, TNF-α, IL-1 and IL-2 as well as the pathogen-associated molecular pattern (PAMP), e.g. LPS and LTA, stimulate the production of NO. Interestingly, *B. pseudomallei* found a way of supressing this mechanism. Macrophages infected with this human pathogen do not have an activated iNOS production. The reason for this effect is that IFN-β and IFN-γ are not produced and the JAK-STAT-pathway is not started. This is a consequence of the special LPS structure of this bacterium (UTAISINCHAROEN *et al* 2003).

1.3.1.5.   Evasion of autophagy

Autophagy is a typical mechanism occurring in all eukaryotic cells and is crucial for regulated degradation of cellular components. This mechanism starts with the enclosure of cytoplasm within a membrane (phagophore), subsequently a double membrane is generated. Afterwards this autophagosome fuses with an endosome before the final assembly with a lysosome. After degradation of the material inside of the autophagosome, the products are exported into the cytoplasm and used for production of new cellular components. Therefore, autophagy is of major importance in context of removing and recycling non-functional and damaged organelles as well as degradation of certain long-lived proteins. Furthermore, this mechanism has been recognized as a part of the innate immune system and is a defence mechanism against bacteria. However, certain bacteria are able to modify autophagy in a manner that they can survive (XIE and KLIONSKY 2007).

As demonstrated by CULLINANE *et al* in 2008, the autophagy marker LC3 seems to be a key factor in this process. This protein co-localizes only with a part of *B. pseudomallei* leading to a decreased level of autophagy and the survival of the bacteria. Nevertheless, studies also revealed a reduced intracellular survival of the bacteria in T3SS *bopA* mutants as a result of the increased co-localization of LC3. This factor can also be recruited directly to the autophagosome by LC3-associated phagocytosis (LAP) (SANJUAN *et al* 2007). However, this defence mechanism of the immune system seems to be ineffective against *B. pseudomallei;* the majority of the bacteria escape to the cytoplasm and elude the canonical autophagy.

1.3.2.   Co-cultivation of *Acanthamoeba* spp. and *B. pseudomallei*

The hypothesis that *B. pseudomallei* can use acanthamoebae as host organisms is supported by several facts. *B. cepacia* has been demonstrated to survive inside *Acanthamoeba* strains during *in vitro* experiments. *Acanthamoeba* spp. are known

to serve as reservoirs for human pathogens. A series of co-culturing experiments revealed, that this interaction results in the rare and unusual coiling phagocytosis (INGLIS *et al* 2000b).

Similar co-culturing experiments with *Acanthamoeba astronyxis* and *B. pseudomallei* showed that adhesion results in vacuole formation. Figure 26 demonstrates

events observed in trophozoites within one hour after the start of the experiment while using SYTO 9 fluorescent stain for the bacteria.
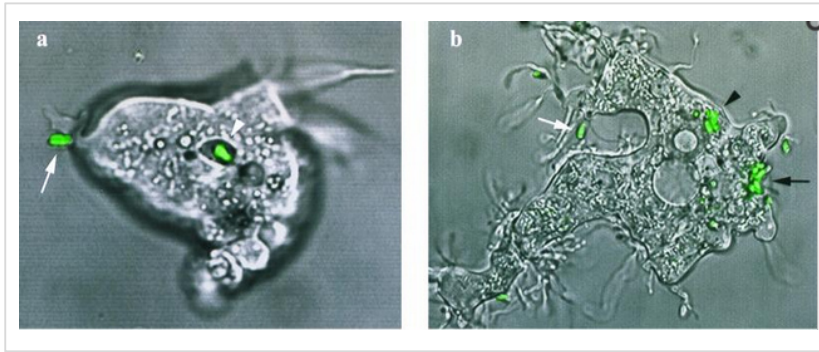
Figure 26. *Acanthamoeba* trophozoites ingesting *B. pseudomallei* (green) and storing them in vacuoles (INGLIS *et al* 2000b).

Interestingly *B. pseudomallei* attached to the trophozoite with their polar end and vacuoles with single to multiple bacilli are produced to the end of forming external bacillary tufts. These events cannot be seen in fully rounded trophozoites or cysts (INGLIS *et al* 2000b).

Transmission electron microscopy has been used to observe the interaction between bacteria and amoebae in more detail. As shown in figure 27, already several minutes after the start of the co-culturing experiment, pseudopodia of the amoebae attached to the bacteria and first vacuoles filled with single bacilli were observed. A large percentage of these vacuoles were filled with multiple bacteria within 30 minutes. As shown in figure 28, in some vacuoles bacteria were surrounded by multiple membrane stacks, typically found in link with coiling phagocytosis (INGLIS *et al* 2000b).

Overall, the co-culturing experiments lasted 72 hours and within this time the majority of the vacuoles were attached to the external plasma membrane of the trophozoites. Some rounded trophozoites had highly motile bacteria in their vacuoles and some trophozoites even had free moving bacteria in their cyto-

plasm. Afterwards, trophozoite lysis with spilling the cytoplasmic contents occurred (INGLIS *et al* 2000b).
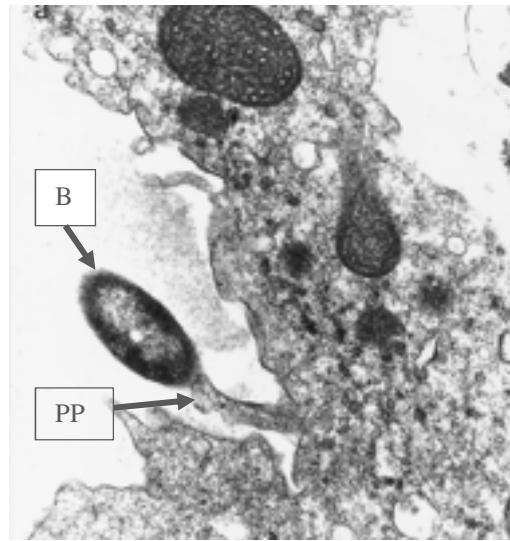
Figure 27. Pseudopodium attaching to *B. pseudomallei*. Pseudopodia (PP) of *Acanthamoeba* spp. trophozoite reaching out to attach to the polar end of the bacterium (B) (INGLIS *et al* 2000b).Modified.
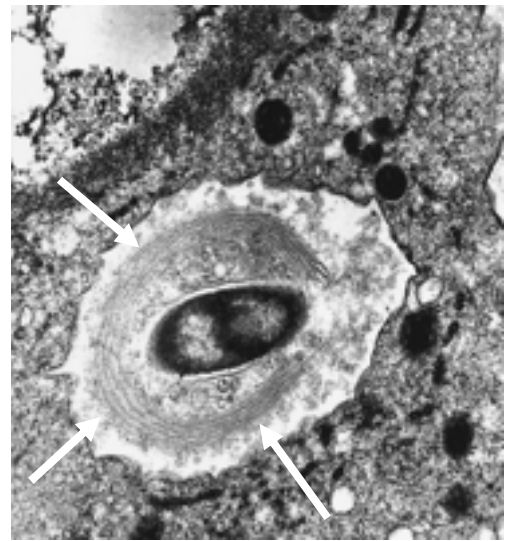
Figure 28. Ingested bacterium inside of a trophozoite. A vacuole harbours a bacterium surrounded by multiple membrane stacks (*see white arrows)* typical for coiling phagocytosis. (INGLIS *et al* 2000b). Modified.

However, this step was not seen in e.g. 2 hours bacterial preparations. Furthermore, viable bacteria could be recovered from trophozoites 1 hour after endocytosis started. Moreover, also after 72 hours of co-culture motile bacteria were seen inside of the trophozoites. Interestingly, the same observations were made in similar experiments with *L. pneumophila* and *Acanthamoeba* spp. Replication of *B. pseudomallei* inside of the trophozoites was not proven. Nevertheless, the gained results give important information of how these bacteria survive in vacuoles and therefore act inside of macrophages. The same experiments conducted with other *Acanthamoeba* strains from different morphological groups also revealed vacuole formation but less pronounced than with *Acanthamoeba astronyxis*, member of morphological group I (INGLIS *et al* 2000b).

Overall, the discovery of coiling phagocytosis between *Acanthamoeba* spp. and *B. pseudomallei* is of high importance. The interaction between *L. pneumophila* and both human monocytes and *Acanthamoeba* spp. is well understood and also

shows this rare form of coiling phagocytosis (HORWITZ 1984). Still, the occurrence of this type of phagocytosis between *B. pseudomallei* and mammalian macrophages has to be proven. One could assume that similar to *L. pneumophila* also *B. pseudomallei* has advantages from using this host. The ability to escape from vacuoles in the late stages of co-culture demonstrates the viability of the intracellular bacteria. These experiments, of course, give an insight into the interactions between these two organisms but further experiments have to answer if this rally is a symbiosis (HORWITZ 1984).

1.3.3. *Acanthamoeba* spp. as virtual training grounds

The interactions between *L. pneumophila* and *Acanthamoeba* spp. are well understood and due to the fact that *L. pneumophila* and *B. pseudomallei* seem to share the same way of entering the amoebae, also the possibility of sharing other features is imaginable (INGLIS *et al* 2000b).

Several studies have shown that *L. pneumophila* grown inside of amoebae is highly resistant to unfavourable environmental conditions as fluctuating temperatures, pH and osmolarity (ABU KWAIK *et al* 1997). Furthermore, passaged bacteria are also able to survive biocides and chemical disinfectants. Next to that, *L. pneumophila* is also able to survive in amoebal cysts and this leads to resistance to biochemical and physical agents (BARKER *et al* 1992).

1.4. Aims of this study

The main aim of this work was to give a first detailed insight into the amoebal diversity in soil. For morphological identification FLA have to be in their cyst stage. To achieve the goal of describing and correctly identifying all *Acanthamoeba* strains, the trophozoites and cysts have to be transferred to fresh agar plates to the end of getting clonal colonies. However, *Acanthamoeba* strains can have one and the same morphotype but belong to different genotypes and vice

versa. With the help of identification key, the morphological groups of the clonal cultures were determined. The molecular analysis e.g. PCR and Sanger-Sequencing were used to identify the amoebal genotypes.

The second aim of this thesis was to answer the question if *Acanthamoeba* spp. indeed can act as host organisms for the pathogen *B. pseudomallei*. The bacterium is facultative intracellular and it is still hardly unknown which organisms act as its hosts. An *in vitro* interaction of Acanthamoeba with *B. pseudomallei* has been described by INGLIS *et al* in 2000b, but the detection of *B. pseudomallei* in amoebae isolated from environmental samples is still missing. Thus, the investigation of *Acanthamoeba* isolates from the environment for natural infections with *B. pseudomallei* will lead to a better understanding of the worldwide distribution of this pathogen.

2. Material and Methods

2.1. Cultivation of *Acanthamoeba* spp. on Non-Nutrient (NN-) agar plates

Every *Acanthamoeba* spp. isolate was grown on non-nutrient (NN-) agar plates (table 4) to the end of getting clonal cultures.

Table 4. Non-Nutrient agar for 500 mL of agar solution resulting in 30 plates.

7,5 g bacteriological agar
475 mL MiliQ H_2O
5 mL NEFF
5 mL $CaCl_2$ x 2 H_2O
5 mL NaCl
5 mL KH_2PO_4
5 mL $MgSO_4$ x 7 H_2O
5 mL Na_2HPO_4

All components were added to a 1 L bottle and mixed on a magnetic stirrer for 2 minutes. Afterwards, the solution was autoclaved for 15 min at 120 °C to ensure its sterility. The plates were poured in the laminar airflow cabinet as soon as the agar solution had a temperature between 70°C-80°C; for each plate, 17 mL were used. The plates were cooled down in the laminar flow and can, if sealed, be stored for up to one week in the refrigerator at 6°C.

2.1.1. Inoculation of samples

To ensure the growth of amoebae, 50 μL of *Escherichia coli* were plated on the agar plates. From every soil sample 0.4 g was inoculated onto NN-agar plates after flame sterilization of the inoculation loop (figure 29). All culture plates were sealed with parafilm.

Subsequently, the plates were labelled with the soil number and date and stored at room temperature protected from direct sunlight.

Picture 29. NN-agar plate with 0.4 g of soil to start the amoebal cultures.

2.1.2. Clonal cultures

The culture plates with the soil were screened under an inverted phase contrast microscope (Nikon Optoteam, Vienna, Austria) for the growth of acanthamoebae daily. In order to prevent the growth of fungi, antimycotic rings 100 LD2 (Abtek Biological Ltd, Liverpool, United Kingdom) were used. To achieve clonal cultures and optimal growth conditions, the amoebae were transferred onto fresh agar plates with *E. coli* as soon as singular amoebae were visible. This step helped to get rid of the fungi possibly growing on the plates and to separate different amoebae strains growing on the same plate.

2.2. Identification of *Acanthamoeba* spp.

Amoebae can be identified, at least to the genus levels, in some cases also to the species level by the morphology of their cysts. A more precise identification can be achieved by sequencing of their 18S rRNA genes, which is termed genotyping.

2.2.1. Morphotyping

In the process of achieving pure cultures notes on the morphotypes of the different acanthamoebae were made in parallel, ensuring that all different strains were isolated and identified.

All isolates were morphotyped with the help of the identification keys introduced by PUSSARD and PONS (1977) and PAGE (1991).

Species identification was achieved following the flow charts shown in figures 30-32. The morphology of the endo and ectocysts are the most important characters for identification.
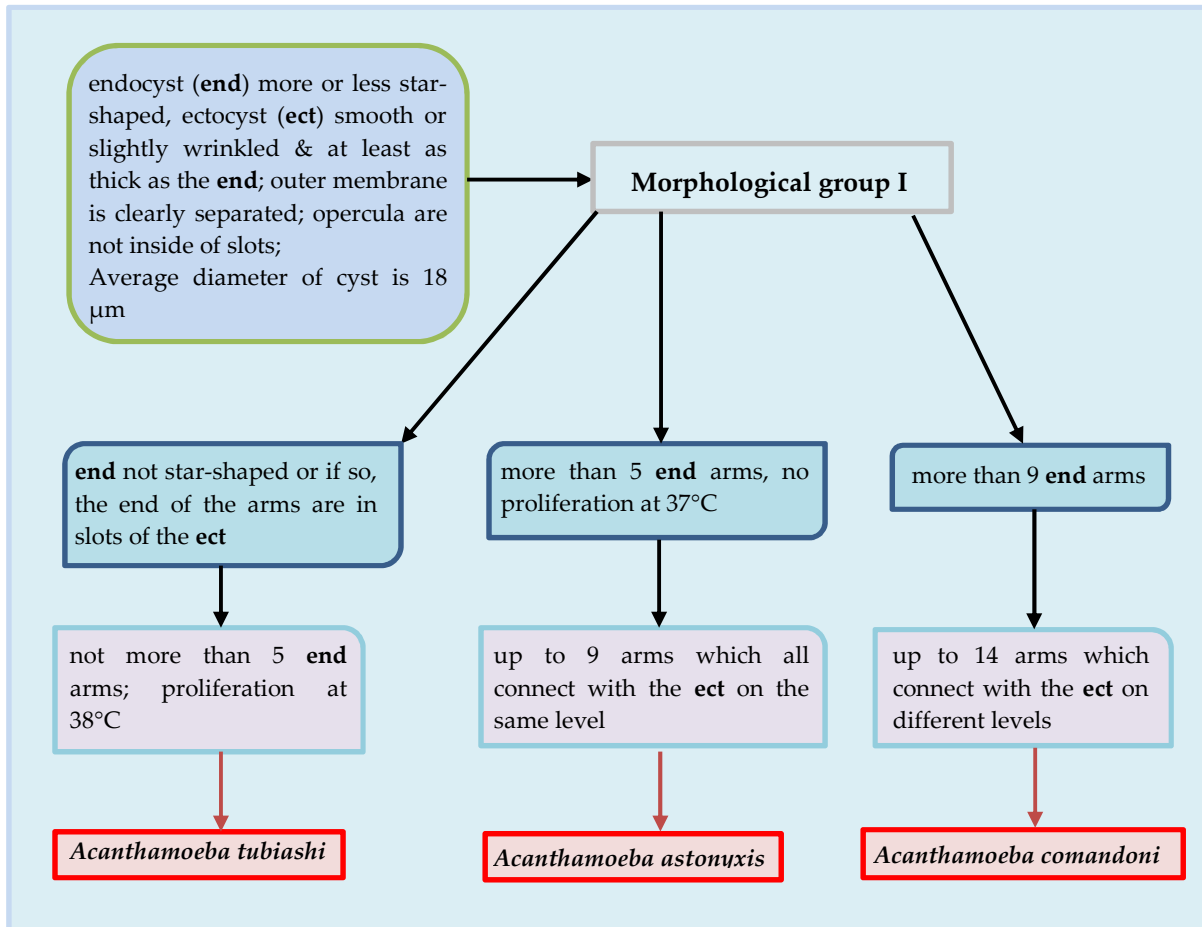
Figure 30. Identification key for morphological group I.
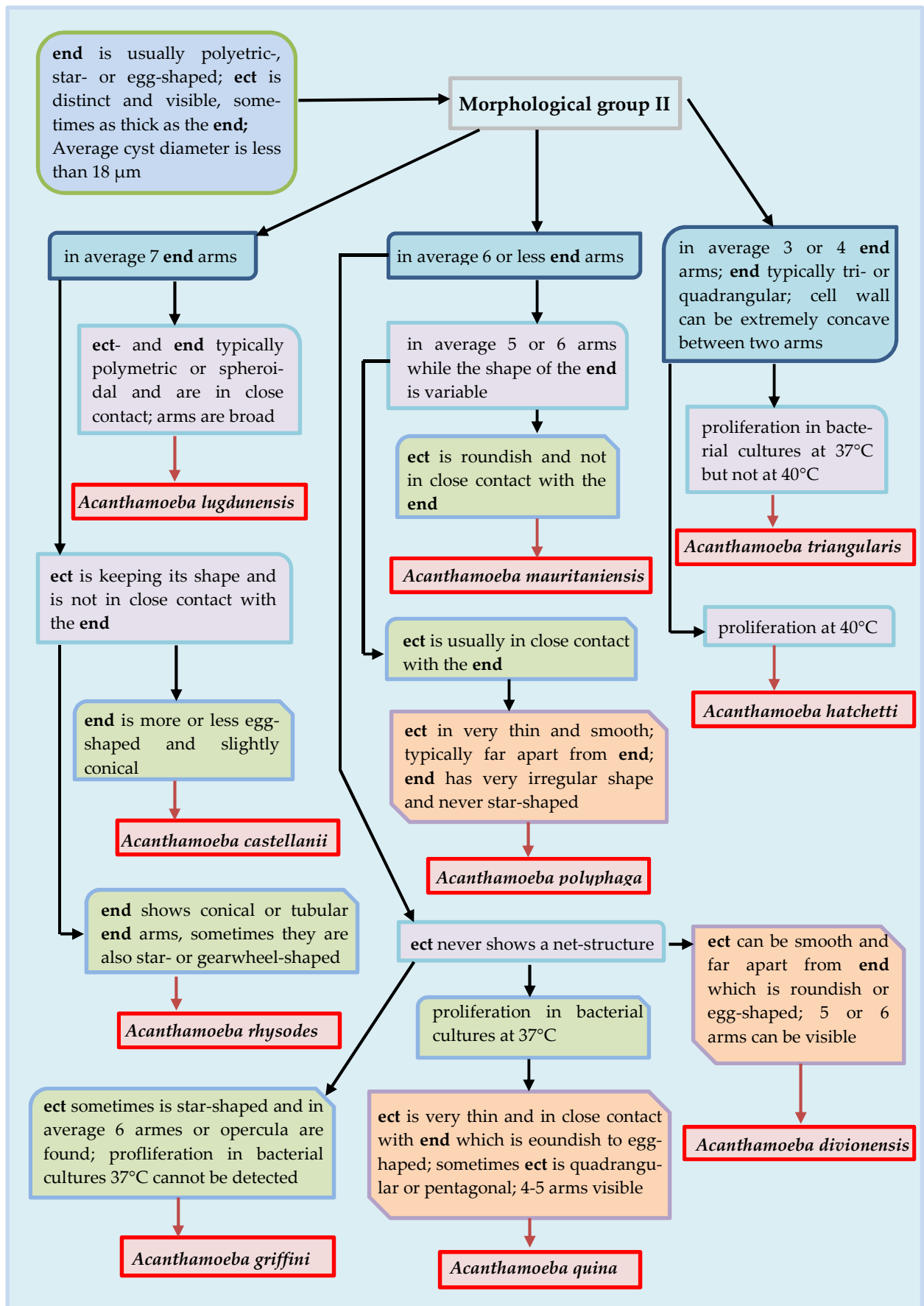
Figure 31. Identification key for morphological group II.
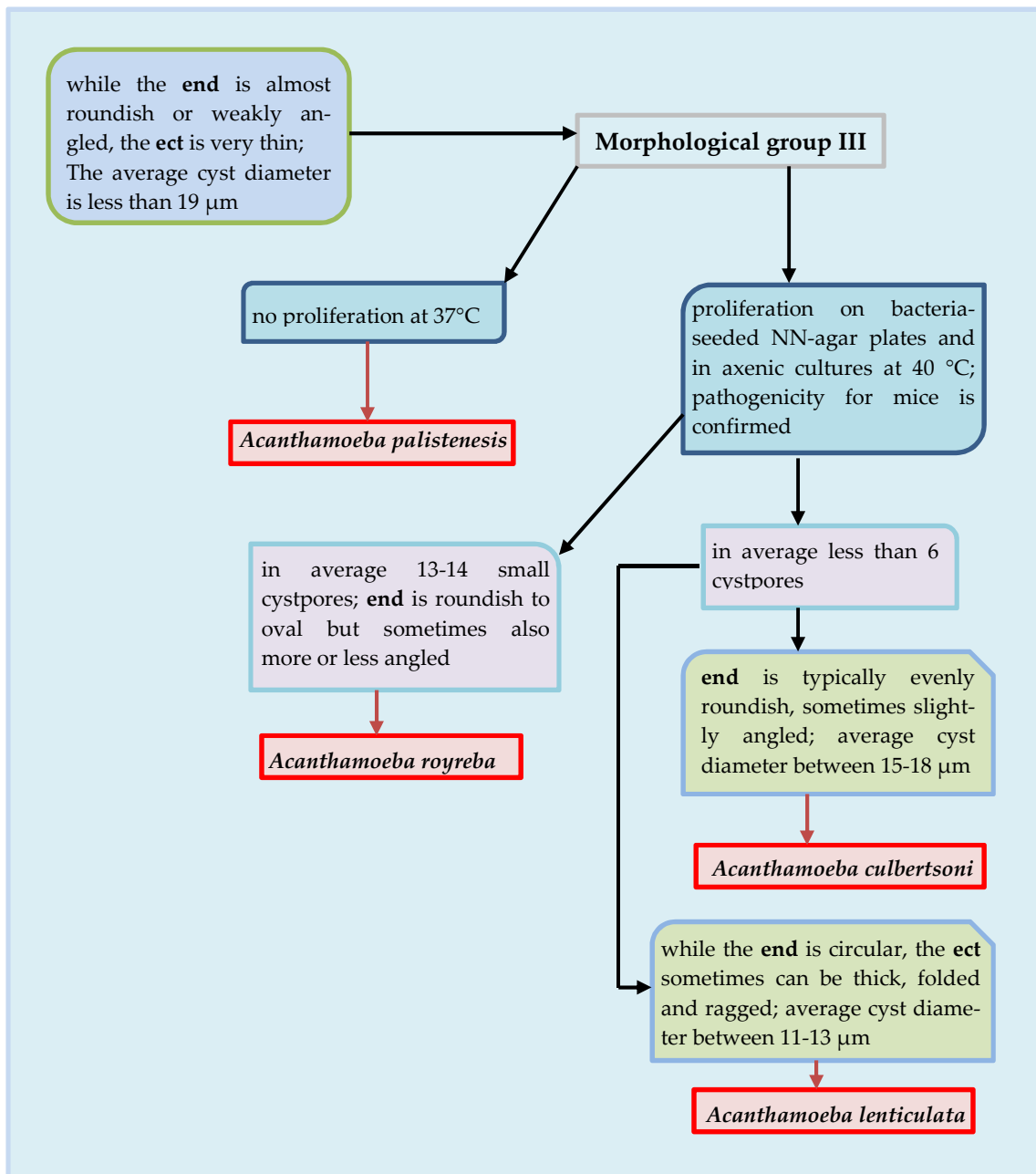
Figure 32. Identification key for morphological group III.

2.2.2. DNA extraction

The pure cultures were used for harvesting the amoebae from the plates as long as they are in their trophozoite stage. For these purpose the plates were processed in the laminar airflow cabin to secure sterile conditions.

For each plate, one 15 mL falcon was filled up with 6 mL of sterile NaCl solution. A sterile Q-tip was moistened with sterile saline and used for detaching

acanthamoebae from the agar by scraping. Afterwards, the Q-tip was washed thoroughly in the saline leaving the amoebae from the Q-tip in the solution. This step was repeated 3 times for each plate. Overall, 3 plates per strain were cultured and harvested. To increase the DNA yield in the end an additional working step was added as demonstrated in figure 33. In total, 3 mL of fresh saline were pipetted onto the agar plate. Subsequently, the solution was rinsed over the plate several times to be finally combined with the saline in the falcon tube.

The falcons were put into the centrifuge (Sigma-Aldrich, St. Louis, USA) for 15 min at 12,000 x g to break up the cell walls and gain the DNA. An additional working step by means of freezing the whole tube over night, was also included here to increase the amount of DNA in the solution.
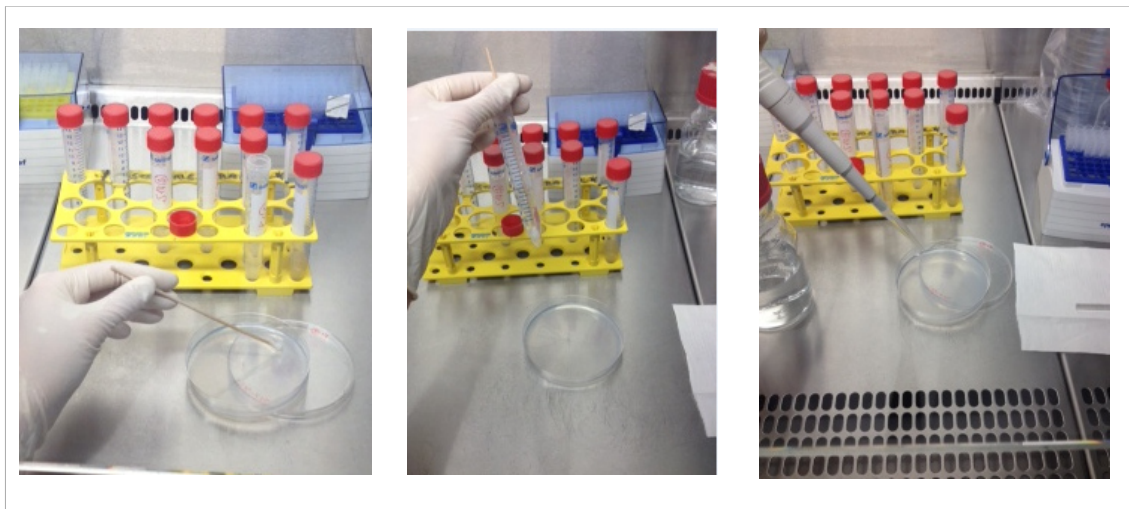
Figure 33. Harvesting of trophozoites from the NN-agar plates using sterile saline, a sterile Q-tip and a micropipette.

Subsequently, the DNA-extraction was performed according to the recommended protocol of QIAamp® DNA Mini Handbook (Qiagen, Hilden, Germany), REF 51306.

The pellet was resuspended in 200 μL of PBS and 20 μL of Proteinase K (Qiagen, Hilden, Germany) were added. Afterwards, 200 μL Buffer AL (Qiagen, Hilden, Germany) were added to the sample and the solution was mixed by pulse vortexing for 15 seconds. The sample was incubated at 56°C for 10

minutes in a thermomixer comfort (Eppendorf, Hamburg, Germany) and the sample was centrifuged briefly to remove drops from the inside of the lid. Subsequently, 200 μL of 96-100% ethanol were added to the sample and mixed by pulse vortexing for 15 seconds and the sample was again centrifuged using 1-15 microcentrifuge (Sigma-Aldrich, St. Louis, USA) to remove drops from inside of the lid.

The mixture was carefully applied to the spin column which was placed in a 2 mL collection tube without wetting the rim. The closed column was centrifuged at 6,000 x g for 1 minute. The filtrate was discarded and the step was repeated until all of the solution was processed. Afterwards, the column (Qiagen, Hilden, Germany) was transferred into a new collection tube and 500 μL of buffer AW1 (Qiagen, Hilden, Germany) were added carefully. A centrifugation step at 6,000 x g for 1 minute followed and the collection tube with the containing filtrate was discarded afterwards. The column was put into a new collection tube and 500 μL of buffer AW2 (Qiagen, Hilden, Germany) was added. The column was placed into a new collection tube and centrifuged at 20,000 x g for 1 minute to get rid of the remaining buffer AW2. Subsequently, the filtrate was discarded. The column was placed into a DNAse-free microcentrifuge tube and 200 μL of buffer AE (Qiagen, Hilden, Germany) were added directly onto the membrane. Afterwards the sample was incubated at RT for 1 minute and centrifuged at 6,000 x g for 1 minute. In the end the DNA sample is stored at -20°C.

2.2.3. Polymerase chain reaction

The total genomic DNA (gDNA) was further used for a polymerase chain reaction (PCR) using a mastercycler Vapo.protect (Eppendorf, Hamburg, Germany). Therefore, different primer pairs were used to amplify the 18S rDNA gene (table 5).

Table 5. Primers used in PCR reactions and their location in the 18S rRNA gene (WALOCHNIK *et al* 2004).

Primer	Approximate location	Sequence
P1fw (=Rhiz I)	624-642	5'-CAAGTCTGGTGCCAGCAGC-3'
P1rev (=RhizII)	642-624	5'-GCTGCTGGCACCAGACTTG-3'
P2fw (=Rhiz 1)	1265-1280	5'-GATCAGATACCGTCGTAGTC-3'
P2rev (=Rhiz 2)	1280-1265	5'-GACTACGACGGTATCTGATC-3'
P3fw (=Rhiz 3)	1871-1852	5'-CAGGTCTGTGATGCCCTTAG -3'
P3rev (=Rhiz II)	1852-1871	5'-CTAAGGGCATCACAGACCTG-3'
ACfw	1032-1052	5'-TGCCACCGAATACATTAGCAT-3'
ACrev	1370-1390	5'-ACAAGCTGCTAGGGGAGTCA-3'

The following primer pairs were combined:

Rhiz I & Rhiz 2, Rhiz 1 & Rhiz 2, Rhiz 1 & Rhiz 3, Rhiz 2 & Rhiz II, ACfw & ACrev (WALOCHNIK *et al* 2004). As indicated in tables 6-9 the use of different primers required also different PCR programmes.

Table 6. & 7.: PCR programmes (Gastb 56 & 58) with adapted annealing temperatures for the use of different primers.

Gastb56		Gastb58	
min	°C	min	°C
15	95	15	95
1	95	1	95
2	56	2	58
3	72	3	72
7	72	7	72
∞	4	∞	4
30 x		30 x	
Rhiz primers		Rhiz primers	

Table 8. PCR programme used for AC primers

Table 9. Composition of the different compounds needed for the PCR mastermix.

AC	
min	°C
15	95
1	95
2	60
3	72
7	72
∞	4
35 x	
AC primer	

Mastermix
11 µL dH$_2$O
5 µL buffer
5 µL MgCl$_2$
5 µL primer forward (10 pmol)
5 µL primer reverse (10 pmol)
1 µL dNTPs
0,25 µL polymerase
31,25 µL per tube

The amount of genomic DNA (gDNA) used was around 20 ng to get the best results. Therefore, NanoDrop ND-1000 spectrophotometer (peqlab, Erlangen, Germany) was used to determine the DNA concentration and to calculate the optimal amount of DNA. According to the calculated DNA-volume, the amount of water, as shown in table 9, was adapted to achieve the final mastermix-volume of 50 µL per tube. Furthermore, a positive and a negative control were prepared.

2.2.4. Gel electrophoresis

The agarose and TAE (Tris-Acetate-EDTA) buffer solution was mixed as indicated in table 10 on a magnetic stirrer and heated until boiling. Afterwards, the 10,000x GelRed™ (Biotium, Hayward, USA) was added and now the clear solution was poured into the gel tank. The comb was inserted into the agarose-solution to form the slots as soon as the gel was cooled and solid. Subsequently, the gel was covered completely with 1 x TAE buffer.

Table 10. Composition of the different compounds needed for an agarose gel.

small 1 x TAE agarose gel (10 slots)	big 1 x TAE agarose gel (20 slots)
1 g agarose	2 g agarose
2 μL GelRed™	4 μL GelRed™
fill up to 50 mL with TAE buffer	fill up to 100 mL with TAE buffer

Afterwards, the marker D3812 Direct Load™ Step Ladder 50 bp (Sigma-Aldrich, St. Louis, USA), as shown in figure 34, was pipetted into the first slot of the gel.

In total, 27 μL of the PCR product were mixed with 3 μL of 10x loading buffer (Sigma-Aldrich, St. Louis, USA) and pipetted into empty slots of the gel. Finally, the gel electrophoresis was started at 100–150 V, 300 mA using the horizontal electrophoresis system (Bio-Rad, Berkeley, USA). As soon as the marker band was at the bottom of the gel, the electrophoresis was stopped.
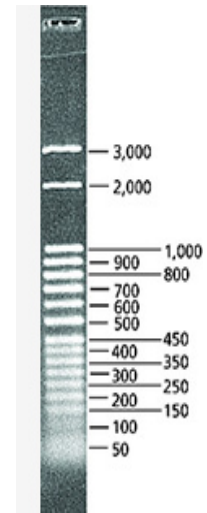
Figure 34. D3812 Direct Load™ Step Ladder, 50 bp, Sigma-Aldrich

2.2.5. DNA-Purification

As shown in figure 35, the DNA bands were visualized displayed under an ultraviolet lamp (UV) using a gel imager (Intas, Göttingen, Germany) and were cut out from the gel.
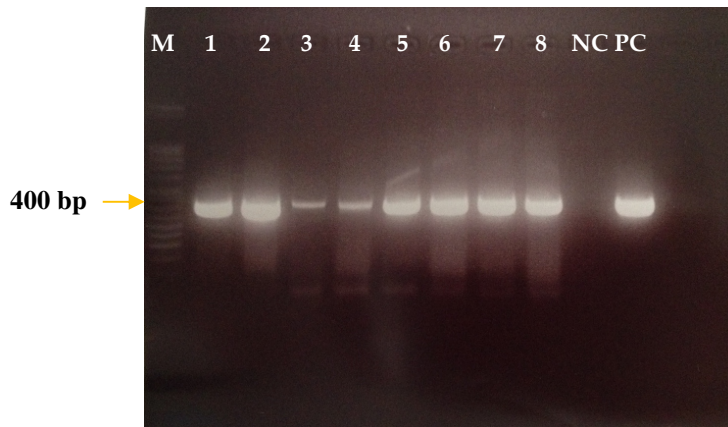
Figure 35. DNA bands in a TAE gel under the UV lamp; M = Marker, NC: negative Control, PC: Positive control, 1-8: DNA samples from different *Acanthamoebae* strains.

For purifying DNA out of the TAE gel, the Illustra ™ GFX™ kit (GE Healthcare, Little Chalfont, England) was used.

Sample capture

The weight of a DNase-free 1.5 mL microcentrifuge tube was determined and a sterile scalpel was used to cut out the DNA bands from the gel while under UV light (Intas, Göttingen, Germany). The weight of the gel piece was calculated. Per 10 mg of gel 10 mL of Capture buffer type 3 (GE Healthcare, Little Chalfont, England) were added to the gel piece. It was especially important to take a minimum of 300 μL of Capture buffer type 3 to sustain the highest amount of DNA. Therefore, also for gel slices weighting less than 300 mg at least 300 μL of buffer were added. Afterwards, the sample was mixed by inversion and incubated at 60°C in a thermo comfort (Eppendorf, Hamburg, Germany) for 15-30 minutes until the agarose was completely dissolved. The solutions were mixed by inversion every 3 minutes. Once the agarose slice was completely dissolved the colour of the solution had to be yellow. For every purification needed one GFX MicroSpin column (GE Healthcare, Little Chalfont, England) had to be placed into one Collection tube.

Sample Binding

The Capture buffer type 3–sample mix was centrifuged to collect also the remaining liquid at the bottom of the tube. The maximum of 800 μL of the buffer-sample mixture was added to the GFX MicroSpin column and Collection tube. Subsequently, the sample was incubated at room temperature (RT) for 1 minute and the column with the collection tube was centrifuged with 1-15 microcentrifuge (Sigma-Aldrich, St. Louis, USA) at 16,000 x g for 20 seconds. The flow through was discarded and the GFX MicroSpin tube was placed back inside of the collection tube. The sample binding steps were repeated until the buffer-sample mixture was fully loaded.

Wash & Dry

In total, 500 μL of Wash buffer type 1 (GE Healthcare, Little Chalfont, England) were added to the MicroSpin column and placed inside of a collection tube. Both were centrifuged at 16,000 x g for 30 sec. The collection tube together with the flow-through was discarded and the MicroSpin column was placed on a new DNase-free 1.5 mL microcentrifuge tube.

Elution

Afterwards 10-50 μl Elution buffer type 4 (GE Healthcare, Little Chalfont, England) OR type 6 (GE Healthcare, Little Chalfont, England) was added directly to the centre of the membrane in the GFX MicroSpin column without touching the membrane with the tip. The sample was incubated at RT for 1 min and centrifugation was conducted at 16,000 x g for 1 minute to gain the purified DNA. The DNA was stored at -20°C.

2.2.6. Sequencing PCR

This working step was conducted to identify the different genotypes of the *Acanthamoeba* strains.

The purified fragments were further used for sequencing PCR as with the ABI PRISM BigDye (Thermo Fisher Scientific, Waltham, USA) sequencing kit suggested. The compounds had to be combined as indicated in table 11.

Table 11. Composition of the different compounds needed for the Sequencing PCR.

ABI Mix	1 μL
PCR Product	1-3 μL
primer	2 μL
sequencing buffer (AB Big Day Terminator)	1 μL
sterile water	fill up to 10 μL

The sequencing PCR was conducted using the PCR programme showing in table 12. The primers were the same ones used during the initial PCR reaction. After 40 cycles the fragments were be purified as described in the next working step.

Table 12. Programme used for Sequencing PCR.

min	°C
30	96
10	96
5	50
4	60
∞	4
40 x	
same primers as with PCR reaction	

Table 13. Buffer used for purifying the DNA after the Sequencing PCR.

1 μL of Natrium-Acetate buffer
10 μL of DNA sample from Sequencing PCR
33 μL ice-cold 100% ethanol
44 μl sample mix

2.2.6.1. Purification of PCR products

After combining the DNA with the components as shown in table 13, the sample mix was vortexed and left on ice for 17 min. Subsequently, centrifugation was done at 12.000 x g, 4°C for 30 min using Heraeus Fresco 17 Centrifuge (Thermo Electron Corporation, Waltham, USA). The supernatant was discarded and 90 μL of ice-cold 70% ethanol was added (optional: freeze overnight at -20°C). Afterwards, centrifugation at 12.000 x g, 4°C for 10 min (when frozen overnight centrifuge for 30 min) was conducted. The supernatant was discarded and dehydration of the sample was performed for 3 min. Afterwards 20 μL Hi-Di™ Formamide (Applied Biosystems, Waltham, USA) was added to the sample and incubated with an open cap at RT for 5 min. After vortexing of the sample, it was incubated at 95°C for 5 min. Following, the samples on ice were incubated for 5 min. Afterwards the tubes inserted into the 48-well plate of the sequencer and Sanger sequencing was performed with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Waltham, USA)

2.3. Data Analysis

The sequences had to be further analysed to identify the amoebal genotypes.
First, the GeneDoc programme (NICHOLAS *et al* 1997) was used to reverse and
complement the reverse sequence. Afterwards, the sequences were transferred
to the Cluster X program (THOMPSON *et al* 1997) where the alignment was
performed. Finally, the aligned sequences were transferred again to GeneDoc
(NICHOLAS *et al* 1997). The sequences were aligned to a reference alignment of all
20 acanthamoebae genotypes. The genotypes were assessed with ≤ 5% sequence
dissimilarity within one genotype as established by GAST *et al* (1996) and STOTHARD *et al* (1998). As demonstrated in figures 36 and 37 the working step was finalized in defining the genotype with the help of the constant and variable regions of the 18S rDNA gene.

Table 14. IUPAC nucleotide code used for bases which cannot be uniquely defined from the sequences.

IUPAC nucleotide code	base
R	A or G
Y	C or T
S	G or C
W	A or T
K	G or T
M	A or C

 It was from major importance to use only reference strains from the gene bank
for which the entire 18S rDNA gene is available. For all nucleotides that could
not be identified doubtlessly the IUPAC code as shown in table 14 was used
(NC-IUB, 1984). As single nucleotide polymorphisms (SNPs) often occur in the
variable regions, in certain cases the exact identification of the genotype was not
possible at first. Therefore a further working step was added.

2.3.1. Sequence analyses

As shown in figure 36, sequences were aligned to reference strain and then the respective dissimilarity was calculated. Red rectangles in the figures indicate dissimilarities e.g. SNPs between the sequences. Green rectangles in the figures mark the parts of the variable regions which are similar between reference strain and amoebal isolate.
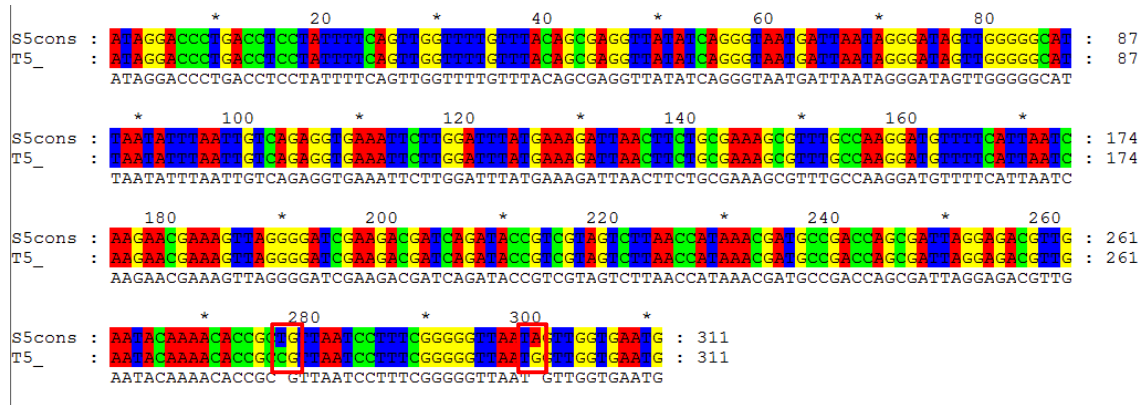
Figure 36. Alignment, showing a sample sequence (S5cons) and a reference sequence of genotype (T5).
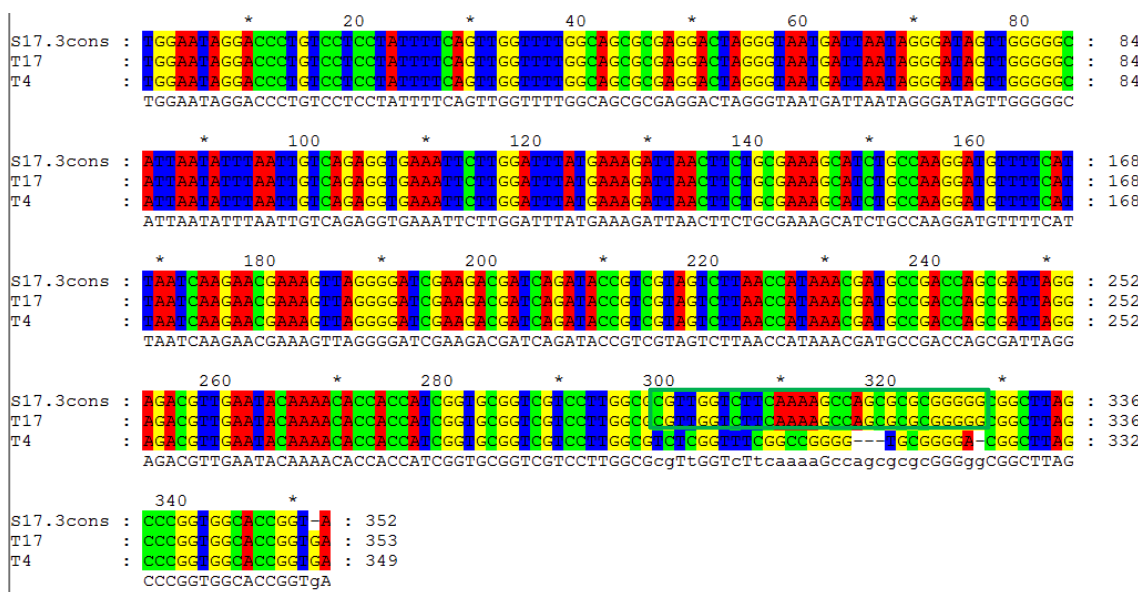
Figure 37. Alignment showing a sample sequence (S17.3cons) closely related to both, genotypes T17 and T4.

Some genotypes are very closely related and thus a clear assignment to one of these genotypes is not always possible. Therefore, multiple sequences from these genotypes were downloaded from GenBank and processes via GeneDoc

(NICHOLAS *et al* 1997) and ClustalX (JEANMOGIN and HIGGIN 1997) as demonstrated in figure 38.
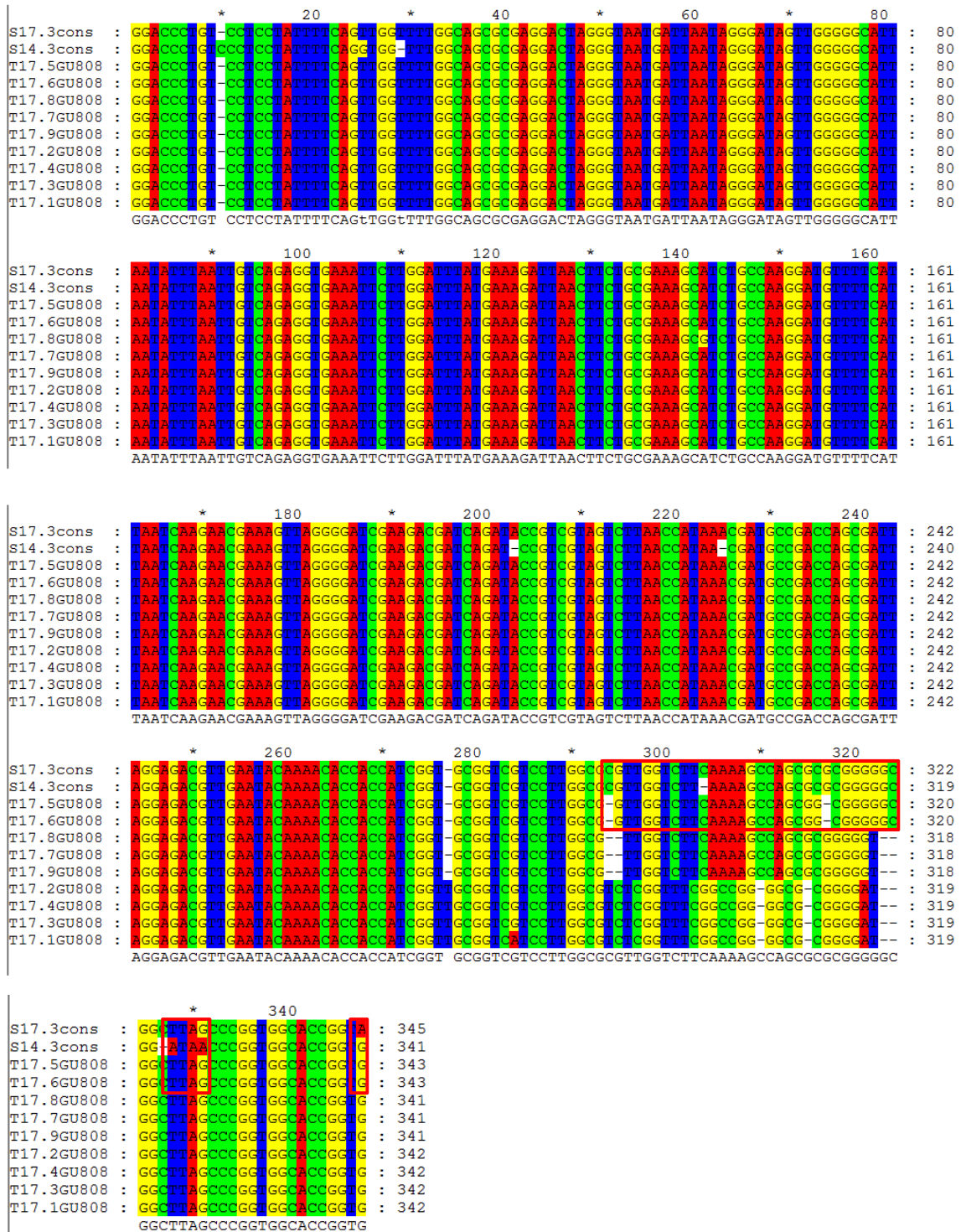
Figure 38. Alignment with several reference sequences: Here, two samples sequences (S17.3cons & S14.3 cons) are compared to a set of reference sequences for genotype T17.

Moreover, the sequences of all isolates from one soil sample were also compared to each another (figure 39). Thereby, also different strains within one genotype could be identified.
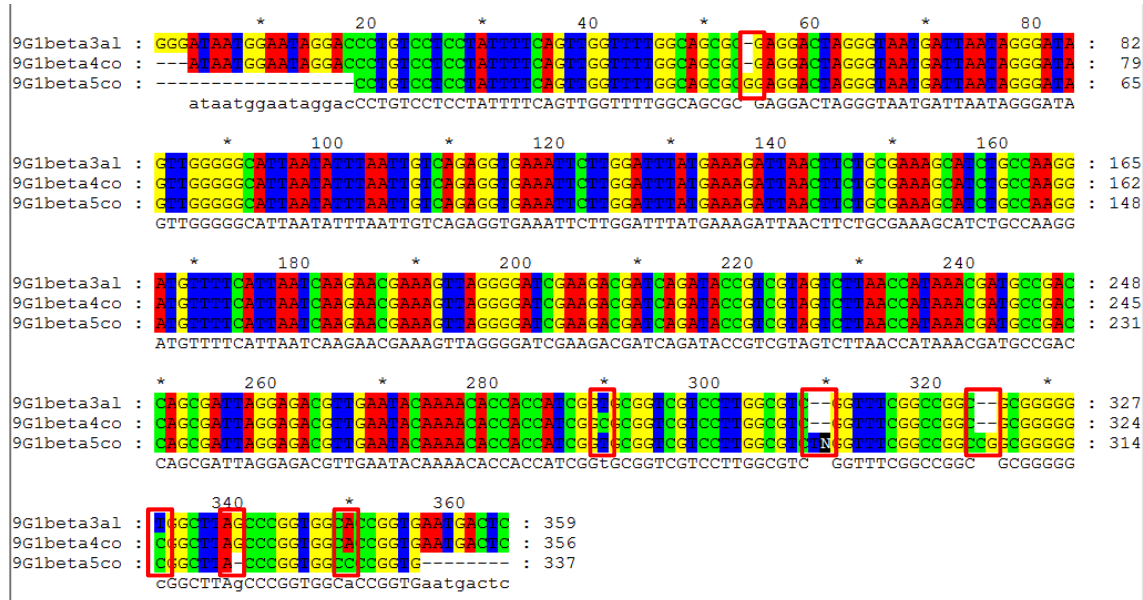
Figure 39 Comparison of sequences from different isolates from one soil sample.

3. Results

3.1. Isolation of *Acanthamoeba* spp.

Overall, 100% of the soil samples investigated were positive for *Acanthamoeba* spp. Most samples revealed more than one strain. In some cases also two different strains of the same genotype were found in one sample.

3.2. Morphotypes

As indicated in figure 40 in total 74 amoebal strains were found in 36 soil samples. The majority of isolates, namely 42 strains, belonged to morphological group II, 27 strains belonged to morphological group III and 5 strains belonged to morphological group I.
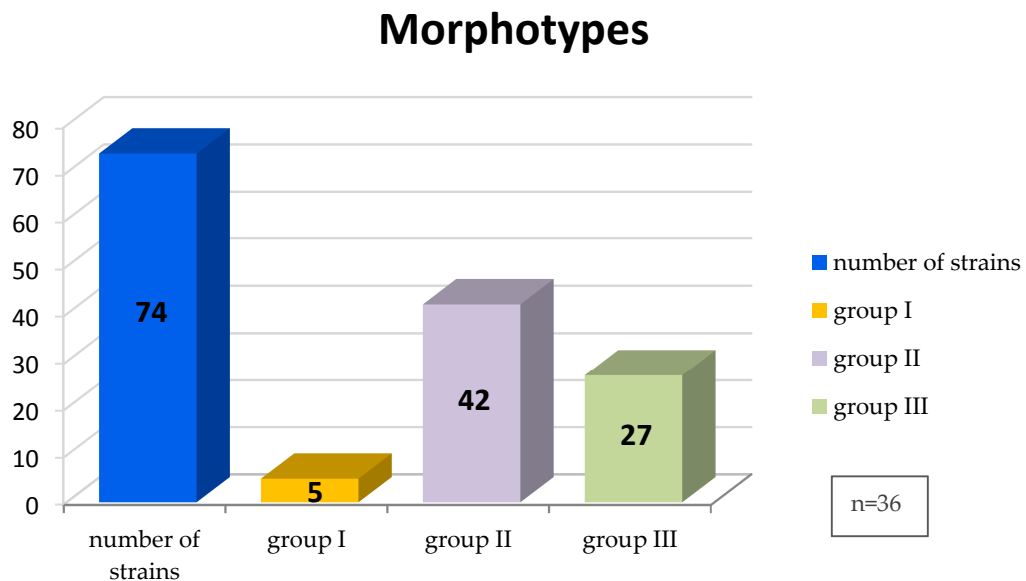
Morphotypes

Figure 40. Distribution of morphological groups: morphological group II and III were the most abundant ones.

3.3.   Genotypes

As demonstrated in figure 41, a high variety of genotypes was found. Genotype T4 was the most abundant genotype in the current study, but also T5, typically found in soil and sand, was frequently isolated. In total 5 strains could not be explicitly identified as they clustered between T4 and T17. Altogether 36 soil samples were processed resulting in 74 amoebal sequences. Interestingly, none of the isolated strains was 100% identical to already sequenced Acanthamoebae strains from the NCBI GenBank.

The isolates belonged to 9 different genotypes, including T4, T5, T11, T13, T14, T15, T16, T17 and T19, of which the latter six are rarely found in the environment.

Genotypes

n= 74

Legend: T4, T5, T4/T17, T17, T11, T13, T14, T15, T16, T19, indefinable
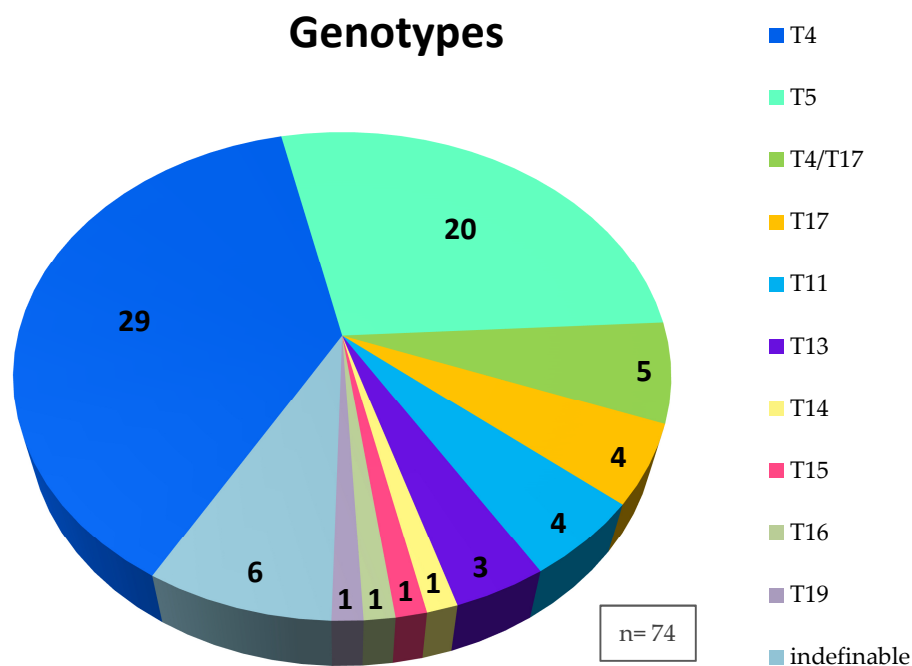
Figure 41. Distribution of genotypes of all examined soil samples.

3.4. Geographical distribution

3.4.1. Madagascar

Altogether, 53 strains belonging to 6 different genotypes were found in the soil. As indicated in table 15, all morphotypes were discovered. While some samples harboured more than one *Acanthamoeba* spp., others had just one. In total morphological group II with 29 different strains and group III with 19 different strains prevailed but also 5 strains showed morphotype I.

Table 15. *Acanthamoeba* strains isolated from soil samples collected in Madagascar.

Sample	Strain	Morphotype	Genotype
S1	S.1	III	T5
S2	S.2	II	T5
S3	S.1	II	T11
	S.2	III	T5
	S.3	II	T11
	S3.4	II	T11
	S3.5	I	T11
S4	S4	III	T5
S5	S5	III	T5
S6	S6.1	III	T5
	S6.3	II	T4/T17
	S6.4	II	T5
S7	S7.1	III	T5
S8	S8	III	T5
S9	S9.1	III	T5
	S9.2α	II	T4
S10	S10	I	T17
S11	S11	II	T4
S12	S12	II	T4
S13	S13.1	III	T5
S14	S14.1	III	T5
	S14.3	I	T17

Sample	Strain	Morphotype	Genotype
S15	S15	II	T4
S16	S16.1	III	T5
	S16.2	III	T5
S17	S17.1	III	T5
	S17.3	III	T17
S18	S18.1α	II	T4
S19	S19.1	III	T5
	S19.2	II	T4
S20	S20.1	III	T5
	S20.3	II	T4/T17
S21	S21	II	T17
9/C2	9/C2.1α	II	T13
	9/C2.1β	II	T14
	9/C2.2	II	T13
	9/C2.3	II	T4
6/G2	6/G2.2	II	T4
	6/G2.3	II	T4
9/G1β	9/G1β.1	III	T5
	9/G1β.3α	II	T4
	9/G1β.4α	II	T4
	9/G1β.5α	II	indefinable
	9/G1β.5β	II	T4
6/B2	6/B2.1	II	T4
	6/B2.2α	II	T4
	6/B2.3	II	T4
6/C3	6/C3.2	I	indefinable
	6/C3.4	II	indefinable
8a/3	8a/3.1	I	T13
	8a/3.2	III	T4
	8a/3.6	III	indefinable
8a/5	8a/5.3	II	indefinable

As shown in figure 42, in total 53 *Acanthamoeba* strains were found in 28 soil samples. The most abundant genotypes were T5 and T4 with together 34 strains, corresponding to 64%. However, all of the other strains found in soil, were exclusively identified in Madagascar. Moreover, in total 14 strains of rare genotypes, namely T11, T13, T14 and T17 were identified. Overall, four strains of T17 and T11 genotypes and three strains of T13 found in two soil samples.
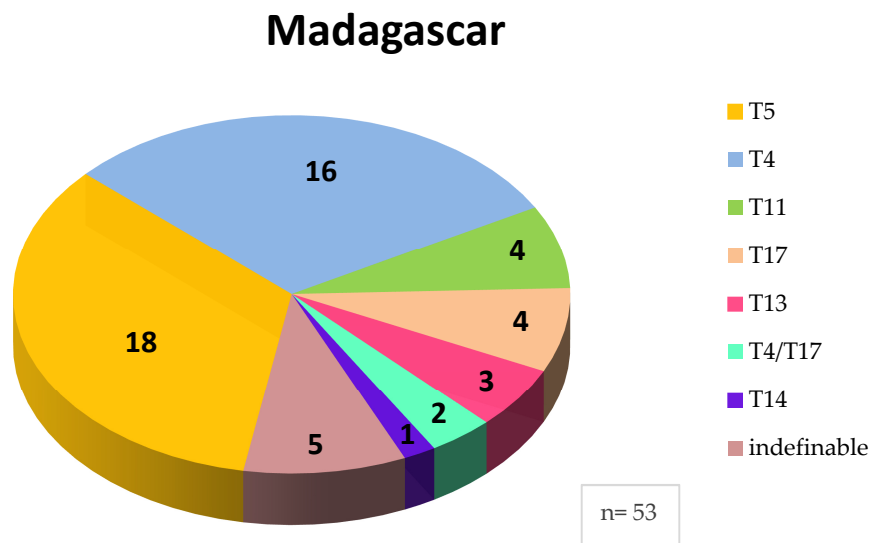
Madagascar

Figure 42. Diversity of the different *Acanthamoeba* isolates from soil collected in Madagascar.

Overall, 2 strains could not be clearly identified and resulted in defining them as clustering between T4 and T17.

3.4.2. Burkina Faso

As demonstrated in table 16, in total 3 soil samples from Burkina Faso were processed and 5 different strains were isolated. Four strains were out of morphological group III. The only strain of morphological group II was identified as the rare genotype T19.

Table 16. *Acanthamoeba* strains isolated from soil samples collected in Burkina Faso.

Sample	Strain	Morphotype	Genotype
BF36	BF36.2	II	T19
	BF36.3	III	T4/T17
BF40	BF40.3	III	T4
BF50	BF50.1	III	T15
	BF50.2	III	T16

Interestingly, all isolates had different genotypes and only one strain belonged to the most abundant genotype T4.

3.4.3.   Congo

As shown in table 17, members of all morphological groups were found in the three soil samples investigated. Next to 6 strains of group II also 3 strains of group III were identified. Two strains could not be genotyped distinctively as they clustered between T4 and T17. One strain, belonging to morphological group III, could not be identified, probably due to contamination of the culture.

Table 17. *Acanthamoeba* strains isolated from soil samples collected in Congo.

Sample	Description	Morphotype	Genotype
K1	K1.1	II	T4/T17
	K1.2	III	T5
K29	K29.1	III	indefinable
	K29.4α	II	T4/T17
	K29.4β (α=β)	II	T4
K30	K30.1	III	T5
	K30.2	II	T4
	K30.5α	II	T4
	K30.5β	II	T4
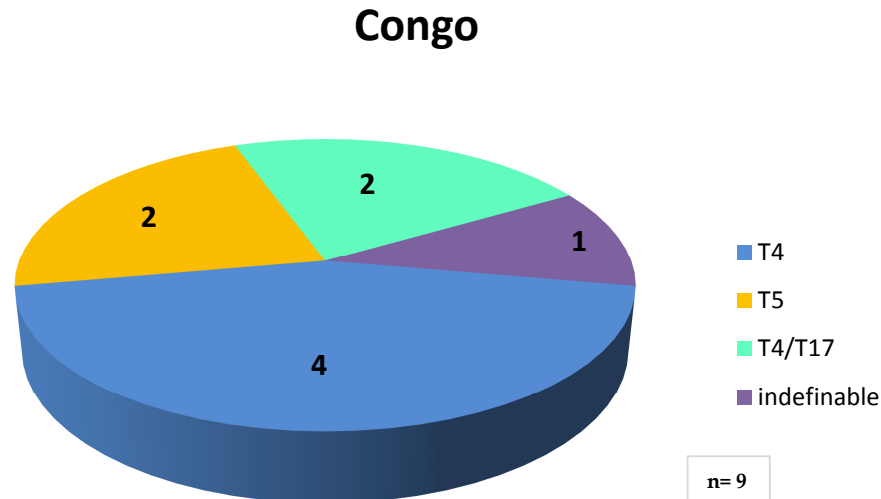
Congo

Figure 43. Diversity of all different *Acanthamoeba* isolates found in two soil samples collected in Congo.

As shown in figure 43 in total 9 isolates, namely belonging to T4 and T5 were identified. Two isolates clustered between T4 and T17. The amoebal diversity in these 3 soil samples was particularly high.

3.4.4.   Ethiopia

Overall, all 8 different strains were found in two soil samples. Except from one isolate showing morphotype III, morphological group II was dominant (table 18). Interestingly, all isolates from Ethiopia had the same genotype, namely T4.

Table 18. *Acanthamoeba* strains isolated from soil samples collected in Ethiopia.

Sample	Strain	Morphotype	Genotype
E101E	E101E.1	II	T4
	E101E.2	II	T4
	E101E.3	II	T4
	E101E.4	III	T4
E103B	E103B.1	II	T4
	E103B.2	II	T4
	E103B.3	II	T4
	E103B.4	II	T4

3.5.   Excystment

During microscopical morphotyping an excystment of a trophozoite was observed and several pictures were made during this 30 second long event which started with a distinct shacking of the cyst. Subsequently, as shown in figure 45, the trophozoite started leaving its cyst by pushing through an ostiole. Next to the cyst numerous rod-shaped bacteria, most probably the on the NN-agar plates seeded *E. coli*, are visible.

Afterwards, as shown in figure 44 and 48, the amoeba left the cyst completely.
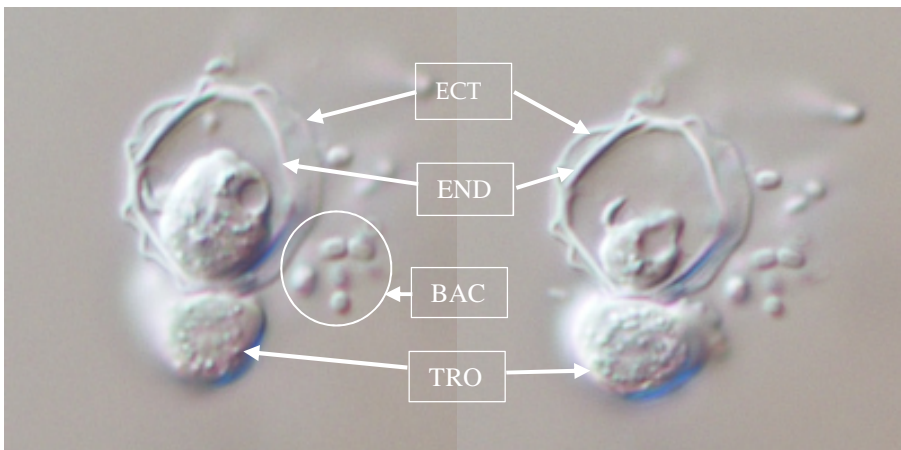
Figure 44. (left) and **figure 45. (right).** Starting excystment of a trophozoite (TRO) with clearly visible ecto (ECT) and endocyst (END); rod-shaped fragments are bacteria. Orig.
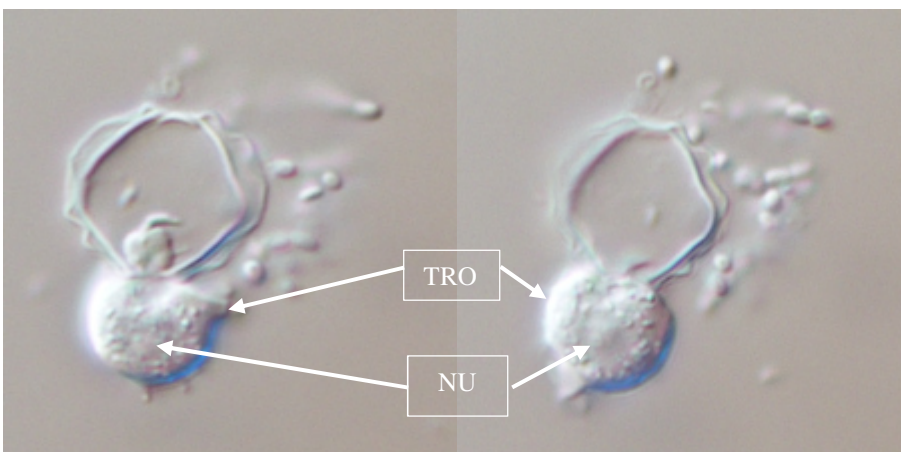
Figure 46. (left) and **figure 47 (right).** Proceeded excystment with a clearly visible nucleus (NU) inside of the trophozoite (TRO). Orig.
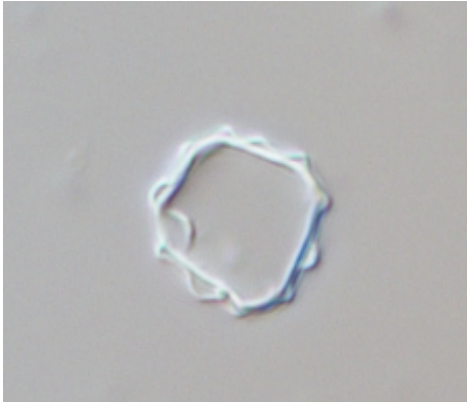
The trophozoite showed numerous granules inside and also the nucleus was clearly distinguishable from the cytoplasm. As demonstrated in figure 48, excystment ends with an empty cyst showing an intact endo (END) and ectocyst (ECT).

Figure 48. The empty cyst is left behind. Orig.

3.6. *B. pseudomallei* positive acanthamoebae isolates

One of 76 *Acanthamoeba* isolates tested was found positive for *B. pseudomallei* by qPCR. This strain had been isolated from a soil sample from Madagascar, where melioidosis is endemic. The interaction between *B. pseudomallei* and *Acanthamoeba* spp. was stable as it persisted for 12 co-cultivations. Interestingly, the *Burkholderia*-DNA resembled only in 94% to the *recA* gene of the *B. pseudomallei* reference strain.

4. Discussion

4.1.　　　High prevalence of *Acanthamoeba* spp.

This study clearly demonstrated that acanthamoebae are highly abundant in soil from African regions endemic or predicted to be endemic for melioidosis. Altogether, 100% of the soil samples, originating from 4 different countries, were positive for *Acanthamoeba* spp. This is an interesting finding as other studies on the amoebal distribution in the African environment revealed lower numbers. One of the first studies in Africa was conducted by HAMADTO *et al* in 1993 showing that 25% of swimming pools, 40% of surface waters and canal samples and 100% of tap water samples were positive for *Acanthamoeba* spp. HASSAN *et al* (2012) demonstrated that almost 43% of water samples from hydraulic systems of dental units and haemodialysis units in Alexandria, Egypt, were contaminated with FLA. Especially surgical categories of dental clinics showed the highest contamination rate with 72.7% while the water samples were tested negative. Similar results were described by HIKAL *et al* in 2015 where air-water syringes and cup fillers were tested and 100% were found contaminated. Moreover, 72% tap water samples were positive. Another more recent study from Egypt by AL-HERRAWY *et al* in 2014 showed that 49.2% of examined swimming pools are contaminated with acanthamoebae. The latest study from Egypt by TAWFEEK *et al* from 2016 showed again different contamination rates, water samples were positive in 33.3% and 40% of the soil were *Acanthamoeba*-positive. In 2014, MUCHESA *et al* found FLA in 87.2% of environmental water samples in South Africa. In that study, 12.8% of the water samples throughout different zones from aerobic to anoxic were tested positive for *Acanthamoeba* spp. Also studies in Sudan showed a high prevalence of these protozoa in water systems, 89% of the samples were positive and an interaction with *Vibrio cholera* was suggested (SHANAN *et al* 2011). A recent study from

Uganda demonstrated that natural as well as communal water sources are contaminated by pathogenic protozoa (SENTE *et al* 2016). Next to finding *Acanthamoeba* spp. with a prevalence of up to 60.7%, also various strains of e.g. *Hartmanella* spp., *Naegleria* spp., *Cryptosporidium* spp. and *Giardia* spp. were found.

The highest number of studies on this topic has been conducted in Tunisia showing that *Acanthamoeba* spp. are found in 39% of the haemodialysis units (DENDANA *et al* 2008). Also after cleaning and before haemodialysis sessions samples were taken, with still 18% being positive. Moreover, TRABELSI *et al* in 2010 tested dental unit waters for acanthamoebae and found 69% of the samples positive. Furthermore, TRABELSI *et al* demonstrated in 2016 that 53.5% of samples from Sfax University hospital were contaminated with *Acanthamoeba* spp. While 80% of the surgical services and 13.3% of the surgical intensive care units were positive, no evidence of these organisms were found in the medical intensive care unit of the Sfax University hospital (TRABELSI *et al* 2016).

Similar studies from other continents have been published in the past years. TODD *et al* demonstrated in 2015 overall 63.9% of soil samples from Jamaica being positive for FLA. SCHEIKL *et al* showed in 2016 that in total 83.3% of the examined hospital cooling towers were positive for free-living amoebae, while *Acanthamoeba* spp. dominated amongst them leading to the assumption that these organisms might be a risk factor for nosocomial infections. During that study it was also demonstrated that several amoebal isolates harboured intracellular bacteria. KAO *et al* tested several water reservoirs in Taiwan in 2015 coming to the results that in 39.5% of the samples the genus *Acanthamoeba* can be found. Moreover, FLA were also detected in water sources of a bone marrow transplant intensive care unit, a transplant ICU and a haemodialysis unit, where typically patients with an altered immune response are located (KHURANA *et al* 2015). A study conducted in Thailand by THAMMARATANA *et al* in 2016 showed that only 15.9% of the water samples were positive for *Acanthamoeba*. In

contrast to that SCHEIKL *et al* demonstrated in 2014 that 72.6% of industrial waters including process waters, cooling tower waters and cooling lubricants in Austria were positive for FLA.

Overall, the global studies of the recent years focused mainly on the amoebal distribution in water systems. However, some studies also focused on the prevalence isolation of acanthamoebae in soil samples. RAHDAR *et al* confirmed the contamination of rivers, swimming pools and agricultural canals with FLA in 2012 by collecting water and soil samples from Southern Iran. In total, 71.6% (43) of water and 26% (13) of the soil samples were found positive for acanthamoebae. During a recent study by KARAMATI *et al* in 2016, soil samples were collected in recreational and public areas of Iran and East Azerbaijan. While sixty soil samples were examined, 41.6% were positive for *Acanthamoeba* spp. A recent study published by REYES-BATLLE *et al* in 2016, examined forty soil samples collected on the island of El Hierro, Canary Islands, Spain. Overall, acanthamoebae were isolated from 47.5% of the samples. WAGNER *et al* (2016) demonstrated high occurrence of FLA in soil from Venezuela. FLA were isolated from 51.8% of 27 samples. However, GEISEN *et al* already showed in 2014 that the diversity of acanthamoebae in soil is very high. A study by TANVEER *et al* (2015) revealed similar results to our study, as almost all samples were positive for FLA. Overall, 94.11% of the soil and 92.31% of the water samples, collected in different cities of Pakistan, were positive for acanthamoebae (TANVEER *et al* 2015).

During our study, in total 74 *Acanthamoeba* clones were cultivated and sequenced while none of the strains was identical to already known sequences of this genus. Comparing our work to the study of GEISEN *et al* from 2014 and TANVEER *et al* 2015, it can be confirmed that the diversity in African soil is as described for the Netherlands, Denmark, Sardinia, Tibet and Paktistan. However, our study was the first study particularly dedicated to the prevalence, distribution and genetic diversity of *Acanthamoeba* spp. in soil samples from diverse

African countries. The finding of all samples being positive is highly interesting and again confirms the amazing ability of acanthamoebae to adapt to the several environments.

4.2.   Country-specific distribution

Overall, a wide range of different morpho and genotypes was found and especially interesting is the fact that most soil samples harbored more than one *Acanthamoeba* strain. Generally, the soil samples showing the highest diversity were moist but still also dry and sandy samples contained members of the genus *Acanthamoeba*. This is of greatest interest as these are the first detailed data on the diversity of *Acanthamoeba* spp. in soil of regions endemic or at least assumedly endemic for melioidosis.

As PODLIPAEVA *et al* demonstrated in 2006, *Acanthamoeba* cysts can even be isolated from 30,000 to 35,000 year old permafrost. The authors observed excystment of trophozoites and detected a heat shock protein of the HSP70 family for the first time in these organisms. PODLIPAEVA and GUDKOV also demonstrated in 2009 that *Acanthamoebae* can be found in arctic soils and they measured high levels of Hsp70 in free-living amoebae from tundra soil. However, FLA can also outlast extremely hot climates e.g. in the Negev desert in Israel as RODRIGUEZ-ZARAGOZA *et al* demonstrated in 2005a&b.

Overall, also species diversity changed throughout the different regions suggesting that temperature and humidity play a role in *Acanthamoeba* survival and proliferation. This was already described in 2015 by KAO *et al* for the prevalence of *Acanthamoeba* throughout the year in environmental studies from Taiwan. Interestingly, in this study, the detection rates were higher in the North than in the central and southern regions during all seasons. Thus, also climatic conditions have to be considered to play a role in the diversity of *Acanthamoeba*. In the current study the greatest species diversity was found in Madagascar. The Meteorological Service in Madagascar defines three climate areas ranging

from tropical to temperate and arid (CIP). The dry and cool season lasts from May to October and the wet and warm season during the remaining months (CLIMATEDATA).

Burkina Faso shows three different climate zones in a basically tropical climate (CIP). While the hot and dry season is combined with hot winds from the Sahara, the rainy season lasts about 4 months from May or June to September. As the Climatic Research Unit (CRU) from the University of East Anglia (UEA) shows, the highest average monthly temperature reaches 32.8°C. However, higher temperatures are occurring as 46°C were reported in Markoye by CRU. Although only three soil samples were examined, the diversity with 4 genotypes was high. Winds have to be taken into account as a possible transport system for *Acanthamoeba* spp. over long distances (INIT *et al* 2010).

In Congo, the CRU of UEA describes the climate as tropical with constant high temperatures and humidity. Typically, one dry and one rainy season alternate over the year. From three soil samples in total 7 strains of *Acanthamoeba* were isolated and 2 genotypes could be identified.

The CRU of UEA describes the climate in Ethiopia being rather diverse with average monthly temperatures of 25°C and alpine climate in the mountains. Overall, the climate zones are diverse and range from tropical savannah over warm desert to humid subtropical climate zones (CIP). Interestingly, although the climate shows high variability, only one genotype was found, namely T4. Although, the sample number was rather low, the difference to the other countries investigated is striking.

The variation of climate is influenced very much by winds, the east coast of Madagascar being mainly exposed to trade winds (BIRD 2010). These factors can have a significant impact on the amoebal diversity. FLA are considered to be cosmopolitan and able to adapt rapidly to changed environmental conditions. Therefore, amoebal communities show shifts throughout the climate zones and the different seasons. As these protozoa are transported over sea and

air, the passage via e.g. the passage winds, seems to put this island in a good position for high *Acanthamoeba* diversity (LORENZO-MORALES *et al* 2005). Different studies by SIMITZIS-LE and CHASTEL in 1982 and LORENZO-MORALES *et al* in 2007 had their main focus on the distribution of FLA via salvage mammalians or rodents. The distribution via animals e.g. birds is a possibility to overcome large distances. Nevertheless, the fact that just one amoeba has to survive the journey is the key factor. One cyst has to survive the unfavourable conditions and as soon as the environment is beneficial again, excystment starts generating a new population acanthamoebae trophozoites able to proliferate rapidly via binary fission and spread over the new environment.

4.3.   Amoebal morphotypes

Overall, morphological group II was the most abundant morphotype found. Altogether, 42 isolates belonging to group II were found. This group, established by PUSSARD and PONS in 1977, also holds the most pathogenic strains. Generally, however, also representatives of group III can cause disease. Overall, 27 members of this group were found in the soil samples from Africa investigated.

Morphological group I typically has extremely large trophozoites and cysts (PUSSARD and PONS 1977). Interestingly, several isolates of this group contained intracellular bacteria. INGLIS *et al* demonstrated in 2000b, that *Acanthamoeba* strains belonging to morphological group I interact best with *B. pseudomallei*. Although, also other amoebal morphotypes showed coiling phagocytosis, the survival of *B. pseudomallei* was not as distinct as with *A. astronyxis* (group I).

4.4.   Finding of rare genotypes

In this study, several rare genotypes were isolated. While 29 isolates were of the world-wide most abundant genotype T4, 4 isolates were of genotypes T17 and

T11 respectively. Moreover, also 3 strains of the rare genotype T13 were found next to 1 strain of each of the genotypes T14, T15, T16 and T19. The particularly rare genotype T17 was first described in Thailand in 2010 by NUPRASERT *et al.* Two years later this genotype was also found in Brazil (MAGLIANCO *et al* 2012). Acanthamoebae from this genotype are members of the morphological group I and show cysts with a distinct star-shape. Furthermore, the trophozoites as well as the cysts are defined by large diameters.

The finding of T17 in soil samples from Madagascar is the third finding of this genotype in environmental samples. This is of great interest as it also helps to understand the global distribution of this genotype. Until today no infections have been described and T17 had been exclusively been described as an environmental genotype (NUPRASERT *et al* 2010, MAGLIANCO *et al* 2012).

Moreover, also the rare genotype T19 was found during this study. This genotype was first described by MAGNET *et al* in 2014 when it was isolated from waste water. Furthermore, one strain of T15 genotype was isolated from soil collected in Burkina Faso. This genotype has been already described as pathogenic, causing *Acanthamoeba* keratitis in an Italian patient (DI CAVE *et al* 2009).

4.5. Medical Relevance

4.5.1. *Acanthamoeba* spp. as pathogens

Acanthamoeba spp. can be pathogenic causing partially severe diseases e.g. AK and GAE. This study clearly demonstrates that soil is an important source of environmentally acquired infections. Although not all strains found could be clearly assigned to a known genotype, the majority of strains have genotypes that are known to be linked to human infections. As described by BOOTON *et al* in 2004, genotype T4 is the genotype most commonly found in keratitis patients. According to the CDC T4, is the most common cause of AK. However, also genotype T3 (LEDEE *et al* 1996), T11 (KHAN *et al* 2002) as well as T6 (WALOCHNIK *et al* 2000), can cause AK. Next to that, also T5 (*A. lenticulata),*

T10 (*A. culbertsoni)* and T11 (*A. hatchetti)* include pathogenic strains (WALO-CHNIK *et al* 2015, WALOCHNIK *et al* 2000).

In the current study, T4 was detected in soil samples from all countries investigated. Therefore, the annual cases of only altogether several hundred worldwide described for especially amoebal skin infections, GAE and AK are most probably highly underestimated (WALOCHNIK *et al* 2015). Even in industrial countries the diagnosis of meningoencephalitis caused by amoebae is often not made fast enough to start a treatment to safe the patient's life.

Nevertheless, with this study I hope to raise awareness for the potential health risk emanating from *Acanthamoeba* spp. also in Africa.

4.6. *B. pseudomallei*

In the current study, *B. pseudomallei* was detected in one Acanthamoeba isolate collected in Madagascar. First of all, this confirms the interaction of these two organisms as already described by INGLIS *et al* in 2000b during *in vitro* studies. Secondly, it describes a stable interaction, as the amoebal strain was sub-cultivated 12 times on agar plates and obviously the amoeba did not lose the endosymbiont. Due to the fact that processing clonal cultures sometimes takes several weeks, there is the chance of losing the bacterium during sub-culturing. As described in Material and Methods, only a small piece of agar is cut out and transferred to a new plate. Thus, a low number of amoebae, optimally a single trophozoite, are transferred.

Interestingly, in a recent study by the ERAfrica collaboration-laboratory in Greifswald, Germany *B. pseudomallei* was detected by qPCR in 13 out of 114 soil samples (11%) from the same sites that had been tested in the current study (unpublished data). As described by WHITHATANUNG *et al* in 2016 also from Northeast Thailand, one of the main endemic regions, *B. pseudomallei* was isolated from 26.7% of the soil samples investigated. Therefore, the findings from the African regions predicted to be endemic for melioidosis with 11% of the soil

samples being positive for *B. pseudomallei* is in fact rather high. LIMMATHU-
ROTSAKUL *et al* (2010b) demonstrated that there is no even distribution of *B.
pseudomallei* in the environment. They examined 100 slots within 625 m^2 in a rice
field and while some slots (2.5x2.5 m) were highly positive for the bacterium,
areas of the same size right next to them, were negative for *B. pseudomallei.*
However, this phenomenon had been described previously in other bacterial
soil communities (WITHATANUNG *et al* 2016). Overall, the detection of *B.
pseudomallei* in soil can be quite challenging. Typical habitats are located next to
houses, schools, community facilities and cultivated fields where melioidosis
cases have been reported (LIMMATHUROTSAKUL *et al* 2010b).

Soil sampling also provides an insight into the interactions between *B. pseudo-
mallei* and protozoan organisms. According to SANDON *et al* in 1927, excysted
protozoa can be found within the first 20 cm of soil. However, amoebae can be
also found until 50 cm of depth but they are always in their encysted stage
(EKELUND *et al* 2001). Thus, the interactions between *B. pseudomallei* and *Acan-
thamoeba* spp. mostly take place in the soil surface. As LIMMATHUROTSAKUL
et al suggested in 2013a, the optimal sampling depth for *B. pseudomallei* is within
the upper 30 cm in soil. In the current study, the collection of the soil was per-
formed according to the guidelines of LIMMATHUROTSAKUL *et al* (2013a). As
documented in this study, acanthamoebae are indeed ubiquitous in African soil.
Moreover, acanthamoebae are also known to be responsible for shaping bacte-
rial communities in soil and water (HAHN *et al* 2001). While the bacteria can be
influenced in their size by means of producing bigger or smaller morphotypes,
also the species composition can be affected by FLA. Depending on tempera-
ture, pH and nutrient compounds the bacterial community, and therefore the
food source for *Acanthamoeba* can be influenced. Overall, the interaction of bac-
teria and FLA seems to be a very fine-tuned system being influenced by various
environmental and weather conditions as well as by the respective species in-
volved.

CHAOWAGUL *et al* described in 1989 that *B. pseudomallei* has an optimal growth temperature of around 40°C, thus it is conceivable that the bacterium survives inside of amoebae during periods of unfavourable environmental conditions. According to INGLIS *et al* in 2000b, *B. pseudomallei* can also replicate inside of acanthamoebae until they burst and expel it to the environment. Then the bacteria can infect the next amoeba.

Although *B. pseudomallei* is mainly endemic in tropical and subtropical regions, melioidosis is of worldwide concern. As LIMMATHUROTSAKUL *et al* demonstrated in 2016, the global warming will have a major impact on the global distribution of *B. pseudomallei.* As soon as the bacterial pathogen finds appropriate growth conditions it can also establish in countries being non-endemic so far. As predicted by the WHO, the risk factors for melioidosis in industrial countries, e.g. Diabetes, will dramatically rise within the next decade. Therefore, the population as well as clinicians have to be aware of this potentially fatal disease.

Moreover, more and more people travel to highly endemic regions e.g. Thailand. The first melioidosis case in Austria was detected in a 33-year old man returning from Southeast Asia (LEITNER *et al* 2015). The patient had worked as a diving instructor in Malaysia when the first symptoms, namely spontaneous and resolving fever, left upper abdominal pain and weight loss started. A sonography and CT was performed in 2012 and showed a slightly bigger spleen and an affected kidney. However, the laboratory results were normal. As the symptoms persisted further tests were made in 2013 and sonography showed a 10 cm big expansion on the spleen. The PET-CT confirmed the finding and revealed liquid inside of the expansion. Subsequently, drainage was conducted obtaining yellow fluid. The liquid was further used for culturing and revealed that the patient was suffering from melioidosis. Treatment was initiated with 3 days of piperacillin/tazobactam followed by 5 weeks of amoxicillin/clavulanic

acid. To prevent a relapse, the patient received doxycycline for 2 months. The patient fully recovered.

As NGAUY *et al* described in 2005 an infection with *B. pseudomallei* can lead to the disease also decades later.

Altogether, this study is the first confirmation of a natural interaction between *B. pseudomallei* and *Acanthamoeba* spp. isolated from environmental samples. This is a significant contribution to understand the global distribution of *B. pseudomallei*.

5.   Abbreviations

µm	-	micrometer
°C	-	Celsius
A549	-	lung epithelial cell line
AHL	-	Acly-homoserine lactones
AK	-	*Acanthamoeba* keratitis
AP	-	Acanthopodia
API 20NE	-	biochemical test kit ©biomerieux
bp	-	base pairs
$CaCl_2$	-	Calcium chloride
CD	-	Cluster of differentiation
CDC	-	Centers for Disease, Control and Prevention
CIP	-	Climate Information Center
CNS	-	Central Nervous System
CpG	-	DNA-islands with Guanine and Cytosine dominating
CSF	-	Cerebrospinal Fluid
CRU	-	Climatic Research Institute
DNA	-	Deoxyribonucleic acid
ECT	-	ectocyst
EN	-	Encystation
END	-	endocyst
EX	-	Excystment
FLA	-	free living amoeba
FV	-	Food vacuole
fsp	-	full speed
g	-	Gram
G-CSF	-	granulocyte colony stimulating factor
GAE	-	Granulomatous amoebic encephalitis
gDNA	-	genomic DNA
H_2O	-	water
HeLa	-	epithelial cell line
HLA	-	human leukocyte antigen
i.v.	-	intra venous
IDSA	-	Infectious Diseases Society of America
IFN	-	Interferon
Ig	-	Immunoglobulin
IL	-	Interleucin
iNOS	-	inducible nitric oxidase synthase
irlR	-	invasion related locus
JAK-STAT	-	signalling pathway
kDa	-	kilo Dalton
KH_2PO_4	-	potassium dihydrogen phosphate
L	-	liter
LD_{50}	-	abbreviation for lethal dose 50%
LPS	-	Lipopolysaccharide
LuxI	-	auto-inducer synthase
LuxR	-	receptor
M	-	molar

Abbreviations

mA	-	milliampere
Mbp	-	Mega base pairs
mg	-	milligram
$MgSO_4$	-	Magnesium sulphate
min	-	minute
mL	-	millilitre
MNGC	-	multinucleated giant cells
mRNA	-	messenger RNA
Na_2HPO_4	-	sodium hydrogen phosphate
NaCl	-	sodium chloride
NC	-	negative control
NFκB	-	transcription factor
ng	-	nanogram
NN	-	non nutrient
NU	-	nucleus
OP	-	operculum
p.o.	-	per oral
PAMP	-	pathogen associated molecular pattern
PBS	-	phosphate buffered saline
PC	-	positive control
PCR	-	Polymerase chain reaction
PP	-	pseudopodia
qPCR	-	real-time polymerase chain reaction
RNA	-	ribonucleic acid
rRNA	-	ribosomal RNA
RT	-	room temperature
sec	-	second
SNP	-	single nucleotide polymorphism
sp.	-	species (singular)
spp.	-	species (plural)
SYTO9	-	fluorescent dye
T1-T20	-	*Acanthamoeba* genotypes
TAE	-	Tris Acetate EDTA
TLR	-	toll like receptor
TRO	-	trophozoite
TTSS	-	type three secretion system
UEA	-	University of East Anglia
UV	-	ultraviolet
V	-	volt
WHO	-	world health organisation

6. Glossary

Adaptive immune system: see *immune system*

Adhesion: mechanism bacteria use to adhere to cells; the proteins used for this mechanism are adhesins

Adhesins: proteins varying not only in their length but also in their diameter

Aerobic: defines organism in a need for oxygen to survive and perpetuating their metabolism

Antigens: molecules e.g. proteins, lipids or polysaccharides which lead to production of antibodies as part of the adaptive immune reaction; antigens can be recognized by the antigen receptors of either B- or T-cells

Autoinducer: molecules produced during quorum sensing; autoinducers are produced in higher amounts as the density of bacteria increases and the growth of the organisms can be adapted

Autophagy: degradation of organelles in the cell-own lysosomes

Binary fissions: cell proliferation where the cell is increasing its length and after the replication of the DNA starts separating in the middle

Cluster of differentiation (CD): defines groups of monoclonal antibodies identifying a similar molecule on the cell surface

Cytokines: are proteins produced by the cell to influence the actions of other cells; lymphocytes produced cytokines which are also labelled as interleukines (IL)

Cytoplasm: the substance with unites the cytosol and the cell organelles between the cell membrane and the nucleus; typically the cytoplasm is colourless and is composed of 80% water

Deoxyribonucleic acid (DNA): molecule which harbours the genetic information (genes) of an organism; DNA can be replicated and also used as a source for generating mRNA which is further used for the synthesis of proteins

Dysentery: inflammatory disease concerning the intestine leading to bloody diarrhoea

Endosome: organelles in animal cells dedicated to transport material newly ingested by endocytosis; transportation to lysosomes leads to degradation of the material

Endosymbionts: one organism lives inside of another organism; special form of symbiosis

Eukaryotes: organisms consisting of a separated nucleus divided from the cytoplasm by membranes

Food vacuole: compartment used by organisms to take up food and digest it

Genomical DNA (gDNA): DNA from the nucleus

Gel electrophoresis: method frequently used in molecular biology; gel is used to separate molecules according to their size

Genotype: genetic composition of organism in terms of combined alleles

Gram negative bacteria: consists of a thicker cell wall composed of an outer and a cytoplasmic membrane; due to the outer membrane the bacteria have another layer for protection; a gram staining helps to distinguish between gram positive and gram negative bacteria

IFN-γ: cytokines mainly needed for the activation of macrophages; IFN-γ is produced by CD8 T cells, NK cells and $CD_4 TH_1$ cells

Immune system: eukaryotic organisms have a system which helps them to eradicate pathogens out of their system. The immune system has two types, namely the innate and the adaptive immune system. Overall, it is composed of tissues, cells and molecules as cytokines to orchestrate the innate adaptive immune system

Immunoblot (=western blot): method used in molecular biology to examine a protein solution; gel electrophoresis is the first step to separate the proteins according to their size. It is followed by blotting them on a microcellulose membrane and a specific antibody labelled with e.g. radioisotope marks the interacting proteins

In vitro: experiments performed with microorganisms or cells outside of an organism

In vivo: experiments performed on a whole and living organism

Intracellular: organism which enters its host and stays there for a certain time; the cellular enzymes of the host are used to maintain the metabolism of the intracellular organism

Keratoplasty: medical surgery where patients damaged cornea is completely replaced by the cornea of a healthy donor

Lipopolysaccharide (LPS): is found in the cell membrane of gram negative bacteria and are known to stimulate Toll-like receptors (TLR) on dendritic cells and macrophages

Lysis: defines the bursting of the cell membrane to the end that the cell content is expelled and the cell is dead

Lysosome: is a organelle surrounded by membranes and filled with hydrolytic enzymes needed for digesting components; typically these organelles are acidic and are used for degradation

Macrophages: are large phagocytic cells with a wide range of different functions; next to being a scavenger cell they are also producing pro-inflammatory cytokines and recognize pathogens. Furthermore, they act as antigen-presenting and effector phagocytic cells. Overall, they are important representatives of both innate and adaptive immune system.

MD-2: also known as lymphocyte antigen 96 with the task to interact with TLR4 to recognize LPS

Mitosis: cell proliferation of one cell into two daughter cells with the exact same set of chromosomes

Multi nucleated giant cells (MNGC): distinct cells e.g. monocytes and macrophages fuse and often develop during infections

Monocytes: precursor leukocytes producing macrophages; they typically have a bean-shaped nucleus

Morbidity rate: also known as prevalence; percentage of population being affected by e.g. a certain disease

Morphotype: differences in shape of an organism from the same species

Mortality rate: number of deaths in a certain time period; measured in units of deaths per 1000 individuals during one year

Motile: organism able to move; bacteria use e.g. flagella to change their direction

Necrosis: cell death due to chemical or physical injuries leading to bursting of the cell. Thus rupture leads to a massive escaping of cell debris which has to be eliminated by phagocytes

Neutrophils: are the biggest leukocyte population in the peripheral blood and also known as polymorphonuclear neutrophilic leukocytes; they have the task to enter infected tissues and phagocyte extracellular pathogens

NFκB: important transcription factor made of two chains; without cell stimulation this factor is inhibited and to be found in the cytoplasm; it is typically activated as the TLR receptors are stimulated

Nitric oxide synthase (iNOS): enzyme family with the task to produce nitric oxide

Operon: cluster of genes typically found in prokaryotes; the genes are organised without spacers in between and are controlled by a single regulatory region

Opsonisation: covering of pathogenic cells with antibodies and therefore prone them for degradation

Pathogen: organism e.g. bacteria and viruses which harms its host by causing diseases

Pathogen associated molecular pattern (PAMP): different components of pathogens e.g. DNA, RNA, LPS are recognized by PAMPs and the immune system is stimulated due to production of cytokines

Pathogenicity: see *pathogen*

Polymerase chain reaction (PCR): methods widely used in molecular biology to amplify certain fragments with the help of specific primer pairs. The fragments can be separated via gel electrophoresis and be further analysed afterwards

Phagocytic cells: neutrophils and macrophages which ingest pathogens as bacteria and viruses and destroy them

Phagocytosis: internalisation of bacteria and viruses by phagocytic cells to the end that the pathogens are destroyed

Phagolysosomes: are produced when phagosomes and lysosomes fuse; lysosomes harbour enzymes and other molecules used to degrade pathogens

Phagosomes: harbours the pathogens ingested during phagocytosis

Pilus: extension used by bacteria to substitute plasmids for antibiotic resistances

Pinocytosis: also known as cell drinking; small particles are ingested and transported in vesicles finally fusing with lysosomes to digested the material

Proteasome: protein complexes of eukaryotic cells used for the degradation of damaged and unwanted proteins

Protozoa: eukaryotic organisms with one nucleus typically having the ability to move and feed

Pseudopodia: eukaryotic cells are able to temporarily produce protrusions of the cell membrane with the task to move and ingest food particles

Quorum sensing: mechanisms used by bacteria and also some insects to regulate their gene expression according to their cell density; molecules used during this process are called autoinducers (see *autoinducers*)

Receptor: signal molecules are able to bind to specific receptor proteins which can transduce this signal from the cell surface to the inside of the cell

Ribosome: machinery of the cell used for synthesises proteins with the help of mRNA. It is constituted out of a small and a big subunit and a large number of proteins. All of the components are required to ensure full functionality

RNA-polymerase: enzyme used to generate RNA out of DNA

Sanger sequencing: method where the exact nucleotide composition of a DNA fragment is examined; this method uses didesoxynucleotides to generate fragments of different length and a variation on the last nucleotide is the fragment which can be detected

Sepsis: immune reaction on infections of the bloodstream caused by bacteria, viruses, fungi and also parasites leading to organ failure

Septicaemia: see *sepsis*

Sequencing: see *sanger sequencing*

Serum: fluidal part of blood

Single nucleotide polymorphism (SNP): single nucleotides in RNA or DNA are changed; according to these variabilities different strains of a species can be described

Symbiosis: two organisms live in close relationship and do not harm one another but more hold and advantage for either one of these partners

T-helper cell 1 (T_H1): subset of T cells (CD4) which produce cytokines and are used to activate macrophages

Toll-like receptor (TLR): pattern recognition receptor found on all cells of the innate immune system with the task to recognize PAMPs (see *Pathogen associated molecular pattern)* e.g. LPS

Transcription factor: proteins needed to regulate the gene expression of eukaryotic cells

Trophozoite: vegetative form of the amoebae which is able to feed, move and divide actively

Ubiquitin: highly conserved protein which can be found throughout all eukaryotic cells; attaches in covalent binding to lysine and depending on the length of the ubiquitin chain different cell actions e.g. degradation of the marked protein with proteasomes are activated

Virulence factor: molecules e.g. capsule, endo- and exotoxins produced by bacteria helping them to colonize the host cell via several mechanisms

Vortexing: mixing a sample with a device generating constant vibration

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8. Appendix

8.1. Abstacts

8.1.1.   Abstract English

Acanthamoeba spp. are ubiquitously found in the environment e.g. in water, soil, dust and air. Furthermore, they are facultative pathogens leading to severe diseases as *Acanthamoeba* keratitis (AK) and granulomatous amoebic encephalitis (GAE). One of the impressive abilities of these protozoa is their ability to produce extremely resistant cysts. On the one hand they form motile trophozoites which feed on bacteria and multiply by binary fission. On the other hand, harsh conditions lead to the formation of cysts. These are defined by an outer ectocyst and an inner endocyst, which are responsible for the typical star-shaped appearance. The cyst morphology is also used to identify their morphological group. Moreover, *Acanthamoeba* spp. are well known reservoirs for human pathogens, e.g. *Legionella pneumophila,* and are described as virtual training grounds for various bacteria. Recently, there has been evidence that *in vitro* they can also act as host for *Burkholderia pseudomallei.*

B. pseudomallei is a gram-negative bacterium with a facultative intracellular life cycle and it is a natural inhabitant of soil in tropical and sub-tropical regions. It can cause the severe and underestimated disease melioidosis having a mortality rate of up to 90%. The infection is acquired either by inhalation of aerosols from contaminated soil and water, by ingestion or through skin lesions. The symptoms can range from skin infections, liver and spleen abscesses, encephalitis and septicemia to pneumonia and clearly depend on the bacterial load as well as on the mode of infection. Thus, diagnosis can be extremely challenging and particularly clinicians in non-endemic regions are not always aware of this potentially fatal disease.

The aim of this study was to identify the diversity of *Acanthamoeba* spp. in soil from African regions predicted to be endemic for melioidosis as no comprehensive data are available yet for this continent. Furthermore, it was aimed to answer the question if *Acanthamoeba* spp. indeed can act as natural host organisms for *B. pseudomallei.* Overall this project will contribute to a better understanding of the global distribution of this bacterium.

Soil samples, were collected at various sites in 4 African countries, namely Madagascar, Burkina Faso, Congo and Ethiopia. Afterwards, acanthamoebae were isolated on NN-agar plates seeded with *E. coli* and sub-cultured to the end

of gaining clonal cultures. Then, the trophozoites were harvested from the plates, washed and whole-cell DNA was extracted. While one aliquot was used to identify the acanthamoebae, the other aliquot was used to run *B. pseudomallei*-specific qPCR. All acanthamoebae isolates were genotyped by sequencing a fragment of their 18S rDNA.

All soil samples investigated were positive for *Acanthamoeba* spp. and in average more than one *Acanthamoeba* strain was detected per sample. Interestingly, all morphological groups and strains from 9 different genotypes were found. Genotype T4 is the most abundant genotype worldwide and it was also the most prevalent one in this study. However, the soil collected in Madagascar showed a different picture, as here T5 was the genotype found most frequently. Moreover, the extremely rare genotype T17, which had been first described in Thailand, was isolated from altogether 4 samples. During this study, T17 was exclusively found in soil samples collected in Madagascar. Soil from Burkina Faso showed the highest diversity of *Acanthamoeba* spp. including also several rare genotypes as e.g. T11, T15 and T19. Overall, the majority of genotypes found are known to be linked to human disease.

Moreover, one *Acanthamoeba* isolate was positive for *B. pseudomallei*, even after 12 cycles of subculture. This is the first proof that *B. pseudomallei* can naturally use acanthamoebae as a host.

8.1.2.   Abstract german

Acanthamoeba spp. sind in der Umwelt omnipräsent und können in Wasser, Erde, Staub und Luft gefunden werden. Außerdem sind sie fakultative Pathogene. Sie können schwere Infektionen, einerseits in Form von Akanthamöben-Keratits (AK) und andererseits von Granulomatöser Akanthamöben Encephalitis (GAE) hervorrufen. Eine der Besonderheiten dieser Protozoen ist es, extrem resistente Zysten auszubilden. Der Trophozoit stellt die bewegliche Form dar, die sich von Bakterien ernährt und sich mittels Zellteilung vermehrt. Ungünstige Umweltbedingungen führen zur Bildung der resistenten Zysten. Diese werden aufgrund der typischen sternförmigen Morphologie ihrer Ekto- und Endozyste in unterschiedliche Gruppen eingeteilt. Des Weiteren sind *Acanthamoeba* spp. bereits als Wirtsorganismen für Humanpathogene wie beispielweise *Legionella pneumophila* von medizinischer Bedeutung. Es ist bekannt, dass Bakterien nach Amöbenpassage eine deutlich gesteigerte Resistenz gegenüber Desinfektionsmitteln und Antibiotika aufweisen. Erst kürzlich gab es Hinweise darauf, dass sie *in vitro* auch als Wirte für *Burkholderia pseudomallei* fungieren können.

B. pseudomallei ist ein gram-negatives und fakultativ intrazelluläres Bakterium, das typischerweise im tropischen und subtropischen Erdreich zu finden ist. Eine Infektion kann zum Ausbruch der Krankheit Melioidose führen, wobei Mortalitätsraten von bis zu 90% beschrieben sind. Die Infektion kann durch Inhalation, mittels Wundeintritt oder auch durch Nahrungsaufnahme erfolgen. Die Manifestationen reichen von Hautinfektionen, Leber- und Milzabszessen, Sepsis bis zu Lungenentzündung. Abhängig von der Bakterienkonzentration und dem Infektionsweg können sich verschiedenste Symptome ausprägen. Besonders in nicht-endemischen Gebieten sind Ärzte über das Krankheitsbild kaum informiert.

Das Ziel dieser Arbeit war es, mehr über die Diversität von *Acanthamoeba* spp. im Boden in afrikanischen Regionen, in denen Melioidose endemisch ist zu erfahren. Bislang gibt es dazu keine Daten. Außerdem sollte die Frage beantwortet werden, ob Akanthamöben natürliche Wirte für *B. pseudomallei* darstellen. Grundsätzlich soll diese Studie auch zu einem besseren Verständnis der globalen Verbreitung dieses Pathogens beitragen.

Dafür wurden in vier für Melioidose endemischen Regionen Afrikas, nämlich Madagaskar, Burkina Faso, Kongo und Äthiopien, Bodenproben gesammelt und auf Akanthamöben getestet. Die isolierten Akanthamöben wurden auf Non-Nutrient-Agarplatten, die mit *E. coli* bestrichen wurden, zu Reinkulturen gezüchtet. Im Anschluss wurden die Trophozoiten geerntet und deren DNA isoliert. Ein Teil davon wurde verwendet, um *B. pseudomallei* mittels qPCR erneut nachzuweisen. Der andere Teil wurde für eine spezifische Akanthamöben-PCR benutzt. Alle Isolate wurden durch Sequenzierung des 18S rRNA-Gens identifiziert.

Alle 36 untersuchten Bodenproben erwiesen sich als positiv auf *Acanthamoeba* spp und insgesamt wurden 74 verschiedene Akanthamöben-Stämme isoliert. Im Durchschnitt wurde mehr als ein Stamm pro Probe isoliert. Dabei wurden alle morphologischen Gruppen gefunden sowie 9 verschiedene Genotypen identifiziert. Der weltweit häufigste Genotyp, T4 war auch in dieser Studie mit 29 unterschiedlichen Isolaten dominierend. Hingegen wurde in Madagaskar hauptsächlich Genotyp T5 nachgewiesen. Des Weiteren wurde der extrem seltene Genotyp T17 aus vier unterschiedlichen Proben aus Madagaskar isoliert. Dieser Genotyp war 2010 zum ersten Mal in Thailand beschrieben worden. Interessanterweise wiesen die Proben aus Burkina Faso, trotz der geringen Probenanzahl, die höchste Diversität auf, es wurden mehrere Stämme der seltenen

Genotypen T11, T15 und T19 gefunden. Insgesamt gelten fast alle der isolierten Genotypen als humanpathogen.

Außerdem konnte in einem Akanthamöben-Isolat *B. pseudomallei* nachgewiesen werden. Da die Interaktion zwischen den beiden Organismen trotz 12 Subkultivierungsschritten bestehen blieb, kann sie als sehr stabil bezeichnet werden. Damit konnte erstmals gezeigt werden, dass Akanthamöben tatsächlich als natürliche Wirte für *B. pseudomallei* dienen können.

8.2.   Index of Figures

Appendix

8.3. Index of tables

8.4.   Acknowledgments